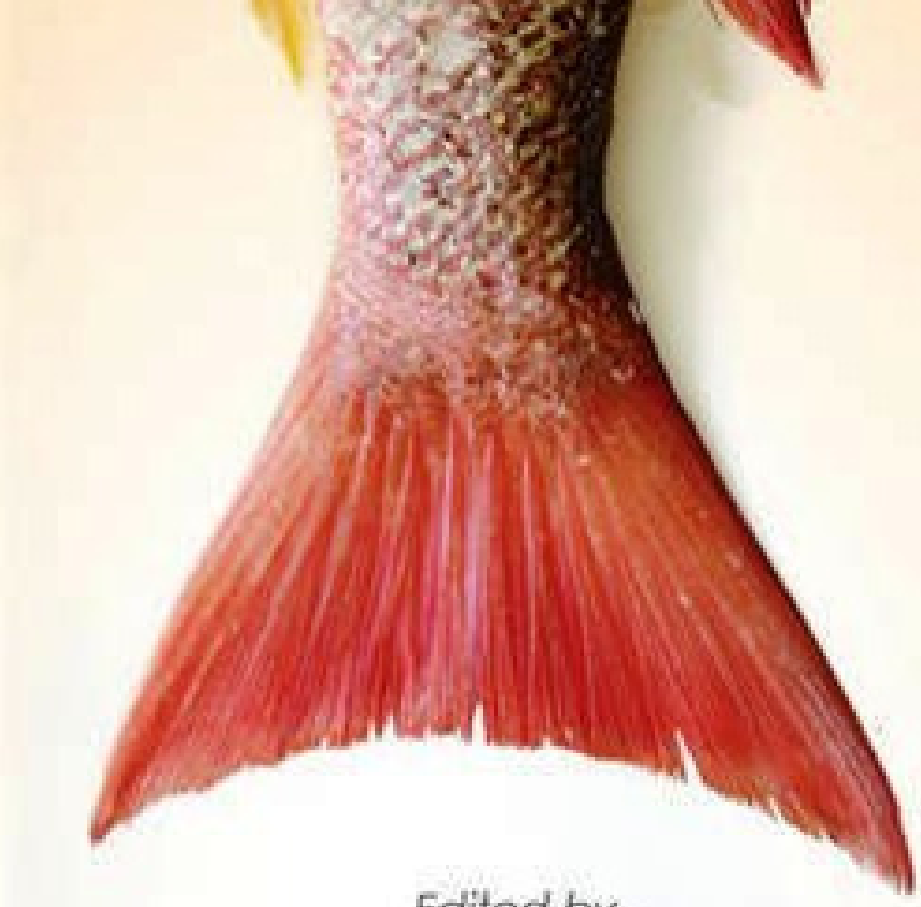

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DIETARY NUTRIENTS, ADDITIVES AND FISH HEALTH

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Dietary Nutrients, Additives, and Fish Health

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Chhorn Lim

This book is dedicated to my wife, Brenda; our children, Cheang Chhun, Lisa, Chhorn Jr., Elizabeth, Brendan and Edelweiss; our grandchildren, Bryent, and Madelyn; our brothers, Korn, Daniel (Eang), Thong Hor, Muoy Hor and Trauy; our sisters, Huoy Khim, Chhay Khim, Seang Hay and Huoy Teang; and our nephews and nieces for their patience and unconditional love.

Carl D. Webster

This book is dedicated to my friend Caroline; my daughters NancyAnn, Catherine, and Emma; my Mom and Dad; my brothers Tom and Pete; my uncle David; my other "children" Lydia, Scout, Stevie, Michael, and Iggy; and to those who are patiently waiting for me Samwise, Barley, Darwin, Misty, Shyron, KC, Tillie, and Poppins. This book is also dedicated to all the teachers and professors who instilled in me the love of learning and the joys of hard work. As the immortal Willy Wonka noted, "We are the music makers. And we are the dreamers of the dreams."

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Preface

The United States Aquaculture Society (USAS) is a chapter of the World Aquaculture Society (WAS), and a worldwide professional organization dedicated to the exchange of information and networking among diverse aquaculture constituencies interested in the advancement of the aquaculture industry through the provision of services and professional development opportunities. The mission of the USAS is to provide a national forum for the exchange of timely information among aquaculture researchers, students, and industry members in the United States. To accomplish this mission, the USAS will sponsor and convene workshops and meetings, foster educational opportunities, and publish aquaculture-related materials important to US aquaculture development.

The USAS membership is diverse, representing researchers, students, commercial producers, academics, consultants, commercial support personnel, extension specialists, and other undesignated members. Member benefits are substantial and include issue awareness, a unified voice for addressing issues of importance to the US aquaculture community, networking opportunities, business contacts, employment services, discounts on publications, and a semi-annual newsletter reported by regional editors and USAS members. Membership also provides opportunities for leadership and professional development through service as an elected officer or board member; chair of a working committee; or organizer of a Special Session or Workshop, special project, program, or publication as well as recognition through three categories of career achievement (early career, distinguished service, and lifetime achievement). Student members are eligible for student awards and special accommodations at national meetings of the USAS and have opportunities for leadership through committees, participation in Board activities, sponsorship of social mixers, networking at annual meetings, and organization of special projects.

At its annual business meeting in New Orleans in January 2005, under the leadership of President LaDon Swann the USAS voted to increase both the diversity and quality of publications for its members through a formal solicitation process for sponsored publications including books, conference proceedings, fact sheets, pictorials, hatchery or production manuals, data compilations, and other materials that are important to US aquaculture development and that will be of benefit to USAS members.

Disease outbreaks have become a major threat to the sustainability of the aquaculture industry, with antibiotics and chemicals historically used to treat aquatic animals ineffective or not allowed today. In this book *Dietary Nutrients, Additives, and Fish Health*, the relationships between dietary nutrients, antinutritional factors/toxins, and non-nutrient dietary additives (e.g., probiotics, prebiotics, plant extracts, and organic acids and their salts) and fish performance, immune system function, and health are comprehensively reviewed. Through collaboration with Wiley-Blackwell Publishing on book projects such as these, the USAS Board aims to serve its membership by providing the most up-to-date and timely information through publications of the highest quality at a reasonable cost. The USAS thanks the editors Chhorn Lim, Carl Webster, Delbert M. Gatlin, and Cheng-Sheng Lee for

donating royalties which will help provide the benefits and services to members and to the aquaculture community, and Justin Jeffryes and Niles Balmforth (Wiley-Blackwell Publishing) for their cooperation. The USAS Publications Committee members include Wade O. Watanabe (Chair), Jeff Hinshaw, Jimmy Avery, Christopher Kohler, Gary Fornshell and Kevin Hopkins.

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Chapter 1

Overview of Fish Immune System and Infectious Diseases

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Introduction

Cultured finfish are an important source of animal protein worldwide (Naylor et al. 2009), and the Food and Agriculture Organization (FAO) reported that over half of the world's supply of fish and shellfish is now from aquaculture (FAO 2008). As fish consumption increases and natural fish stocks decrease, aquaculture practices will need to intensify in order to meet global demand. Intensification will likely lead to an increase in disease problems, due to a higher number of animals in a limited and confined environment and the influence of poor environmental conditions (i.e., water quality) on the fish immune system. For example, limited disease-related problems were reported in the channel catfish (*Ictalurus punctatus*) industry prior to 1980 because stocking densities were less than 10,000 fish/ha and maximum feeding allowances were about 50 kg/ha/day with most farms using a single crop system (Hawke and Khoo 2004). Production intensity increased following that time with >12,000 fish/ha stocked, and feeding increased accordingly to 90–112 kg/ha/day. Multi-cropping systems (i.e., various sizes of fish cultured together) that utilized limited water exchange were also employed (Hawke and Khoo 2004). As a result, up to 45% of on-farm losses were reported to be due to infectious disease (USDA/APHIS 1997). The emergence or re-emergence of pathogens will likely be seen in many aquaculture ventures as production intensifies and degrades environmental parameters.

Immunity is the inherited ability to recognize and respond defensively against foreign living and non-living agents. The immune response is a coordinated response of immune cells and molecules and memory in vertebrate animals (including fish) that occurs as a result of recognition of foreign agents. Fish have evolved with both non-specific (innate immunity) and adaptive (acquired) immune mechanisms. The innate immune response is limited in specificity via germline encoded pathogen recognition receptors (PRRs) that respond to pathogen-associated molecular patterns (PAMPs) such as bacterial or fungal glycoproteins and lipopolysaccharides (Kawai and Akira 2010; Boltana et al. 2011). The innate response is an important first line of defense, especially in larval fish. Research suggests that the innate immune response is important in priming and regulating adaptive immunity (Fearon and Locksley 1996). Adaptive immunity allows for specificity and memory (Pilström

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2005; Secombes et al. 2005). This chapter provides an overview of the fish immune system and the infectious diseases of fish (bacterial, viral, parasitic, and fungal).

Immune Organs and Tissues

Thymus

The thymus of fish is composed of lymphoblasts (early immune cells) in a reticular endothelial cell network; it is the first organ to obtain mature lymphocytes during immune maturation (Manning 1994; Rombout et al. 2005). Evidence in fish supports the notion that the thymus is responsible for the development of T-lymphocytes (T-cells), as is the case in other jawed vertebrates. T-cell selection occurs in the thymus, and only T-cells that recognize foreign antigenic peptides in the context of self major histocompatibility complex (MHC) molecules are released (Kuby 1994). T-cells that recognize self antigen and self MHC are killed via programmed cell death or apoptosis. Mature T-cells are then released from the thymus and become distributed in the immunological organs and tissues (Rombout et al. 2005). In adult fish, as in mammals, the thymus decreases significantly in size.

Kidney

The kidney is important in hematopoiesis and contains two segments: the anterior or head kidney and the posterior or trunk kidney. Blood cell differentiation occurs here instead of in bone marrow, as in mammals. Early in development, the entire kidney is involved in production of blood cells and early immune responses. The anterior kidney is considered the primary B-lymphocyte (B-cell) organ and is where the B-cells develop. As the fish matures, the posterior kidney is primarily involved in filtration and/or urinary functions. The kidney also contains the reticuloendothelial system, which is a network of sinusoids lined with phagocytic cells that have roles in antigen presentation. There is usually a concentration of melanomacrophage centers consisting of macrophages, lymphocytes, and plasma cells, and these centers are involved in antigen trapping and immune responses (Agius and Roberts 2003).

Spleen

The spleen is a secondary immune organ in fish and is involved in antigen processing, antibody production, and memory. Most fish spleens are not organized into red and white pulp, as in mammals. Manning (1994) demonstrated in carp (*Cyprinus carpio*) that the proliferative response to antigen was scattered and not organized into thymus-dependent and -independent regions. Melanomacrophage centers are also located in the spleen and are primarily responsible for the breakdown of erythrocytes. However, as discussed above, they may be involved in antigen presentation and immunologic memory. In rainbow trout (*Oncorhynchus mykiss*), Hadidi et al. (2008) demonstrated that the spleen size predicted the resistance to *Flavobacterium psychrophilum*, suggesting a role in innate immunity.

Gut

Gut associated lymphatic tissue (GALT) consists of lymphoid aggregates and follicles in the lamina propria of the intestine (Rombout et al. 1989). Immunoglobulin (Ig) + and Ig – cells (B- and T-cells) are present in the intestinal epithelium, suggesting importance as an immune tissue (Rombout et al. 1993). Antigen-specific antibody is secreted onto mucosal surfaces of the intestine. Fish do not have lymph nodes; most likely, their kidney, spleen, and GALT play an equivalent role to the lymph system in mammals with respect to antigen processing and presentation. Rombout et al. (2011) published an excellent review of the present state of fish intestinal immunology.

Natural Defense Barriers

Skin and Mucus

The mucus and skin/scales of fish act as a natural barrier to foreign substances and pathogens. Mucus consists of glycoproteins (lectins) or mucopolysaccharide proteins produced by goblet cells in the skin epidermis, gills, and mucosa of the gut (Dalmo et al. 1997; Sadovy et al. 2005). The mucus can serve as a non-specific defense mechanism, as it can result in sloughing off the

gills, skin, or gut lining, thereby preventing colonization by fish pathogens. The mucus also contains non-specific humoral molecules and specific antibodies.

Innate Immunity and Disease Resistance

Non-specific Immune Cells

Fish phagocytes (macrophages and neutrophils) express receptors on their surface that recognize invading pathogens and activate an innate immune response. These receptors are termed pathogen recognition receptors (PRRs), and these will sense the presence of pathogens through recognition of pathogen-associated molecular patterns (PAMPs). The interaction between PRRs on phagocytic cells and PAMPs of pathogens leads to the initiation of the innate immune response. Recent reviews provide current status of PRRs in fish (Boltana et al. 2011; Hansen et al. 2011; Palti 2011) and the antimicrobial mechanisms of fish that can be induced through PRR and PAMP interactions (Rieger and Barreda 2011).

Monocytes/macrophages

Monocytes and/or tissue macrophages are probably the single-most important cell in the immune response of fish. Not only are they important in inflammation and the production of cytokines (Clem et al. 1985), but they are also the primary cells involved in phagocytosis and killing of pathogens upon initial recognition and subsequent infection (Shoemaker et al. 1997). Macrophages also have an important role in antigen-presentation, thus linking the non-specific and specific immune responses. Forlenza et al. (2011) recently provided an excellent review of macrophage activation in fish.

Neutrophils

Neutrophils (granulocytes) are the primary cells involved in the initial stages of inflammation (12–24 hours) in fish (Manning 1994); their function includes phagocytosis and production of cytokines to recruit immune cells to the damaged and/or infected area. In channel catfish the neutrophil is phagocytic, but it appears to kill bacteria by extracellular mechanisms rather than via intracellular mechanisms (Ellis 1981; Waterstrat et al. 1991). The role of the neutrophils in immunity likely varies among different species of fish.

Non-specific Cytotoxic Cells

Non-specific cytotoxic cells (NCC) are present in fish (Evans and Jaso-Friedman 1992) and their functions are closely related to those of mammalian natural killer cells. These cells can kill a variety of target cells including tumor cells, virally infected cells, and protozoan parasites. NCCs function by lysis of target cells following receptor binding and signaling of the lytic cycle. These cells are important in parasite (Evans and Gratzek 1989) and viral (Hogan et al. 1996) immunity.

Non-specific Humoral Molecules of Fish

The serum, mucus, and eggs of fish contain a number of non-specific humoral molecules that can act against invading pathogens. Magnadottir (2006) and Whyte (2007) provide good reviews of these, a few of which are discussed in the following.

Lectins

Lectins are glycoproteins that non-specifically bind to sugars located on the surface of bacteria, viruses, and parasites, resulting in precipitation and agglutination reactions. Some lectins can act as opsonins for phagocytosis and can also be involved in activation of the complement system. Known lectins in fish include C-type lectins, mannose-binding lectin, pentraxins (C-reactive protein and serum amyloid protein), and ficolin. Sharon and Lis (1993) suggest that lectins are also involved in cell recognition and binding, and are also important in cellular communication. Xu et al. (2001) demonstrated their potential defensive action against parasites.

Lysozyme

Lytic enzymes, such as lysozyme, have been described in fish. Lysozyme is an antibacterial molecule that cleaves the 1-4-β-linkages between N-acetylmuramic

acid and N-acetylglucosamine in the cell wall of bacteria, resulting in cell lysis. Similar to lectins, lysozyme can also act as an opsonin for phagocytosis and activate the complement system. The molecules are also important in opsonization of target cells and in the attraction and activation of cells that are essential in inflammation.

Complement

The complement system is a group of serum molecules involved in the control of inflammation, opsonization of immune complexes and microorganisms, and lysis of pathogens. The liver is responsible for the production of components of the complement cascade. Fish complement can generally be activated through the classical pathway (i.e., specific immunoglobulin or IgM), alternative pathway (i.e., bacterial cell wall components, viral components, or surface molecules of parasites), and lectin pathway (i.e., interaction of mannose-binding lectin with surface molecules of microbes) (Boshra et al. 2006). Even though all three pathways exist in fish, full characterization of all components and their actions are incomplete (Sakai 1992; Boshra et al. 2006). The most important components of the complement system, namely C1r, C2, C3, C4, and C5–C9 (formation of the membrane attack complex), have been characterized and, for the most part, function demonstrated (Boshra et al. 2006). Each of the components reacts in an enzymatic cascade and generates products that are able to clear antigenic molecules and immune complexes, participate in the inflammatory response, lyse microorganisms, and aid in phagocytosis by macrophages and neutrophils. Nakao et al. (2011) provide a review of the current status of research on the teleost complement system.

Transferrin

Transferrin, which is found in the serum of fish, is an iron-binding glycoprotein that plays an important role in transporting iron. This protein acts as a bacterial defense mechanism by binding iron within the fish in order to make this essential element inaccessible to bacterial pathogens, thus preventing their growth.

Protease Inhibitors

Different fish species have protease inhibitors, such as α-2-macroglobulin. Such enzymes may have important roles in non-specific immunity by neutralizing proteolytic enzymes that are produced by different bacterial pathogens.

Signaling Molecules

A number of different cytokines are produced in fish and function to modulate both innate and adaptive immune responses. These include tumor necrosis factors, interleukins (IL), interferons, and chemokines. Recent reviews have been provided on these cytokines (Goetz et al. 2004; Robertsen 2006; Alejo and Tafalla 2011; Secombes et al. 2011).

Adaptive Immunity

Adaptive immunity is characterized by its specificity and association with immunological memory; however, these responses take time to develop in comparison to the innate response. The adaptive immune system of fish is divided into two branches: cell-mediated and humoral immunity, discussed in the following sections.

Cell-mediated Immunity

Cell-mediated immunity is important for defense against intracellular pathogens (i.e., intracellular bacteria, parasites, and viruses). Cell-mediated immune components consist of thymus-dependent lymphocytes, or T-cells, which express T-cell receptors (TCR) on their surface and provide specificity. T-cells consist of cytotoxic T-cells (Tc) and T-helper cells (Th). Both types recognize foreign antigen presented to them (from antigen-presenting cells) in the context of major histocompatibility complexes (MHC), and the interaction between the TCR and MHC molecule results in activation of the cells. Tc cells are identified by surface glycoprotein CD8, which is a co-receptor for binding to MHC class I molecules. These cells kill intracellular pathogens, viral infected cells, and foreign cells. Th cells are identified by the surface glycoprotein CD4 that binds to MHC class II molecules; these cells are further subdivided to Th1 and Th2 cells. Th1 cells secrete gamma interferon, tumor necrosis

factor beta, and IL-2, which activate antimicrobial activity in macrophages. Th2 cells secrete IL-4, IL-5, and IL-13, which promote strong antibody responses. Similar to humoral immunity, research is advancing the understanding of these processes in fish and the current state of knowledge on fish T-cells was recently reviewed (Laing and Hanson 2011).

Humoral Immunity

Antibody, or immunoglobulin (Ig), is the primary effector molecule of humoral immunity; it provides specificity. Antibody circulates in the serum of fish, and can also be found at mucosal sites and inside of some fish eggs. The primary antibody in fish serum has been described as a tetrameric IgM-like molecule (i.e., structurally similar to mammalian IgM). In teleosts, the antibody molecule comprises eight heavy chains and eight light chains. Hikima et al. (2010) describe the organization of fish Ig genes, the expressed Ig isotypes and their transcriptional control. Additional Ig isotypes were recently identified in fish: IgD in channel catfish (Edholm et al. 2011), and IgT in rainbow trout (Hansen et al. 2005), and IgZ in zebrafish (*Danio rerio*) (Flajnik 2005). Zhang et al. (2010) recently described the protein structure of IgT as monomeric in serum and polymeric in the gut of rainbow trout. The authors also demonstrated B-cells expressing surface IgT and suggested their importance in the GALT of rainbow trout.

The primary function of an antibody is to bind antigen. One effector mechanism of an antibody is to bind to bacterial pathogens, viruses, and toxins, which can result in neutralization. Binding of an antibody to a pathogen can also serve as an opsonin, in which macrophages can recognize the antibody via receptors and phagocytose the pathogen. Fish IgM activates complement and is also efficient at agglutinating bacterial cells that can aid phagocytosis.

Fish possess B-cells (surface Ig positive cells), which are considered similar to mammalian B-cells. However, Li et al. (2006) demonstrated that rainbow trout B-cells were both phagocytic and microbiocidal. The surface immunoglobulin of B-cells serves as the receptor for antigen recognition and has the same specificity of the antibody molecule produced. Peptide, protein, polysaccharide, lipopolysaccharide, and lipoprotein, but not lipids, are potential antigens. In a general sense, B-cells become activated following antigen binding to the immunoglobulin surface, either from circulating antigen or from presentation by antigen presenting cells (i.e., macrophages). Different antigens can invoke different mechanisms of B-cell activation. T-independent antigens can stimulate B-cells to produce antibody following binding without needing to interact with T-helper cells. T-dependent antigens need to be recognized by both B-cells and T-helper cells to elicit an antibody response. The activated B-cells then proliferate, differentiate, and generate a population of antibody secreting cells.

Immunologic memory and affinity maturation has been suggested in fish; however, the mechanisms for these are different than in mammals. An interesting difference between primary and memory humoral responses of fish and mammals is that fish do not switch to class IgG. The memory response of fish is IgM, which is same as the primary response. Ye et al. (2011a) suggest affinity maturation of fish Ig; however, the mechanism may be structural changes rather than germline or class switching (i.e., isotype changes). Fish IgM possesses eight antigen binding sites and, while affinity at each site may be relatively low, the molecule has a greater effective binding strength or avidity due to the presence of these multiple binding sites. Research is advancing the understanding of these processes in fish and different models have been proposed (Ye et al. 2011a, b; Zwollo 2011; Costa et al. 2012).

Bacterial Pathogens of Fish

Bacteria are microscopic prokaryotes that are grouped into two categories – Gram-negative or Gram-positive – based on differential staining using the Gram stain. Bacterial cells are generally grouped into three categories based on shape: coccus (round, oval, circular), bacillus (rod), and spirillium (spiral). Bacterial pathogens are typically identified following isolation in pure culture by growth in or on media, and are characterized using Gram-staining, acid fast-staining, and biochemical (nutrient) tests. New tests include serological or antibody-based techniques (immunofluorescence antibody test or enzyme-linked immunosorbent assay, IFAT or ELISA, respectively), fatty acid profiles, and nucleic acid probes such as polymerase chain reaction (PCR) methods. Fish

pathogens may be grouped into primary and secondary pathogens. Primary pathogens are capable of causing disease in a healthy host without other pathogens or environmental problems (e.g., *Edwardsiella ictaluri*, *Renibacterium salmoninarum*, *Francisella* spp.). Secondary pathogens typically cause disease due to environmental problems and/or co-infection (e.g., *Streptococcus* spp., *Aeromonas* spp., *Flavobacterium* spp., *Vibrio* spp.). To establish the ability of an isolated microorganism to cause disease, Koch's postulates need to be fulfilled as follows: (1) pathogen is present in all diseased animals; (2) pathogen is isolated and grown in pure culture; (3) pathogen from pure culture was placed back into fish and reproduced the same disease; and (4) the pathogen is re-isolated from the diseased fish in pure culture.

External/behavioral signs of bacterial disease may include anorexia or lack of feeding response, lethargy, abnormal swimming, excess mucus production, darkened body coloration, necrotic lesions and/or fin erosion, swollen abdomen (ascites), increased opercular movement, and pale or necrotic gills. Internal clinical signs may include reddening of intestine, swollen organs (enlarged spleen), pale or mottled organs (liver), white nodules (granulomas), and hemorrhagic organs (swim bladder).

Table 1.1 lists the bacterial pathogens that are responsible for major economic losses to all cultured fish species worldwide. Numerous books discuss in detail the etiological agents, disease signs, epizootiology, pathology, diagnosis, and control of bacterial pathogens important to cultured fish (Austin and Austin 2007; Plumb and Hansen 2010; Woo and Bruno 2011). The focus of the next sections of this chapter will be on emerging or re-emerging diseases considered by the authors to currently have a negative impact on aquaculture.

Flavobacterium spp.

Flavobacterium spp. are Gram-negative, rod-shaped, filamentous, and yellow-pigmented bacteria that are believed to be ubiquitous in freshwater environments (Bernardet and Bowman 2006). These bacterial species require specialized low-nutrient media for growth because they do not grow effectively on standard bacteriological media. Numerous formulations have been used in culture; some of the more common types include tryptone-yeast extract salts (TYES), Shieh or modified Shieh, Anacker and Ordal (also referred to as Cytophaga), and Hsu-Shotts (Cain and LaFrentz 2007). Two main bacterial species are responsible for most of the diseases affecting farm-raised fish: *F. columnare* and *F. psychrophilum*. However, new species are being identified and can also have negative impacts on fish and potentially aquaculture (Loch and Faisal 2013).

Flavobacterium psychrophilum is the causative agent of bacterial coldwater disease (CWD) (Borg 1960) or rainbow trout fry syndrome (RTFS), and is probably one of the most significant bacterial disease agents of trout and salmon in freshwater worldwide. *Flavobacterium psychrophilum* has a broad geographic distribution and all salmonid species, as well as some non-salmonid fish species, are believed to be susceptible (Starliper 2011). CWD typically occurs in young fish at low temperatures in the range 4–16 °C. Clinical signs of *F. psychrophilum* infections often depend on the size of fish affected and may vary between epizootics. In general, *F. psychrophilum* causes a septicemic infection that can be isolated from most organs of heavily infected fish. In alevins, the epithelial tissue covering the yolk sac may become eroded and, in some cases, the yolk sac may rupture. Common clinical signs in fry and fingerlings include yellow-pigmented lesions on the caudal peduncle, frayed and eroded fins, and dark coloration. If lesions appear on the caudal peduncle, necrosis may progress deep into the muscle tissue and expose the vertebrae. Although these are the classical clinical signs, fish may not exhibit any external lesions but instead may display general disease signs such as loss of appetite, lethargy, exophthalmia, and hanging at the water surface. Internally, petechia hemorrhaging may be visible on the pyloric caeca, adipose tissue, heart, swim bladder, and the peritoneal lining. The spleen of infected fish is commonly enlarged. Fish surviving an epizootic of CWD may exhibit spiral swimming and spinal compression types of deformities.

Preventative measures for CWD include the use of management strategies to reduce risk factors such as stress, poor water quality, and cutaneous lesions, since these factors tend to increase disease transmission. Removal of mortalities and morbid fish from rearing units is important to reduce the potential for bacterial shedding. Due to the ubiquitous

Table 1.1 Economically important bacterial pathogens of fish.

Disease	Bacteria	Fish affected	Distribution
Vibriosis	*Vibrio anguillarium* *Vibrio* spp.	Freshwater and marine	Worldwide
Coldwater vibriosis	*Vibrio salmonicida*	Salmonids	Worldwide
Wound disease	*Moritella viscosa*	Salmonids	Northern Europe
Furunculosis	*Aeromonas salmonicida salmonicida*	Salmonids	Worldwide
	Aeromonas salmonicida achromogens	Freshwater	
Enteric redmouth disease (ERM)	*Yersinia ruckeri*	Salmonids	Worldwide
Piscirickettsiosis	*Piscirickettsia salmonis*	Salmonids	Worldwide
Columnaris disease	*Flavobacterium columnare*	Freshwater	Worldwide
Coldwater disease/Rainbow trout fry syndrome (RTFS)	*Flavobacterium psychrophilum*	Salmonids	Worldwide
Bacterial kidney disease	*Renibacterium salmoninarum*	Salmonids	Worldwide
Enteric septicemia of catfish	*Edwardsiella ictaluri*	Catfish species	USA/Asia
Edwardsiellosis	*Edwardsiella tarda*	Freshwater and marine	Worldwide
Motile *Aeromonas* septicemia (MAS)	*Aeromonas hydrophila*, *A. caviae*, *A. sobria*	Freshwater	Worldwide
Pasteurellosis	*Photobacterium damsela piscida*	Marine	Worldwide
Streptococcosis	*Streptococcus iniae*, *S. agalactiae*, *S. dysgalactiae*, *S. phocae*	Freshwater and marine	Worldwide
Francisellosis	*Francisella noatunensis*, *F. asiatica*	Freshwater and marine	Worldwide
Tenacibaculosis	*Tenacibaculum maritimum*	Marine species	Worldwide

nature of *F. psychrophilum*, CWD can even occur with management strategies in place. External treatments have been used to reduce mortality associated with the bacteria, including bath administration of salt (sodium chloride) and potassium permanganate. However, due to the systemic nature of *F. psychrophilum* infections, CWD is commonly treated with antibiotics. In the United States, florfenicol (AQUAFLOR®) and oxytetracycline (Terramycin®) are approved for use in freshwater-reared salmonids to control mortality due to CWD. The development of an efficacious vaccine for CWD has been difficult and there are no commercially available vaccines at this time, largely due to the lack of consistent protection in fish immunized with killed whole-cell preparations using mass delivery methods. Recently, LaFrentz et al. (2008) developed an attenuated strain of *F. psychrophilum* that was able to confer protective immune responses to rainbow trout following immersion vaccination, making it a promising candidate for vaccine development. Such a strategy was further supported by Alvarez et al. (2008) and Lorenzen et al. (2010).

Flavobacterium columnare is the causative agent of columnaris disease (Bernardet et al. 1996). The bacterium is a significant pathogen of cultured fish species due to its worldwide distribution and its ability to infect most freshwater fish and cause disease over a wide range of temperatures (>15°C). Columnaris disease is responsible for large economic losses in aquaculture and is one of the leading causes of mortality in the channel catfish industry in the United States. All ages of fish are susceptible to columnaris disease, but the disease is more prevalent in young fish. In general, clinical signs of columnaris disease are easily recognized and include frayed fins, depigmented lesions on the skin, and necrotic gill lesions. Wet mounts of gill tissue or skin lesions from diseased fish will reveal long slender rods with gliding

movement, and the cells will aggregate into columns or 'haystacks' of cells (thus the name columnaris disease). Skin lesions often begin around the dorsal fin and then increase in size, eventually resulting in a gray to white lesion that has the appearance of a saddle. In some cases the margin of the lesion is yellow in appearance due to the proliferation of the yellow-pigmented bacterium. Gill tissue can exhibit severe necrosis and may appear white to brown and also yellowish due to the presence of large quantities of the bacterium. Internal pathology is rarely present. Although these are the classical clinical signs of disease, some diseased fish may exhibit no external lesions but instead will appear dark in color and lethargic.

As with the other *Flavobacterium* spp., preventative measures include the use of good management practices to provide proper environmental conditions for fish and reduce risk factors such as stress, poor water quality, and cutaneous lesions. Treatment for columnaris disease generally includes external bath treatments and/or antibiotics. External treatments that have been used include bath administration of salt, copper sulfate, potassium permanganate, hydrogen peroxide, chloramine-T, and quaternary ammonium compounds (i.e., Roccal®, Hyamine, Diquat). In the United States, hydrogen peroxide (35% PEROX-AID®) is approved for use in freshwater-reared coolwater finfish and channel catfish to control mortality due to external columnaris disease. Additionally, florfenicol (AQUAFLOR®-CA1) and oxytetracycline (Terramycin®) are approved for the control of columnaris disease in channel catfish and freshwater-reared rainbow trout, respectively. Two vaccines are available in the United States for the prevention of columnaris disease. One is a *F. columnare* bacterin (FryVacc1) (Bowker et al. 2012) approved for use in salmonids, and the other is a modified live vaccine (AQUAVAC-COL®) approved for use in channel catfish (Shoemaker et al. 2009, 2011).

Francisella spp.

Francisella spp. have probably caused disease in fish for a number of years; however, difficulty to culture on standard media likely lead to the under-reporting of these bacteria to date (Birkbek et al. 2011). *Francisella* spp. are aerobic, facultative intracellular Gram-negative coccobaccilli (Foley and Nieto 2010). The media required to culture *Francisella* spp. require cysteine and a source of iron from blood (Mikalsen and Colquhoun 2010) and/or supplemental hemoglobin (Soto et al. 2009). The two species commonly associated with disease in fish are *Francisella noatunensis noatunensis* and *F. noatunensis orientalis* (*F. asiactia*). *Francisella noatunensis noatunensis* is associated with disease of Atlantic cod (*Gadus morhua*; Nylund et al. 2006) and Atlantic salmon (*Salmo salar*; Birkbeck et al. 2007). *Francisella noatunensis orientalis* is associated with disease in tilapia (*Oreochromis* spp.), three-lined grunt (*Parapristipoma trilineatum*; Kamaishi et al. 2005; Hsieh et al. 2006; Birkbeck et al. 2007; Mikalsen and Colquhoun 2010), and hybrid striped bass *(Morone chrysops* x *M. saxitalis*; Ostland et al. 2006). Disease signs include lack of appetite and emaciation. The most notable internal signs are white–cream-colored nodules or granulomas present in the spleen, heart, kidney, and liver (Olsen et al. 2006; Mauel et al. 2007). Control strategies for *Francisella* include vaccination and antibiotics. Oral administration of florfenicol at the early stages of *Francisella* infection was effective at treating francisellosis in tilapia (Soto et al. 2010). Soto et al. (2011) produced an attenuated *F. asiatica* iglC (gene of the intracellular growth pathogenicity island that aids intracellular survival of *Francisella* sp. in macrophages) mutant that showed vaccine potential in laboratory trials. Due to the nature of *Francisella* spp. as intracellular pathogens, modified live vaccines will probably be needed to induce adequate protection in the field.

Aeromonas spp.

Motile *Aeromonas* septicemia (MAS) is usually an infectious disease of warmwater fish (channel catfish, cyprinids, eels, centrarchids, striped bass); however, trout and salmon can also be affected. The most common species isolated and characterized from fish are *A. hydrophila*, *A. sobria*, and *A. caviae* (Austin and Austin 2007). The bacteria are motile, cytochrome oxidase-positive Gram-negative rods that have the ability to ferment glucose and are resistant to vibriostat (0/129). Definitive identification requires a battery of tests (Plumb and Hansen 2010). Rimler–Shotts

selective media may aid in the identification by yielding orange-yellow colonies when incubated at 35°C (Shotts and Rimler 1973). Clinical signs include poor feeding response, lethargy, pale gills, exophthalmia, and hemorrhagic eyes. In scaleless fish, fins are often frayed and necrotic lesions develop. Internally, organs are friable and hyperemic, the liver may be mottled, ascites may be present, and intestine is generally void of food. Since 2009, *A. hydrophila* has emerged as a significant pathogen in the channel catfish industry in the United States. Between June and October of 2009, an estimated loss of more than 1.36 million kg of food-size channel catfish was reported in West Alabama alone (Hossain et al. 2013). Infections are often associated with stress including temperature shock, low dissolved oxygen (DO), high ammonia, handling or hauling, and co-infection. Control of MAS infections may rely on husbandry practices (e.g., maintain high DO levels and reduce parasite load), feeding antibiotics, and/or vaccination. At present, there are no commercially licensed vaccines for MAS in fish.

Streptococcus spp.

Streptococcal disease is caused by three main species of facultative anaerobic encapsulated Gram-positive streptococci. *Streptococcus iniae*, *S. agalactiae*, and *S. dysgalactiae* are responsible for disease in more than 30 species of freshwater, estuarine, and marine fish worldwide. The bacteria are typically grown on sheep blood agar and may or may not be beta-hemolytic. Other standard microbiological media – such as tryptic soy broth, brain heart infusion broth, or agar – may be employed to routinely culture the bacteria. The growth temperature is 28–30°C with a range of 10–45°C. Lancefield grouping is commonly employed to differentiate *Streptococcus* spp. (Lancefield 1933). *Streptococcus iniae* is not groupable, *S. agalactiae* is Group B (Vandamme et al. 1997), and *S. dysgalactiae* is group C (Nomoto et al. 2008). Disease signs typically include loss of appetite, dark skin pigmentation, erratic or C-shaped swimming, eye opacity, and exophthalmia. Eye opacity or cloudiness appears to be associated with chronic disease. Other disease agents also cause similar clinical signs; definitive diagnosis should therefore rely on culture and identification of the bacteria using biochemical (Facklam et al. 2005; Shoemaker et al. 2006) or molecular techniques such as PCR (Zlotkin et al. 1998; Berridge et al. 2001; Kawata et al. 2004; Mata et al. 2004). The significance of *S. iniae* as a fish pathogen was reviewed by Agnew and Barnes (2007); all three bacteria may be potential zoonotic agents and may therefore possess the ability to infect humans (Weinstein et al. 1997; Lau et al. 2006). Shoemaker et al. (2001) suggest the greatest zoonotic risk appears to be associated with older or immunocompromised people who incur a puncture wound while handling or preparing fresh, whole fish for cooking.

Control of streptococcal disease is via culture management, antimicrobial treatments, and vaccination strategies. Shoemaker et al. (2000) demonstrated that a reduction in fish density resulted in decreased mortality, and suggested this was a result of less fish-to-fish transmission. Feeding of medicated diets is practiced and reduces overt disease signs (Gaunt et al. 2010). However, disease often reoccurs upon discontinuing of feeding. Various vaccination strategies using killed and attenuated vaccine candidates have been practiced (Eldar et al. 1997; Klesius et al. 2000; Evans et al. 2004; Buchanan et al. 2005; Locke et al. 2010; Shoemaker et al. 2010). The strategies have demonstrated effectiveness in the laboratory, and success has also been documented in the field. Work in Israel and Australia has demonstrated the emergence of new serotypes after continued use of autogenous killed vaccines in the field (Bachrach et al. 2001; Agnew and Barnes 2007). Martins et al. (2011) demonstrated that coinfection with parasites led to decreased vaccine performance in tilapia, thereby verifying in part the reported ineffectiveness of vaccines used on commercial farms in the United States.

Viral Pathogens of Fish

Viruses are submicroscopic particles (i.e., 18–300 nm by electron microscopy) that require a host cell to replicate. Most individual virus particles (virion) consist of a single type of nucleic acid, either DNA or RNA (not both), contained within a protein shell or coat (capsid) that may or may not be enveloped (Smail and Munro 1989). Viral infections are typically confirmed following isolation of the virus in tissue culture (Plumb and Hanson 2010). Cytopathic effect

(CPE) or cell injury is often unique to each virus type and may be indicative of the type or group of virus. Following isolation in cell culture, viruses can be characterized by electron microscopy and/or serum neutralization with antiserum specific to the virus. New technologies for virus identification include serological tests (IFAT and ELISA) and nucleic acid probes (e.g., PCR and RT-PCR).

Disease signs in fish infected with viruses may range from exophthalmia, hemorrhagic lesions, distended fluid-filled abdomens, pigment changes, and lethargy to no signs and/or rapid death. Viral pathogens may also result in tumor-like growths both internally and externally. An important consideration for viral pathogens is the limited treatment options available due to the requirement for a living cell for replication. Effective biosecurity measures are prudent to prevent pathogen introduction and spread (Lee and O'Bryen 2003). Effective vaccines have been developed and used for some fish viral agents, and one of the first DNA vaccines approved and commercially available in Canada was developed for use against infectious hematopoietic necrosis virus (Salonius et al. 2007). Another equally effective DNA vaccine has been developed against viral hemorrhagic septicemia virus, but it is not yet commercially available (Lorenzen et al. 2001).

Viral pathogens that are of economic importance to cultured fish are listed in Table 1.2. Numerous available books discuss in detail the viral agents, disease signs, epizootiology, pathology, diagnosis, and control of the viruses that cause loss in cultured fish (Smail and Munro 1989; Plumb and Hansen 2010; Woo and Bruno 2011). The main focus of this section is emerging or re-emerging viral diseases affecting fish that are significant or potentially significant for aquaculture species.

Infectious Salmon Anemia Virus

Infectious salmon anemia (ISA) is a relatively new viral disease caused by infectious salmon anemia virus, which has a virion around 100 nm in size and is of the orthomyxoviridae family. The viral genome is composed of eight single-strand RNA segments (Cottet et al. 2011). The disease was first seen in Norway in the late 1980s and has caused devastating losses in Atlantic salmon (*Salmo salar*) culture in Scotland, Chile, North America, and the Faroe Islands (Miller and Cipriano 2003). Clinical signs of ISA usually appear about 2 weeks post-infection and include severe anemia, swelling and hemorrhaging in the kidney and other organs, pale gills, protruding eyes, darkening of the posterior gut, fluid in the body cavity, and lethargy. Hematocrits can be reduced from around 35–48% to 12–25% (Dannevig et al. 1993), indicative of anemia. The spread of ISA virus may occur as a result of the purchase of infected smolts that did not exhibit clinical disease, farm-to-farm transfer, or from fish processing plants or industries where organic material (especially blood and processing water) from ISAV-infected fish is discharged without treatment (Bruno et al. 1995). Both wild and cultured Atlantic salmon are susceptible to infection. ISA virus also infects brown trout in the marine environment but apparently does not cause disease; instead, it may serve as a reservoir for infection (Nylund and Jakobsen 1995). Extreme strategies including eradication have been employed to control this viral disease; however, there is some evidence that other fish species may carry the virus and can transmit it to susceptible fish. ISA is a World Organization for Animal Health Office International des Épizooties (OIE) reportable disease (OIE 2010a).

Spring Viremia of Carp Virus

Spring viremia of carp virus (SVCV) is caused by *Rhabdovirus carpio*, a bullet-shaped RNA virus around 70 × 180 nm in the *Lyssavirus* genus of the family Rhabdoviridae. SVCV infects a broad range of fish species and causes high mortality in susceptible hosts in cold water (12–17°C; Ahne et al. 2002). Infections have occurred in common and koi carp (*Cyprinus carpio*); grass carp (*Crenopharyngodon idella*); silver carp (*Hypophthalmichthys molitrix*); bighead carp (*Aristichthys nobilis*); cruian carp (*Carassius carassius*); goldfish (*C. auratus*); roach (*Rutilus rutilus*); ide (*Leuciscus idus*); tench (*Tinca tinca*); and sheatfish (*Silurus glanis*) (Plumb and Hansen 2010). Long indigenous to Europe, the Middle East, and Asia, the disease was reported recently in North and South America. In the spring of 2002, SVCV was isolated from koi carp farmed in North Carolina (Goodwin 2002). In the same year the virus was also reported from carp in lakes and rivers in

Table 1.2 Economically important viral diseases of fish. World Organization for Animal Health, Office International des Épizooties (OIE) reportable viruses are marked with an asterisk (*).

Disease	Virus (type)	Fish affected	Distribution
Infectious hematopoietic necrosis*	Infectious hematopoietic necrosis virus (rhabdovirus)	Salmonids	Worldwide
Infectious pancreatic necrosis	Infectious pancreatic necrosis virus (birnavirus)	Salmonids, marine species	Worldwide
Infectious salmon anemia*	Infectious salmon anemia virus (orthomyxovirus)	Atlantic salmon	Worldwide
Pancreas disease	Pancreas disease virus (togavirus)	Salmonids	Europe
Viral hemorrhagic septicemia*	Viral hemorrhagic septicemia virus (rhabdovirus)	Freshwater and marine	Worldwide
Spring viremia of carp*	Spring viremia of carp virus (rhabdovirus)	Carps	Worldwide
Koi herpes*	Koi herpes virus (herpesvirus)	Carps	Worldwide
Channel catfish virus disease	Channel catfish virus (herpesvirus)	Channel catfish	USA, Asia
Viral nervous necrosis	Viral nervous necrosis virus (betanodavirus)	Marine species	Worldwide
Iridovirus disease*	Red sea bream (iridovirus)	Marine species	Asia
Epizootic hematopoietic necrosis*	Epizootic hematopoietic necrosis virus (ranavirus)	Redfin perch, rainbow trout	Australia

Wisconsin and the Mississippi River. SVCV causes impairment of the salt–water balance in fish, resulting in edema and hemorrhages. Liver, kidney, spleen, gill, and brain are the primary organs containing the virus during infection. Horizontal transmission most likely occurs when waterborne virus enters via the gills, whereas vertical transmission may be possible as adult carp shed virus during spawning (Bekesi and Csontos 1985). Reservoirs of SVCV are infected fish and carriers from either cultured, feral, or wild fish populations (Goodwin et al. 2004). This virus may remain infective for long periods of time in water or mud. Once the virus is established in a pond or farm, it may be difficult to eradicate without destruction of all fish at the farm. SVC is an OIE (OIE 2010b) reportable disease. Recommendations for preventing the disease and its spread include the use of a water source free of virus, disinfection of eggs and equipment, and proper disposal of dead fish (Schlotfeldt and Alderman 1995). The OIE (2010) Manual for Aquatic Animal Disease has specifications for surveillance programs to achieve and maintain the biosecure status of aquaculture facilities.

Viral Hemorrhagic Septicemia Virus

On a worldwide scale, viral hemorrhagic septicemia (VHS) is probably the most significant disease. VHS is caused by an enveloped bullet-shaped rhabdovirus in the *Novirhabdovirus* genus of the family Rhabdoviridae that is about 65 nm in diameter and 180 nm long, called viral hemorrhagic septicemia virus (VHSV; Zwillenberg et al. 1965). The genome of VHSV is single-stranded RNA. VHSV has been genetically grouped into four genotypes (I, II, III, and IV) based on the N and G genes (Einer-Jensen et al. 2006; Nishizawa et al. 2006). Genotype I is typically associated with freshwater farmed trout in Europe (Al-Hussinee et al. 2011). Genotype II and III are associated with marine fish from the Baltic Sea and North Atlantic Ocean (Nishizawa et al. 2006). Genotype IV was reported from marine fish in the Pacific Northwest, Japan, and Korea (Hedrick et al. 2003). In 2005, VHSV was isolated and characterized from freshwater fish in the Great Lakes Basin of North America (Elsayed et al. 2006). Interestingly, the viral isolate was genetically related to VHSV genotype IV, but distinct enough to be considered genotype IVb

with the other viral isolate from the marine fish designated IVa (Al-Hussinee et al. 2011). Clinical signs of VHS include severe hemorrhaging in the musculature and internal organs of the fish; however, definitive diagnosis relies on isolation of the virus in tissue culture and subsequent viral neutralization and/or molecular confirmation via RT-PCR (OIE 2010c). VHS has historically been responsible for severe fish losses. The recent identification of VHSV in freshwater in the United States is significant because at least 28 fish species are reported to be susceptible. Due to VHSV being a reportable virus, the national and international trade of baitfish and cultured fish could be severely restricted. USDA-APHIS has outlined a number of regulatory requirements that must be followed if VHSV is suspected (Bowser 2009).

Koi Herpes Virus

Koi herpes virus (KHV) disease is an important viral disease of koi and cultured carps worldwide. The causative virus is koi herpes virus or cyprinid herpes virus 3 (Hedrick et al. 2000). The virus is an enveloped icosahedron about 200 nm in size with a DNA genome (Hedrick et al. 2000; Miyazaki et al. 2008). Hedrick et al. (2000) first reported the disease in Israel and the Unites States in both carp and koi (ornamental carp). Since the first description it has been detected in many countries, most likely as a result of the live koi trade (Haenen and Hedrick 2006). Mortality events can be significant with 80–100% death rate occurring (Haenen et al. 2004). Clinical signs include loss of equilibrium and erratic swimming, gill discoloration, and necrosis (Sano et al. 2011). Other signs include anorexia, exophthalmia, fin erosion, hemorrhage on skin and base of fins, and patchy-appearing skin related to mucus production. The disease is an OIE reportable disease (OIE 2011d). Diagnostic methods rely on tissue culture (i.e., virus isolation), viral antigen detection (IFAT), and PCR methods (Bercovier et al. 2005; Yuasa et al. 2005). The PCR assay to detect KHV genomic DNA followed by sequencing the PCR product is considered to be the best method for virus detection currently available (OIE 2010d). A newly licensed vaccine (Cavoy™-Norvartis Animal Health) against KHV (Cyprinid herpes virus-3) is now available through veterinarians for prophylactic management of KHV disease.

Iridovirus Diseases

Epizootic hematopoietic necrosis disease (EHND) and red sea bream iridoviral disease (RSIVD) are caused by viruses in the Iridoviridae family (Whittington et al. 2010; Sano et al. 2011). Iridoviruses possess icosahedral virions about 120–200 nm in size with a single linear double-stranded DNA genome (Chinchar et al. 2005). Epizootic hematopoietic necrosis virus is in the genus *Ranavirus* and affects mainly wild redfin perch (*Perca fluviatilis*) and cultured rainbow trout in Australia (Langdon et al. 1986; Whittington et al. 1999). Red sea bream (*Pagrus major*) iridovirus is in genus *Megalocytivirus* and mainly affects high-value marine aquacultured fish in Asia. EHND and RSIVD are both OIE reportable diseases and have been shown to infect a number of economically important freshwater (EHND) and marine fish (RSIVD) experimentally. Diagnostic methods for detection of EHND rely on tissue culture and subsequent immunological analysis or PCR with DNA sequencing (Marsh et al. 2002; OIE 2010e;). Detection of RSIV is also based on immunological analysis and PCR methods from fish tissue, as viral isolation in tissue culture is difficult (OIE 2010f).

Parasitic Diseases

Parasites may be microscopic or macroscopic in size and need a fish host for survival and/or to complete their life cycle. Parasites in general gain some benefit (e.g., nutrients) from the fish host. Most fish parasites may cause mechanical injuries to the gill, skin, or internal organs. Some parasites may inhibit the function of vital organs in fish, such as digenetic trematodes and tapeworms. Parasites may secrete harmful substances and cause toxic effects (Sindermann 1990; Zhang et al. 1999). The mechanical injury caused by the parasite provides a portal of entry for pathogenic microorganism, and may enhance fish susceptibility to bacterial diseases (Sindermann 1990; Xu et al. 2007; Martins et al. 2011).

Protozoan Diseases

Diseases caused by protozoans are among the most significant of all parasitic diseases. Cultured fish losses due to parasites are mainly caused by protozoan

parasites (Rogers 1985). In freshwater fish, white spot disease caused by *Ichthyophthirius multifiliis* (Ich), whirling disease, and proliferative gill disease caused by Myxozoan protozoan are some of the severe parasitic diseases that often lead to significant losses of cultured fish. In marine fish, marine white spot disease (caused by *Cryptocaryon irritans*) and marine velvet (caused by the dinoflagellate, *Amyloodinium ocellatum*) both lead to serious mortalities.

Ciliate Protozoans

The protozoan parasite *Ichthyophthirius multifiliis* (Ich) infects virtually all freshwater fish and causes damage to their gills and skin. Epizootics have been reported worldwide and result in severe economic losses for aquaculture producers. The life cycle of the parasite includes three stages: an infective theront, a parasitic trophont, and a reproductive tomont. The parasitic trophont lives completely within the host and feeds on damaged cells and body fluids of fish. The movement of theront and trophont (penetration, rotation, and relocation) cause severe tissue damage (Xu et al. 2000). The parasite feeds on host cells until it is mature; the mature trophont then drops off the host, attaches to substrates, and undergoes multiple divisions to produce 512–1024 infective theronts in less than 24 hours. At optimum temperature (22–24°C), Ich can reproduce rapidly and cause high fish mortality within a short period of time (Matthews 2005; Dickerson 2006). *Cryptocaryon irritans* is a ciliate protozoan that parasitizes marine fish and is one of the most common causes of disease in marine aquaria. The symptoms and life cycle are generally similar to those of *Ichthyophthirius* in freshwater fish, including white spots, so the disease is also known as marine white spot disease. *Cryptocaryon* requires a much longer time for tomont division than in *Ichthyophthirius*. *Cryptocaryon* tomonts take 8 days at 25°C and longer at lower temperatures to divide and produce infective theronts (Dickerson 2006).

Other commonly reported ciliated protozoans are listed in Table 1.3. Trichodinid species that infect freshwater and marine fish include *Trichodina*, *Trichodinella*, and *Tripartiella* species. Most of the clinically important species infect fish skin and/or gills (Shoemaker et al. 2006). Parasitism of the gills is more detrimental than of the body. Signs of disease include respiratory distress, loss of appetite, depigmentation, or loss of scales. *Chilodonella* is a leaf or heart-shaped ciliate that has distinct parallel rows of cilia along the body margin. *Chilodonella* infects the skin, fins, and gills of freshwater fish. A heavy infection can cause detached scales, necrosis of branchial epithelium, and mass mortalities. *Apiosoma* and *Ambiphyra* are similar ciliates; both have a barrel-shaped body with a ring of cilia. The major difference is the shape of macro-nucleus, with the rounded nucleus in *Apiosoma* and the ribbon-shaped nucleus in *Ambiphyra*. These protozoans have a direct life cycle and reproduce by binary fission on the skin and gills. *Epistylis* attaches to fish with its stalk and commonly forms branched colonies. A severe infection causes erosion of skin, scales, and spines and bloody lesions, thus the common name is "red sore disease." Most of these parasites may be treated with salt, formalin, or other approved parasiticides; however, it may take multiple treatments to effectively control these ciliates.

Flagellated Protozoans

Marine velvet is caused by the dinoflagellate *Amyloodinium ocellatum* and leads to serious mortality in brackish and marine warmwater fish at aquaculture facilities worldwide (Noga and Levy 2006). Outbreaks occur rapidly and may result in 100% mortality within a few days. The total life cycle for this parasite approximates 3 weeks and is very similar to *Cryptocaryon irritans*. It has a free-swimming infective dinospore stage, a parasitic trophont stage that feeds on fish skin and gill epithelium, and an encysted tomont stage that divides to produce infective dinospores (Noga and Levy 2006). Dinoflagellate *Piscinoodinium* is similar to *Amyloodinium* in morphology and causes freshwater velvet in tropical freshwater fish (Noga and Levy 2006). *Ichthyobodo necatrix* (*Costia*) is an important flagellated parasite of freshwater fish. Transmission of *Ichthyobodo* is by direct fish-to-fish contact. Common signs indicating the presence of this parasite are respiratory distress and flashing. *Ichthyobodo* can cause severe losses in a short time if not treated. *Hexamita* is flagellate protozoan found in the gastrointestinal tracks of many freshwater and marine fish in the world. Among them, *Hexamita salmonis* may be present wherever trout or salmon are reared. The disease is commonly found in fingerlings and outbreaks are usually sporadic in aquaculture facilities (Woo 2006). Effective treatments for *Ichthyobodo*

Table 1.3 Economically important parasitic and fungal diseases of fish. World Organization for Animal Health, Office International des Épizooties (OIE) reportable parasitic and fungal diseases are marked with an asterisk (*).

Disease	Parasite	Fish affected	Distribution
"White spot" disease	Ciliated protozoan: *Ichthyophthirius multifiliis*	Freshwater fish	Worldwide
Marine white spot disease	Ciliated protozoan: *Cryptocaryon irritans*	Marine fish	Worldwide
Trichodinosis	Ciliated protozoan: *Trichodina* spp., *Trichodonella* spp., *Tripartiella* spp.	Freshwater fish	Worldwide
Trichophrya infestation	Ciliated protozoan: *Trichophrya* spp.	Freshwater fish	Worldwide
Ambiphyra and Apiosoma infestation	Ciliated protozoan: Ambiphyra spp., Apiosoma spp.	Freshwater fish	Worldwide
Chilodonella infestation	Ciliated protozoan: *Chilodonella* spp.	Freshwater fish	Worldwide
Epistylis infestation	Ciliated protozoan: *Epistylis* spp.	Freshwater fish	Worldwide
Dinoflagellate infestation	Flagellated protozoan: *Amyloodinium* spp., *Piscinoodinium* spp., *Crepidoodinium* spp., *Ichthyodinium* spp.	Marine, brackish, and freshwater fish	Worldwide
Hexamitosis and Sprironucleosis	Flagellated protozoan: *Hexamita* spp., *Spironucleus* spp.	Marine and freshwater fish	Worldwide
Ichthyobodosis	Flagellated protozoan: *Ichthyobodo* spp. (*Costia* spp.)	Freshwater fish	Worldwide
Whirling disease	Myxozoan protozoan: *Myxobolus cerebralis*	Salmonids	USA, Europe Asia
Proliferative gill disease	Myxozoan protozoan: *Aurantiactinomyxon ictaluri*	Channel catfish	USA
Proliferative kidney disease	Myxozoan protozoan: *Tetracapsula renicola* n. sp	Salmonids	Europe, N. America
Henneguyiasis	Myxozoan protozoan: *Henneguya* spp.	Freshwater fish	Worldwide
Gill flukes	Mongenetic trematodes: *Dactylogyrus* spp.	Freshwater and marine fish	Worldwide
Gyrodactylus	Mongenetic trematodes: *Gyrodactylus* sp., *Gyrodactylus salaris**	Freshwater and marine fish	Worldwide
Trematode *Bolbophorus*	Digenetic trematodes: *Bolbophorus* spp.	Freshwater fish	USA
White grub and Yellow grub	Digenetic trematodes: *Posthodiplostomum* spp., *Clinostomum* spp.	Freshwater fish	Worldwide
Asian tapeworm infestation	*Bothriocephalus acheilognathi*	Freshwater fish	Worldwide
Thorny-headed worm infection	*Acanthocephalus* spp.	Freshwater fish	Worldwide
Red worm	Nematode: *Camallanus* spp.	Freshwater fish	Worldwide
Fish louse	Crustacean parasite: *Argulus* spp.	Freshwater fish	Worldwide

Table 1.3 (*Continued*)

Disease	Parasite	Fish affected	Distribution
Copepod ectoparasite	Copepod ectoparasite: *Ergasilus* spp.	Freshwater fish	Worldwide
Anchor parasite	Crustacean parasite: *Lernaea* and *Lernaeocera* spp.	Freshwater and marine fish	Worldwide
Sea lice	*Lepeophtheirus salmonis* and *Caligus elongatus*	Marine fish	Worldwide
Water molds	Fungi: *Saprolegnia* spp., *Achlya* spp.	Fresh, brackish water fish	Worldwide
Epizootic ulcerative syndrome*	Fungi: *Aphanomyces invadans* or *A. piscicida*	Fresh, brackish water fish	Asia–Pacific region
Branchiomycosis or "gill rot"	Fungi: Branchiomyces spp.	Freshwater fish	Europe, Asia

include copper sulfate and formalin. Management practices, such as quarantine to avoid transmission to un-infected fish, should be practiced.

Myxozoan Protozoan

Whirling disease is a severe parasitic disease that was first described in rainbow trout in Germany in 1893 (Bartholomew and Reno 2002). Since then, whirling disease caused by *Myobolus cerebralis* has been reported in 26 different countries, including some African and European countries (i.e., Russia), the United States, and other countries. Whirling disease in the United States was first reported in Pennsylvania in 1956 (Hoffman 1990; Bartholomew and Reno 2002). Presently, whirling disease has occurred in 22 states located in the eastern and western United States. The spread of the whirling disease parasite has been attributed to transfer of live fish (Hoffman 1990; Bartholomew and Reno 2002).

Myxobolus cerebralis has a two-host life cycle that involves fish and an alternate host: a common bottom-dwelling tubifex worm. *Myxobolus cerebralis* mainly infects farmed salmonid fish, but is also found in wild fish populations. When an infected fish dies, large numbers of spores are released into the water and become myxospores. The myxospores are then ingested by the tubifex worm and develop into infective triactinomyxon (TAM). Fish can be infected by TAM attaching to fish bodies and/or by fish eating infected tubifex worms. Whirling disease affects juvenile fish and causes fish skeletal deformation and neurological damage. The parasitized fish show "whirling" swimming behavior instead of normal swimming, have difficulty feeding, and are more vulnerable to predators. The mortality rate for fingerlings can reach 90% or higher in the infected populations (Hoffman 1990; Bartholomew and Reno 2002).

Management strategies to prevent and control whirling disease include control of the worm host and its habitats, disinfection of water containing triactinomyxons spores, stocking larger fish into infected waters, eliminating infected fish, enforcement of disease regulation, and stocking less-susceptible species or strains of fish (Wagner 2002).

Proliferative kidney disease (PKD) is an economically important disease of salmonids in Europe and North America. PKD of salmonid fishes is caused by *Tetracapsuloides bryosalmonae*, a myxozoan parasite of salmonid fishes. *T. bryosalmonae* uses a bryozoan as an alternate host, rather than an oligochaete or polychaete worm. Five bryozoan species belonging to the genera *Fredericella* and *Plumatella* have been found to develop infection with *T. bryosalmonae*, resulting in the development of spherical sacs that release spores (Anderson et al. 1999). These spores are released into the surrounding water where they can infect salmonid fish. PKD primarily occurs during summer (April–June), and mortalities may range from 10% to 95% in infected populations (Noga 1996). *T. bryosalmonae* affects mainly the kidney and spleen but can become systemic in most susceptible fish hosts. Other diseases caused by myxozoan parasites include ceratomyxosis, which is a disease of trout and salmon caused by *Ceratomyxa shasta*. Ceratomyxosis has caused disease and deaths in both juvenile and adult hatchery and wild salmonids. Aside from

disinfection and quarantine, there are no good control methods for myxozoan infections. Myxozoan spores are long-lived with some surviving for well over 1 year, so disinfection is mandatory for eradication (Noga 1996).

Monogenetic Trematodes

Among monogenetic trematodes in fish, *Gyrodactylus* spp. and *Dactylogyrus* spp. are the most common. *Dactylogyrus* sp. is often found on the gills, whereas *Gyrodactylus* is found either on the gill or skin of freshwater fish. Monogenetic trematodes use haptors at the posterior end to attach to fish. They have large centrally located anchors and hooks around the margin of the haptor. Anchors and hooks of monogenetic trematodes penetrate into the surface layer of skin, fins, and gills, causing tissue damage. These worms move on the body surface and feed on dermal and gill debris (Post 1983). Monogenetic trematodes are hermaphroditic and contain both male and female reproductive organs. The main difference between the two is that *Gyrodactylus* is viviparous (produce live offspring) and *Dactylogyrus* is oviparous (produce eggs). One of the species that is of concern to salmon fisheries and fish farms is *Gyrodactylus salaris*, which has been responsible for heavy losses of Atlantic salmon in European countries (Buchmann and Bresciani 2006). *Gyrodactylus salaris* is the only parasitic disease at present that is OIE reportable (OIE 2010g). Other monogenetic trematodes are usually not a severe problem unless they increase to high numbers. In heavy gill infections, fish become lethargic, swim near the surface while experiencing partial suffocation, and do not feed (Post 1987). With large numbers of monogenetic trematodes on the skin, fish show excessive mucus, rubbing against sides of holding tanks and occasionally jumping out of water. Formalin, potassium permanganate, and copper sulfate may be used as prolonged treatments for monogenetic trematodes. Recent work has demonstrated the importance of these pathogens in increasing the susceptibility of infected fish to bacterial infections (Xu et al. 2007).

Digenetic Trematodes

There are many digenetic trematodes that infect both freshwater and marine fish. Digenetic trematodes require more than one host to complete their life cycle. Fish may serve as final hosts or intermediate hosts in their life cycle. Very few adult-stage digeneans can cause major damage to the fish host. Metacercarial (larval) infection is the main source of mechanic damage in fish, which leads to economic loss (Paperna and Dzikowski 2006). In recent years, a freshwater digenetic trematode, *Bolbophorus damnificus* or *confusus*, has caused problems in commercially raised channel catfish in Louisiana, Mississippi, and Arkansas (Terhune et al. 2003; Flowers et al. 2005). The trematode is vectored by pelicans (*Pelecanus* spp.), the final host, and snails (*Planorbella trivolvus*), the first intermediate host. *Bolbophorus* infections have caused high mortality and decreased production in channel catfish (Terhune et al. 2003). Other digenetic trematodes, such as white grubs (*Posthodiplostomum* spp.) and yellow grubs (*Clinostomum* spp.), occur in wide-ranging fish populations. These digenetic trematodes usually cause minimal effects on growth, reproduction, and survival of fish (Post 1987) unless fish are heavily infected. Methods employed to keep pelicans (aquatic birds) away from aquaculture facilities and reduce snail populations (Hoffman 1999) are helpful in controlling digenetic trematodes.

Tapeworm, Acanthocephalons, and Nematodes Infection

Asian tapeworm is one of the most serious cestodes that affects fish. The body of a tapeworm is ribbon-shaped and divided into short segments. Asian tapeworm has an unusually wide range of hosts including various carp species, minnows, golden shiner, catfish, and many other aquarium fish. It can cause up to 90% mortality in grass carp and juvenile common carp. The adult tapeworms live in the intestines of many species of fish. Eggs of this tapeworm pass out of the fish with its feces and into water. After hatching, the larvae are eaten by crustaceans (copepods); fish are then infected when they eat the copepods.

Acanthocephalan comprises worms with an anterior proboscis covered with many hooks (Thorny-headed worm). These acanthocephalans are widely distributed in various fish species and almost all are endoparasites in the digestive tract of fish. Fish can serve as final hosts for those acanthocephalans, which become

sexually mature while in host fish. Fish can also serve as intermediate hosts for acanthocephalans, which spend their adulthood in marine mammals (Post 1987). Acanthocephalan epizootics are rare in cultured fish and these parasites are not usually regarded as economically important pathogens of fish.

There are many parasitic nematodes in fishes. These nematodes infect freshwater, marine, and brackish water fish species. Fish nematodes usually have a life cycle involving one or two intermediated hosts (Yanong 2002). Most nematodes infect fish as adults, but some occur as larval stages (Molnár et al. 2006). Adult nematodes usually occur in the intestine while larval stages can be found in almost any part of the fish, including the body cavity, internal organs, external muscle layers, and deeper layers of the skin or fins (Yanong 2002). Disease related to nematodes is surprisingly rare among fish. In fact, a fish may live a relatively normal life with hundreds of nematodes in various organs and body tissues (Post 1987). However, fish may show mortality when they are heavily infected, especially juveniles. Juvenile fish infected by nematodes are more likely to show signs of illness and also have reduced growth rates (Yanong 2002).

Cleaning and sterilizing ponds is an effective way of reducing the numbers of the intermediate hosts of some helminthes (Noga 1996; Yanong 2002). Control strategies for helminthes are generally aimed at disrupting the life cycle and eradicating the helminthe intermediate hosts (Noga 1996). Quarantine and restricting the movement of infected fish will prevent the spread of helminthes and reduce infection loads. Live foods, such as oligochaete worms (e.g., tubifex worms), may act as carriers, and fish should not be fed live feeds if possible (Noga 1996; Yanong 2002).

Crustacean Parasites

There are many crustacean parasites that can infect freshwater and marine fish. Crustacean parasites possess attachment organs that are deeply embedded in the host's tissue. Some species that move freely on the surface of the fish can rupture the protective skin, destroy the mucus cover, and open wounds for subsequent bacterial infections. Anchor worms (*Lernaea* spp.), fish louse (*Argulus* spp.), and copepod ectoparasite (*Ergasilus* spp.) are some commonly seen crustacean parasites in freshwater fish. A more severe crustacean parasite affecting marine cultured fish is sea lice, which has caused heavy losses in salmon culture farms in European countries. *Lepeophtheirus salmonis* and *Caligus elongatus* are the most important species affecting farmed fish (Lester and Harward 2006). Other species of parasitic copepods are also becoming a problem as finfish aquaculture expands worldwide and new species of fish are being cultured.

Fungal Diseases

Saprolegniasis

Saprolegniasis, commonly called "water mold," is a fungal disease of fish and fish eggs. Water molds are caused by aquatic fungi, primarily *Saprolegnia* spp., *Achlya* spp., and *Aphanomyces* spp. These fungi are common in fresh or brackish water and affect all species and ages of freshwater and estuarine fish. The likelihood of infection by fungi is increased when fish are injured physically or infected by parasites. Saprolegniasis also commonly occurs when the water temperature drops below 15°C, and often after a cold front rapidly reduces the water temperature (Noga 1996). Infected fish show a gray or whitish growth in and on the skin and/or fins. Eventually, these growths look like cotton. Once fungus is established on fish, it may grow and spread to healthy tissue and eventually kill its host. Epizootic ulcerative syndrome (EUS) is one of the most destructive diseases of fresh- and brackish-water farmed and wild fish in the Asia–Pacific region (an OIE reportable disease). The EUS is caused by the oomycete pathogen, *Aphanomyces invadans* (also called *A. piscicida*), a non-septate, broad, sparsely branching type of fungus (Vishwanath et al. 1998). The invading fungus causes significant necrotic changes in the skin and muscle tissue, produces granulomas, and ultimately results in the formation of dermal ulcers. If EUS outbreaks occur in small or closed water bodies, liming of water, improvement of water quality, and removal of infected fish are helpful in reducing fish mortality (OIE 2010h).

Branchiomycosis

Branchiomycosis or "Gill rot" is caused by the fungi *Branchiomyces sanguinis* and *Branchiomyces demigrans* in freshwater fish in Europe and Asia (Noga

1996). Infections are located primarily in the blood vessels (intravascular) of the gill, and are confined to the gill arches and the base of the primary lamellae. The pathogen destroys the branchiate membranes and gills (Post 1987). Diseased fish refuse food, congregate near the edge of rearing units, and rise to the surface of the water. The disease usually occurs in the summer and results in high mortality of fish (Post 1987). Prevention of all fungal infections can be accomplished by good management such as maintaining good water quality, removing dead fish, and preventing the accumulation of decomposing organic matter (Post 1987).

Conclusion

This chapter provided a brief overview of the fish immune system and of the emerging or re-emerging bacterial, viral, parasitic, and fungal diseases considered by the authors to negatively impact aquaculture. Maintaining good water quality, minimizing stress, and providing adequate nutrition are major factors impacting the immune system of fish, and ultimately the innate and acquired immune responses to pathogens. The knowledge presented in this chapter provides basic information to enable the understanding of specific examples that are presented throughout the remainder of this book.

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Chapter 2

Protein, Amino Acids, and Ingredients

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Introduction

Proteins are the most abundant substances in living organisms and cells. All proteins are made up of the 20 different amino acids that are linked together by covalent bonds (peptide bonds). Shorter chains of two or more amino acids can be linked by covalent bonds to form polypeptides. The 20 amino acids are: alanine, arginine, asparagine, aspartic acid, cysteine (cystine), glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Only glycine does not have an asymmetric carbon atom; the alpha carbon of all other amino acids have four different groups comprising a carboxyl group, an amino group, a hydrogen atom, and an R-group. Amino acids can be classified based on the polarity of their different R-groups. From amino acids, organisms can synthesize a wide array of biologically important products such as enzymes, hormones, feathers, scales, and antibodies. Proteins differ from one another due to their own unique sequence of amino acid units.

Since there are many different proteins in an organism, they have many different biological functions. Enzymes are highly specialized proteins that are responsible for catalytic activity. Transport proteins found in blood plasma bind/carry specific molecules or ions from one organ to another; hemoglobin is one such protein that carries oxygen to tissues in an organism. Nutrient proteins are exemplified by casein; storage proteins are found in the seeds of plants; contractile proteins allow an organism to change shape, locomote, or move, and are represented by actin and myosin found in skeletal muscle; motile proteins, such as tubulin, are components of cilia and flagella which allow for movement; structural proteins serve to strengthen biological tissues and cells and are represented by collagen, elastin, and keratin; regulatory proteins assist in cellular or physiological activities and are represented by hormones; and proteins can serve to protect the organism against infection or injury and are represented by immunoglobulins.

Generally, fish fed diets with increased protein levels have higher final weights than those fed lower percentages of protein until an optimal protein level is reached, after which final weight either remains the same or declines (Fig. 2.1). There are a number of factors that affect the dietary protein requirement of fish and crustaceans. The size of the organism is one factor, as smaller animals require higher-protein diets. Protein quality is another factor, as diets formulated with high-quality, highly digestible ingredients allow for a lower protein level than diets comprising less-digestible ingredients. Feeding rate is a third factor that affects protein requirement, as animals that are fed to satiation require lower dietary protein than those fed a restricted ration. Presence of natural

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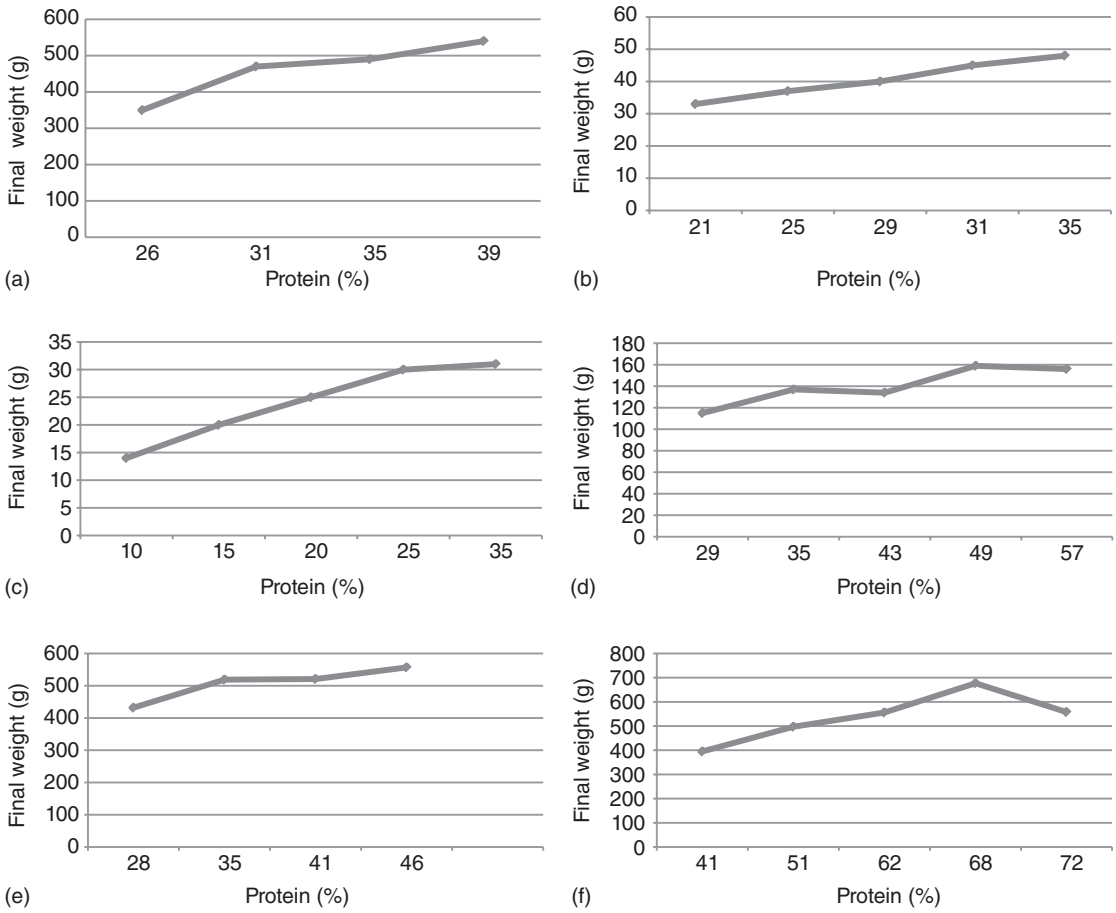

Figure 2.1 Effect of different protein levels on final weight of several fish species. (a) Channel catfish (data adapted from Reis et al. 1989); (b) golden shiner (data adapted from Lochmann and Phillips 1994); (c) rainbow trout (data adapted from Kim et al. 1991); (d) turbot (data adapted from Lee et al. 2003); (e) sunshine bass (data adapted from Webster et al. 1995); and (f) halibut (data adapted from Aksnes et al. 1996).

foods, water quality (temperature, dissolved oxygen, etc.), and dietary energy also affects the protein requirements of fish and crustaceans.

Immunity is the ability of an organism to resist attack from a pathogen. Generally, fish have an immune system that has shorter response times, is less specific, has weaker recognition memory, and has a smaller immunoglobulin array than humans. Further, the mucus of fish has a more prominent response in defending the organism from pathogens. While fish have humoral response and cellular responses, as do mammals, shrimp do not have a specific immune response and instead rely on a rudimentary system that includes phagocytosis and the use of lectins.

Fish have three levels of defense. The first line consists of physical barriers such as scales, skin, mucus, stomach acid (in species with true stomachs), and chemical mediators (such as lysozyme, transferrin, and complement system). The second line of defense involves cells (phagocytes and natural cytotoxic cells) and an inflammatory response. The third line of defense is the development and deployment of a specific immune response by either production of antibodies to the antigen or development of a T-cell response. Innate immunity (the first two lines of defense) has a much more rapid response, taking effect in hours to days. Specific immunity requires days to weeks to develop.

In shrimp and fish, there are cellular and humoral immune functions; different types of hemocytes (defense cells) have the ability to phagocytize foreign cells while other types of hemocytes can utilize oxidative burst, secrete lectins, use the phenoloxidase system, or coagulate to attack pathogens. Lectins, lysozyme, plasma proteins, and penaeidins (antibacterial peptides) comprise humoral factors in shrimp.

The purpose of the immune system is to protect an organism from infection from pathological agents, whether they are viruses, bacteria, or parasites. A properly functioning immune system can mean the difference between survival or death, and/or preventing the spread of the pathogen if a non-lethal infection does occur. There are two components of the immune system of a fish: innate immunity and adaptive immunity. These components are linked so that a response to a pathogen by the innate immune component stimulates an adaptive immune response, while an adaptive immune response can use many of the effector mechanisms of innate immunity to eliminate pathogens.

Innate immunity is also called natural or native immunity and consists of responses that are present prior to infection. Innate immunity is the first line of defense for the organism against possible infection/infestation of pathogens and consists of rapid, generalized responses that generally react in the same manner to any subsequent infection. Innate immunity consists of external physical barriers in fish, such as scales, skin, and mucus; enzymes and other secretions present in mucus, tissues, and gastric juices; blood proteins, such as complement and acute phase proteins; macrophages and neutrophils; and non-specific cytotoxic cells.

Adaptive immunity, also known as acquired immunity, is completely different from innate immunity. Adaptive immunity is specific for each antigen, has many responses for a diverse number of antigens, has "memory" of previous pathogens so that the response to repeated infections is greater, and has self/non-self recognition. Adaptive immunity does not operate independently of innate immunity, but rather compliments the generalized defense of the innate immune response with a specific response to the pathogenic organism. There are two types of responses that adaptive immunity can utilize: humoral immunity and cell-mediated immunity. Both components utilize "memory" so that previous pathogens are "remembered" and a more effective immune response can be offered.

Humoral immunity is mediated by antibodies called immunoglobulin that circulate in the serum. In fish, these antibodies are produced by B-lymphocytes, which are thought to be similar to mammalian B-lymphocytes. However, only one type of immunoglobulin is produced in most fish species and that is a form of immunoglobulin M (IgM). For cell-mediated immunity, T-lymphocytes mediate the response and are directed at intracellular pathogens, such as viruses or pathogens that are inside host cells. Cell-mediated immunity cannot be transferred with serum and must be transferred by T-lymphocytes.

Protein Effects on Immunity in Mammals

Severe protein deficiency can lead to increased bacterial infections in mammals. When rats were fed protein-free diets, cell-mediated and humoral immunity was greatly reduced (Kenney et al. 1968). Bounous and Kongshavn (1978) reported that Swiss mice fed a 12% casein diet that contained only 0.4% phenylalanine (Phe) and 0.2% tyrosine (Tyr) (the requirement of Swiss mice) grew normally and had a 100% increase in plaque-forming cell (PFC) response to sheep red blood cell compared to mice fed a 12% casein diet that contained 0.6% Phe and 0.3% Tyr. Likewise, they noted that humoral immunity had a similar response when Phe and Tyr were restricted to 0.2% and 0.1%, respectively, although it was not known if it was the ratio of each amino acid or the actual availability of each amino acid that was responsible for the results. In a subsequent study, the researchers reported that it was not the actual amount of Phe in a diet that caused the increased immune activity, but rather the ratio of Phe to one or more undetermined amino acids; however, they could not identify the possible amino acid(s) based upon the diets used in their study (Bounous and Kongshavn 1982).

It has been shown that young animals must develop an immune response to various infection agents. Nutritional status of the host can influence

the severity of impact to parasites and influence the rate of immunity acquisition. In rodents, dietary protein is extremely important in resisting nematode infection (Michael and Bundy 1991, 1992), while in ruminants (sheep), Bawden (1969) reported that animals fed a low-protein diet had more nematodes than those fed a diet containing a higher percentage of protein. Abbott and Holmes (1990) demonstrated that sheep fed a diet containing soybean meal (to increase dietary protein percentage) had significantly enhanced immune response and decreased pathology in the host to nematode, *Haemonchus contortus*, infection. It may be that protein reduces pathogenicity by increased synthesis of essential proteins, and/or may influence the mechanisms utilized to remove the nematodes from the body. Van Houtert et al. (1995) fed diets containing fish meal to sheep and reported that animals fed higher-protein diets had higher nematode expulsion rates compared to sheep fed a low-protein diet; however, higher protein intake did not improve circulating antibody levels to the parasite or non-parasite antigens. Conversely, increased protein intake decreased circulating anti-ovalbumin antibodies and lymphocyte response.

Bown et al. (1991) stated that the development of resistance in sheep to gastrointestinal parasites was dependent upon protein status because many immune components, such as immunoglobulin and lymphokines, are largely composed of a proteinaceous nature. Kambara et al. (1993) fed two age classes of sheep either a high-protein (20%) diet or a low-protein (11%) diet and compared the resistance to infection by the nematode *Trichostrongylus colubriformis*. They found that young sheep (8–26 weeks old) fed the diet containing 20% protein had a higher resistance to infection than sheep fed the diet containing 11% protein; however, no such trend was observed in older (33–51 weeks old) sheep.

In growing lambs, enhanced immune response was associated with feeding diets containing increased protein. Coop et al. (1995) fed lambs a diet with added protein and reported that expulsion of the gastrointestinal nematode, *Ostertagia circumcincta*, was due to a higher concentration of mucosal mast cells compared to lambs that were fed only the basal diet with reduced protein.

During immunological challenge, nutritional requirements of an organism may be changed. During challenge, nutrients can be directed away from growth and maintenance and towards supporting immune system functioning, while amino acids are formed from muscle catabolism and are used to synthesize acute phase proteins in the liver; they are also used as energy. However, when weanling pigs fed diets containing different percentages of protein were challenged with lipopolysaccharide, there were no interactive effects between immune challenge and dietary protein, indicating no differences in protein requirements for growth and feed conversion between control pigs and those undergoing an immunological challenge (van Hougten et al. 1994). Further, the authors did not observe any effect on lymphocyte number or antibody response among the different dietary treatments during challenge.

In humans, chronic protein insufficiency (CPI) has a bipolar effect on immunological response, enhancing cell-mediated immunity while either reducing humoral immunity or having no effect. Cooper et al. (1974) reported that mice fed diets deficient in protein had lower weight gain and final average weight than mice fed a diet with sufficient protein, and also showed a decrease in globulin levels for alpha-1, alpha-2, beta, and gamma globulins. Further, there was no effect on humoral immune response to an antigen (*Brucella abortus*). However, an increased macrophage response was observed in mice fed a protein-deficient diet and their resistance to pseudorabies virus infection was greater compared to mice fed a protein-sufficient diet, but their resistance to bacterial infection with streptococci decreased (Cooper et al. 1974). The results indicate that CPI does not affect humoral immunity, primarily due to no change in B-cell-dependent immune response to an antigen. However, response of thymus-derived antigen-reactive cells (T-cell) seems to be enhanced by CPI due to an increase in thymic hormone output. This could be the result of a general decrease in production of short-lived B-cells, but long-lived cells (such as T-cells) appear to persist and remain for long periods of time, even when the organism has a protein deficiency (Bell and Hazell 1975).

It has been shown in mice that the type of dietary protein has a profound influence on the development of humoral immunity on T-cell-dependent (TD) and T-cell-independent (TI) antigens (Bounos et al. 1983). The type of protein does not however seem to have an

effect on some cell-mediated immunity aspects such as graft-versus-host reaction, delayed hypersensitivity reactions, resistance to *Salmonella* infection (Bounos and Kongshavn 1985), or phagocytosis by peritoneal macrophages (Bounos et al. 1981). With these observed effects, it has been theorized that protein type influences the ability of B-lymphocytes to respond to an immunogenic event. In mice B-lymphocytes, the newly-formed cells supplied by the bone marrow, are the primary response cells in the spleen and other peripheral lymphoid tissues. Bounos et al. (1985) reported that the observed effects of dietary protein type on humoral immune response is not due to the rate of primary B-lymphocytes production in the bone marrow, but may be due to changes in the activity of the B-lymphocytes or in alterations to the activation and differentiation of the peripheral lymphoid tissues. Their findings indicate that individual amino acids may be the influencing factor on B-cell response, but have little effect on T-lymphocytes (Bounos and Kongshavn 1985).

Protein Effects on Immunity in Fish

There appear to be numerous similarities in the influence of protein on immunity in mammals and that in fish and shrimp. There are many factors that directly influence the dietary protein requirement of fish and shrimp, such as size of the organism, protein quality, energy supply, feeding rate, water physico-chemical parameters, trophic level of the organism, culture system and level used (extensive versus intensive culture methods), stocking density, diet formulation, and quality of dietary ingredients. As in mammals, insufficient protein has been reported to adversely affect the immune system of fish. Kiron et al. (1995) fed rainbow trout, *Oncorhynchus mykiss*, various percentages (10, 35, and 50%) of protein and found that fish fed the diet containing 10% protein had reduced lysozyme activity and a reduced level of C-reactive proteins compared to fish fed diets containing 35% and 50% protein. However, all immune parameters measured in fish fed the two higher-protein diets were not significantly different from each other. This was in agreement with an earlier study in which Kiron et al. (1993) reported that rainbow trout fed diets with low (0% and 20%) protein levels had reduced humoral immune responses and resistance to infectious hematopoietic necrosis (IHN) virus compared to fish fed diets containing 35% and 50% protein. However, higher mortalities were recorded in fish fed a diet containing 50% protein. Both studies stated that antibody titers were unrelated to dietary protein levels and that it is possible that globulin synthesis in fish is maintained preferentially, regardless of dietary protein level. While fish fed diets deficient in protein had reduced health parameters and were more susceptible to viral infection and mortality than fish fed diets containing adequate levels of protein, feeding diets containing protein levels in excess of requirement also impaired immune function. This supports data from Hardy et al. (1979), who reported that Chinook salmon, *Oncorhynchus tshawytscha*, fed diets that contained either too low or too high a protein level had increased susceptibility to viral infection.

In contrast, some studies have found inconclusive results when fish were fed diets containing various protein levels. Omar et al. (1996) stocked Nile tilapia, *Oreochromis niloticus*, at either 1 fish/L, 3 fish/L, or 4 fish/L, fed them diets containing 10%, 30%, and 40% protein, and exposed them to *Aeromonas hydrophila*. They reported that antibody production at 28 days post-immunization with formalin-killed *A. hydrophila* and survival after disease challenge was highest in fish fed a diet containing 30% protein and stocked at 1 fish/L, while fish with the lowest antibody production and lowest survival to disease challenge were fed the diet containing10% protein and stocked at 4 fish/L.

Lim and Klesius (1998a) immunized channel catfish, *Ictalurus punctatus*, with formalin-killed *Edwardsiella ictaluri*, and fed the fish diets containing various percentages of protein. They then challenged the fish with *E. ictaluri* and found that macrophage migration was higher in fish fed a diet containing 28% protein compared to fish fed diets with lower percentages of protein, but that there was no improvement in immune response in fish fed diets containing more than 28% protein. However, these data are in contrast to Lim and Klesius (1998b) who reported no influence of dietary protein level on macrophage migration in channel catfish fed diets containing either 25% or 40% protein.

Arginine is important in many metabolic pathways, such as protein synthesis and metabolism of glutamic acid and proline. As was mentioned in the preceding

section on the effects of protein on mammalian immunity, amino acids can affect immunological function of the organism. The addition of amino acids appears to have similar effects to varying protein level in fish. Buentello and Gatlin (2001) fed channel catfish diets containing graded levels of crystalline arginine (0.0, 1.0, 2.0, and 4.0%), and reported that fish fed 2.0% crystalline arginine had a significantly higher percentage of survival when exposed *to E. ictaluri* compared to all other treatments, suggesting that the enhanced survival was due to an increased capacity of macrophages to destroy bacteria.

In largemouth bass, *Micropterus salmoides*, serum lysozyme activity and respiratory burst of head kidney leukocytes were amplified with increasing levels of arginine (Zhou et al. 2012). For lysozyme, highest values were achieved when arginine was added in excess of the requirement, while there was no additional benefit to respiratory burst activity once arginine was added to the requirement level. Complement hemolysis was not significantly affected by any level of arginine.

Fish Meal Replacement by Plant Protein Mix

Use of different ingredients in fish diets can affect the immune system, even when dietary protein levels are maintained. Wood (1968) reported that Pacific salmon fed a diet containing corn gluten had significantly lower percentage survival compared to fish fed a diet containing cottonseed meal when exposed to bacterial kidney disease. Sitja-Bobadilla et al. (2005) fed juvenile gilthead sea bream, *Sparus aurata*, diets containing a mix of plant-protein sources as partial or total replacement of fish meal (FM). Supplemental amino acids were also added to the diets containing the plant-protein mix to balance the essential amino acid composition and make the diet similar to that containing 0% of the plant-protein mix (control). Growth data indicated that inclusion of a plant-protein mix reduced growth as higher inclusion levels were added; however, the effect on immune function was contradictory. Plasma lysozyme levels were not affected, but respiratory burst of head kidney leukocytes were significantly increased in fish fed diets containing 75% plant-protein mix compared to fish fed the control (0% plant-protein mix), but not significantly different compared to fish fed diets containing 50% and 100% plant-protein mix. Complement alternative pathway (ACH_{50}) was increased in fish fed the diet with 50% FM replacement, but this activity decreased in fish fed diets replacing 75% and 100% of the FM. Plasma myeloperoxidase activity (MPO) was significantly increased in fish fed diets in which 75% and 100% of the FM was replaced with the plant-protein mix, but MPO of head kidney leukocytes was similar among all treatments. This is in agreement with Rumsey et al. (1994), who reported that rainbow trout fed soybean protein also had enhanced leukocyte intracellular killing activity. However, these findings are in contrast to other reports where leukocyte and serum MPO had various changes depending upon the dose, timing, and diet (Cuesta et al. 2002; Ortuno et al. 2002; Rodriguez et al. 2003).

It was also reported that replacing 50% of FM with plant proteins enhanced plasma complement, which was significantly reduced in fish fed diets containing higher percentages of FM replacement with a plant-protein mix (Sitja-Bobadilla et al. 2005). Since hepatocytes are the main source of the C_3 component, any degeneration or disruption of the liver could result in reduced complement production. Indeed, it was reported that as FM was replaced by a plant-protein mix, livers of gilthead sea bream fed higher inclusion levels showed an increase in steatosis (accumulation of fat in the liver), which could have led to decreased complement production.

Wedemeyer and Ross (1973) demonstrated that different protein ingredients in isonitrogenous diets did not affect susceptibility to infection in coho salmon, *Oncorhynchus kisutch*; however, Rumsey et al. (1994) reported that rainbow trout fed isonitrogenous diets containing FM had reduced serological and non-specific immunity compared to fish fed diets containing soybean meal. Further, it has been reported that Atlantic salmon, *Salmo salar*, fed diets containing various soybean ingredients had increased lysozyme activity and total immunoglobulin, but that disease resistance was dependent upon the soy protein source.

Krogdahl et al. (2000) observed increased lysozyme activity of intestinal mucosa in Atlantic salmon fed soybean molasses. This could indicate an increase in macrophage and eosinophil activation. It was also reported that IgM increased in the mid and distal mucosa of the intestine. When fish were challenged with *Aeromonas s. salmonicida*, increased resistance

to infection was shown by fish fed diets containing soy protein concentrate, suggesting that local immune response in the mid-intestinal mucosa to soybean products increased resistance to the bacteria because an immunological response had already been activated due to the soy protein concentrate.

The systemic stimulation of immunity by soy protein concentrate has also been reported in rainbow trout; fish fed a diet containing soy protein concentrate had increased circulating leukocytes, increased activity of macrophages, and elevated immunoglobulin levels compared to fish fed a FM-based diet (Rumsey et al. 1994) and also increased resistance to *A. salmonicida* challenge (Siwicki et al. 1994). This is in contrast to Neji et al. (1993) who found that rainbow trout fed diets containing animal proteins (poultry by-product meal and blood meal) had increased resistance to infection from *A. salmonicida* compared to fish fed diets containing plant-source proteins (corn gluten and soybean meal).

Bransden et al. (2001) showed that there were no significant differences in lysozyme activity, antiprotease activity, neutrophil oxygen radical production, plasma total immunoglobulin, total plasma protein, plasma glucose levels, or mortality from *Vibrio anguillarum* after challenge in Atlantic salmon fed diets containing FM or experimental diets containing either poultry feather meal, lupin meal, or a mixture of the two ingredients as partial replacement for FM.

Poultry by-product meal (PBM) is a high-quality animal-source protein ingredient that has been successfully added to aquaculture diets to partially or totally replace FM; however, there have been inconsistent results on its ability to totally replace FM due to differences in product quality, digestible and utilizable nutrient composition, and price. Sealey et al. (2011) stated that when rainbow trout fry (0.5 g) were fed diets which had totally replaced FM with either chicken protein concentrate, a PBM blend, or chicken and egg protein concentrate, weight gain was significantly higher for fish fed either the chicken protein concentrate or chicken and egg protein concentrate compared to fish fed the control (FM) diet. However, among the diets, there was no difference in percentage survival (average 14%) of fish challenged with *Flavobacterium psychrophilum*, which is the causative agent of bacterial coldwater disease. There did not appear to be a benefit from adding poultry protein ingredients for disease resistance in rainbow trout, but the addition of poultry proteins did not depress immune response. This is consistent with findings of Kumar et al. (2010) who reported that rainbow trout fed a diet containing jatropha meal, *Jatropha curcas*, as a 50% replacement of the FM had similar weight gain and feed conversion ratio as fish fed a control diet containing 68.7% FM without adversely affecting hematological parameters such as red and white blood cell counts, hemoglobin, hematocrit, globulin, and lysozyme activity.

When soybean meal and PBM were used to totally replace FM in diets for sunshine bass, *Morone chrysops* X *M. saxatilis*, it was reported that there were no adverse effects on growth or most immunological parameters measured. Fish fed a diet containing 34% protein with 0% FM had lower total serum protein compared to fish fed a diet containing 42% protein with 0% FM, but serum protein was not different from fish fed a diet containing 38% protein with 0% FM or a diet containing 44% protein with 30% FM (Rawles et al. 2011). However, no differences were found among treatments in total immunoglobulin, lysozyme activity, and natural hemolytic complement activity after 459 days of feeding. Serum protein, immunoglobulin, and lysozyme activity did increase linearly with increasing protein level, while serum protein and lysozyme activity also increased quadratically with increasing dietary protein level. Since lysozyme and complement activity indicate general health and humoral immune responses in fish, total replacement of FM with soybean meal and PBM (while concomitantly reducing protein level) did not appear to hinder overall health and growth of sunshine bass grown in ponds. Disease challenge studies are however required to corroborate the effects of reduced protein level and FM replacement on immune function.

Fish Hydrolysate

Kotzamanis et al. (2007) fed larval European sea bass, *Dicentrarchus labrax*, diets containing two different fish protein hydrolysates at either 10% or 19% inclusion to determine their effects on growth, enzyme production, and disease resistance. When challenged with *Vibrio anguillarum*, larvae fed sardine hydrolysate containing 54% di- and tri-peptides had

significantly lower mortality compared to larvae fed a commercial protein hydrolysate comprising mostly (51.4%) of oligopeptides. However, it is not possible to discern a reason for these results as the inclusion of fish protein hydrolysates affected the gut microbiota with *Vibrio* spp. being present in the larvae. Improvement in larval resistance of *V. anguillarum* might therefore be due to settled strains of *Vibrio* in the culture environment preventing the pathogenic *Vibrio* from invading the larvae. An alternative hypothesis is that the settled strains stimulated the larval immune system, preventing infection by the pathogenic *V. anguillarum*.

Macrophages are one of the most active leukocytes in innate immunity and defend the organism in early stages of infection from pathogenic bacteria. Bogwald et al. (1996) reported increased respiratory burst activity and superoxide anion production of kidney macrophages of Atlantic salmon that had been previously injected with protein hydrolysate from cod muscle when compared to control cells. However, no beneficial effects of hydrolyzed fish protein on immune system were reported in Atlantic salmon (Gildberg et al. 1995) or cod, *Gadus morhua* (Gildberg and Mikkelsen 1998) challenged with *A. samonicida* and *V. anguillarum*, respectively. Similarly, Murray et al. (2003) reported that addition of either 29.1% cooked fish with bones, 30.3% fish hydrolysate with bones removed, or 31.4% fish hydrolysate with bones added had no effect on hematocrit, leucocrit, complement fixation (ACH_{50}), lysozyme activity, total serum immunoglobulin, MPO, and percentage phagocytosis in juvenile coho salmon. Further, there was no difference in survival of coho salmon after challenge with *V. anguillarum* among treatments. The hydrolyzed fish comprised 63.5% intact proteins, 29.4% polypeptides, 4.2% free amino acids, and 3.0% ammonia. The average length of polypeptides was 11.3 amino acids.

Japanese sea bass, *Lateolabrax japonicus*, fed diets containing various percentages (8.1% and 13.6%) of fish protein hydrolysate (FPH) had significantly higher weight gain, lysozyme activity, complement hemolytic activity, and phagocytic activity after 60 days of feeding compared to fish fed a diet without FPH (control diet); however, no differences in the number of NBT-positive cells were found and there were no differences in survival in fish exposed to *V. anguillarum* for 14 days (Liang et al. 2006). The latter finding is consistent with other reports that did not find improvement in survival of fish fed diets containing FPH (Gildberg et al. 1995; Gildberg and Mikkelsen 1998). It could be that the molecular weight of the peptide used in the FPH by Liang et al. (2006) was not of correct size. Peptides ranging in size from 500 to 3000 Da were reported to improve superoxide anion production in Atlantic salmon, and peptide fraction >500 Da improved growth and survival of carp larvae (Carvalho et al. 2004).

Yeast

Brewer's yeast, *Saccharomyces cerevisiae*, is a by-product from the brewing industry and has various compounds that may have immunostimulatory effects, such as β-glucans, nucleic acids, and mannan oligosaccharides. Li and Gatlin (2004) fed sunshine bass, *Morone chrysops* X *M. saxatilis*, diets containing either 1% or 2% brewer's yeast and 1% or 2% of a commercial prebiotic. No significant differences were found in weight gain, neutrophil oxidative production test, serum lysozyme, and intracellular superoxide anion of sunshine bass fed the various diets; however, extracellular superoxide anion was significantly higher in fish fed 1% brewer's yeast, 2% brewer's yeast, and 1% prebiotic compared to fish fed the unsupplemented (basal) diet. Further, challenge to *Streptococcus iniae* resulted in higher mortality in fish fed the basal diet compared to fish fed either brewer's yeast or the commercial prebiotic. This is in agreement with Reyes-Becerril et al. (2008) who fed larval grouper, *Mycteroperca rosacea*, a diet containing 1.10% yeast (*Debaryomyces hanserii*) for 4 weeks before challenging them with *Amyloodinium ocellatum*. They found that mortality was 40%, while fish fed a diet without yeast had 90% mortality at the end of the 7-day challenge period. Currently, specific mechanisms by which yeast and prebiotics influence immune function are unknown, although it is believed that the microflora of the intestine is a determinant for the development of the immune system in fish possibly due to increased levels of IgM in fish fed a diet containing yeast (Reyes-Becerril et al. 2008).

β-glucans are polysaccharides that are composed of glucose molecules linked by either a β-1,3 and β-1,6 bond, or a β-1,3 and β-1,4 bond. The influence of

β-glucans on immune function has been inconsistent; while non-specific immune parameters appear to be enhanced by feeding diets with β-glucans (either added or through yeast inclusion), resistance to infection does not appear to be improved. Welker et al. (2007) reported no differences in growth, lysozyme, spontaneous hemolytic complement (SH50), plasma bactericidal activity, and respiratory burst (NBT) of phagocytes in channel catfish fed diets containing either a polysaccharide with β-1,3 and β-1,6 bonds, a mannan oligosaccharide derived from yeast, *S. cerevisiae*, or whole-cell live *S. cerevisiae* compared to a diet without addition of any yeast product. Further, no difference in survival following *E. ictaluri* challenge was found among fish in any treatments. This is in contrast to Chen and Ainsworth (1992), who reported that channel catfish fed β-glucan and baker's yeast had increased antibody production and higher survival percentage following *E. ictaluri* challenge due to an increase in phagocytic and bactericidal activity. However, the β-glucan was injected into the fish in the study of Chen and Ainsworth (1992) and not provided in the diet. Thus, even though yeast and yeast supplements (β-glucans) have shown enhanced immune parameters, these do not translate into increased disease resistance when included in a diet.

The addition of yeast fermentation products to diets fed to hybrid tilapia, *Oreochromis niloticus X O. aureus*, grown in cages resulted in an increase in serum lysozyme activity, levels of C_3 and C_4 (serum alternative complement pathway), head kidney macrophage phagocytic activities, and macrophage respiratory burst activity compared to fish fed a diet without the yeast product (He et al. 2009). However, there was no disease challenge component in the study so it is unclear if the increased levels of non-specific immunity would have biological significance.

In another feeding trial, addition of various percentages of baker's yeast to diets of small (0.3 g) Nile tilapia significantly reduced mortality (40–60%) after interperitoneal injection of *A. hydrophila* compared to fish fed a diet without baker's yeast (70%) (Abdel-Tawwab et al. 2008).

Distiller's Dried Grains with Solubles

Distiller's dried grains with solubles (DDGS) is a by-product of ethanol production, either from the fuel ethanol industry or the beverage (bourbon) industry. Historically, DDGS was produced by the bourbon distillery industry and used in diets for dairy cows; however, 99% of the DDGS currently produced in the United States is produced by the fuel ethanol industry. DDGS has a modest protein content (27–32%) and contains no antinutritional factors that are present in some plant-protein ingredients, such as cottonseed meal, soybean meal, and rapeseed meal. Use of DDGS in aquaculture diets is limited because of the low-lysine and high-fiber content; however, it has been shown to be a viable ingredient in fish diets when used at moderate (<35%) inclusion levels.

Lim et al. (2007) fed experimental diets containing various (0, 10, 20, and 40%) percentages of DDGS as partial replacement of soybean meal and corn meal to juvenile (9.4 g) Nile tilapia. At the end of 10 weeks, weight gain and feed efficiency ratio (FER) of fish fed diets containing 10% and 20% DDGS was not significantly different compared to fish fed the control diet; however, fish fed the diet containing 40% DDGS were significantly smaller than fish fed the control diet, even when the diet was supplemented with 0.4% lysine. There were no differences in red blood cell count, white blood cell count, hemoglobin, hematocrit, serum protein, lysozyme activity, and antibody titer among treatments. Likewise, there was no difference in percentage mortality of Nile tilapia exposed to *Streptococcus iniae*.

Distiller's dried grains with solubles contain yeast cells in which 5.3% of the protein content is contributed by yeast protein. Yeast is a nutritious ingredient containing protein, B-complex vitamins, and β-glucans. β-glucans have been reported to stimulate immune responses in humans, terrestrial animals, and fish; however, no differences in any immune parameter measured was found by Lim et al. (2007). Non-specific immune response, such as macrophage and neutrophil migration and phagocytosis, have been reported to be enhanced by β-glucans (Duncan and Klesius 1996a) while yeast glucan has been shown to sometimes improve lysozyme activity (Engstad et al. 1992; Jorgensen et al. 1993), but not all the time (Duncan and Klesius 1996a; Whittington et al. 2005). Prolonged feeding of β-glucan has been reported to increase the susceptibility to bacterial infection (Robertsen et al. 1990; Couso et al. 2003).

Lim et al. (2009) fed channel catfish diets containing various percentages (0, 10, 20, 30, and 40%) of DDGS for 12 weeks and found no differences in weight gain, red blood cell count, white blood cell count, serum protein, lysozyme activity, alternative complement activity, superoxide anion production, or macrophage chemotaxis ratio among treatments. However, diets containing DDGS, regardless of inclusion percentage, had significantly higher hemoglobin, hematocrit, and total immunoglobulin levels, and had significantly less mortality from *E. ictaluri* challenge due to higher antibody titers compared to fish fed the diet without DDGS (control) after 21 days post-challenge. Ainsworth et al. (1994) also reported increased antibody titers to *E. ictaluri* in channel catfish fed 0.1% β-glucan but did not observe improved survival when fish were challenged, although a yeast species different from baker's yeast was used as the source of the β-glucan.

Failure to improve immune parameters and survival against disease challenge by DDGS has also been reported in Nile tilapia. Shelby et al. (2008) reported that Nile tilapia fed a diet containing 60% DDGS had significantly lower final weight and percentage weight gain compared to fish fed the control diet (0% DDGS) and the diet containing 30% DDGS; however, there were no significant differences in the number of erythrocytes and leucocytes, respiratory burst activity, total plasma protein, globulin, lysozyme, total hemolytic complement, and percentage survival in fish after challenge with *S. iniae* among treatments.

Spirulina

Spirulina, *Arthrospir platensis*, is a freshwater blue-green alga that contains a high percentage (60–70%) of protein and is used in diets for larval and juvenile fish. It also has numerous bioactive components that have been found to have antioxidant capabilities, generally attributable to the biliproteins such as phycocyanin. Nile tilapia fed diets containing 0.5–1.0% spirulina had significantly higher weight gain, final weight, red blood cells, white blood cells, lymphocytes, and superoxide anion production than fish fed a diet without spirulina (control); however, fish fed diets containing spirulina had lower monocytes and granulocytes compared to fish fed the control diet (Abdel-Tawwab and Ahmad 2009). Cumulative fish mortality was significantly reduced with addition of Spirulina to the diet of Nile tilapia challenged with *A. hydrophila* for 10 days. It is theorized that Spirulina stimulated the immune system by increasing phagocytic and natural killer cell activities (Duncan and Klesius 1996b), and enhancing leucocyte activities such as phagocytosis and production of superoxide and cytokine (Watanuki et al. 2006).

Probiotic Bacteria

Most of the research on the use of probiotics in aquaculture diets has focused on Gram-positive bacteria, although Gram-negative bacteria, microalgae, and yeasts have also received some attention. Use of pre- and probiotics are covered in detail in Chapter 13; however, one study worthy of mention was conducted on grouper, *Epinephelus coioides*, which were fed a basal diet or a diet containing the bacteria *Psychrobacter* sp SE6 for 60 days. At the conclusion of the study, no significant differences in any growth parameter or enzyme activities were reported (Sun et al. 2011). Further, no differences in phagocytic activity, phagocytic index, lysozyme, C_3, and total superoxide dismutase between fish fed the two diets was reported, although fish fed the diet containing the probiotic did have elevated levels of the serum complement C_4. While the authors concluded that the probiotic enhanced the immune responses of grouper, this conclusion does not appear to be supported by the data presented by the authors.

Cottonseed Meal

Cottonseed meal (CSM) is a high-protein (41%) plant ingredient that has been used in diets of a variety of fish species, but the presence of the toxic compound, gossypol, and its low-lysine content limit inclusion percentages in aquaculture diets. In a feeding trial with crucian carp, *Carassius auratus gibelio* X *Cyprinus carpio*, diets containing various percentages (0, 20, 40, and 56%) of CSM to partially or totally replace rapeseed meal/soybean meal/peanut cake had no effect on hemoglobin, lysozyme, superoxide dismutase, alanine aminotransferase, and aspartate aminotransferase (Cai et al. 2010). The lack of any significant differences in non-specific immunity parameters is in contrast to Yildirim et al. (2003), who fed channel catfish diets containing gossypol and reported increased serum

lysozyme activity at a dietary level of or exceeding 900 mg gossypol/kg of diet. It may be that gossypol improves non-specific immune response, but no mechanism has been postulated (Barros et al. 2002).

Effect of Protein on Immunity in Shrimp and other Crustaceans

As in mammals and fish, when white shrimp, *Litopenaeus vannamei*, were fed diets containing inadequate levels of protein, reduced immune function was reported. Pascual et al. (2004) fed white shrimp diets containing 5, 15, and 40% protein. They reported that shrimp fed the diets containing 5% and 15% protein diets had reduced numbers of hemocytes and reduced respiratory burst activity compared to shrimp fed a diet containing 40% protein, indicating that cell number and phagocytic ability was compromised when shrimp were fed diets containing reduced levels of protein and amino acids.

Unlike fish, a crustacean's immune system is mainly non-specific and uses phagocytes, encapsulation, and agglutination to defend against infection. β-glucan from yeasts has been shown to enhance disease resistance in shrimp (Sung et al. 1994; Song et al. 1997; Chang et al. 2003; Sajeevan et al. 2006). Sajeevan et al. (2009) fed shrimp, *Fenneropenaeus indicus*, for 40 days using either a control diet, a diet containing 10% yeast (*Candida sake* S165), or a diet containing 0.2% glucan from *Candida sake* S165. At the conclusion of the study, it was reported that shrimp fed the diet containing 10% yeast had higher total hemocyte count (THC), phenoloxidase activity, peroxidase activity, and respiratory burst activity compared to shrimp fed the control diet. Shrimp fed the diet containing 0.2% glucan had elevated THC values compared to shrimp fed the control diet, yet the other three measured immune parameters were not different from the control treatment. Shrimp were then challenged with white spot syndrome virus and those fed the control diet had 96% mortality; shrimp fed the diet containing 0.2% glucan had 64% mortality; and shrimp fed the diet containing 10% yeast had 36% mortality. All means were significantly different from each other.

The differences in immune function and survival in shrimp fed whole yeast compared to shrimp fed diets containing cell wall glucan may be due to the process used to produce glucan. Since extraction of cell wall glucan from yeast involves treatments with alkali, acid, and heat, these procedures may remove, destroy, or deactivate valuable nutrients from the yeast, leaving the cell wall glucan as the main by-product. It may be that nucleotides (Sajeevan et al. 2006), vitamins, minerals, or carotenoids (Scholz et al. 1999) may be important in maintaining optimal health in shrimp. It may therefore be more beneficial to feed shrimp diets containing whole yeast as compared to feeding diets with cell wall glucan.

The ability of white shrimp to tolerate stress increased with increasing dietary protein up to 43% protein and decreased thereafter (Xia et al. 2010). This is in agreement with Liu et al. (2005) who reported that survival of white shrimp fed diets containing 30% protein was significantly higher after a salinity change compared to shrimp fed a diet containing 20% protein. This result could be due to the fact that shrimp use amino acids to maintain the osmotic pressure balance in water with low salinity.

Yeast

Pacific white shrimp were fed a basal diet (35% protein and 8% lipid) or experimental diets containing either 2% brewer's yeast, 5% brewer's yeast, 2% commercial prebiotic, or 5% commercial prebiotic for 6 weeks. At the conclusion of the study, no differences in final weight, weight gain, survival, total hemocyte count, hemolymph phenoloxidase, respiratory burst, or hemolymph protein was found among any treatments. However, survival of shrimp fed 2% commercial prebiotic and 5% brewer's yeast was significantly higher after a low-salinity (2 ppt) test compared to shrimp fed the basal (unsupplemented) diet (Li et al. 2009).

Nucleotides

Nucleotides have been shown to increase phagocytic activity, T-cell-dependent antibody production, neutrophil levels, and interleukin-2 production in mammals. Freshwater prawn, *Macrobrachium rosenbergii*, were fed a diet containing various (0, 1.5, 2.25, and 3.0%) percentages of a nucleotide mixture with equal amounts of AMP, IMP, CMP, and GMP. After 60 days of feeding, prawn fed a diet containing 1.5%

Table 2.1 Effect of protein, amino acids, and ingredients on immune responses and disease resistance in fish and shrimp.

Organism	Variable	Immune responses	Disease challenge	Literature cited
Sunshine bass, *Morone chrysops X M. saxatilis*	Brewer's yeast	Use of brewer's yeast: Lysozyme 0 Neutrophil oxidative production 0 Extracellular superoxide anion + Intracellular superoxide anion 0	*Streptococcus iniae* Survival +	Li and Gatlin (2004)
Sunshine bass, *Morone chrysops X M. saxatilis*	Protein level	Total serum protein + Immunoglobulin 0 Lysozyme 0 Hemolytic complement 0		Rawles et al. (2011)
Rainbow trout, *Oncorhynchus mykiss*	Protein level	Increasing protein level: Antibody production 0	IHNV Survival[1]	Kiron et al. (1993)
Rainbow trout, *Oncorhynchus mykiss*	Protein level	Increasing protein level: Lysozyme – C-reactive protein – Antibody production 0	Survival[2]	Kiron et al. (1995)
Rainbow trout, *Oncorhynchus mykiss*	Physic nut, *Jatropha curcas*	Replacement of fish meal: Lysozyme 0 Red blood cells 0 White blood cells 0 Hemoglobin 0 Hematocrit 0 Corpuscular hemoglobin 0 Globulin 0		Kumar et al. (2010)
Rainbow trout, *Oncorhynchus mykiss*	Poultry products	Use of various poultry products as replacement of fish meal	*Flavobacterium psychrophilum* Survival 0	Sealey et al. (2011)
Atlantic salmon, *Salmo salar*	Soy products	Lysozyme in fish fed alcohol-extracted soybean meal diet was higher than in fish fed control diet or diet with soy protein concentrate. IgM +	*Aeromonas salmonicida* Survival 0	Krogdahl et al. (2000)
Atlantic salmon, *Salmo salar*	Lupin and feather meals	Use of lupin and feather meals as replacement of fish meal Lysozyme 0 Neutrophils 0 Immunoglobulin 0 Total plasma protein 0	*Vibrio anguillarum* Survival 0	Bransden et al. (2001)

Coho salmon, *Oncorhynchus kisutch*	Fish hydrolysate	Replacement of fish meal: Lysozyme 0 Complement 0 Serum Ig 0 Antibody production 0 Respiratory burst 0 Plasma protein –	*Vibrio anguillarum* Survival 0	Murray et al. (2003)
Nile tilapia, *Oreochromis niloticus*	Protein level	Increasing protein level: Antibody production + (low density) Antibody production – (high density)	*Aeromonas hydrophila* Survival + (low density)	Omar et al. (1996)
Nile tilapia, *Oreochromis niloticus*	Fish hydrolysate	Replacement of fish meal Respiratory burst + Lysozyme enzyme acid phosphatase +		Bogwald et al. (1996)
Nile tilapia, *Oreochromis niloticus*	Distiller's dried grains with solubles	Replacement of fish meal: Lysozyme 0 Red blood cells 0 White blood cells 0 Hemoglobin 0	*Streptococcus iniae* Survival 0	Lim et al. (2007)
Nile tilapia, *Oreochromis niloticus*	Baker's yeast	Increasing percentage of baker's yeast led to: Red blood cell + Hemoglobin + Hematocrit + Globulin +	*Aeromonas hydrophila* Increasing percentage of baker's yeast reduced mortality	Abdel-Tawwab et al. (2008)
Nile tilapia *Oreochromis niloticus*	Distiller's grains	Replacement of soybean meal: Lysozyme 0 Red blood cells 0 White blood cells 0 Respiratory burst 0 Total plasma protein 0 Globulin 0 Hemolytic complement 0	*Streptococcus iniae* Survival 0	Shelby et al. (2008)
Nile tilapia, *Oreochromis niloticus*	Spirulina	Addition to diet: Red blood cells + White blood cells + Lymphocytes + Monocytes – Granulocytes – Superoxide anion + Hematocrit 0 Serum protein 0 Antibody titer 0	*Aeromonas hydrophila* Increased survival +	Abdel-Tawwab and Ahmad (2009)

(*continued*)

Table 2.1 (*Continued*)

Organism	Variable	Immune responses	Disease challenge	Literature cited
Hybrid tilapia	Yeast	Increasing percentage of yeast: Lysozyme + C_3 + C_4 + Phagocytic activity + Phagocytic index 0 Respiratory burst +		He et al. (2009)
Channel catfish, *Ictalurus punctatus*	Protein level	Increasing protein level: Serum protein 0 Antibody production 0 Macrophage production +	*Edwardsiella ictaluri* Survival 0	Lim and Klesius (1998a)
Channel catfish, *Ictalurus punctatus*	Protein level	Increasing protein level: Macrophage migration 0 Antibody production 0 Antibody production after 2-week booster +		Lim and Klesius (1998b)
Channel catfish, *Ictalurus punctatus*	Arginine	Increasing levels of arginine: Phagocytosis +	*Edwardsiella ictaluri* Survival +	Buentello and Gatlin (2001)
Channel catfish, *Ictalurus punctatus*	Yeast	Inclusion of yeast or yeast: Lysozyme 0 Hemolytic complement 0 Plasma bactericidal activity 0 Respiratory burst (NBT) 0	*Edwardsiella ictaluri* Survival 0	Welker et al. (2007)
Channel catfish, *Ictalurus punctatus*	Distiller's dried grains with solubles	Replacement of soybean meal: Lysozyme 0 Red blood cells 0 White blood cells 0 Hemoglobin + Hematocrit + Serum protein 0 Total immunoglobulin + Alternative complement 0 Superoxide anion production 0 Macrophage chemotaxis 0 Antibody titer +	*Edwardsiella ictaluri* Survival +	Lim et al. (2009)
Olive flounder, *Paralichthys olivaceus*	Kelp	Inclusion of kelp: Lysozyme + Activated neutrophils (NBT) + Myeloperoxidase activity (MPO) +		Kim and Lee (2008)

Leopard grouper, *Mycteroperca rosacea*	Yeast	Inclusion of yeast: Hemoglobin 0 Plasma protein 0 Superoxide dismutase + IgM +	*Amyloodinium ocellatum*	Reyes-Becerril et al. (2008)
Japanese sea bass, *Lateolabrax japonicus*	Fish hydrolysate	Replacement of fish meal: Lysozyme + Complement + Phagocytic activity + NBT 0		Liang et al. (2006)
Gilthead seabream, *Sparus aurata*	Feeding regime	Restricted versus satiation feeding: Lysozyme 0 Complement 0 Respiratory burst 0		Sitja-Bobadilla et al. (2003)
Gilthead seabream, *Sparus aurata*	Plant protein mix	Replacement of fish meal: Lysozyme 0 Complement (50% replacement) + Respiratory burst (75% replacement) + Myeloperoxidase (100% replacement) + Plasma protein (75 and 100% replacement) − Complement (75 and 100% replacement) −		Sitja-Bobadilla et al. (2005)
Grouper, *Epinephelus coioides*	Yeast	Inclusion of *Psychrobacter* sp: Lysozyme 0 Phagocytic activity 0 Phagocytic index 0 C_3 0 C_4 + Superoxide dismutase 0		Sun et al. (2011)
European sea bass, *Dicentrarchus labrax*	Sardine silage	Replacement of fish hydrolysate	*Vibrio anguillarum* Survival +	Kotzamanis et al. (2007)
Crucian carp	Cottonseed meal	Replacement of soybean meal: Lysozyme 0 Hemoglobin 0 Superoxide dismutase 0		Cai et al. (2010)

(*continued*)

Table 2.1 (*Continued*)

Organism	Variable	Immune responses	Disease challenge	Literature cited
Largemouth bass, *Micropterus salmoides*	Arginine	Arginine level: Lysozyme + Respiratory burst + Complement 0		Zhou et al. (2012)
White shrimp, *Litopenaeus vannamei*	Protein level	Increased protein led to Granular cells + Hialin cells + Hemocytes + ProPO 0 ProPO: granular cells − Basal respiratory burst + Activated respiratory burst + Hemaglutinating activity −		Pascual et al. (2004)
White prawn, *Fenneropenaeus indicus*	Yeast	Inclusion of yeast: Hemocyte count + NBT + Phenoloxidase + Peroxidase + (+ signifies an enhanced effect; − signifies a decreased effect; 0 signifies no effect)	WSSV Survival +	Sajeevan et al. (2009)

of the nucleotide mixture had significantly higher percentage weight gain than prawns fed the basal (unsupplemented) diet. Further, prophenol oxidase activity, super oxide anion production, total hemocyte count, and total serum protein in prawn fed diets with the nucleotide mixture were significantly higher than prawn fed the basal diet (Shankar et al. 2012). Mortality of prawn fed 1.5% and 2.25% of the nucleotide mixture was significantly lower than prawn fed the basal diet and the diet containing 3.0% nucleotide mixture when challenged with white muscle virus and *A. hydrophila*.

Conclusions

Aquacultured organisms fed diets deficient in protein and/or amino acids may be more susceptible to pathogenic infection. When protein/essential amino acid requirements are met, feeding higher levels of protein or essential amino acids does not appear to increase immune response. While it is important that intensively cultured organisms be fed diets containing protein/amino acid at levels that meet requirements, protein ingredients included in the diets also have a significant effect on the immune system (Table 2.1). It is therefore imperative to know what ingredients comprise the diet when evaluating immune response, although it appears that contradictory results can occur. These differences may be due to species cultured, age/stage of species, culture method and management, genetic factors, experimental conditions, and diet formulation.

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Chapter 3

Lipids and Fatty Acids

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Introduction

Lipids represent a diverse range of compounds that are classified on the basis of their solubility in organic solvents; each has contrasting functional roles. Fatty acids are closest to being the "building blocks" of lipids, as they are components of many lipid classes collectively called complex lipids. These include acylglycerols (glycerides) and sphingolipids, in which fatty acids are esterified to alcohol or amino groups, respectively. Simple lipids that do not contain fatty acids comprise a much smaller group, with cholesterol and other sterols being the major representatives. However, as most lipids are complex, fatty acids generally account for the bulk of dietary lipid.

Lipids can be divided into two groups based on polarity: polar lipids, such as phospholipids, play predominantly structural roles; and neutral lipids are primarily responsible for storage of energy in the form of triacylglycerols (TAG) and other storage components such as steryl esters. All fatty acids have important roles in the provision of energy and as structural components, but some specific fatty acids also have key roles in the control and regulation of metabolism. Some lipids, including polyunsaturated fatty acids (PUFA), cannot be synthesized endogenously and are therefore essential components of the diet. A balanced diet must not only provide sufficient lipid/fatty acid to satisfy gross energy requirements, but must also satisfy specific requirements for critical functional lipids such as essential fatty acids (EFA), phospholipids, and sterols.

Interest in dietary lipid and fatty acids has increased in recent years due to the fact that supplies of marine resources for feeds are now limiting aquaculture development (Naylor et al. 2009). Declining global fisheries has resulted in aquaculture now supplying an increasing proportion of fish and seafood, approximately 50% in 2010 (FAO 2011). However, fishmeal and fish oil (FO), the major feed ingredients for farmed carnivorous and marine species, are derived from reduction fisheries based on small pelagic species such as anchovies and sardines that have reached their sustainable limits. In the last three decades, annual catches of feed fish have been 20–25 million tonnes (Mt), which reduces to around 6–7 Mt of fishmeal and 1.0–1.4 Mt of FO; there is little prospect of these increasing. In 1992, aquaculture accounted for around 15% and 20% of global fishmeal and FO supplies, respectively, and around 68% and almost 89% in 2006 (Tacon and Metian 2008). The finite supply therefore means the high use of marine products in aquaculture is unsustainable and increasing the pressure to reduce dependency on marine fish meals and oils (Naylor et al. 2009). The limited supply of FO combined with the rising demand is leading to increasing and unstable prices, along with environmental pressures to use alternative sustainable resources. Alternatives

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to marine FO are urgently required for sustainable development of aquaculture.

Biochemistry and Metabolism

Fatty Acid Acid Structure and Nomenclature

Fatty acids have a basic structure consisting of a long aliphatic chain with a carboxyl group at one end and a methyl group at the other (Fig. 3.1). The hydrocarbon chain can be "saturated" (i.e., all available carbon bonds taken or saturated with hydrogen) or unsaturated, containing carbon-carbon double bonds. Fatty acid nomenclature is complicated by several systems. The most commonly used and the International Union of Pure and Applied Chemistry (IUPAC) accepted system is the n-*x* (or "omega-*x*") nomenclature, where fatty acids are described by the general formula C:Dn-*x*, where C = chain length, D = number of ethylenic/double bonds, and n-*x* (or ω*x*) indicates the position of the first double bond relative to the methyl end. Thus, 16:0 represents a saturated fatty acid containing a 16-carbon aliphatic chain with no double bonds, and 18:1n-9 (18:1ω9) denotes a monounsaturated fatty acid with an 18-carbon aliphatic chain with a single *cis* double-bond nine carbons from the methyl group (Fig. 3.1). PUFA contain two or more double bonds, most commonly separated by single methylene (CH_2) groups. Thus, 22:6n-3 (22:6ω3) is a 22-carbon chain containing six double bonds with the first situated three carbons from the methyl group (Fig. 3.1). In the alternative Δ^x (delta-*x*) nomenclature, commonly used for specifying fatty acyl desaturase (Fads) activities, the double bonds are numbered from the carboxyl end of the molecule and so 22:6n-3 is written as 22:6Δ4,7,10,13,16,19. A Fads that introduces an ethylenic (double) bond six carbons from the carboxyl end of the aliphatic chain has Δ6 activity.

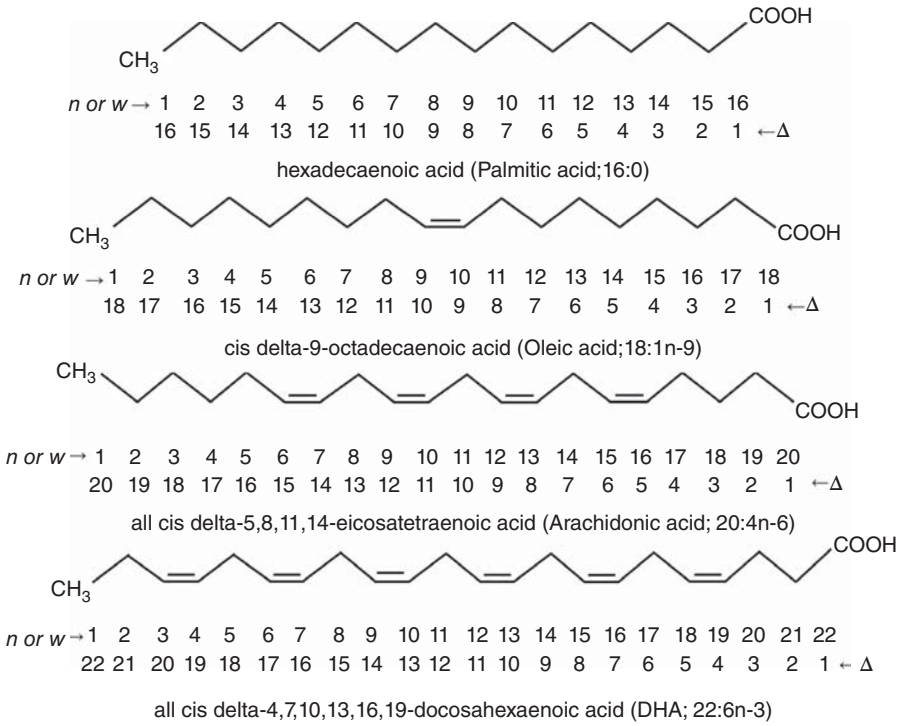

Figure 3.1 Fatty acid structures illustrated by major saturated (16:0), monounsaturated (18:1n-9), and polyunsaturated (20:4n-6 and 22:6n-3) fatty acids showing systematic names (trivial names or common abbreviations) and the n-*x* (or ω*x*) and Δ^x carbon numbering systems.

Fatty acids, particularly PUFA, are often described by their semi-systematic names such as eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3), which still indicate the numbers of carbons and double bonds present. Trivial names often reflect their sources, such as palmitic acid (16:0) from palm oil, oleic acid (18:1n-9) from olive oil, and arachidonic acid (ARA; 20:4n-6) from peanut oil. Another term that requires defining is long-chain PUFA (LC-PUFA), which describes PUFA with a chain length of C_{20} or greater and two or more double bonds. A similar term, HUFA (highly unsaturated fatty acids), has also been used extensively in aquaculture-related literature.

Lipid Class Structures

Phospholipid is a generic term for all lipids containing phosphorus, but the term is commonly used to mean phosphoglycerides, the predominant polar lipids that are derived from phosphatidic acid (PA), which is L-glycerol 3-phosphate esterified with two fatty acids. The major phosphoglycerides – phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) – contain the "bases" choline, ethanolamine, serine, and inositol, respectively, esterified to the phosphate group of PA (Fig. 3.2). Although there are exceptions, saturated and monounsaturated fatty acids are often preferentially esterified at *sn*-1, with PUFA preferentially esterified on position *sn*-2 (Tocher 1995). Sphingolipids are a group of polar lipids based on the aliphatic (C_{18} or, less commonly, C_{20}) amino alcohol sphingosine, with a long-chain saturated or monounsaturated fatty acid linked to the amino group to form the basic sphingolipid, ceramide. Other groups that can be esterified to the alcohol group of sphingosine include phosphocholine to form sphingomyelin (Fig. 3.2), or sugar moieties to form the glycosphingolipids such as cerebrosides, sulfatides, and gangliosides.

The major neutral lipid, TAG, contains three fatty acids esterified to the alcohol groups of glycerol

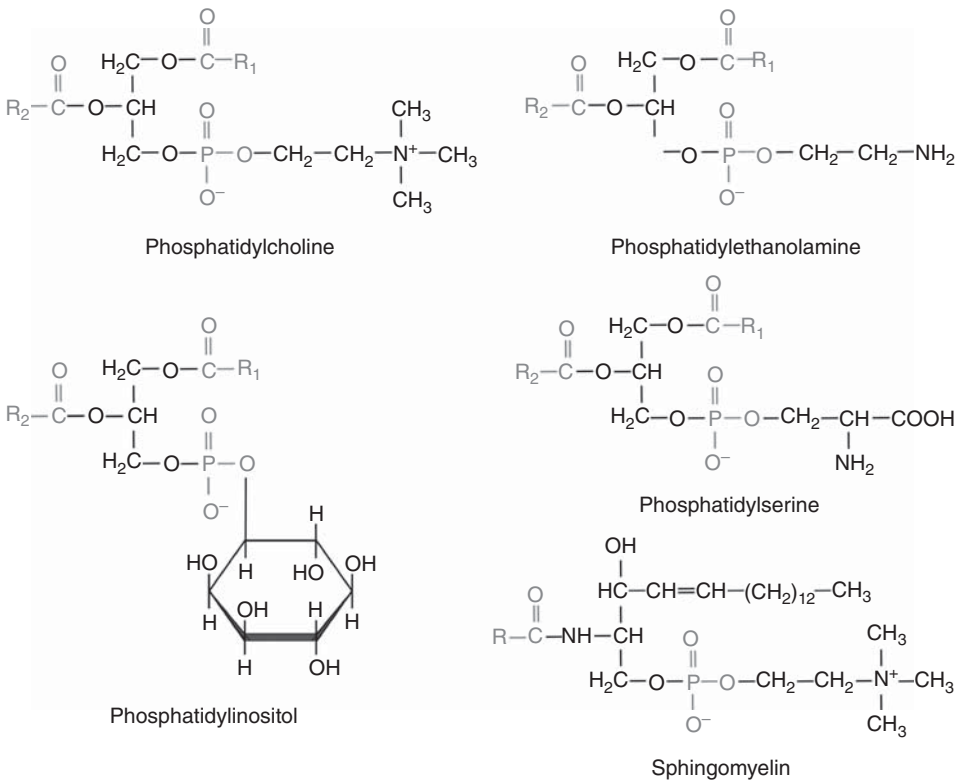

Figure 3.2 Structures of major phospholipid classes.

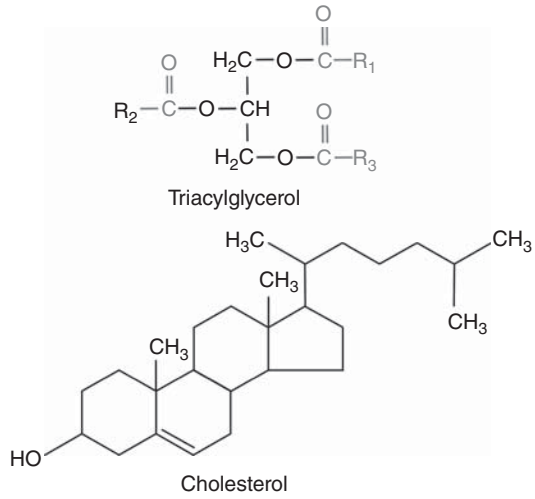

Figure 3.3 Structures of triacylglycerol and cholesterol.

(Fig. 3.3). Saturated and monounsaturated fatty acids are generally preferentially located in the *sn*1 and *sn*3 positions, whereas PUFA are preferentially located in the *sn*2 position (Tocher 2003). This high fatty acid content makes TAG the most efficient form of energy storage. Other storage lipids are wax esters; these are abundant in marine zooplankton, particularly calanoid copepods and euphausids that are natural foods for many marine fish. Wax esters consist of a fatty acid esterified to a fatty alcohol, the latter being predominantly saturated or monounsaturated and often rich in $C_{20/22}$ monounsaturated chains. The large amounts of 20:1n-9 and 22:1n-11 fatty acids in northern FO are derived from the oxidation of the corresponding fatty alcohols during digestion and absorption of wax esters by zooplanktonivorous fish (Tocher 2003).

The most important simple lipid in animals including fish is cholesterol, a member of the sterol family of tetracyclic hydrocarbon alcohols (Fig. 3.3). Cholesterol is an essential component of all cellular membranes, and is also the precursor of bile salts and steroid hormones; it can be stored esterified to fatty acids, particularly in steroidogenic tissues.

General Lipid Metabolism

The major pathways of lipid metabolism in fish including digestion and absorption, lipogenesis, transport, and oxidation have been the subjects of several reviews (Sargent et al. 1989, 2002; Olsen and Ringø 1997; Tocher 2003; Mourente et al. 2007; Tocher et al. 2008). In general, the pathways in fish are similar to those in mammals.

Digestion and Absorption

Lipid digestion occurs largely in the proximal intestine and, if present, the pyloric caeca with the main digestive enzymes, TAG lipases, and phospholipases, which are assumed to be secreted by the pancreas or hepatopancreas. The primary products of lipid digestion are free fatty acids, partial acylglycerols mainly 2-monoacylglycerols, lyso-phospholipids, cholesterol, and fatty alcohols. The hydrolysis products are solubilized/emulsified in bile salt micelles, diffuse to the intestinal mucosa, and then taken up by passive diffusion into enterocytes where free fatty acids are predominantly reesterified with glycerol, partial acylglycerols, and lyso-phospholipids to reform TAG and phospholipids. Steryl esters may reform, but cholesterol is mainly transported from the mucosal cells unesterifed, and fatty alcohols from wax ester hydrolysis are oxidized to the corresponding fatty acid in the intestine. These fundamental physical and biochemical processes of lipid digestion and absorption are generally similar to those in other vertebrates.

Lipid Transport

Free fatty acids can be transported between tissues bound to albumin-like proteins in the blood of many species of fish. However, most lipids are transported as serum lipoproteins, which vary in the relative proportions of lipid and protein similar to those in mammals. Lipoproteins with a high lipid: protein ratio, such as chylomicrons, and very-low-density lipoproteins (VLDL) therefore transport lipid away from the intestine and liver, respectively. Extraction of lipids from VLDL at peripheral tissues results in the formation of low-density lipoprotein (LDL) and then high-density lipoprotein (HDL), which have progressively lower lipid: protein ratios. The proportions of the different serum liporoteins vary between fish species and with sexual maturity. During vitellogenesis, females produce another lipoprotein, vitellogenin, specifically to deliver lipid for oocyte development. Excess dietary lipid in fish is typically

deposited in adipose cells/tissue, although the precise corporeal locations vary with species. Most species have intraperitoneal (intestinal) adipose tissue, and some have significant subcutaneous lipid deposits between the skin and flesh. However, considerable lipid is also deposited in the flesh in "oily" fish such as herring and salmon; in other species, such as cod and halibut, lipid is stored predominantly in the liver. Within cells, fatty acid transport is facilitated by low-molecular-weight tissue-specific cytoplasmic fatty-acid-binding proteins (FABP) that bind long-chain fatty acids and other hydrophobic ligands.

Lipogenesis

The carbon source for the endogenous biosynthesis of new lipid via lipogenesis is acetyl-CoA, which is produced in mitochondria by the oxidative decarboxylation of pyruvate (carbohydrate source) or from the oxidative catabolism of some amino acids (protein source). The primary pathway of lipogenesis is fatty acid synthesis, catalyzed by the cytosolic, multienzyme fatty acid synthetase (FAS) complex, which produces saturated fatty acids, mainly 16:0 and 18:0. The key step is the condensation of malonyl-CoA, formed by acetyl-CoA carboxylase, with the growing acyl chain. The reducing equivalents NADPH, required for lipogenesis, are produced by enzymes in pathways of carbohydrate metabolism including the pentose phosphate pathway (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase), tricarboxylic acid cycle (NADP-dependent isocitrate dehydrogenase), and malic enzyme. The relative importance of the different sources of NADPH varies with species. Monounsaturated fatty acids, such as 18:1n-9 and 16:1n-7, are produced through the activity of microsomal Δ9 stearoyl-CoA desaturase (SCD). Longer-chain saturated and monounsaturated fatty acids, such as 20:0 and 20:1n-9, are produced by the action of fatty acyl elongases (Elovl or elongation of very long fatty acids). Monounsaturated fatty acids cannot be desaturated further because PUFA cannot be synthesized *de novo* by any vertebrate; therefore, PUFA must be obtained in the diet. However, dietary PUFA can be further elongated and desaturated by the action of specific fatty acyl desaturase (Fads) and Elovl enzymes (Fig. 3.4). The other main lipogenic

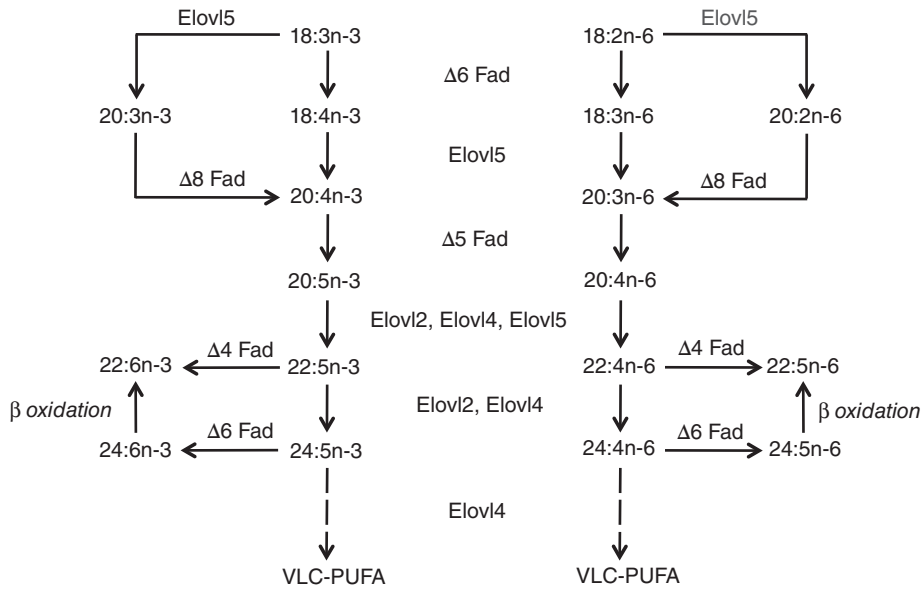

Figure 3.4 Possible biosynthetic pathways for long-chain polyunsaturated fatty acids (LC-PUFA) and very-long chain PUFA (VLC-PUFA; $C_{26}-C_{36}$) in fish. Evidence suggests that the same Δ6 Fad operates on both C_{18} and C_{24} fatty acyl substrates. The precise pathways operating in a particular species depend upon the genome complement of Fad and Elovl genes, and so not all steps are possible in all fish species. Elovl2, Elovl4, and Elovl5, PUFA elongases; Δ6, Δ5, and Δ4 Fad, front-end fatty acyl desaturases.

process is esterification. Fatty acids are therefore esterified to form complex lipids, including membrane phospholipids and TAG. The lipogenic pathways in fish are essentially the same as in mammals.

Fatty Acid Oxidation

Whereas fatty acid biosynthesis occurs in the cytosol, the oxidative catabolism of fatty acids, which is the major source of energy in many species of fish, occurs in mitochondria and peroxisomes. The process, termed β-oxidation, involves the sequential cleavage of two-carbon acetyl-CoA units through a cyclic series of reactions catalyzed by discrete enzyme activities rather than by a multienzyme complex, as in lipogenesis. Fatty acids are transported into the mitochondrion in the form of fatty acylcarnitine esters formed through the action of carnitine palmitoyl (acyl) transferase (CPT-1); they are converted by CPT-II inside the mitochondrion back to fatty acyl-CoA, and then undergo a cycle of dehydrogenation, hydration, second hydrogenation, and cleavage reactions to produce acetyl-CoA and NADH. A major fate of acetyl-CoA is catabolism in the tricarboxylic acid cycle to produce more NADH, which then serves as an electron donor to the electron transport chain driving ATP synthesis through oxidative phosphorylation. The relative oxidation rates of different fatty acids, as determined through *in vitro* studies, has shown the following orders of preference: saturated/monounsaturated > PUFA > LC-PUFA, shorter chain > longer chain, and n-6 > n-3. Despite this preference hierarchy, studies of fatty acid deposition *in vivo* generally show that the higher the dietary concentration of a fatty acid, the lower its relative deposition, implying increased oxidation. The conclusion is that fatty acid oxidation is a balance between substrate fatty acid concentrations (competition) and enzyme specificities. A possible exception to this is that some long-chain n-3 fatty acids (e.g. DHA) appear to be retained in tissues, independent of dietary concentrations. This possibly occurs as a result of the Δ4 double bond in DHA, which requires peroxisomal oxidation to be removed, resulting in poor oxidation of this fatty acid in mitochondria. However, even DHA can have relatively low retention when fed in high concentrations (Stubhaug et al. 2007; Glencross and Rutherford 2011).

Lipid Peroxidation

Lipids, particularly double bond-rich PUFA, are highly susceptible to attack by oxygen and organic radicals. The resultant damage to PUFA in phospholipids can have serious consequences for cell membrane structure and fluidity, with potential pathological effects on cells and tissues (Sies 1991). Fish lipids that are rich in LC-PUFA, specifically membrane phospholipids, are therefore protected from peroxidation by endogenous systems including enzymes such as catalase, glutathione peroxidase, glutathione S-transferase, and glutathione reductase, as well as antioxidant compounds that include glutathione, vitamins E and C, and possibly carotenoid pigments. The processes of peroxidation and antioxidant protection are complex and beyond the scope of this chapter, but are similar to those in other vertebrates (Yu 1994; Jacob 1995) and described in more detail elsewhere (Sargent et al. 2002; Tocher 2003; Martınez-Alvarez et al. 2005).

Functional Roles

Functions of Fatty Acids

Energy

Lipids, specifically TAG, represent the densest form of energy supply ($38.5\,kJ\,g^{-1}$), providing around twice as much as protein ($23.6\,kJ\,g^{-1}$) or carbohydrate ($17.3\,kJ\,g^{-1}$); lipid is therefore the most efficient nutrient for maximizing both energy intake and storage (Bureau et al. 2002). As the major constituents of lipids, fatty acids are the most important chemical form of energy storage in the body. The extent to which a specific fatty acid is used for energy is partly dependent upon its dietary concentration, with the higher the dietary concentration, the higher the oxidation (Tocher 2003). Possible exceptions include EPA and especially DHA, which tend to be preferentially retained, and 22:1n-11 which tends to be preferentially oxidized irrespective of dietary concentration (Sargent et al. 2002). It has been suggested that this phenomenon can be exploited to provide an "omega-3 sparing effect" in fish through optimizing dietary saturated/monounsaturated fatty acid levels and EPA/DHA ratio carefully to maximize n-3 LC-PUFA retention (Turchini et al. 2011a;

Codabaccus et al. 2012). Fatty acids and lipids have particularly important roles as energy sources in fish reproduction, including spawning migrations and embryonic and larval development, and are covered in more detail in other reviews (Sargent et al. 2002; Tocher 2003).

Structural

Fatty acids also play key roles in cell structure as they are integral constituents of phospholipids, which are the fundamental components of the lipid bilayer of cellular membranes (Gylfason et al. 2010). The fatty acid composition of membrane phospholipids affects the physical properties of the membrane (fluidity, etc.) and can also affect the function of membrane-associated proteins (carriers, receptors, enzymes, etc.) and processes. Changes in membrane phospholipid fatty acid composition are therefore important in homeostatic mechanisms (Farkas et al. 2001); adaptation to lower water temperature and increased hydrostatic pressure result in increased proportions of PUFA and monoenes and reduced saturated fatty acids (Tocher 2003). However, redistribution of fatty acids between phospholipid classes and within *sn* positions can also affect physical properties of membranes without gross changes in fatty acid composition (Farkas et al. 1994; Farkas and Halver 1996). The specificities of acyltransferase enzymes may also be altered, with temperature contributing to membrane restructuring (Tocher 1995). The key role that some specific fatty acids can play in membrane structure and function is highlighted by the critical role of DHA in neural membranes (Feller 2008; Wasall and Stillwell 2008). Several studies have demonstrated the important role of DHA in the development of neural tissues in larval fish. Specifically, dietary deficiency of DHA resulted in reduced ability to capture prey at low light intensities in larval Atlantic herring, *Clupea harengus* (Bell et al. 1995a); compromised schooling behavior in yellowtail, *Seriola quinqueradiata* (Masuda et al. 1998; Ishizaki et al. 2001) and Pacific threadfin *Polydactylus sexfilis* (Masuda et al. 2001); delayed response to visual stimuli in larval gilthead sea bream, *Sparus aurata* (Benitez-Santana et al. 2007); and impaired feeding behavior in Asian sea bass/barramundi, *Lates calcarifer* (Glencross and Rutherford 2011).

Metabolic

Relatively small amounts of specific PUFA are required in the diet to support optimal growth and to prevent mortality and a range of other pathologies, suggesting that these fatty acids have critical functional roles in controlling and regulating cellular metabolism and animal physiology (Tacon 1996; Glencross 2009). Examples include the highly biologically active compounds, generically termed eicosanoids and including prostaglandins (PG), leukotrienes (LT), and lipoxins, which are produced in cells of most tissues by the regulated dioxygenase-catalyzed oxidation of ARA, EPA, and some other LC-PUFA (Fig. 3.5; Schmitz and Ecker 2008). Eicosanoids function as short-lived autocrine hormones in the immediate vicinity of the cells and have a wide range of physiological actions important in the control and regulation of blood clotting and cardiovascular tone, reproduction, renal, and neural function, and immune and inflammatory responses (Schmitz and Ecker 2008). There is competition between the fatty acid precursors for the cyclooxygenase (COX) and lipoxygenase (LOX) enzymes that produce PG and LT, respectively, with proinflammatory 2-series PG and 4-series LT being produced from ARA (Fig. 3.5), and less potent or relatively antiinflammatory 3-series PG and 5-series LT produced from EPA (Tocher 1995). Inflammation is an essential protective response to injury and infection, and this is reflected in the fact that ARA is the primary precursor for eicosanoids despite EPA genererally predominating in fish tissue lipids (Tocher 1995). However, excessive or inappropriate inflammation, driven by ARA-derived eicosanoids, results in or contributes to various acute and chronic pathologies (see ‘Effects of Diet’ and ‘Lipids, Fatty Acids, and Health’).

Gene Regulation

Lipids, including fatty acids and particularly PUFA, play key roles in lipid homeostasis through the regulation of gene transcription. Several lipids act as ligands for adopted orphan nuclear receptors that function as heterodimers with the retinoid X receptor (RXR), including oxysterols and liver X receptors (LXR), bile acids and farnesoid X receptor (FXR), and fatty acids and peroxisome proliferator-activated receptors (PPAR) (Chawla et al. 2001). However, PUFA can

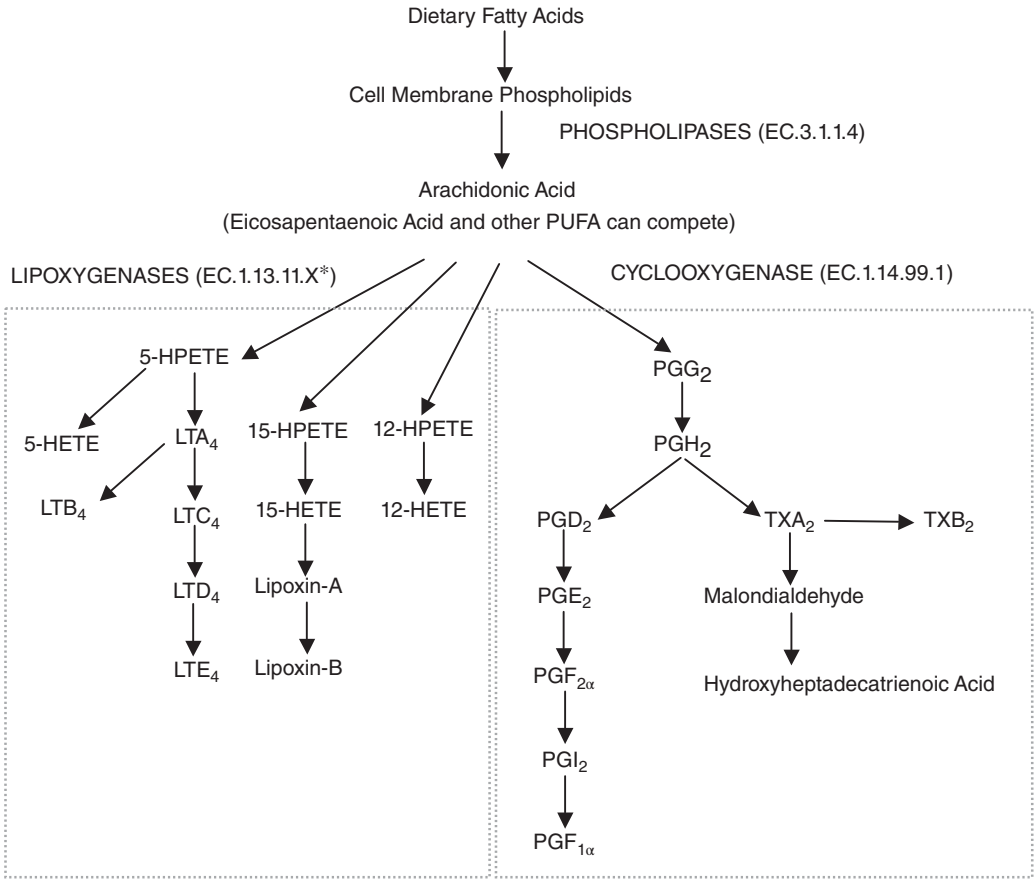

Figure 3.5 Synthesis of eicosanoids from arachidonic acid. Regulation in their synthesis occurs primarily via competition among the polyunsaturated fatty acids (PUFA) for access to the cyclooxygenase and lipoxgenase enzymes. Adapted from Hwang (1989). HPETE: hydroxyperoxyeicosatetraenoic acid; HETE: hydroxyeicosatetraenoic; LTA_4: Leukotriene A_4; LTB_4: Leukotriene B_4; LTC_4: Leukotriene C_4; LTD_4: Leukotriene D_4; LTE_4: Leukotriene E_4; PGG_2: Prostaglandin G_2; PGE_2: Prostaglandin E_2; $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$; PGI_2: Prostaglandin I_2; PGH_2: Prostaglandin H_2; PGD_2: Prostaglandin D_2; $PGF_{1\alpha}$: Prostaglandin $F_{1\alpha}$; TXA_2: Thromboxane A_2; TXB_2: Thromboxane B_2.

potentially affect gene expresssion by a number of other mechanisms including changes in membrane composition and eicosanoid production (Jump 2002). Lipid metabolic pathways are also regulated by both cholesterol and PUFA through sterol regulatory element binding protein (SREBP) transcription factors (Sampath and Ntambi 2005). These nuclear receptors and transcription factors appear to have generally similar roles in the regulation of lipid and fatty acid metabolism in fish as they have in mammals (Leaver et al. 2008a; Cruz-Garcia et al. 2009; Minghetti et al. 2011).

Functions of Phospholipids

Structural

Phospholipids have an amphipathic structure consisting of a polar hydrophilic "head" and a hydrophobic "tail," which is key to their roles as structural components of cell membranes and lipoproteins. The different phospholipid classes are asymmetrically distributed in mammalian cell membranes, with choline-containing phospholipids, PC, and sphingomyelin concentrated in the outer leaflet, and amine-containing PE and PS concentrated in the

inner leaflet; this also appears to be the case in fish membranes (Kagan et al. 1984). Membrane phospholipid compositions in fish are regulated in response to environmental factors as part of a homeostatic mechanism maintaining fluidity (Hochachka and Mommsen 1995). In cold acclimation, the proportions of PE increase and that of PC decrease in response to reduced temperature (Tocher 1995). In plasma lipoproteins, lipid/water interfaces are formed by phospholipids, along with cholesterol and proteins that enable hydrophobic lipids such as TAG and steryl esters to be transported in the aqueous environment of the blood (Tocher 1995).

Metabolic

Phospholipids and molecules derived from phospholipids also have important roles in metabolism as intra- and intercellular lipid mediators involved in key signaling pathways. Although phospholipid metabolism is poorly studied in fish, evidence suggests that many of these pathways occur in fish and that the phospholipid-derived mediators play roles similar to those in mammals (Tocher 1995, 2003).

Phosphoinositides and Protein Kinase C

In mammals, the phospholipid PI is the precursor of a range of intracellular mediators collectively termed phosphoinositides, which include phosphorylated derivatives such as PIP_2 that have important roles in regulating ion channels and transport proteins involved in golgi/lysosome/endosome trafficking, cell proliferation, survival, and migration (Gamper and Shapiro 2007; Hirsch et al. 2007). Phosphorylated PI derivatives are also sources of other intracellular secondary messengers, such as DAG and inositol trisphosphate (IP_3) formed by the cleavage of PIP_2 by phospholipase C-b in response to various stimuli (Berridge 2005; Michell 2007). Inositol trisphosphate stimulates calcium mobilization from the endoplasmic reticulum; increased intracellular Ca^{2+} and DAG are, in turn, activators of a threonine/serine kinase, protein kinase C (PKC), which is an important regulator of metabolism (Gomez-Fernandez and Corbalan-Garcia 2007). Additionally, DAG can influence a range of biological responses through other proteins, including phospholipases, and modulate other membrane associated-proteins and processes simply by altering the biophysical properties of membranes (Gomez-Fernandez and Corbalan-Garcia 2007). Acidic lipids, particularly the phospholipid PS, also have a metabolic role as activators of PKC, along with DAG and Ca^{2+} ions (Newton 2009). Although these signaling pathways are generally poorly understood in fish, inositol phospholipid metabolism was investigated in electrocytes from the electric ray, *Discopyge tschudii*; PS-activation of PKC was studied in rainbow trout and dogfish, *Scyliorhinus canicula*; and PKC was implicated in the stimulation of steroidogenesis in goldfish, *Carassius auratus* (Tocher et al. 2008).

Eicosanoids

Membrane phospholipids are the source of LC-PUFA substrates for the synthesis of eicosanoids (see above) through the activity of phospholipase A_2 enzymes, whose activation results in the release of PUFA from the *sn*2 position. The principal enzyme in mammals is type IV cytosolic phospholipase A_2 ($cPLA_2$), which is specific for phospholipid molecules containing ARA (or EPA) at the *sn*2 position. However, little is known about the specificity of phospholipase enzymes in fish or crustaceans (Tocher 1995, 2003).

Platelet-Activating Factor

An ether analogue of PC, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine or platelet-activating factor (PAF), is a potent intercellular mediator of leukocyte functions such as platelet aggregation, inflammation, and anaphylaxis (Snyder 1990). It is produced by inflammatory cells via acetylation (utilizing acetyl-CoA) of lyso-PAF by lyso-PAF acetyltransferase, and degraded through the action of phospholipase A_2-like enzymes, PAF acetylhydrolases. PAF biosynthesis and degradation have been demonstrated in fish (Tocher et al. 2008). In mammals, reacylation of lyso-PAF is highly specific for ARA and, in turn, 1-alky-2-arachidonyl-glycerophosphocholine is substrate for the synthesis of PAF via an ARA-specific phospholipase A_2 that also produces ARA for eicosanoid synthesis (Snyder 1990). However, the relationship between PAF metabolism

and eicosanoid synthesis has not been studied in fish.

Other Intercellular Lipid Mediators and G-Protein Coupled Receptors

Phosphatidic acid (PA), the base diacyl phospholipid, plays several roles in cellular metabolism. In addition to being a key intermediate in the biosynthesis of many other lipids and phospholipids, its unique biophysical properties, with anionic phosphate head group linked as a phosphomonoester, are central to its functional roles in both membrane/vesicle fission and fusion, as well as protein recruitment (Kooijman and Burger 2009).

Although it can be rapidly converted to DAG, it is now accepted that PS itself is likely an important intracellular signaling molecule involved in the regulation of cellular processes such as proliferation, differentiation, transformation, tumor progression, and survival signaling (Wang et al. 2006).

Many intercellular lipid mediators, including eicosanoids and PAF, are now known to function by interacting with G-protein-coupled receptors (GPCR) in the plasma membrane (Im 2009). Others lipid mediators that act via GPCR include lyso-PA and sphingosine-1-phosphate, which have essential roles in many cell processes (Makide et al. 2009). Several other lyso-phospholipids, including lyso-PS, lyso-PE, lyso-PI, lyso-phosphatidylglycerol, and lyso-phoshatidylthreonine may also function as lipid mediators, but their roles are not fully understood (Makide et al. 2009). Recently, various other lipids have been recognized as possible GPCR-linked intercellular signaling mediators; these include fatty acids, bile acids, resolvin E1, and acylethanolamides such as the endogenous cannabinoid, arachidonylethanolamine, or anandamide (Im 2009). Currently, little is known about these signaling mechanisms in fish, although it is possible that many of the lipid mediators will be important regulators in all vertebrates.

Functions of Cholesterol

Cholesterol has important structural, functional, and metabolic roles that are likely to be very similar in fish and shellfish to that in mammals. Cholesterol therefore has a major structural role as a critical component of cell membranes, reducing fluidity and permeability of the plasma membrane to both protons and sodium ions (Lange and Steck 2008). Membrane cholesterol has high affinity to sphingolipids, which partly regulate its distribution between cellular compartments (Ikonen 2008). In addition, membrane cholesterol has several functional roles including cell signaling through the formation of lipid rafts, intracellular transport as an important component in caveola-dependent and clathrin-dependent endocytosis, and nerve conduction as a major constituant of the myelin sheath (Simons and Ikonen 2000).

Depending upon tissue, cholesterol is also the precursor of various metabolically active molecules. In adrenal/interrenal glands and gonads, cholesterol is the precursor of steroids, nuclear hormones that have both genomic, affecting gene transcription, and more rapid non-genomic effects (Wehling 1997). Adrenal steroids include: mineralocorticoids, such as aldosterone and 11-deoxycorticosterone, which regulate water and electrolyte balance including sodium and potassium retention and excretion; and glucocorticoids such as cortisol, corticosterone, and cortisone, which are involved in the regulation of carbohydrate and protein metabolism and, in the case of cortisol, serve an important role in stress responses (see 'Effects of Diet'; Jobling 1994). Gonadal steroids, including estrogens (e.g., estradiol and progesterone), androgens (e.g., testosterone), and their derivatives have roles in sex differentiation, gametogenesis, and the control of reproduction including vitellogenesis (Jobling 1994). In addition, cholesterol is the substrate for ecdysterone, a key hormone involved in regulating the molting process in crustacea. In liver, cholesterol is the precursor for bile acid synthesis via cytochrome P450-mediated oxidation catalyzed by cholesterol-7α hydroxylase (Moschetta et al. 2005). Bile acids such as cholate are then conjugated, for instance with taurine, before storage in the gallbladder as the major components of bile, which is secreted into the intestine to aid emulsification of dietary lipids and fat-soluble vitamins (Olsen and Ringø 1997). In skin, UV radiation converts 7-dehydrocholesterol, the cholesterol precursor, into the secosteroid cholecalciferol or vitamin D_3, which is the precursor of the active form of vitamin D_3 (Halver 2002).

Lipid and Fatty Acid Requirements

Lipid Requirement

A dietary lipid requirement cannot be defined for any species, as it is influenced by several other nutritional factors. Lipid is principally a source of energy so the amount required in the diet will depend upon the contents of other dietary energy sources, such as protein and carbohydrate. However, as protein (flesh) deposition is the primary objective in animal production, including aquaculture, and protein sources are usually the most costly feed ingredients, the aim is to minimize the use of dietary protein for energy. With an appropriate amount of energy supplied by lipids or carbohydrates, use of protein for energy can therefore be reduced or "spared." Carbohydrates can only be efficiently digested and utilized by some species (often herbivorous/omnivorous), whereas other species have limited capabilities of digesting carbohydrates, restricting their viability as an energy source. The amount of lipid that can be effectively included in a diet for any particular species is also limited by the capacity of the digestive system of that species to emulsify and digest lipid. When lipids are included at too high a level, their emulsification and digestion are impaired and the lipid is poorly utilized and excess is excreted (Glencross et al. 2002a). There are distinct differences among fish and crustacean species in their capacities to deal with high dietary lipid levels.

The amount of dietary lipid required is also influenced by the need to satisfy EFA requirements (see below). Therefore, although an "optimum" level cannot be defined, there is a range within which dietary lipid should or can be supplied. Within this, the lower limit will largely be dictated by the amount required to supply EFA requirements, which will depend upon the lipid source(s) and their fatty acid profiles. Early life stages of some species may have a dietary requirement for cholesterol and/or phospholipid that would also have to be included. Certain physiological stages, such as reproduction (migration, spawning, etc.) in some species, may also demand higher dietary lipid levels to support deposition and accumulation of lipid stores. Higher growth rates can be achieved by increasing dietary lipid above this minimum level, probably as a result of protein sparing; however, there will also be an upper limit that, if exceeded, will result in unwanted deposition of lipid in the tissues due to an imbalance of nutrient and energy supply (Company et al. 1999; Craig et al. 1999; Gaylord and Gatlin 2000). Deposited lipid contributes to increased weight in many species, but not to yield. Atlantic salmon, *Salmo salar*, are a notable exception, as a significant amount of lipid is deposited in the flesh. This means that salmon are able to tolerate and utilize higher dietary lipid levels, but excess lipid deposition can still cause problems for fish processors (Sargent and Tacon 1999). With most species, excess dietary lipid deposited in the peritoneal cavity or liver (e.g., Atlantic cod, *Gadus morhua*) represents wasted energy, as it is inefficient to supply nutrients that are essentially not used.

The relationships between dietary lipid contents, growth, and lipid deposition have been investigated in various fish species and upper limits, at least, are beginning to be defined relative to nutrient (e.g., amino acid) supply. Weight gain was increased in brown trout, *Salmo trutta*, and rainbow trout as dietary lipid increased up to 29% (Arzel et al. 1993; Luzzana et al. 1994) and, similarly, weight gain in Atlantic salmon was higher in fish fed diets containing up to 47% lipid compared to fish fed 31% lipid (Hemre and Sandnes 1999). Consequently, the upper level for dietary lipid in salmon feeds has increased over the years, and Einen and Roem (1997) suggested an "optimal" dietary lipid level of 35%. Although so-called "high-energy" (high-lipid) feeds have become widespread in aquaculture, increased flesh lipid levels have been reported in freshwater fish and salmonids including rainbow trout (Dias et al. 1999) and Atlantic salmon (Bell et al. 1998; Hemre and Sandnes 1999) when the protein: energy balance of the diet is inappropriate for the size of fish being fed. In salmon, increased flesh lipid levels can alter lipid and fatty acid metabolism with health and welfare implications, and may influence fish physiology such as inducing early sexual maturation in males (Sargent and Tacon 1999; Shearer and Swanson 2000). Excessive flesh lipid also affects carcass and product qualities, including texture and pigmentation, which has negative impacts on both processors and consumers (Bell et al. 1998; Hillestad et al. 1998); however, this can be alleviated by feeding a low-fat "finishing" diet prior to harvest (Rasmussen et al. 2000).

Compared to salmonids, particularly Atlantic salmon, there is a lower limit to the growth-promoting

effect of high-fat diets in marine fish species (Borges et al. 2013). Although weight gain increased with increasing dietary lipid up to about 24% in European sea bass, *Dicentrarchus labrax*, growth rate then declined in fish fed 30% lipid (Manuel Vergara et al. 1996; Lanari et al. 1999; Peres and Oliva-Teles 1999). In contrast, work with barramundi (Asian sea bass) demonstrated that high growth performance could also be achieved provided that the use of high lipid (30%) diets was applied to fish of the appropriate size (and the appropriate protein to energy ratio was not too low) and surpassed growth of fish fed diets with lower (10% and 20%) lipid levels (Glencross et al. 2008). However, increased dietary lipid levels were reflected in increased tissue lipid contents in turbot, *Psetta maximus* (Saether and Jobling 2001) and small Asian sea bass (Catacutan and Coloso 1995), indicating that high-fat diets may also promote the development of fatty liver pathology (Caballero et al. 1999). Of course, global finfish aquaculture production is dominated by freshwater species that inhabit a predominantly low trophic level, including carps and tilapia (Tacon et al. 2010). The natural diets of these species generally contain lower levels of lipid and, possibly, higher levels of carbohydrates, which the fish are adapted to effectively and efficiently utilize. As a result, these species cannot tolerate or utilize high dietary lipid, so commercial feeds typically contain less than 10% dietary lipid (Lim et al. 2011).

Similar to low trophic level fish species, shrimp and other crustaceans appear to have a limited ability to utilize dietary lipid (Glencross et al. 2002a, b). Highest weight gain responses were usually achieved with feeds containing 6–8% dietary lipid, whereas higher levels often retarded growth, most likely due to inefficient digestion and/or reduced consumption caused by the high caloric content (Kanazawa et al. 1977; Davis and Robinson 1986; Sheen and D'Abramo 1991; Glencross et al. 2002a).

Essential Fatty Acids

All vertebrate and crustacean species have a dietary requirement for specific n-3 and n-6 fatty acids (EFA), as they cannot synthesize PUFA from monounsaturated fatty acids *de novo*. The essentiality of PUFA reflects their critical metabolic and physiological roles (see above) and, in this respect, it should be noted that the biologically active PUFA are primarily the LC-PUFA, ARA, EPA, and DHA (Das 2006). The C_{18} PUFA, typified by linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), have no specific metabolic roles in fish and can therefore function as EFA only as precursors for n-6 and n-3 LC-PUFA (Sargent et al. 1995a; see Fig. 3.4). However, many species cannot produce C_{20} and C_{22} LC-PUFA from C_{18} precursors and instead require LC-PUFA in the diet, and so C_{18} PUFA cannot satisfy EFA requirements. In species that have the capacity to convert C_{18} PUFA to LC-PUFA, both C_{18} PUFA and C_{20-22} LC-PUFA can be termed EFA, although the LC-PUFA is often more effecient. No vertebrate or crustacean species are able to interconvert the n-6 and n-3 PUFA families. Definition of EFA requirements for normal growth and development has been a well-studied area of fish nutrition, driven by the needs of the aquaculture industry that involve determining the absolute requirements of specific PUFA, the optimal balance between different PUFA, and how the requirements vary with ontogenesis. The area has been well covered by recent articles containing thorough reviews of the literature pertaining to dietary requirements (Glencross 2009; Tocher 2010). The following section summarizes the main points. In discussing EFA requirements, it is still convenient to subdivide fish into freshwater and marine species, although this may change as more species are studied.

Freshwater and Diadromous Fish Species

The EFA requirements of juvenile and subadult freshwater and diadromous species can usually be satisfied by the C_{18} PUFA, 18:3n-3, and 18:2n-6 at about 1% of diet dry weight (Table 3.1). It is likely that all freshwater/diadromous fish require both n-3 and n-6 PUFA, but coldwater species including salmonids may have a higher requirement for 18:3n-3 while warmwater species such as tilapia, *Oreochromis* sp., may have a higher requirement for 18:2n-6, although tilapia do require n-3 PUFA for maximal growth (Lim et al. 2011). In some species, n-3 LC-PUFA can satisfy EFA requirements at lower levels than 18:3n-3 and growth can be improved by dietary n-3 LC-PUFA (Santha and Gatlin 1991; Chou and Shiau 1999; Ruyter et al. 2000a). A specific requirement for DHA has been identified in the diadromous barramundi, but a critical requirement for EPA was

Table 3.1 Reported essential fatty acid (EFA) requirements of freshwater fish and crustaceans (J: juvenile; L: larvae). Source: Adapted from Tocher, D. R., D. S. Francis and K. Coupland. 2010. n-3 Polyunsaturated fatty acid-rich vegetable oils and blends. Pp. 209–244 in Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds, G. M. Turchini, W.-K. Ng and D. R. Tocher, eds. Boca Raton: Taylor & Francis, CRC Press.

Species	Latin name	Stage	EFA	Requirement (% dry weight)	Reference
Arctic char	*Salvelinus alpinus*	J	18:3n-3	1.0–2.0	Yang et al. 1994
Atlantic salmon	*Salmo salar*	J	18:3n-3	1.0	Ruyter et al. 2000b
		J	n-3 LC-PUFA	0.5–1.0	Ruyter et al. 2000a
Ayu	*Plecoglossus altivelus*	J	18:3n-3 or EPA	1.0	Kanazawa et al. 1982
Channel catfish	*Ictalurus punctatus*	J	18:3n-3	1.0–2.0	Satoh et al. 1989
Cherry salmon	*Oncorhynchus masou*	J	18:3n-3 or n-3 LC-PUFA	1.0	Thongrod et al. 1990
Chum salmon	*Oncorhynchus keta*	J	18:2n-6 and 18:3n-3	1.0 of each	Takeuchi et al. 1979
Coho salmon	*Oncorhynchus kisutch*	J	18:2n-6, 18:3n-3	1.0 of each	Yu and Sinnhuber 1979
Common carp	*Cyprinus carpio*	J	18:2n-6	1.0	Takeuchi and Watanabe 1977
		J	18:3n-3	0.5–1.0	Takeuchi and Watanabe 1977
		L	n-6 PUFA	1.0	Radunzneto et al. 1996
		L	n-3 PUFA	c. 0.05	Radunzneto et al. 1996
Eel	*Anguilla japonicus*	J	18:2n-6 and 18:3n-3	0.5 of each	Takeuchi et al. 1980
Grass carp	*Ctenopharyngodon idella*	J	18:2n-6	1.0	Takeuchi et al. 1991
		J	18:3n-3	0.5	Takeuchi et al. 1991
Milkfish	*Chanos chanos*	J	18:2n-6 and 18:3n-3	0.5 of each	Bautista and de la Cruz 1988
Rainbow trout	*Oncorhynchus mykiss*	J	18:3n-3	0.7–1.0	Castell et al. 1972a, b
		J	n-3 LC-PUFA	0.4–0.5	Takeuchi and Watanabe 1976
		L	DHA essential	?*	Wirth et al. 1997
Sheatfish	*Silurus glanis*	J	18:3n-3	1.0	Borgut et al. 1998
Striped bass	*Morone chrysop* x *M. saxatilis*	J	n-3 LC-PUFA	1.0	Gatlin et al. 1994
Tilapia	*Tilapia zilli*	J	18:2n-6	1.0	Kanazawa et al. 1980
	Oreochromis nilotica	J	18:2n-6	0.5	Takeuchi et al. 1983
	O. nilotica x O. aureus	J	n-3 required	?	Chou and Shiau, 1999
Whitefish	*Coregonus laveratus*	J	18:3n-3	>1.0	Thongrod et al. 1989
			n-3 LC-PUFA	0.5–1.0	Watanabe et al. 1989
Crustaceans					
Giant river prawn	*Macrobrachium rosenbergii*	J	ARA	0.08	D'Abramo and Sheen 1993
		J	DHA	0.075	D'Abramo and Sheen 1993
		J	18:3n-3:18:2n-6	0.083 (ratio)	Teshima et al. 1994

*A question mark in the 'Requirement (% dry weight)' column indicates that the nutrients were reported to be required, but that this requirement could not be quantified.

also determined irrespective of the inclusion of DHA (Glencross and Rutherford 2011). Studies examining the manipulation n-3 and n-6 fatty acids in the diet of this species also suggest that the animal responds to provision of both fatty acid families and can use these when provided as short-chain PUFA (Williams et al. 2006). Requirements of freshwater fish for ARA or n-6 LC-PUFA are unknown (Bell and Sargent 2003). There are few data on the EFA requirements of early life stages of freshwater fish species, but n-3 LC-PUFA and DHA may be more important and essential in larvae compared to adults or juveniles of some species (Webster and Lovell 1990; Wirth et al. 1997). Broodstock nutrition affects egg fatty acid compositions in freshwater species (Santiago and Reyes 1993; Abi-ayad et al. 1997), and is critical for producing high-quality eggs with EFA optimized for the requirements of developing embryos and larvae (Tandler et al. 1995; Izquierdo et al. 2001; Quintero et al. 2011).

Marine Fish Species

The C_{18} PUFA cannot satisfy EFA requirements of juvenile and subadult marine fish, and so the n-3 LC-PUFA, EPA, and DHA are required (Table 3.2). For many species, n-3 LC-PUFA of around 1% of diet dry weight can meet the requirements, although levels above 1% appear to be required by some species. The quantitative requirement for n-6 LC-PUFA has not been fully determined, but studies suggest ARA is essential (around 0.3% of diet dry weight) in weaned turbot (Castell et al. 1994; Bell et al. 1995b). Quantitative EFA requirements of marine fish can vary with the dietary DHA: EPA ratio, consistent with DHA generally having a higher EFA value than EPA (Kalogeropoulos et al. 1992; Watanabe 1993; Ibeas et al. 1994; Brinkmeyer and Holt 1998; Wu et al. 2002).

Defining the EFA requirements of larval marine fish is hampered by their small size, poorly developed digestive system, difficulties of preparing microdiets, and the use of live feeds; values can also vary with dietary lipid level and the criteria measured, such as growth and survival (Salhi et al. 1994; Furuita et al. 1996a; Izquierdo et al. 2000; Cahu and Zambonino-Infante 2001; Koven et al. 2001a; Robin and Vincent 2003; Kvale et al. 2006; Conceição et al. 2007, 2010; Yufera and Darias 2007). Generally, larvae tend to have a higher requirement for n-3 LC-PUFA than juveniles and pre-adult fish, with the requirement decreasing as the DHA: EPA ratio increases (Rodriguez et al. 1994a, 1998a). The greater effect of DHA is possibly related to its role in visual and neural tissue development (Sargent et al. 2002). Dietary ARA improved growth in larval marine fish (Rodriguez et al. 1994a; Estevez et al. 1997; Bessonart et al. 1999), and improved survival after handling stress in sea bream larvae (Koven et al. 2001b), although it inhibited growth, increased mortality, and had negative effects on pigmentation in yellowtail flounder larvae (Ishizaki et al. 1998). Consequently, increasing attention has been paid to the role of EFA in metamorphosis, including pigmentation and eye migration, in marine flatfish (Lund et al. 2007, 2008). During premetamorphic stages the absolute and relative amounts of EFA, as well as duration of feeding, are important during critical periods, which vary among species. Generally, DHA and n-3 LC-PUFA were associated with promoting correct metamorphosis and pigmentation (Reitan et al. 1994; Estevez and Kanazawa 1996; Estevez et al., 1997, 1999; Villalta et al. 2005; Hamre and Harboe 2008a, b). Although there is evidence that dietary ARA is essential for optimal growth and development of marine fish larvae, precise requirements are not defined and excess can negatively affect metamorphosis in flatfish (Rodriguez et al. 1994a; Ishizaki et al. 1998; Bessonart et al. 1999; Estevez et al. 1999; Hamre et al. 2007; Lund et al. 2007, 2008).

Optimal broodstock nutrition is necessary to produce high-quality eggs in marine fish that contain sufficient and appropriate EFA contents to supply developing embryos and larvae at a time of increased requirement (Tandler et al. 1995; Izquierdo et al. 2001). Many studies have demonstrated that egg fatty acid compositions are affected by broodstock diets in marine species (Fernandez-Palacios et al., 1995; Silversand et al. 1995; Verakunpiriya et al. 1996; Bell et al. 1997; Vassallo Agius et al. 1998; Almansa et al. 1999), and egg quality criteria including hatching, fertilization rate, and early survival were positively correlated with increased egg n-3 LC-PUFA, ARA, and DHA: EPA ratio (Harel et al. 1992; Fernandez-Palacios et al. 1995; Pickova et al. 1997; Rodriguez et al. 1998b; Bruce et al. 1999; Salze et al. 2005).

Table 3.2 Reported essential fatty acid (EFA) requirements of marine species of fish and crustaceans. (L: larvae; EJ: early juvenile; J: juvenile; SA: sub-adult.) Source: Adapted from Tocher, D. R., D. S. Francis and K. Coupland. 2010. n-3 Polyunsaturated fatty acid-rich vegetable oils and blends. Pp. 209–244 in Fish Oil Replacement and Alternative Lipid Sources in Aquaculture Feeds, G. M. Turchini, W.-K. Ng and D. R. Tocher, eds. Boca Raton: Taylor & Francis, CRC Press.

Species	Latin name	Stage	EFA	Requirement (% dry diet)	Reference
Atlantic cod	*Gadus morhua*	L/EJ	EPA required	?	Zheng et al. 1996
		L/EJ	DHA	c. 1.0	Takeuchi et al. 1994
Barramundi	*Lates calcarifer*	J/SA	DHA	1.0	Glencross and Rutherford 2011
California halibut	*Paralichthys californicus*	L	DHA	1.21	Vizcaino-Ochoa et al. 2010
Cobia	*Rachycentron canadum*	J	DHA	0.8–1.2	Trushenski et al. 2012
European sea bass	*Dicentrarchus labrax*	J/SA	n-3 LC-PUFA	1.0	Coutteau et al. 1996b
Gilthead sea bream	*Sparus aurata*	J/SA	n-3 LC-PUFA	0.9 (DHA:EPA = 1)	Kalogeropoulos et al. 1992
		J/SA	n-3 LC-PUFA	1.9 (DHA:EPA = 0.5)	Ibeas et al. 1994
		J/SA	DHA:EPA	0.5	Ibeas et al. 1997
		L/EJ	n-3 LC-PUFA	5.5 (DHA:EPA = 0.3)	Rodriguez et al. 1994a
		L/EJ	n-3 LC-PUFA	1.5 (DHA:EPA = 2.0)	Rodriguez et al. 1998a
		L/EJ	n-3 LC-PUFA	1.5 (in phospholipid)	Salhi et al. 1999
		L/EJ	DHA:EPA	c. 2	Rodriguez et al. 1994b
Grouper	*Epinephelus malabaricus*	J/SA	n-3 LC-PUFA, DHA > EPA	1.0	Wu et al. 2002
Japanese flounder	*Paralicthys olivaceus*	J/SA	n-3 LC-PUFA	1.4	Takeuchi, 1997
Korean rockfish	*Sebastes schlegeli*	J/SA	n-3 LC-PUFA	0.9	Lee et al. 1993
		J/SA	EPA or DHA	1.0	Lee et al. 1994
Mahimahi	*Coryphaena hippurus*	L/EJ	n-3 LC-PUFA	0.6–1.0	Ostrowski and Kim 1993
Red drum	*Sciaenops ocellatus*	J/SA	n-3 LC-PUFA	0.5–1.0	Lochman and Gatlin 1993
		J/SA	EPA + DHA	0.3–0.6	Lochman and Gatlin 1993
Red sea bream	*Pagrus major*	J/SA	n-3 LC-PUFA or EPA	0.5	Yone 1978
		J/SA	EPA	1	Takeuchi et al. 1990
		J/SA	DHA	0.5	Takeuchi et al. 1990
		L/EJ	n-3 LC-PUFA	2.1 (with 1.0 as DHA)	Furuita et al. 1996a
		L/EJ	DHA	1.0–1.6	Furuita et al. 1996a
		L/EJ	EPA	2.3	Furuita et al. 1996a
Silver bream	*Rhabdosargus sarba*	J/SA	n-3 LC-PUFA	1.3	Leu et al. 1994
Starry flounder	*Platichthys stellatus*	J/SA	n-3 LC-PUFA	0.9	Lee et al. 2003
Striped bass	*Morone chrysop x M. saxatilis*	J/SA	n-3 LC-PUFA	1.0	Gatlin et al. 1994
		L/EJ	18:3n-3	?	Webster and Lovell 1990
		L/EJ	n-3 LC-PUFA	<0.5	Webster and Lovell 1990
Striped jack	*Pseudocaranx dentex*	J/SA	DHA	1.7	Takeuchi et al. 1992
		L/EJ	DHA	1.6–2.2	Takeuchi et al. 1996
		L/EJ	EPA	<3.1	Takeuchi et al. 1996

(*continued*)

Table 3.2 (*Continued*)

Species	Latin name	Stage	EFA	Requirement (% dry diet)	Reference
Turbot	*Psetta maxima*	J/SA	n-3 LC-PUFA	0.8	Gatesoupe et al. 1977
		J/SA	ARA	c. 0.3	Castell et al. 1994
		L/EJ	DHA required	?	Reitan et al. 1994
Yellowtail flounder	*Pleuronectes ferrugineus*	J/SA	n-3 LC-PUFA	2.5	Whalen et al. 1999
Yellowtail/ kingfish	*Seriola sp.*	J/SA	n-3 LC-PUFA	2.0–2.4	Deshimaru et al. 1982
Yellowtail	*Seriola quinqueradiata*	L/EJ	n-3 LC-PUFA	3.9 (DHA:EPA = 0.5)	Furuita et al. 1996b
		L/EJ	DHA	1.4–2.6	Furuita et al. 1996b
		L/EJ	EPA	3.7	Furuita et al. 1996b
Crustaceans					
Black tiger shrimp	*Penaeus monodon*	J	18:3n-3	1.2	Glencross and Smith 1999
		J	18:2n-6	1.2	Glencross and Smith 1999
		J	EPA	0.9	Glencross and Smith 2001
		J	DHA	0.9	Glencross and Smith 2001
		J	n-3:n-6	2.5 (ratio)	Glencross et al. 2002a
Blue shrimp	*Penaeus stylirostris*	J	n-3:18:2n-6	1.18 (ratio)	Fenucci et al. 1981
Brown shrimp	*Penaeus aztecus*	J	18:3n-3	1–2	Shewbart and Mies 1973
Common prawn	*Palaemon serratus*	J	18:3n-3:18:2n-6	0.45 (ratio)	Martin 1980
Fleshy prawn	*Penaeus chinensis*	J	18:3n-3	0.7–1.0	Xu et al. 1993
		J	DHA	1	Xu et al. 1994
		J	18:3n-3 > 18:2n-6	–	Xu et al. 1994
Karuma shrimp	*Penaeus japonicus*	J	EPA	1.1	Kanazawa et al. 1978
		J	DHA	1.1	Kanazawa et al. 1979b
		J	18:3n-3 > 18:2n-6	–	Kanazawa et al. 1977
Whiteleg shrimp	*Litopenaeus vannamei*	J	EPA or DHA	0.5	González-Félix et al. 2003
		J	ARA	0.5	González-Félix et al. 2003

Crustaceans

Although all crustaceans have an absolute requirement for specific PUFA and/or LC-PUFA, most data are for marine (penaeid) shrimps (prawns) (Table 3.2). Weight gains of *Penaeus japonicus* fed diets containing PUFA or LC-PUFA were higher than prawns fed 18:1n-9 alone, with effectiveness being in the following rank order: EPA > DHA > 18:3n-3 > 18:2n-6, with best growth achieved with either 1.0% (of diet dry weight) 18:2n-6 or 18:3n-3 in combination with n-3 LC-PUFA (Kanazawa et al. 1979a–c). The requirement for DHA in *Penaeus chinensis* was 1.0%, whereas the requirement for 18:3n-3 was between 0.7 and 1.0% (Xu et al. 1993, 1994). Other nutrient deletion studies

with marine shrimp demonstrated that LC-PUFA, particularly EPA, elicited significantly higher growth rates than PUFA, and DHA had the highest EFA activity in tiger shrimp, *Penaeus monodon* (Merican and Shim 1997). Further studies with *P. monodon* showed that addition of 1.2% of either 18:2n-6 or 18:3n-3, or 0.9% of either EPA or DHA, enhanced weight gain (Glencross and Smith 1999, 2001). Combined requirements for EPA and DHA were around one-third of the level required as exclusive sources, suggesting synergistic effects of EFA, and the ideal ratio of n-3 to n-6 PUFA was estimated at 2.5 (Glencross et al. 2002c), supporting early studies showing that best growth responses were elicited by a combination of n-3 and n-6 PUFA (Deshimaru et al. 1979). An evaluation of the requirement for ARA of *P. monodon* showed a progressive decline in growth with each incremental inclusion level (Glencross et al. 2001b). It was suggested that this was primarily a response to the alteration in the n-3 to n-6 PUFA balance, but it has been demonstrated that there was no apparent benefit to ARA inclusion for juvenile animals. In contrast, a dietary ratio of 18:3n-3: 18:2n-6 of under 0.5 was suggested for the common prawn *Palaemon serratus* (Martin 1980). Further studies suggested that EFA requirements were related to dietary lipid level, and that the proportion of EFA in dietary lipid was critical for satisfying EFA requirements rather than the absolute level (Glencross et al. 2002b). In contrast, the absolute requirement for LC-PUFA was not dependent upon dietary lipid levels in *Litopenaeus vannamei* (González-Félix et al. 2002, 2003). In summary, marine shrimp fed diets containing about 6.0% lipid require around 1.0% 18:2n-6, and 1.5% 18:3n-3 at a ratio of 0.7, along with 0.3% EPA and DHA, to achieve the highest growth rates (Glencross et al. 2002b).

There are few studies of freshwater shrimp, but the qualitative EFA requirements of the caridean shrimp, *Macrobrachium rosenbergii*, have been studied (D'Abramo and Sheen 1993; Table 3.1). Dietary DHA, ARA, and an EPA/DHA mixture all increased weight gain when supplemented to feeds containing 6% lipid, with ARA possibly showing greater EFA activity than n-3 LC-PUFA. Neither 18:2n-6 nor 18:3n-3 increased growth, yet they may be metabolized to their corresponding LC-PUFA, albeit at a rate insufficient to sustain the highest growth rates. Despite this, higher activity for 18:2n-6 was suggested and improved growth may be possible by adding both C_{18} PUFA to the diet, with an optimal n-6 to n-3 ratio above 1. In summary, for freshwater crustaceans there appears to be an EFA requirement for LC-PUFA, either ARA or EPA at levels around 0.1 and 0.6%, respectively, which are significantly lower than those required by marine shrimp, and ARA alone may be sufficient to achieve optimal growth. Although both marine and freshwater crustaceans require dietary LC-PUFA, requirements differ dramatically, possibly reflecting the fatty acid composition of their natural foods.

Requirements for Phospholipids

Crustaceans

Dietary phospholipid has been demonstrated to be required for growth and survival of larvae and juveniles of various crustacean species (Table 3.3). Although there is little difference in requirements between larval and juvenile stages within a particular species, there appears to be no requirement in adults. This suggests that the requirement may be based on an insufficient rate of synthesis to specifically satisfy demands of the rapid growth rate of early life stages. Furthermore, the apparent lack of a requirement for dietary phospholipids of juveniles of freshwater species *M. rosenbergii* and *Cherax quadricarinatus* suggests that the requirement may be unique to marine crustaceans.

A phospholipid requirement in shrimp was first demonstrated through the addition of phospholipids, derived from short-necked clam, to the diet of *P. japonicus* (Kanazawa et al. 1979c). The addition to the diet of 3.5–6% soybean lecithin, containing 23.6% PC, improved growth and survival in *P. japonicus* larvae (Kanazawa et al. 1985). The inclusion of phospholipids at 1.0–1.5% of diet improved digestion of lipids and increased the growth of juvenile *P. monodon* (Glencross et al. 1998; Paibulkichakul et al. 1998), and addition of 2.5% of soybean lecithin to the diet increased weight gain in juvenile banana shrimp, *P. merguiensis* (Thongrod and Boonyaratpalin 1998). The effects of dietary phospholipid vary with class. Thus, dietary PC significantly reduced mortality compared to PE (ovine) and PI (soybean) in juvenile lobsters, *Homarus americanus* (D'Abramo et al. 1981).

Table 3.3 Reported phospholipid requirements of crustaceans and fish. Abbreviations: J: juvenile; PL: postlarvae; L: larvae; BPC: purified bonito egg PC; BPL: bonito egg polar lipid; CPL: corn polar lipid; EL: chicken egg lecithin; EPC: purified egg PC; GPL: fish gonal phospholipids; PL: various phospholipid sources supplemented to supply 2 % dietary phospholipids including EL; SL: sunflower, rapeseed and marine phospholipids; SL: soybean lecithin; SPC: purified soybean PC; SPE: purified soybean PE; G: growth; S: survival; M: malformations; R: stress resistance. Source: Adapted from Tocher, D. R., E. Å. Bendiksen, P. J. Campbell and J. G. Bell. 2008. The role of phospholipids in nutrition and metabolism of teleost fish. Aquaculture 280: 21–34.

Species	Stage	Phospholipid supplement and levels studied	Optimal requirement and criteria used	Reference
Crustaceans				
Banana shrimp (*Penaeus merguiensis*)	J	SL	2.5% (G)	Thongrod and Boonyaratpalin 1998
Black tiger shrimp (*Penaeus monodon*)	L/PL	PL	1.0–1.5% (G)	Paibulkichakui et al. 1998
	J	SPC	1.25% (G)	Chen 1993
Karuma shrimp (*Penaeus japonicus*)	J	Shortneck clam PC and PE	1.0% (G)	Kanazawa et al. 1979c
	J	SPE and SPI	3.0% (G)	Teshima et al. 1986c, b
	L	SL	3.0% (G)	Kanazawa 1983
	L	BPC and SPI	0.5 to 1.0% (G)	Kanazawa et al. 1985
Redtail prawn (*Penaeus penicillatus*)	J	SPC	1.25% (G)	Chen and Jenn 1991
Whiteleg shrimp (*Litopenaeus vannamei*)	PL	deoiled SL	6.5% (G)	Coutteau et al. 1996a
	PL	SPC or EPC	1.5% (G)	Coutteau et al. 1996a
Fish				
Atlantic salmon (*Salmo salar*)	J (180 mg)	0, 2, 4, 6, and 8% SL/CPL	6% (G)	Poston 1991
	J (180 mg)	0 and 4% SL	4% (G)	Poston 1990b
	J (1.0 g)	0 and 4% SL	4% (G)	Poston 1990b
	J (1.7 g)	0 and 4% SL	4% (G)	Poston 1990b
	J (7.5 g)	0 and 4% SL	0% (no requirement)	Poston 1990b
Ayu (*Plecoglossus altivelus*)	L	0 and 3% SL or EL	3% (G,S,M)	Kanazawa et al. 1981
	L	0, 1, 3 and 5% SL	3% (M), 5% (G,S)	Kanazawa et al. 1983a
	L	0 and 3% EL or BPL	3% (G,S,M)	Kanazawa et al. 1983a
	J	0 and 3% SL or BPL	3% (G)	Kanazawa et al. 1981
	J	0, 1, 3 and 5% EL	3% (G)	Kanazawa et al. 1981
Common carp (*Cyprinus carpio*)	L	0 and 2% EL	2% (G,S)	Geurden et al. 1995b
	L	0 and 2% PL	2% (G,S)	Geurden et al. 1995b
	L	0 and 2% SPC, SPI or EL	2% (G,S,M except EL)	Geurden et al. 1997b

Table 3.3 (*Continued*)

Species	Stage	Phospholipid supplement and levels studied	Optimal requirement and criteria used	Reference
European sea bass (*Dicentrarchus labrax*)	L	3, 6, 9, and 12% SL	12% (G,S,M)	Cahu et al. 2003
	J	0 and 3% SL	3% (G)	Geurden et al. 1995a
	J	0 and 2% EPC or SPC	2% (G)	Geurden et al. 1995a
Gilthead sea bream (*Sparus aurata*)	L	9, 11, and 15% SL	> 9 % (G,S)	Seiliez et al. 2006
	L	PC/PI 1.28, 1.6, 2.37, 3.07	PC/PI = 1.28 (G,S,M)	Sandel et al. 2010
Japanese flounder (*Paralichthys olivaceus*)	L	0, 3, 5, and 7% SL	7% (G,S)	Kanazawa, 1993
	J	0, 3, 5, and 7% SL	7% (G)	Kanazawa, 1993
Knife jaw (*Oplegnathus fasciatus*)	L	0, 2.5, 5, and 7.4% SL	7.4% (G,S)	Kanazawa et al. 1983b
	L	0, 3, 5, and 7% SL	5% (G,S,R)	Kanazawa, 1993
	J	0, 3, 5, and 7% SL	3% (G)	Kanazawa, 1993
Pikeperch (*Sander lucioperca*)	L	1, 5, and 9% SL or GPL	9% (G,M)	Hamza et al. 2008, 2012
Rainbow trout (*Oncorhynchus mykiss*)	J	0, 2, 4, and 8% SL	4% (G)	Poston, 1990a
	J	0 and 14%	14% (G)	Rinchard et al. 2007
Red sea bream (*Pagrus major*)	L	0 and 5% SL	5% (G,S)	Kanazawa et al. 1983b
Striped jack (*Pseudocaranx dentex*)	J	0, 0.5, 1, 1.5, and 2% SPC	1.5% (G,S,R)	Takeuchi et al. 1992
	J	0 and 1.5% SPE	1.5% (G)	Takeuchi et al. 1992
Turbot (*Psetta maximus*)	J	0 and 2% EL	2% (G)	Geurden et al. 1997c
White sturgeon (*Acipenser transmontanus*)	J (5–10 g)	0 and 8% SL	0% (no req.)	Hung and Lutes 1988

Levels of 1.0% of PC or PI derived from bonito eggs and soybean, respectively, most effectively improved growth and survival of larval *P. japonicus*, whereas PC from chicken egg and PE from bonito eggs and ovine brain were less effective (Kanazawa et al. 1985).

Dietary phospholipid requirements are commonly reported to be between 1.2 and 1.5% of the diet for juvenile penaeid shrimps (Chen and Jenn 1991; Chen 1993; Kanazawa 1993; Coutteau et al. 1996b). Most studies have used relatively crude phospholipid preparations including soybean and egg yolk lecithins which contain several phospholipid classes, making the effects of an individual class difficult to determine. However, some estimates were based

on investigations using highly purified sources of phospholipids, such as 80% pure PC from soybean (Chen and Jenn 1991; Chen 1993). Addition of 1.5% of PC from either a 95% pure soybean source, 94% pure chicken egg source, or deoiled soybean lecithin (23% PC) increased growth of *P. vannamei* relative to a PC-deficient diet (Coutteau et al. 1996a). Based upon the collective results, a relatively conservative estimate for PC requirement is between 0.5 and 1.5%.

Fish

Dietary phospholipids improve growth in both larvae and early juveniles, reduce mortality and incidence of malformation in larvae, and perhaps increase stress resistance of various freshwater and marine finfish species (Table 3.3). As described above, however, defining dietary phospholipid requirements is compromised by the variable phospholipid content and class composition of different phospholipid preparations. Studies with larval fish generally require phospholipids to be supplied through enrichment of live feeds, with remodeling of the phospholipid and fatty acid composition in the feed organisms further complicating data interpretation. However, based on the available studies, quantitative phospholipid requirement can be rather high for larval fish (up to 12% of diet dry matter), but usually less for juvenile fish (2–4%) although higher levels of up to 14% have been reported (Cahu et al. 2009). The apparent order of efficacy is generally PC > PI > PE > PS, with PC more effective on growth and PI more influential for survival and preventing deformities (Geurden et al. 1998a; Tocher et al. 2008). The efficacy of sphingolipids or other phospholipids is not known and, so far, no requirement for dietary phospholipids has been reported for adult fish (Tocher et al. 2008).

Requirements for Cholesterol

Crustaceans

Crustaceans have a requirement for cholesterol, and insufficient dietary cholesterol results in reduced growth and/or increased mortality (Teshima 1997). The cholesterol requirement of different shrimp species ranges from 0.2% to 1.0% of diet, and it can sometimes be satisfied through feed ingredients without supplementation (Duerr and Walsh 1996; Smith et al. 2001). Furthermore, higher levels may adversely affect growth (Thongrod and Boonyaratpalin 1998). The requirement for cholesterol may be partly satisfied in some shrimp species through a limited ability to convert phytosterols, plant sterols contained in some feed ingredients, to cholesterol. Thus, a combination of cholesterol (0.22%) and a phytosterol mix (1.39%) containing predominantly sitosterol could partially spare the cholesterol requirement of freshwater crayfish, *Pascifasticus leniusculus*, but not of juvenile lobsters, *H. americanus* (D'Abramo et al. 1984, 1985a). Although sitosterol could partially replace dietary cholesterol in *P. japonicus*, it could not completely replace cholesterol nor partially spare the cholesterol requirement when included at high sitosterol: cholesterol ratios (Teshima and Kanazawa 1986; Teshima et al. 1989). Similarly, no other sterol (cholesterol precursor) could completely replace dietary cholesterol in *P. japonicus* (Teshima et al. 1983).

There is some evidence suggesting an interaction or relationship between cholesterol requirements and the level of dietary phospholipid. In the absence of lecithin, the dietary cholesterol requirement of *P. vannamei* was reported to be 0.35%; when deoiled soybean lecithin was included at 1.5% and 3.0% however, the cholesterol requirement decreased to 0.14% and 0.13%, respectively (Gong et al. 2000). However, no interaction between the dietary PC and cholesterol requirement was observed in *P. monodon* (Chen and Jenn 1991; Chen 1993).

Fish

There is no known requirement for cholesterol in finfish. However, although dietary cholesterol level had no effect on mortality, SGR, and apparent digestibility coefficients of macronutrients in Atlantic salmon, liver cholesterol concentration and hepatosomatic index were both increased by cholesterol supplementation (Bjerkeng et al. 1999). Furthermore, genes of the cholesterol biosynthesis pathway were upregulated in liver of salmon fed vegetable oil (VO) compared to fish fed FO (Taggart et al. 2008). As tissue cholesterol levels were unaffected, the reduced dietary cholesterol intake in fish fed VO appeared to be compensated by increased synthesis (Leaver et al. 2008b). As dietary cholesterol levels decrease and phytosterol levels increase, more attention to sterol requirements and

metabolism may be warranted and result in increasing inclusion of plant meals and oils in feed formulations.

Biochemical/Molecular Basis of Requirements

Essential Fatty Acids

The EFA requirements of both fish and crustaceans appear to vary qualitatively with environment, dietary preference, or trophic level (Sargent et al. 2002). This may reflect evolutionary adaptation, with marine fish and crustaceans experiencing less evolutionary pressure to retain LC-PUFA biosynthesis due to high levels of EPA and DHA produced by phytoplankton (microalgae) in the marine environment (Sargent et al. 1995b). In contrast, freshwater food webs usually display lower levels of EPA and, particularly, DHA (Sargent et al. 1995b); evolutionary pressure for retaining LC-PUFA biosynthesis has therefore been higher in freshwater species. Although experimental data generally support this hypothesis, there are confounding factors. For instance, a similar generalization as above could possibly be made for dietary preference/trophic level with LC-PUFA biosynthesis being lost from carnivorous/piscivorous species obtaining high levels of EPA and DHA in their prey, whereas herbivorous species consuming low dietary EPA and DHA have retained endogenous LC-PUFA biosynthesis pathways. However, an exception to this generalization was Northern pike, the piscivorous freshwater species that was shown to be able to produce EPA and DHA from 18:3n-3 in hepatocyte assays using radiolabeled fatty acid substrates (Buzzi et al. 1997a). Considering the number of diadromous and euryhaline species, it is not particularly appropriate to define many fish species as marine or freshwater. A recent feeding study suggested that rabbitfish, *Siganus canaliculatus*, a rare example of an exclusively herbivorous marine species that consumes benthic algae and seagrasses, could produce EPA and DHA endogenously (Li et al. 2008). It was subsequently shown that rabbitfish possess all of the enzyme activities required for the biosynthesis of EPA and DHA, suggesting that trophic level and/or feeding preference can be factors determining the status of the LC-PUFA synthesis pathway in some species (Li et al. 2010; Monroig et al. 2012; Morais et al. 2012a).

Until recently, the pathway for synthesis of LC-PUFA in vertebrates was thought to occur in two distinct phases with rather different kinetics (Fig. 3.4). The first phase, synthesis of EPA, occurs entirely in microsomes and is achieved by Δ6 desaturation of 18:3n-3 to produce 18:4n-3, which is elongated to 20:4n-3 followed by Δ5 desaturation (Cook and McMaster 2004). The same enzymes and pathway are responsible for the synthesis of ARA from 18:2n-6. The second phase, DHA synthesis from EPA, is slower and requires two further elongation steps, a second Δ6 desaturation, and transport of C_{24} intermediates to peroxisomes for a final chain-shortening step (Sprecher 2000). This proposed mechanism for the second phase was originally described in rat tissues, but there is biochemical evidence supporting the involvement of C_{24} PUFA intermediates in the production of DHA from EPA in rainbow trout (Buzzi et al. 1996, 1997b). Recent evidence suggested that DHA production from EPA may be possible by direct Δ4 desaturation of the EPA elongation product 22:5n-3, at least in some fish species (Li et al. 2010; Morais et al., 2012a; Fig. 3.4).

Whatever the precise pathway, the conversion of C_{18} PUFA to LC-PUFA is determined by the presence or absence of appropriate fatty acyl desaturase (Fads) and elongase (Elovl) enzymes (Bell and Tocher 2009b; Fig. 3.4). The EFA requirements described above suggest that species able to produce LC-PUFA from C_{18} PUFA must express the necessary biosynthetic activities, whereas other species, including many marine fish and crustaceans, cannot or have only limited capacity to produce LC-PUFA (Tocher 2010). The general hypothesis that enzyme (gene) deficiencies in the LC-PUFA synthesis pathway are the basis for qualitative differences in EFA requirements between fish species was supported by biochemical studies. An early *in vivo* study showed that EPA and ARA were produced from radiolabeled 18:3n-3 and 18:2n-6, respectively, in rainbow trout, but not in turbot (Owen et al. 1975), and *in vitro* cell culture studies indicated that turbot and sea bream lines had very low Δ5 Fads or Elovl activities compared to salmonid lines (Ghioni et al. 1999; Tocher and Ghioni 1999). Early work with marine crustaceans indicated that they had little or no capability to biosynthesize LC-PUFA from C_{18} PUFA (Kanazawa et al. 1979d), but it appears that the freshwater shrimp *M. rosenbergii* can convert C_{18} PUFA to their corresponding LC-PUFA,

albeit at a rate insufficient to sustain the highest growth rate (D'Abramo and Sheen 1993). However, addition of C_{18} PUFA as the sole EFA to diets of the marine shrimp *P. monodon* produced growth rates that equaled or exceeded those obtained with diets containing an EPA and DHA rich FO (Glencross and Smith 1999). This suggested that either sufficient LC-PUFA synthesis capacity existed in this species to utilize those shorter-chain EFA, or the demands for LC-PUFA were lower than previously thought.

Recent work has focused on elucidating the molecular basis of these biochemical differences. Whereas cDNAs for Δ6 Fads have been cloned from all species of fish investigated including freshwater (common carp), salmonid (rainbow trout and Atlantic salmon), and marine (turbot, Atlantic cod, gilthead sea bream, meagre *Argyrosomus regius*, cobia *Rathycentron canadum*, Atlantic bluefin tuna *Thunnus thynnus*, European and Asian sea bass), cDNA for Δ5 Fads has only been cloned from Atlantic salmon and, so far, no discrete Δ5 Fads has been found in marine fish (Hastings et al. 2001; Seiliez et al. 2001, 2003; Zheng et al. 2004a, 2005a, 2009a; Tocher et al. 2006; González-Rovira et al. 2009; Mohd-Yusof et al. 2010; Morais et al. 2011a; Monroig et al. 2013). Analyses of sequenced Acanthopterygii genomes revealed a single Δ6-like Fads in medaka, *Oryzias latipes*, two very closely related Fads-like genes in the stickleback, *Gasterosteus aculeatus*, and no Fads homologs in pufferfish, *Tetraodon nigroviridis* and *Takafugu rubripes* (Leaver et al. 2008a).

Some fish species express bifunctional desaturases, previously unknown in vertebrates. The first bifunctional desaturase reported was a zebrafish, *Danio rerio*, Δ6/Δ5 Fads that also showed activity towards C_{24} PUFA, indicating that it could function at two steps in the LC-PUFA synthesis pathway, which is consistent with it being the only PUFA Fads represented in the zebrafish genome (Hastings et al. 2001; Tocher et al. 2003a). Recently, a similar bifunctional Δ6/Δ5 Fads was isolated from the marine herbivore rabbitfish, *S. caliculatus*, which was also shown to express a distinct Δ4 Fads (Li et al. 2010). In addition to providing the molecular mechanisms to support the previously reported ability of rabbitfish to produce n-3 LC-PUFA (Li et al. 2008), the Δ4 Fads enables direct desaturation of 22:5n-3, providing a possible alternative pathway for the synthesis of DHA, which has previously not been reported in vertebrates, in this particular species (Fig. 3.4). A similar Δ4 Fads was recently isolated from the Senegalese sole, *Solea senegalensis*, a marine species with an unexpectedly low requirement for dietary LC-PUFA, which suggested that this alternative (more direct) pathway for DHA biosynthesis from EPA may be more widespread in teleost species (Morais et al., 2012a). Recently, the recognition that Δ6 Fads enzymes in some marine fish species that also display high levels of Δ8 activity, including cobia, cod, sea bream, turbot, and Asian sea bass, suggests a possible alternative pathway for the biosynthesis of EPA in some fish (Monroig et al. 2011a; Tu et al. 2012; Fig. 3.4). Irrespective of activity (Δ6, Δ5, Δ4, or bifunctional), all teleost PUFA desaturases are Fads2 enzymes whereas, in contrast, elasmobranchs have Fads1 and Fads2 enzymes possessing Δ5 and Δ6 activities, respectively, as in mammals (Castro et al. 2012). Fads1 has therefore been lost during evolution of teleosts with expansion and functional diversification of Fads2.

There are seven Elovl genes known in mammals with Elovl2, Elovl4, and Elovl5 all involved in LC-PUFA biosynthesis (Jakobsson et al. 2006). In fish, cDNAs for Elovl5 have been isolated from freshwater (zebrafish, common carp, tilapia, and pike), salmonid (rainbow trout and Atlantic salmon), and marine (cod, turbot, gilthead sea bream, cobia, Asian sea bass, Atlantic bluefin tuna, rabbitfish, and Senegal sole) species (Agaba et al. 2004, 2005; Hastings et al. 2005; Zheng et al. 2009a; Mohd-Yusof et al. 2010; Morais et al. 2011a, 2012a; Monroig et al. 2012, 2013; Carmona-Antoñanzas et al. 2013), whereas Elovl2 cDNAs have only been isolated from zebrafish and Atlantic salmon (Monroig et al. 2009; Morais et al. 2009). Recenty, Elovl4 cDNAs have been cloned from zebrafish, cobia, and Atlantic salmon (Monroig et al. 2009, 2011b; Carmona-Antoñanzas et al. 2011). Fish Elovl5 elongates predominantly C_{18} and C_{20} PUFA, Elovl2 elongates predominantly C_{20} and C_{22} PUFA, and Elovl4 is primarily involved in the production of very-long-chain PUFA (VLC-PUFA) from C26 to C36 (Fig. 3.4). Analyses of the sequenced Acanthopterygii genomes showed medaka, stickleback, and pufferfish do not possess Elovl2 homologs, suggesting that the Elovl5 may be the only PUFA Elovl gene in species such as sea bream and turbot (Leaver et al. 2008a).

The varying competencies of different fish species to biosynthesize LC-PUFA probably reflect their genome complement of both Fads and Elovl genes.

In addition to differences in genome complement, differences between species also exist with respect to expression and activity of LC-PUFA synthesis genes and their products. Thus, the highest levels of Δ6 Fads expression and desaturation activity were in liver and intestine in salmon, whereas Δ6 Fads expression and desaturation activity were very low in Atlantic cod liver and intestine (Tocher et al. 2006). In marine fish, including cod, cobia, and Asian sea bass, the highest expression of Δ6 Fads was in the brain, suggesting that the retention of Δ6 Fads in marine fish may be to maintain membrane DHA levels in neural tissues at times of high demand, such as embryonic and larval development (Zheng et al. 2005a, 2009a; Tocher et al. 2006; Mohd-Yusof et al. 2010). There is nutritional regulation of LC-PUFA biosynthesis in freshwater and salmonid fish, with LC-PUFA biosynthesis activity increased by EFA deficiency and modulated by different C_{18} PUFA in a carp cell line (Tocher and Dick 1999, 2000). Dietary trials with salmonids in freshwater showed that LC-PUFA biosynthesis was increased (Tocher et al. 1997, 2002, 2003b), and expression of LC-PUFA biosynthesis genes, especially Δ6 Fads, was induced in salmon fed diets containing VO lacking LC-PUFA when compared to fish fed diets containing FO rich in EPA and DHA (Zheng et al. 2004b, 2005a, b; Leaver et al. 2008b; Taggart et al. 2008). In contrast, there were little differences in liver and intestinal Δ6 Fads expression and activity in cod fed diets containing either VO or FO (Tocher et al. 2006), possibly reflecting differences in the Fads gene promoters in cod and salmon (Zheng et al. 2009b).

The above differences in complement and expression of genes affect the biochemical markers of EFA deficiency in fish. Mead acid (20:3n-9) is the biochemical marker of EFA deficiency in mammals, based on the specificity of Fads being n-3 > n-6 > n-9; only in the absence of dietary PUFA can 18:1n-9 serve as a substrate for the LC-PUFA synthesis pathway with 20:3n-9 being produced (Tocher et al. 1998). In species of fish that can produce EPA, 20:3n-9 is also a marker of EFA deficiency with a 20:3n-9 to DHA ratio of 0.4 in tissue phospholipids indicating EFA deficiency (Castell et al. 1972a; Watanabe et al. 1974, 1989). Production of 20:3n-9 is not possible in species of fish that cannot produce EPA, but 18:2n-9 and 20:2n-9 have been reported in sea bream fed low EFA feeds (Kalogeropoulos et al. 1992), and 18:2n-9 accumulated in a turbot cells cultured in medium lacking PUFA (Tocher et al. 1988).

Phospholipids

Phospholipid requirements are unique in that any proposed mechanism must not only explain the requirements in larval and early juvenile shrimp and fish, but must also explain their lack of effect in adults. Although there are few data on crustaceans, the existing evidence indicates that the essentiality of phospholipids (PC being the most effective) is not due to any specific component of the molecule such as choline or EFA (Kanazawa et al. 1979c, 1985). Dietary phospholipids may increase the physical stability of feeds in water and reduce losses of water-soluble nutrients; one beneficial effect of dietary lecithin in crustaceans could therefore be as an emulsifier enhancing digestion of lipids (Glencross et al. 1998). The requirement of phospholipids in crustaceans has also been associated with lipid transport and the role of phospholipids as components of lipoproteins, specifically HDL (Teshima and Kanazawa 1980). Lipoproteins transport lipids from the gut epithelia into the hemolymph and from there to the tissues. When soy lecithin was removed from the diet, lower levels of PC and cholesterol and reduced rates of cholesterol transport from the midgut gland to the hemolymph were observed in juvenile lobsters (D'Abramo et al. 1982, 1985b). Similarly, dietary phospholipids enhanced cholesterol transport from the midgut gland (hepatopancreas) to the hemolymph and then muscle in juvenile *P. japonicus* (Teshima et al. 1986a, b).

The mechanism of phospholipid requirement has been studied in more detail in fish. As sources of EFA to larval fish, phospholipids are more effective than TAG due to higher proportions of EFA and increased digestibility (Tocher 1995; Sargent et al. 1997, 1999a, b), but increased larval growth and survival in response to dietary phospholipid appears independent of EFA requirements and the delivery of other essential nutrients such as choline and inositol (Geurden et al. 1995a). Furthermore, although dietary phospholipids increased digestibility in juvenile fish

(Craig and Gatlin 1997; Kasper and Brown 2003), the growth-promoting effect was not due to enhanced emulsification and digestion (Geurden et al. 1997a, 1998b). However, several studies have reported intestinal steatosis in larvae fed phospholipid-deficient diets, suggesting a limitation in transport of dietary lipids from the intestine, possibly through impaired lipoprotein synthesis (Fontagné et al. 1998; Geurden et al. 1998b; Olsen et al. 1999; Salhi et al. 1999). Larvae therefore have a limited ability to biosynthesize phospholipids *de novo*, indicating that growth was stimulated by the provision of intact phospholipids in the diet (Geurden et al. 1995a, 1999; Coutteau et al. 1997; Fontagné et al. 1998). It has been suggested that synthesis of the glycerophosphobase backbone is impaired, with the production of PC and PE from DAG via CDP-choline and CDP-ethanolamine phosphotransferases (CPT and EPT) being the deficient steps (Sargent et al. 2002; Tocher et al. 2008). However, PI can also have beneficial effects and it is not formed via the CPT/EPT pathways, suggesting another limiting step in the phospholipid synthesis pathway (Tocher et al. 2008). Whatever the precise mechanism, dietary phospholipids are required for the efficient transport of dietary fatty acids and lipids from the gut to the rest of the body, possibly by facilitating lipoprotein synthesis.

Cholesterol

As with most dietary requirements, the requirement of crustaceans (shrimp) for cholesterol is due to an inherent inability to synthesize this sterol *de novo*. However, the biochemical basis and molecular mechanisms underpinning this metabolic deficiency, including which enzyme(s) or activities are limited or which genes are absent or non-functional, have not yet been determined.

Effects of Diet

As indicated in the previous section, lipid and fatty acid metabolism in fish has become of increasing importance in recent years due to progressive changes in formulations affecting the major lipid-containing ingredients. One major change has been the drive to produce feeds with higher dietary lipid content in order to increase growth rates, which has been enabled by advances in extruder technologies that allow for production of feeds with high lipid contents (up to 40%). This has been largely successful, but can also cause problems if physiological limits are exceeded as described in the previous section, 'Lipid and Fatty Acid Requirements'. In contrast to the deliberately targeted changes in lipid content, the alterations in feed fatty acid composition have been the unwanted consequences of the necessity to reduce the aquculture industry's dependence upon finite marine resources of fishmeal and FO, and to replace these major feed ingredients with more sustainable alternatives (Bell and Tocher 2009a; Bendiksen et al. 2011). There is extensive literature describing the effects of the replacement of dietary FO in a range of fish species (Turchini et al. 2010; Sales and Glencross 2011). The following provides a brief summary of the effects, specifically on fatty acid compositions and metabolism in fish in particular relation to health.

Currently, the most sustainably available alternatives to FO are VO, but no terrestrial plant produces EPA or DHA (Bell and Tocher 2009a). Many studies have shown that VO, either isolated or as blends, can replace some or all of the added FO in fish feeds with minimal effects on fish growth (Turchini et al. 2009; Sales and Glencross 2011). However, replacement of FO, rich in n-3 LC-PUFA, EPA, and DHA, with VO devoid of n-3 LC-PUFA but rich in C_{18} PUFA, such as 18:2n-6 and 18:3n-3, can reduce tissue n-3 LC-PUFA concentrations in fish by more than 50% if total FO replacement is implemented (Turchini et al. 2010; Sales and Glencross 2011). This effect on tissue fatty acid composition occurs regardless of species and is the major consequence of FO replacement with VO in freshwater, salmonid and marine fish, and crustaceans (NRC 2011). Similarly, the reduction in tissue n-3 LC-PUFA is largely independent of the fatty acid composition of VO used to replace FO (Turchini et al. 2010; Nanton et al. 2012). Generally, n-6 fatty acids are the predominant PUFA in the majority of VO including soybean oil, rapeseed (Canola), and palm oil commonly used to replace FO, and so tissue n-6/n-3 PUFA ratio is increased and EPA and DHA are greatly reduced (Brown and Hart 2010; Ng and Gibon 2010; Turchini and Mailer 2010). Although it has been shown that species able to endogenously produce EPA and DHA, such as salmonids, can be net producers of n-3 LC-PUFA (Crampton et al. 2010;

Sanden et al. 2011; Turchini et al. 2011b), substitution with VO rich in 18:3n-3, such as linseed or camelina oils, does not prevent the decrease in the levels of n-3 LC-PUFA in tissues (Tocher et al. 2010).

The detailed metabolic effects of replacement of dietary FO with VO in fish have been recently reviewed (Torstensen and Tocher 2010). One consistent effect observed is that the LC-PUFA biosynthesis pathway is upregulated in fish fed VO diets compared to fish fed FO (Leaver et al. 2008a). This has been shown in several species but the most complete data is in Atlantic salmon, where increased activity of the pathway has been associated with increased expression of Δ6 and Δ5 Fads and, in some cases, elongase genes (Leaver et al. 2008a, b; Bell and Tocher 2009b). However, the increased biosynthetic capacity is not able to compensate for the low levels of LC-PUFA in the diet. Interestingly, it was recently shown that Fads expression was also influenced by dietary lipid content with lower expression of Δ6 and Δ5 Fads in Atlantic salmon fed higher lipid/energy feeds independent of dietary LC-PUFA levels (Martinez-Rubio et al. 2013a). Although feeding VO has the same overall effect in all fish at a gross level, recent research showed that the phenotypic response was influenced by genotype; levels of flesh n-3 LC-PUFA therefore varied significantly between families of salmon all fed the same VO diet (Morais et al. 2012b). Analyses of data from around 100 families showed that the flesh n-3 LC-PUFA phenotype was a highly heritable trait ($h^2 = 0.77$) in Atlantic salmon (Leaver et al. 2011). However, the final fatty acid composition of farmed fish is influenced by diet and its interaction with many metabolic pathways and physiological processes, including intestinal digestion and absorption, transport and tissue uptake pathways, and cellular fatty acyl oxidation and acylation processes. The biochemical and molecular mechanisms underpinning tissue fatty acid compositions in fish and the effect of diet are complex and yet to be fully elucidated. Genomic studies through transcriptomic (oligo microarray) and proteomic analyses of gene expression in key tissues, such as liver and intestine, are beginning to address these issues and provide the data to assist diet formulation (Morais et al. 2011b, c, 2012c, d). Furthermore, advances in sequencing technology (RNAseq, etc.) will enable omic analyses to be applied to species that currently have relatively few genomic resources.

Lipids, Fatty Acids, and Health

Although many, if not all, dietary components can influence health of fish, lipids and fatty acids are among the most studied. As with all dietary components, nutritional pathologies in a lipid context range from deficiency of lipids as energy-providing macronutrients leading to under-nutrition and eventually starvation, to deficiency of "micronutrients" with critical roles in cellular functions, such as EFA, and nutritional imbalances in key nutrients. This last point reflects the fact that even if all nutrients are supplied above individual requirement levels, the complicated physiological interactions that occur between nutrients within integrated metabolic pathways can have profound effects. This can be seen most clearly in relation to dietary changes such as replacement of n-3 LC-PUFA-rich FO with n-6 PUFA-rich VO (Oliva-Teles 2012). However, it has often been difficult to draw definitive conclusions due to the number of confounding factors including species, dietary lipid content, the type and level of VO employed, other dietary nutrient compositions, duration of feeding, and many environmental conditions (Montero and Izquierdo 2010).

As decribed above, it is well known that EFA are required to maintain optimal growth in fish, although the precise fatty acids that can satisfy EFA requirements vary among fish species according to trophic level and environment (Glencross 2009). Growth retardation occurs without adequate levels of EFA, and long-term deprivation results in critical deficiencies that lead to degeneration of animal health (Glencross 2009). The most obvious signs of EFA deficiencies usually include poor growth rate and increased mortality (Castell et al. 1972a; Watanabe 1982; Sargent et al. 1995b; Ruyter et al. 2000a; Glencross and Rutherford 2011). Rainbow trout showed a range of other pathologies including erosion of the caudal fin, myocarditis, and shock syndrome (Castell et al. 1972a). Death typically followed the shock syndrome reaction to handling, and there was also an increased sensitivity of the fish to stress (Castell et al. 1972a, b; Watanabe 1982).

Fatty acids are unique among the macronutrients in that they lose little in their primary form through the digestion process. The result of this is well known, as the fatty acid composition of the consumer

closely reflects that of what has been consumed. Any modifications to the fatty acid profile of what is eaten will inevitably lead to changes in fatty acid deposition within the consumer, including the composition of cellular biomembranes. This is one of the main ways that changes in dietary fatty acid composition can affect the metabolism and health of the consumer, via alteration of the physical and functional properties of the cellular membranes into which they are incorporated. Specifically, changes in cellular membrane biochemistry and function can, in turn, affect a range of physiological processes including the circulatory, pulmonary, reproductive, and immune systems (Verlhac Trichet 2010).

Lipids and Fatty Acids and Stress Responses

All animals are subject to stressors that can affect them in a variety of ways, including directly and indirectly influencing their metabolism. Fish stressors can be environmental, such as sudden changes of water temperature or salinity, physical challenges including confinement or handling, and nutritional issues involving either changes in dietary nutrient content or composition and/or feeding regime (Withers 1992). Fish exhibit stress reponses in a range of ways depending on the stimulus. For instance, levels of activity can either be rapidly increased or dramatically reduced, pigmentation display can change, and feeding activity will typically be reduced, despite the fact that energy expenditure may be increased (Glencross and Bermudes 2011). The increase in energy demand can be manifested in an increase of oxygen consumption and, over a longer period of time, an accelerated rate of loss of body tissues (notably protein and lipids) is observed (Withers 1992; Glencross and Bermudes 2011).

At a physiological level, stress acts via two different endocrine-mediated systems depending on whether the stimuli are acute or chronic. For acute stressful stimuli, the key mediator of the response in fish is via the adrenergic endocrine system, which is initiated by a neural trigger stimulating the secretion of the catecholamines, epinephrine (or adrenalin), and norephineprine from the chromaffin tissues that are associated with the head kidney in fish. The key physiological responses that occur as a result of the secretion of catecholamines include hyperglycaemia, elevated cardiac output, and altered blood flow that can be increased or decreased depending upon the organ (Withers 1992). Adrenergic responses have also been shown to activate phospholipase A_2, which hydrolyzes essential fatty acids from membrane phospholipids (Tocher 2003). These fatty acids have a key role in eicosanoid metabolism, as will be discussed in the following section ('Lipids and Fatty Acids and Immune Responses').

With prolonged or chronic stimuli in fish the stress response is mediated by the interrenal glands, which function much as the adrenal cortex in other vertebrates through the synthesis of steroid hormones that are involved in regulating several metabolic pathways. In particular, the major glucocorticoid steroid cortisol has a critical role in eliciting the chronic effects of stress on the animal's physiology, and is the most commonly used biomarker of stress level (Withers 1992). The primary metabolic function of cortisol is to increase blood glucose level through increased gluconeogenesis and inhibition of insulin-stimulated glucose uptake into muscle via the GLUT4 transporter. However, cortisol has a number of other metabolic effects on lipid and protein metabolism, as well as cardiovascular physiology. Furthermore, it has well-known roles in suppressing inflammatory responses by inhibiting PLA_2-stimulated release of ARA from phospholipids, COX activity, and leukotriene synthesis, thus inhibiting eicosanoid production (Montero and Izquierdo 2010). Cortisol also inhibits immune responses through blocking macrophage cytokine (Interleukin-1β) production (Montero and Izquierdo 2010). Studies examining the replacement of FO rich in n-3 LC-PUFA with VO lacking LC-PUFA show varying effects on fish stress responses. The replacement of dietary FO with linseed oil (high 18:3n-3) in feeds showed that, despite an overall increase in total n-3 PUFA, the reduced n-3 LC-PUFA (EPA and DHA) induced a chronic elevation of plasma cortisol and post-confinement stress levels in gilthead sea bream (Montero et al. 2003). It was suggested that this indicated that n-3 LC-PUFA had a role as modulators of the stress response (Montero and Izquierdo 2010). However, blending of a series of VO (rapeseed, soy, and linseed) resulted in a lower n-3 to n-6 ratio in the diet, and also reduced the post-stress cortisol levels (Montero and Izquierdo 2010). A reduction in dietary ARA has

also been linked with increased cortisol response in this same species after acute stress events (Van Anholt et al. 2004).

The physiological responses to stress in fish involve various alterations and/or modulations of lipid and fatty acid metabolism. At an energetic level, there is an increased rate of catabolism of lipid reserves to sustain the higher energy demands associated with the stress response, such as that observed with high temperatures (Glencross and Bermudes 2011). Selective retention of certain fatty acids during lipid catabolism, as described previously, still occurs when the increased catabolic rate is stress-induced. The retention of DHA, particularly in neural and visual tissues, has implied that DHA plays an important functional role in these tissues (Mourente et al. 1991; Sargent et al. 1993); however, the presence or absence of DHA in retinal cells has also been linked to behavioral changes in fish, most notably those affecting feeding and schooling behavior, but also including stress responses (Masuda et al. 1998). Recent work by Glencross and Rutherford (2011) showed that when fed a DHA-deficient diet, Asian sea bass exhibited a distinct change in their behavior relative to fish fed diets with higher levels of DHA or DHA plus EPA; the DHA-deficient fish became more cryptic, stressed, and reluctant to feed. Furthermore, the same study showing stress responses in relation to dietary EFA levels also demonstrated that, even in the presence of dietary DHA, the retention of EPA was enhanced, suggesting that there is selective pressure to retain EFA types that may implicate important and distinct roles for both DHA and EPA in stress responses (Glencross and Rutherford 2011).

Ionic balance in aquatic animals is another homeostatic metabolic process that is modulated in response to stress. Essential fatty acids are involved in ionic regulation through at least two mechanisms. The first involves changes in membrane EFA composition of key tissues, such as gills, which can result in altered cell membrane fluidity and possibly permeability (Rawn 1989; Castell et al. 1994). A study examining the fatty acid composition of the amphipod, *Gammarus duebeni*, when challenged with varying water salinities reported considerable changes in the levels of EFA in the animal's total lipids (Dawson et al. 1984). Specifically, as salinity decreased the levels of PUFA and LC-PUFA in the gill lipids increased. Takeuchi et al. (1989) also found that the lipids of gill tissue of Atlantic salmon changed when the fish were exposed to different water salinities. In particular, the n-3 LC-PUFA content of the gill phospholipids decreased and the level of ARA increased with increasing salinity. More recently, Glencross and Rutherford (2011) observed a suite of pathologies possibly related to disrupted ion balance associated with the elevation of DHA in the diet of juvenile Asian sea bass. An increase in sub-cutaneous hemorhaging, imbalances in potassium and sodium in the plasma, and elevated plasma creatinine and urea levels were all indicative of disruption to the animal's ability to effectively regulate ionic balance. Interestingly, these pathologies were mitigated when EPA was also provided in the diet, but not at higher DHA inclusion levels (equivalent to the levels of DHA and EPA combined) or with the inclusion of ARA in the diet, which actually resulted in the highest plasma urea levels of the study (Glencross and Rutherford 2011). In another recent study examining high levels of dietary DHA, it was found that high inclusion levels (5% of diet/30% of total fatty acids) resulted in an increase in muscular lesions and the presence of ceroid pigment in the liver of European sea bass, again suggesting that an excess of certain EFA can actually be deleterious to the health of the animal (Betancor et al. 2011).

The interaction between EPA and ARA is indicative of the second mechanism whereby EFA can affect ionic balance: through the regulation of eicosanoid production. Eicosanoids are involved in regulating the active transport of ions across the gill membranes (Beckman and Mustafa 1992). An early study demonstrated that, compared to EPA, ARA was the preferred substrate for formation of prostaglandin (PGE_2) in gill tissue of turbot (Henderson et al. 1985). In Atlantic salmon, there was also a strong relationship between dietary n-6 PUFA intake and eicosanoid production in gill tissue (Bell et al. 1991, 1993). Eicosanoid production was greater in gill tissue than in liver or intestinal tissues, and eicosanoids such as PGE_2 have also been shown to stimulate active ionic transport in the kidney (Stokes 1981). However, eicosanoids have been shown to have a major role not only in ionic regulation and response to stress in fish, but also in immune system function (see following section;

Ghioni et al. 2002; Petropoulos et al. 2009; Verlhac Trichet 2010).

Lipids and Fatty Acids and Immune Responses

To understand how lipids and fatty acids can affect immune responses, it is important to first appreciate the complexity of the different elements of the immune system. As in most vertebrates, the immune system in fish consists of both innate and adaptive responses (Corbel 1975; Verlhac Trichet 2010). The innate response is non-specific, mediated by cellular and humoral components, and involves no immulogical "memory" (Corbel 1975). The cellular components of the innate system include phagocytes and natural cytotoxic cells (NCC). Phagocytes are involved in the production of enzymes, reactive oxygen species, and nitric oxide as defence mechanisms, whereas NCC possess receptors that recognize cell wall proteins expressed at the surface of viral infected cells and then destroy those cells (Verlhac Trichet 2010). The humoral components of the innate system include interferons and inflammatory response factors, which involve a range of chemical mediators such as histamine, prostaglandins, cytokines (e.g interleukin (IL) 1β, 6, 8, 10, and 12), and tumor necrosis factor-α (TNF-α).

In contrast, the adaptive immune response is pathogen-specific and mediated by an immunoglobin response to specific antigens, which then stimulates lymphocyte (B-cell and T-cell) activation. In fish, the adaptive immune system is less specific than that of other vertebrates, and has a shorter response and limited immunolglobin repertoire (Verlhac Trichet 2010). It is the antigen specificity that allows for the generation of responses to particular pathogens or pathogen-infected cells (Corbel 1975). The ability to generate antigen-specific responses is also a key part of what is referred to as immunolgical "memory." Thus, should a pathogen infect the animal more than once, the immunolgical memory from the previous infection will rapidly reactivate the specific immune system to respond to and eliminate the threat. Fish have a limited array of immunoglobins that they generate in their immune response to antigens, typically just IgM and IgG analogs (Corbel 1975).

In contrast to fish, crustaceans have no adaptive immune response and rely solely on an innate system. Like most invertebrates, shrimp have the basic mechanims of self versus non-self recognition, cells with phagocytic capacity, and a system of lectins that acts somewhat like antibodies. However, it is the lack of an adaptive immune system that makes shrimp and other crustaceans particularly susceptable to viral pathogens (Verlhac Trichet 2010).

Lipids and fatty acids play important roles in the immune response of fish by affecting the functions of different elements of both the innate and adaptive systems (Fig. 3.6). Both inflammatory and immune cells are sensitive to changes in dietary fatty acid composition (Calder 2001; Fritsche 2007). There have also been reports of selective retention of certain fatty acids in immune cells, such as head kidney macrophages (Montero et al. 2003). An increase in dietary short-chain n-6 PUFA has been observed to lower leucocyte production in Atlantic salmon (Thompson et al. 1996; Petropoulos et al. 2009), but had no effect on phagocytic activity of the head kidney macrophages (Gjøen et al. 2004). In addition, some autoimmune and inflammatory disorders were exacerbated by an increase in the dietary n-6 to n-3 PUFA ratio or with excess provision of particular fatty acids (Terano et al. 1986; Calder 2001; Betancor et al. 2011; Glencross and Rutherford 2011). Alterations in cellular immunity, particularly macrophage, phagocytic, and bacteriocidal activities, have been reported to be reduced in channel catfish and gilthead sea bream fed diets with either soybean or rapeseed oil replacing FO (Sheldon and Blazer, 1991; Montero et al. 2003). Although the reduction in phagocytic activity in gilthead sea bream was observed with the use of single VO, a mixture of VO blended to produce a similar balance to the saturates, monounsaturates, and PUFA found in FO had no major effect on macrophage activity (Montero et al., 2008).

A study by Xu et al. (2010) demonstrated that supplementation of ARA to the diet of Japanese sea bass, *Lateolabrax japonicus*, resulted in enhanced levels of activity of immune system marker enzymes including serum lysozyme, components of the alternative complement pathway, and superoxide dismutase. However, the substitution of dietary FO with VO in feeds for gilthead sea bream did not affect serum lysozyme activity (Montero et al. 2003, 2008). Montero et al. (2003) also demonstrated that certain pathologies and immunological parameters can be

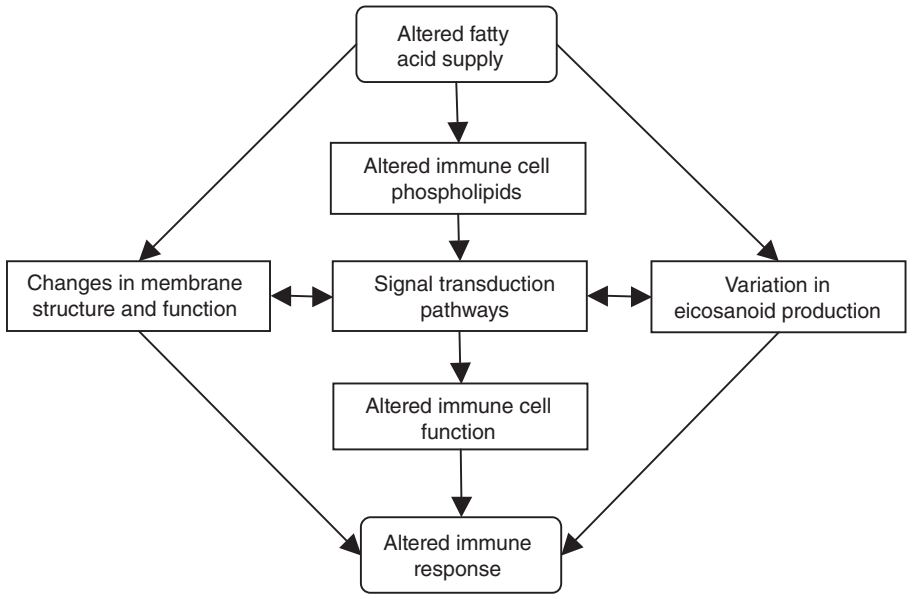

Figure 3.6 Mediation of effects on immune function through changes in dietary fatty acid supply. Derived from Calder (2007).

induced in European sea bass by elevating the ratio of n-6 to n-3 fatty acids with short-chain n-6 PUFA rather than long-chain. Particularly, the alternative complement activity in the serum appeared to be sensitive to changes in dietary lipids, most notably an increase in 18:2n-6 through the use of soybean oil (Montero et al. 2003). It was also indicated that high levels of substitution by single VO had greater impact on the immune system than the use of VO blends or lower levels of substitution (Montero et al. 2003, 2008; Montero and Izquierdo, 2010). The overall outcome of these studies is that it is generally regarded that dietary supplementation with n-3 LC-PUFA is beneficial for health of marine fish through modulation of both inflammation and immune cell function (Calder 2001; Montero et al. 2003; Xu et al. 2010). However, there were some differences among species, with salmonids having a more negative response to n-3 PUFA compared to n-6 PUFA (Montero and Izquierdo 2010).

The LC-PUFA eicosanoid derivatives incuding PGs, prostacyclins (PGI), thromboxanes (TX), LTs, lipoxins (LX), and hydroxy- and epoxy-fatty acids are all implicated in immune response and tissue inflamation processes, and the role that fatty acids play in regulating the production of these hormones is a critical facet of regulation/modulation of the immune system by lipids (Fig. 3.5). Eicosanoids are synthesized by almost all cells in the body and genenerally recognized as having autocrine or paracrine roles; they act through receptor-mediated G-protein-linked signaling pathways (Funk 2001). Production of eicosanoids by macrophages via both the COX and LOX pathways is one example of the major importance of these locally acting hormones in the immune response (Hwang 1989; De Caterina and Basta 2001). The production of PGs via COX inhibits both T and B lymphocyte functions and, as such, it is proposed that these hormones are negative feedback modulators of the immune response (Hwang 1989; De Caterina and Basta 2001). Similarly, the application of COX inhibitors has been reported to enhance both cell-mediated and humoral immune responses in animals (Hwang 1989; Funk 2001). However, the production of LOX-derived derivatives (e.g., LTs and hydroxyperoxyeicosatetraenoic acids, HPETE) is thought to result in immunostimulatory effects. An example of this is the production of LTs by neutrophils, which are also important to the inflammatory response (De Caterina and Basta 2001). As such, these two pathways act in an antagonistic manner, somewhat balancing the immune status of

the animal (Hwang 1989; De Caterina and Basta 2001). Modulation of the production of eisosanoids is therefore a major mechanism whereby the fatty acid profile of membrane phospholipids of various cells can play an important role in regulating the immune system in fish (Tocher 1995).

As described above, the regulation of eicosanoid synthesis is partly based on competition between fatty acid precursors for the COX and LOX enzymes that produce PG and LT, respectively (Henderson et al. 1985; Calder 2001). The proinflammatory 2-series PG and 4-series LT is produced from ARA (Fig. 3.5), and less potent or relatively anti-inflammatory 3-series PG and 5-series LT is produced from EPA (Tocher 1995). As inflammation is an essential protective response to injury and infection, ARA is the primary precursor for eicosanoids despite that general preponderance of EPA in fish (Hwang 1989; Bell et al. 1995b; Villalta et al. 2008). However, the availability of the precursor acid is a key factor in the regulation of eicosanoid synthesis (Hwang 1989), and the presence of EPA regulates conversion of ARA to PG and LT via competitive inhibition (Terano et al. 1986; Garg et al. 1990; Garg and Li 1994). It has been suggested that EPA may be the preferred fatty acid substrate for some LOX enzymes, but that ARA also competes as a substrate for these enzymes (Hwang 1989). Furthermore, it has been postulated that other PUFA may also play a role in competitive access for these enzymes; the addition of 18:4n-3 and 20:4n-3 to salmonid cell cultures resulted in the inhibition of $PGF_{2\alpha}$ production from ARA (Ghioni et al. 2002), while the addition of 18:3n-6 to the diet of rats resulted in reduced proinflamatory Il-1 and TNF-α (Harbige 2003). In another study examining immunomodulatory actions of PUFA in rat leukocytes, ARA stimulated the production of PGE_2 while the addition of DHA or EPA both reduced PGE_2 production (Peterson et al. 1998). Replacing dietary 18:3n-3 with EPA reduced lymphocyte proliferation, whereas replacing18:3n-3 with DHA had no effect (Peterson et al. 1998). Similar effects with supplementation of EPA and DHA have also been observed on IL-2 production, natural killer cell activity, and cytokine and TNF production by macrophages (Calder 1999). Although it is implied that increasing the relative amounts of n-3 fatty acids and reducing n-6 fatty acids suppresses tissue levels of ARA and reduces prostaglandin synthesis, there is evidence that not all n-3 PUFA have the same effect on this process (Peterson et al. 1998; Calder 1999).

Other inflammatory processes have been shown to be mediated by the n-3 LC-PUFA, EPA, and DHA through mechanisms that are not fully understood. In particular, the resolvins, which are further COX-derived products produced from n-3 LC-PUFA such as resolvin E1 from EPA and D-series resolvins and protectin D1 from DHA, have been shown to possess potent anti-inflammatory properties (Seki et al. 2009). The DHA-derived resolvins and protectins have also been identified in rainbow trout, *Oncorhynchus mykiss* (Hong et al. 2005).

Recently, genomic technologies such as transcriptomic analysis via oligo microarray are providing data that are beginning to elucidate the complex interactions between dietary nutrients and immune and inflammatory responses in fish. It was shown that the expression of inflammatory/immune genes was reduced in salmon fed a functional feed with lower lipid content and the EPA/ARA ratio increased in fish experimentally infected with Atlantic salmon reovirus (ASRV), the causative agent of Heart and Skeletal Muscle Inflammatory disease (HSMI; Martinez-Rubio et al. 2012). The effects on gene expression correlated with the intensity of the innate immune response and the severity of the lesions in heart tissue (reduced viral load and heart histopathology scores), and were associated with tissue fatty acid compositions and eicosanoid metabolism (Martinez-Rubio et al. 2012, 2013b).

Lipids and Fatty Acids and Disease Resistance

Disease or stress can be identified as resulting in a loss in immuno-competence. It is the ability of an animal to resist this disease or stress-induced loss of immuno-competence that can be decribed as disease resistance (Verlhac Trichet 2010). Disease resistance in fish is dependent on a wide range of factors including environmental variation, type and duration of pathogen exposure, age, temperature, stress, and dietary factors such as nutritional deficiencies and imbalances (Montero and Izquierdo 2010). Lipids and fatty acids in particular play various roles in the disease resistance of fish. At the most fundamental level, gross energetic deprivation is a significant cause

of immune depression. Deprivation of dietary protein and lipid can therefore lead to an alteration in cellular immunity and also depletion in lymphocytes and their functions (Verlhac Trichet 2010). In contrast, a high dietary fat intake can also suppress lymphocyte function, although this is dependent on the level and type of fat consumed (Calder et al. 2002).

As described above, changes in cell membrane fatty acid composition can alter membrane functions that, in turn, affect a range of physiological processes including the immune system (Verlhac Trichet 2010). Changes in fatty acid composition of the lipids within the cell and of the cellular membrane can also affect gene transcription. It is postulated that this occurs via transcription factor regulation and a series of intracellular signaling cascades (Sweeny et al. 2005). An example of the multiple effects of fatty acids on different systems was shown when elevation of the n-6 and n-3 PUFA ratio in the diet of Atlantic salmon post-smolts had marked effects on the incidence of atherosclerotic lesions, altered ability of the liver to detoxify xenobiotics, and also decreased resistance to bacterial infection (Thompson et al. 1996). Dietary n-3 LC-PUFA have also been observed to affect disease resistance in fish through an increase in the efficacy of some vaccines (Salte et al. 1988). In some cases immunosuppressive effects, possibly associated with increased dietary n-3 PUFA, have been reported in Atlantic salmon after the fish were experimentally infected with *Yersinia ruckerii* (Erdal et al. 1991). Replacement of FO with corn oil (rich in 18:2n-6) reduced mortalities in channel catfish after they were experimentally infected with *Edwardsiella ictaluri*, whereas dietary linseed oil (rich in 18:3n-3) had no effect on survival (Fracalossi and Lovell 1994). There are also numerous studies reporting both effects and absence of effects of VO substitution on immunization efficacy and disease resistance in fish (see Montero and Izquierdo 2010). There are clearly a series of species-specific responses and oil-specific/fatty-acid-specific effects at play, and further studies are required to better define the mechanisms involved.

In addition to general alterations in membrane structure and function, perhaps the most notable impact that dietary fatty acids have on disease resistance is through alterations to the synthesis of PGs and LTs (discussed in 'Lipids, Fatty Acids, and Health'). In other vertebrates, studies have shown that fatty acids have specific roles in key immune responses such as modulation of T-cell signaling (Brassard et al. 2007), and have also been shown to affect inflammatory mediators and splenocyte cytokine gene expression as well as change the expression of IL-2 and IL-10 genes (Tsou et al. 2008). All of these effects of fatty acids can also lead to possible changes in disease resistance.

Conclusions

Lipids are important in energy supply, cellular structural integrity and function, and as substrates for key hormones involved in stress (e.g., thermal, behavioral, ionic) managment and immune response. Starvation alone depletes lipids and retards an animal's stress response and immune function, leading to a reduction of disease resistance. In addition, there are many studies demonstrating that changes in dietary fatty acid compositions can affect fish health and welfare. Many of the reported effects could be a direct consequence of altered cellular fatty acid compositions, particularly of crucial biomembranes, with associated important functional consequences. Specifically, alterations to dietary n-3 PUFA: n-6 PUFA ratios and, particularly dietary EPA: ARA ratios, have important consequences for eicosanoid metabolism. Indeed, the role of EFA including DHA in eicosanoid synthesis is a pivotal one in the function of the immune system. In particular, it affects the balance between immunosuppression and immunostimulation via substrate competition, principally from ARA and EPA, but other LC-PUFA can also contribute to this competitive process. Although a range of effects of dietary lipid and fatty acid on the health and welfare of fish have been reported, they are often not consistent between species or between different trials using the same species. The often conflicting and inconclusive nature of the data suggests that there are many interacting and confounding factors involved that preclude the drawing of general conclusions.

In summary, it is clear that dietary lipid and fatty acids have a range of effects that could influence fish health and welfare; however, these are variable depending upon factors including fish species, level and duration of dietary alteration, other dietary components, and environmental conditions. As a result,

further studies are required to fully understand the impact that changes in dietary lipid and fatty acid may have in different fish and crustacean species.

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Chapter 4

Carbohydrates

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Introduction

Carbohydrates are natural constituents in fish feed due to the binding properties of starch, which highly affect pellet quality, and also due to the contribution of carbohydrates to dietary energy. As for other animals, the healthy function of fish brain/nervous tissue, red blood cells, and gonads will depend on the available amount of glucose (Wilson 1994; Moon 2001). Fish fed diets without carbohydrates will still have near-normal blood glucose levels through the stimulation of increased gluconeogenetic activity from precursors such as glucogenic amino acids (Hemre et al. 2002). Carbohydrates have therefore been found to have protein-sparing effects in most fish species studied. The expansion of aquaculture production combined with a lack of available fish meal resources has led to an increased focus on the inclusion of plant protein ingredients in fish diets (Torstensen et al. 2008). Replacement of fish meal by plant protein ingredients has resulted in diets containing highly variable amounts of carbohydrates, depending on the cultivated species. Different plant ingredients also contain different amounts and qualities of carbohydrates.

In this chapter, only the carbohydrates available for metabolism (digestible carbohydrates) will be discussed. Fish have the total enzymatic system to digest and absorb carbohydrates, but the capacity of the system might be limited depending on the species, life stage, water temperature, the level and type of carbohydrate in the diet, and the degree of processing. There is still much debate if it is better to strictly limit dietary carbohydrates to levels below a species' metabolic capacity to digest, regulate, and utilize carbohydrates, thereby naturally avoiding diabetic-like conditions, or to maximize carbohydrates (a natural, cheap source of energy) to the tolerance level of the species.

Glucose plays a part in energy homeostasis, feeding control, and functions that are regulated by levels of circulating nutrients; although the importance of glucose as a regulator has not yet been fully identified (Sandoval et al. 2008), it is discussed in this chapter. The capacity of fish to rapidly clear circulating glucose seems slow when compared to mammals, which has resulted in the notion that fish are glucose intolerant (Moon 2001). Hemre et al. (2002) reported increased insulin and glucagon as a response to high carbohydrate intake, followed by increased plasma glucose levels, even in carnivorous fish. However, insulin secretion is more responsive to amino acids, especially arginine. Unlike in mammals, insulin and glucagon seem to cooperate in fish (Suarez and Mommsen 1987). Glucagon is known to stimulate gluconeogenesis in mammals, while insulin stimulates energy storage, e.g. fatty acid synthesis and glycogen synthesis. In isolated salmon hepatocytes

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however, both glucagon-like peptide and glucagon stimulate gluconeogenesis. Glucagon is also found to have an effect on glycogenolysis, which supports a mammal-like function of glucagon in fish. Glucose tolerance tests in cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) had shown a similar pattern as found in diabetic humans, where up to 96 hours was required before blood glucose returned to basal levels (Hemre et al. 2002). Still, reports have indicated that glucose may affect feed intake and function as a sensor in fish brains (Polakof et al. 2008). Several fish species have demonstrated a necessity for glucose for brain function (Soengas and Aldegunde 2002). Glucokinase, the main glucose-sensing marker in mammals, has been identified in rainbow trout brain and could be related to food intake regulation (Polakof et al. 2009). Several glucose transporter proteins exist to facilitate glucose uptake into tissues, especially the low-affinity transporter GLUT2 (glucose transporter 2) identified in rainbow trout (Marty et al. 2007). Recently, several glucose transporters are identified in Atlantic salmon given high plant diets; GLUT1, 2, and 3 are highly expressed in liver and GLUT4 are highly expressed in fast muscle. GLUT1 and GLUT3 are expressed in brain but at lower levels, and GLUT1 in mid gut (Sissener et al. 2013). This data is preliminary and must be confirmed in the future research. However, results are similar to earlier findings with rainbow trout and it can be stated without doubt that glucokinase in brain is significantly affected by diet (Sissener et al., 2013; Polakof et al. 2008).

Fish might grow well with diets containing excessive levels of digestible carbohydrate, but may require a feed intake adjustment to meet protein and energy requirements to sustain growth (Lekva et al. 2010). If excessive levels of dietary carbohydrates are included, the results will be decreased feed utilization and changes in carbohydrate metabolism. Carbohydrates exceeding the metabolic capacity of the fish might also result in long-term metabolic disturbances that lower resistance to stress, alter the immune function, and reduce disease resistance.

Biochemistry

Chemically, carbohydrates belong to the polyhydroxy aldehydes or ketones, substances that yield such compounds after hydrolysis. Their basic formulae is $C_n(H_2O)_n$. There are three major classes of carbohydrates: monosaccharides, oligosaccharides, and polysaccharides. The most abundant monosaccharide is glucose, $C_6(H_2O)_6$. Short chains of monosaccharides joined together by covalent bonds are called oligosaccharides. Disaccharides consist of two monosaccharides, for example, sucrose consists of one glucose linked to one fructose. The most abundant disaccharides in fish diets are maltose and isomaltose, products resulting from the processing of starch (from plants in the fish diet mixture). Polysaccharides consist of long chains of monosaccharide units. The polysaccharide chains can be linear, with glucose linked together in α-1,4-glucosidic linkages (amylase), or branched, with branch points linked together in α-1,6-glucosidic linkages (amylopectin). Both starch and glycogen are homopolysaccharides consisting only of glucose. In nature, there also exists heteropolysaccharides, which contain two or more different kinds of monosaccharide units. For example, hyaluronic acid of connective tissue contains alternating residues of two different sugar units. Glucosamine is also classified as sugar; it is a major part of the exoskeleton of crustaceans, and is a part of the natural diets of many fish. Glucosamine is a sugar molecule containing nitrogen with the formulae $C_6H_{13}NO_5$.

Plants store their carbohydrate energy as starch and animals store theirs as glycogen. Stored carbohydrate energy in plants occurs as large clusters or granules that are highly hydrated with many exposed hydroxyl groups. When these are extracted from the cells, they form turbid colloidal solutions or dispersions, important characteristics for the physical quality of fish diets. Different plant starches vary in their amounts of amylase and amylopectin, which affects their physical properties. The type of plant ingredient added to the diet mixture will therefore affect both the physical and nutritional quality of the pellet. The fiber fraction and antinutrients found in plants also belong to carbohydrates; these are not discussed here however.

Carbohydrates can be linked to protein (or amino acid) molecules, glycoproteins (or glucosamines; see above), or lipids called glycolipids (e.g., myelin and sphingomyelin). Polysaccharides are broken down by α- and β-glucosidases, whereas di- and oligosaccharides are hydrolyzed by various brush-border enzymes into their constituent monosaccharides. Fish species

differ greatly in their ability to digest carbohydrates. This variability reflects anatomical and functional differences of the gastrointestinal tracts and associated organs of fish. The capacity to hydrolyze a great variety of carbohydrates is more developed in herbivorous and omnivorous fish than in carnivorous fish, although all fish species investigated so far have the full enzymatic apparatus necessary to hydrolyze complex carbohydrates. However, the magnitude of the apparatus varies. Details on carbohydrate digestibility coefficients for various fish species has been reviewed by Krogdahl et al. (2005). Amylase, which hydrolyzes α-1,4- glucosidic linkages, has been localized throughout the entire gastrointestinal tract (GI) of many fish species, with higher amounts observed in herbivorous and omnivorous compared to carnivorous (Krogdahl et al. 2005).

The ability to adapt amylase secretion to match carbohydrate intake may be restricted to herbivorous and omnivorous fish, and some carnivorous even demonstrated reduced amylase activity when fed high-starch diets (Spannhof and Plantikow 1983). Amylase inhibitors are reported to be present in several grains (Sturmbauer and Hoffer 1985). Di-saccarides are present in fish mucosa of pyloric, mid, and distal segments of herbivorous, omnivorous, and carnivorous fish (Krogdahl et al. 2005). These are specific to the disaccharide they hydrolyze, for example maltose will hydrolyze maltase. Generally, the highest activity of amylase and disaccaridases can be found in the proximal parts of the GI. This may partly explain why most of the glucose (70%) absorption will take place in this region (Buddington et al. 1987).

Intestinal transport plays an important role in intermediary carbohydrate metabolism and the regulation of intestinal hormone release (Collie and Ferraris 1995). Monosaccharides, mainly D-glucose but also some D-galactose and D-fructose, may cross the brush-border membrane by the aid of specific transporters or by simple diffusion. The piscine D-glucose transporters of the brush border show characteristics similar to those found in mammals, for example they are dependent on energy and Na^+ (Collie and Ferraris 1995). D-glucose and D-galactose uptake are energy- and Na^+-dependent with close to similar affinity to the glucose transporter, while most of the D-fructose is absorbed by means of facilitated diffusion (Collie and Ferraris 1995). Glucose transporters identified in fish are GLUT2 (Marty et al. 2007), and preliminary data show that GLUT1, 2, 3, and 4 all exist and are expressed differently in mid-gut, liver, fast muscle, and brain (Sissener et al. 2013).

Functions and Metabolism

Although carbohydrates are not a nutritional requirement of fish, they are still an important nutrient in fish feeds. First, carbohydrates are the most abundant and cheapest energy source compared to protein and lipids. Diets that do not contain carbohydrates typically use protein and lipids for energy, which may also serve as precursors for biological compounds that are traditionally derived from carbohydrates (NRC 1993). Including carbohydrates to spare the use of protein and/or lipids has several advantages. Utilization of carbohydrates reduces catabolism of protein and decreases the amount of ammonia being released into the aquatic environment through protein oxidation, thereby minimizing environmental pollution. Increased use of carbohydrates to spare lipids can also make feeds less vulnerable to oxidation during processing and storage. Maximizing carbohydrate inclusion in fish feed is therefore not only cost effective for feed production but can also ease management related to feed and culture environment.

The second important function of carbohydrates is to serve as binding agents, viscosity builders, and suspending agents in fish feeds. These functions play important roles in determining the physical criteria of pellets, such as water stability, hinder leaching of other water soluble nutrients, and affect density and durability. Pellet water stability determines how stable a pellet is in the aquatic system. Leaching of nutrients is always a major concern and/or challenge in aquatic feed processing. The density of a pellet determines its buoyancy, which is critical for aquatic animals with different feeding behaviors. Durability is related to the hardness of pellets and is important during transportation, handling, and feeding. These physical parameters can be affected by the level or type of carbohydrate used in the feed pellet formulation.

Third, some carbohydrates such as polysaccharides or oligosaccharides have been found to function as prebiotics to improve growth, digestibility, feed conversion ratio, and immunity of fish

(Burr et al. 2008; Gatlin and Burr 2009; Merrifield et al. 2010). Several oligosaccharides, such as fructo-oligosaccharides (FOS), mannan-oligosaccharides (MOS), xylo-oligosaccharides (XOS), and inulin and polysaccharides such as ß-glucan (Denev et al. 2009; Xu et al. 2009) have been well recognized as functional prebiotics. Research in this area is still in its infancy however, and will need further comprehensive investigation to understand the related mechanism.

Finally, a carbohydrate can link with a polypeptide or a fatty acid to form a glycoprotein or a glycolipid, respectively. These biological compounds serve as cell membrane structures and are important in cell-cell communication, tissue connection, and other varied biological activities.

Influx of exogenous glucose into an organism mainly depends on digestion, absorption, and transportation of dietary carbohydrate. Digestion of carbohydrate is the first step for carbohydrate metabolism after feeding. Similar to the digestion in other animals, the major product of carbohydrate digestion in fish is glucose, which serves as a carbon substrate for different metabolic purposes. Other monosaccharides may only account for small quantities (NRC 2011). All enzymes needed for carbohydrate digestion are present in fish but their activities may be limited in some species, especially coldwater carnivorous. This limitation varies among species, dietary quality, culture environment, and differences in life stage. Glucose transport is the second step affecting the influx of glucose into the bloodstream and subsequently its utilization. Glucose transporters (GLUT, types 1, 2, and 4) delivering glucose among different tissues have been documented in fish. Their low affinity to glucose, when compared with the same transporters in mammals, might partially account for the prolonged hyperglycemia that occurs in fish after they are fed with carbohydrates (NRC 2011).

Glucose absorption is generally lower in fish than that in mammals (Collie and Ferraris 1995; Bakke-McKellep et al. 2000). Lower densities of sodium glucose transporter and smaller amounts of absorptive tissue are the reasons for the lower capacity of glucose uptake in intestine (Krogdahl et al. 2004). Among different species of fish, transport of glucose across the basolateral membrane of intestine is adaptive to glucose/carbohydrate levels in herbivorous and omnivorous species by either changing the density of the transporter or altering tissue mass (Collie and Ferraris 1995; Krogdahl et al. 2004). No adaptation of glucose uptake capacity is found for carnivorous fish, however (Buddington et al. 1987; Buddington and Hilton 1987). This could partially explain why some carnivorous species of fish have poor ability to utilize carbohydrate. In Atlantic salmon however a regulation of gut transporters has been observed in response to different diets, but the capacity to further utilize carbohydrate after absorption is limited (Sissener et al. 2013). It is therefore hypothesized that the carbohydrate utilization for this fish is regulated after absorption. A recent study by Panserat et al. (2009) found that metformin, an anti-diabetic drug, relieved hypoglycemia phenomena without affecting glucose transport but induced lipogenic enzyme activities. This suggests that poor utilization of glucose by peripheral tissues may be one of the reasons for prolonged postprandial hyperglycemia in fish.

Glucose homeostasis is a balance between exogenous intake, metabolism, and clearance of glucose. Metabolism of carbohydrate consists of catabolic and anabolic processes, which includes glucose oxidation through glycolysis, glucose storage by glycogen synthesis, and glucose biosynthesis (glucogeneogenesis and glycogenolysis). Fish begin to utilize dietary carbohydrate after digestion, starting with glycolysis to generate energy from glucose or glycogen through the Krebs cycle. Excess glucose may be converted to glycogen through glycogenogenesis, transformed into lipid by lipogenesis, or excreted into urine and/or through the gills (Hung and Storebakken 1994; Deng et al. 2001; NRC 2011). Excretion of glucose through urine in fish is relatively low, but glucosuria has been documented in a few species such as yellowtail, tilapia, and white sturgeon (Furuichi et al. 1986; Lin et al. 2000; Deng et al. 2001). Glycosuria was significantly elevated when the plasma glucose concentration exceeded 8 mmol L^{-1} in white sturgeon intubated with glucose. The total urinary glucose excretion in white sturgeon only amounted to 5.2% of the intubated dosage, however (Deng et al. 2001).

The major tissues involved in glucose metabolism include liver, muscle, kidney, adipose, and intestine. Liver is the main organ for glucose metabolism and glycogen storage. White muscle, kidney, heart, brain, and intestine also store glycogen but at a much lower level. These tissues depend on the bloodstream to

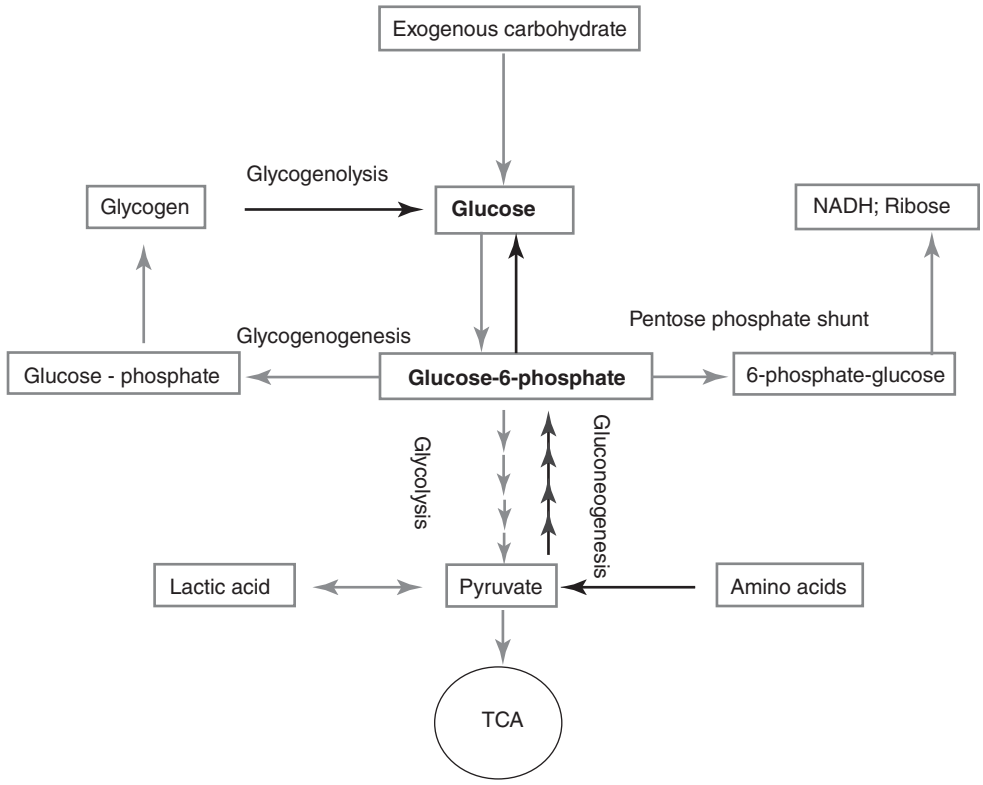

Figure 4.1 The main routes of exogenous carbohydrate utilization and endogenous glucose production, simplified. Each route may dominate differently depending on exogenous and endogenous supply. Glycolysis, glucogenogenesis, and pentose phosphate shunt are pathways involved in glucose utilization. Glycogenolysis and gluconeogenesis are pathways for glucose production. Biochemical textbooks are recommended for more detailed information on each pathway.

transfer glucose for their energy, and function differently in maintaining glucose homeostasis. Kidney and liver are glucose producers and muscle is a glucose user (Suarez and Mommsen 1987). White adipose tissue may be involved in lipogenesis, and also possibly glucose homeostasis (Polakof et al. 2012). The poor utilization of glucose by peripheral tissue is postulated to be one of the reasons for hyperglycemia in coldwater carnivorous fish fed a carbohydrate diet, but the mechanism for the function of those tissues in glucose metabolism is still not well understood. Recently, Polakof et al. (2010) identified that intestine of rainbow trout was a metabolically active tissue involved in glucose homeostasis regulation and glycogen storage, and the intestinal muscle probably served as energy for local movement or activity. This study also suggested that glucagon-like peptide-1 and C-peptide play major hormonal regulatory roles on glucose metabolism in fish gut, and insulin does not. Regulation of glucose metabolism in different tissues might be different and the understanding of their mechanism is still very limited. Research in this area will help to unravel the mystery of the poor utilization of dietary carbohydrate in fish.

Different pathways are involved in the regulation of glucose homeostasis in fish, including glycolysis, lipogenesis, glycogenogenesis, glycogenolysis, and gluconeogenesis (Fig. 4.1). Among these pathways, glycolysis is the major metabolic pathway for glucose catabolism to generate energy or other intermediate products that are used for pentose phosphate pathway, glycogen, or lipid synthesis. The key enzymes regulating this pathway have been shown to respond to dietary carbohydrate intake. For example, glucokinase, the

first enzyme of the glycolytic pathway in liver of many fish species, has been found to be inducible by dietary carbohydrate (Tranulis et al. 1996; Panserat et al. 2000; Enes et al. 2009). The glucokinase activity is induced due to the increased gene expression of this enzyme after the fish are fed with dietary carbohydrate. Glycolysis is therefore not considered as the limiting step for the poor carbohydrate utilization for most fishes, as has been previously suggested (Enes et al. 2009, 2011).

Increased liver glycogen storage in response to dietary carbohydrate intake has been observed in many species of fish (Lee et al. 2003; Enes et al. 2011). Similarly, induced lipogenesis in fish fed carbohydrate have been documented, although the induction may vary among species, complexity, and levels of carbohydrate (Hung and Storebakken 1994; Shiau 1997; Hung and Deng 2002; Brauge et al. 1994). In contrast, glycogenolysis and gluconeogenesis, which involve production of glucose, are not regulated by dietary carbohydrate intake (Panserat et al. 2000, 2001; Enes et al. 2009). The poor regulation of endogenous glucose production is one of the reasons for the limited ability of starch utilization, but not the ineffective regulation of the glycolytic pathway. This may partly explain prolonged hyperglycemia and poor carbohydrate utilization in fish that have adapted to limited exogenous carbohydrate in the natural environment. In Figure 4.1 the major pathways in glucose metabolism are illustrated.

Teleost fishes are generally considered to be glucose intolerant based on a glucose tolerance test (Moon 2001). The prolonged hyperglycemia is more severe in carnivorous fish than herbivorous and omnivorous fish (NRC 2011). Carbohydrate metabolism is regulated by the endocrine system and some hormones, such as insulin and glucagon, have similar regulatory control over carbohydrate metabolism in fish as in mammals (Chan and Woo 1978; Puviani et al. 1990; Mommsen and Plisetskaya 1991). In fish liver, insulin stimulates glycogen synthesis and decreased glycogenolysis and gluconeogenesis; glucagon and glucagon-like peptide-1 increase glycogenolysis, glucose oxidation, and gluconeogenesis (Hemre et al. 2002). Insulin has shown to have an important role in stimulating hepatic glucose uptake, enhancing glucokinase activity, and leading to an increase in liver glycogen content to maintain glucose homeostasis in some fish fed a high carbohydrate diet (Enes et al. 2010). Accumulated evidence has suggested that the persistent hyperglycemia is not due to a low level of insulin in fish as hypothesized. Insulin levels in fish are actually comparable to (or even higher than) those found in mammals, but the insulin response to digestible carbohydrate intake varied among species of fish (Mommsen and Plistskaya 1991; Krogdahl et al. 2005). It is hypothesized that the poor response of plasma glucose to insulin, especially in carnivorous fish such as rainbow trout, is related to the low sensitivity of the Brockmann Bodies insulin secretion system in response to glucose (Polakof et al. 2010). Increased carbohydrate utilization is exhibited in growth hormone transgenic coho salmon (*Oncorhynchus kisutch*) (Leggatt et al. 2009). This demonstrates the importance or potential involvement of growth hormone in carbohydrate metabolism. Other hormones, such as insulin-like growth factor (IGF-1), cortisol, triiodothyronine (T3), and thyroxine (T4), are also involved in carbohydrate metabolism, although their exact mechanisms are still not well understood (Leung and Woo 2010).

Utilization

Carbohydrate utilization in fish is affected by different factors which can be categorized as: (1) biological factors; (2) dietary factors; and (3) environmental factors. Biological factors mainly include species of the animal, different life stages, and nutritional status of the animals. Among the different species of fish, carnivorous fish normally have lower abilities to utilize carbohydrate than omnivorous and herbivorous fish. The poor ability of carnivorous is due to low activity of digestive enzymes and a limited number of and less-adaptive glucose transporters. Activity of amylase that breaks down polysaccharides varies among different species of fish, with higher amylase activity in omnivorous compared to carnivorous fish (Hidalgo et al. 1999). Disaccharidase activities are also significantly different among species of fish; herbivorous fish have the highest level while carnivorous fish have the lowest (Harpaz and Uni 1999). Glucose absorption is found to be low in carnivorous fish due to less abundance of sodium glucose transporter sites (Collie and Ferraris 1995; Bakke-McKellep et al. 2000). Moreover, differently from omnivorous or herbivorous fish, carnivorous fish do not show

adaptive regulation of endogenous production of glucose by gluconeogenesis in response to dietary carbohydrate (Enes et al. 2008a; Kirchner et al. 2008). Within the same fish species, larvae tend to have poorer capacity to digest carbohydrate compared to juvenile and grow-out stages, partly explained by a change in digestive enzyme activities at different life stages (Cahu and Infante 2001; Savona et al. 2011).

Carbohydrate utilization by fish is affected by different dietary factors, which include the quality and quantity of carbohydrate. Other dietary nutrients such as protein and lipid levels and feeding regime have also been shown to affect carbohydrate utilization. Different types of carbohydrates have different chemical structure, granule size, the ratio of amylopectin and amylose, and level or type of anti-nutrients (NRC 2011). For example, the starch granule size is 22 μm for wheat starch and 35 μm for corn starch. Corn contains a high level of trypsin inhibitors compared to wheat (Venou et al. 2003). These characteristics make corn starch less digestible than wheat starch to fish. The molecular complexity of different carbohydrates is wide-ranging and affects carbohydrate utilization, which varies among the species of fish that have been studied. Some fish have better ability to utilize polysaccharides and oligosaccharides than mono- and disaccharides, such as carps (Erfanullah and Jafri 1995; Singh et al. 2006); red sea bream (Furuichi and Yone 1982); tilapia (Shiau and Peng 1993); flounder (Lee et al. 2003); gilthead sea bream (Enes et al. 2010); and cobia (Cui et al. 2010). It has been shown that glucose is better utilized than complex carbohydrates in white sturgeon (*Acipenser transmontanus*) (Hung et al. 1989), rainbow trout (*Oncorhyncus mykiss*) (Bergot 1979), and grass carp *(Ctenopharyngodon idella*) (Tian et al. 2010). Utilization of glucose and starch, however, was similar in grouper (Shiau and Lin 2001).

The quality of other nutrients in the test diets, such as the dietary protein quality and quantity, level of lipid, and the method used to process test diets, need to be considered when results from different studies are compared. For example, it has been reported that insulin secretion in fish is more responsive to amino acid, such as arginine, than glucose level in bloodstream. The quality of dietary protein/amino acid profiles could therefore affect insulin action and thus change levels of blood glucose, which can interfere with the feed intake of fish (Polakof et al. 2008). If dietary protein levels are used to balance the change of carbohydrate levels for test diets, poor growth in fish fed high carbohydrate diets may be due to protein/amino acid deficiency instead of only dietary carbohydrate. To address these confounding factors, a pair-feeding strategy has been conducted utilizing diets in which carbohydrate was the only nutrient that varied (Moreira et al. 2008). Other dietary nutrients, such as lipid levels, have also been found to affect carbohydrate digestibility, with high lipid levels resulting in decreased carbohydrate digestibility (Fountoutake et al. 2005). In rainbow trout, poor dietary carbohydrate utilization is partially related to increased hepatic glucose production under conditions of high dietary fat intake (Panserat et al. 2002).

The effect of nutrient supplementation levels on carbohydrate utilization has been extensively studied. Digestible starch can be added to up to 50% in feeds for omnivorous species, but only 15–25% for salmonids and carnivorous marine fish (NRC 2011). These levels are also dependent on the complexity of carbohydrate. For instance, if more digestible carbohydrate such as dextrin is used, an inclusion level of more than 30% can be utilized in feed formulations for some tropical marine fish without any adverse effect on growth (Deng et al. 2010). The protein sparing effect by dietary carbohydrate may vary among different species of fish, and some discrepancy has also been observed in the same species of fish (Hemre et al. 2002). This variation can be explained not only by different species of fish, but also by experimental conditions such as diet formulation, water temperature, and feeding strategy. Lipogenesis of dietary carbohydrate has been reported in some species but the mechanism is still unclear and under debate (Hemre et al. 2002). Dietary carbohydrate is considered to play more roles in the production of NADPH (from $NADP^+$) to stimulate lipid synthesis rather than provide carbon backbones for lipid biosynthesis. On the other hand, carbohydrate is considered to be used for energy and may prevent lipid from oxidation, therefore leading to an increase in lipid accumulation.

When nutrient utilization is compared between different feeds containing different types and levels of carbohydrates, water stability of feed pellets is a critical factor. Increased level of complex carbohydrates often results in increased stability and reduced leakage

of water-soluble nutrients. Feed water stability is a critical criterion that affects the nutrient utilization of fish, for example in fish larvae, or in fish with slow feeding habits such as sturgeon. Also related to this, feeding regime has been found to be important for carbohydrate utilization. When continuous feeding was compared to a meal-feeding strategy for rainbow trout, white sturgeon, and grass carp, continuous feeding resulted in enhanced growth and carbohydrate utilization (Hung and Storebakken 1994; Lin et al. 1997; Tian et al. 2010). Increased feeding frequency (small meals) may have avoided an overload of carbohydrates in the fish and thus digestion and absorption of carbohydrate was increased and urinary glucose excretion decreased. Feeding regime can therefore affect carbohydrate utilization in some species and life stages of fish.

Feed processing method is another factor affecting carbohydrate utilization. The common methods for feed processing of experimental diets include meat grinder, pellet mill, and extruding methods. Extrusion cooking improves starch gelatinization, which affects pellet physical quality including binding and durability of feed pellets. Gelatinization of starch can affect the rate of starch hydrolysis by enzymes, and therefore enhances its digestibility (Venou et al. 2003). On the other hand, different processing methods involve the use of different moisture levels and temperatures, which can cause nutrient damage, for example, maillard reaction between glucose and amino acid (Deng et al. 2005). The severity of maillard reaction is also dependent on the type and level of carbohydrate used in the feed, and is more severe with simple sugars than complex carbohydrates. Moreover, ingredient particle size can affect carbohydrate utilization because reduction in particle size of ingredients increases their surface area within a pellet and improves gelatinization of carbohydrates, thereby increasing pellet durability, water stability, and digestion (Obaldo 1998; Venou et al. 2003).

Although published information is limited, it has been shown that carbohydrate utilization is affected by environmental factors such as salinity, temperature, and photoperiod. Higher starch digestibility was documented for Atlantic salmon and rainbow trout when held in freshwater compared to seawater (Storebakken et al. 1998; Krogdahl et al. 2004). Increased temperature has been shown to enhance digestibility of carbohydrates in European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*; Enes et al. 2006; Moreira et al. 2008). Aside from the improved digestibility of carbohydrate, the enhanced carbohydrate utilization at a higher temperature was partially due to the augmented capacities of glycolytic, lipogenic, and gluconeogenic enzymes in liver (Enes et al. 2006, 2008b). An increase in water temperature also improved carbohydrate utilization for energy in rainbow trout (Médale et al. 1991; Brauge et al. 1995) and Atlantic salmon (Hemre et al. 1995). Atlantic salmon fed either 10% or 20% of gelatinized corn or wheat, and cultured under different photoperiod conditions, exhibited different glucose metabolism (Hemre et al. 2001). This study also demonstrated the interaction between photoperiod, level, and type of starch. Liver glycogen values reflected dietary starch level and were influenced by light regime, but the muscle glycogen was influenced only by light regime. High starch level in the diet also resulted in lowered saltwater tolerance in parr of salmon when challenged.

Carbohydrate and Stress Responses

Optimal and tolerable levels of dietary carbohydrate for fish are different as discussed by Hilton et al. (1987) and Hemre et al. (2002). A tolerable level is often defined as having no impairment to growth and survival. An optimal level is based on maximum utilization of carbohydrate for energy or other biological functions without adverse effects on metabolic functions, and then often benefits from secondary effects such as protein or lipid sparing. Therefore, the tolerable and optimal levels of carbohydrate in a fish diet, and their resulting formulations, are not necessarily the same. The carbohydrate level that does not affect growth performance of fish may cause impairment to physiological reactions and metabolic aspects, such as prolonged postprandial blood glucose, impaired liver function, or immune modulation (Hemre et al. 2002; Kumar et al. 2007). When cod was fed diets with either no or increasing starch levels, the elevated inclusion of carbohydrate did not result in any growth depression. However, starch levels above 7% of dry matter resulted in metabolic stress as indicated by increased plasma glucose and blood lactate, and lowered ability to regulate plasma glucose back to basal levels after handling stress (Hemre et al.

1990, 1991). Fingerlings of L. *rohita* could tolerate around 50% of dietary gelatinized corn starch, but immune status of the fish was impaired after being challenged with *Aeromonas hydrophila* as indicated by lower survival (Alexander 2011).

Besides, different levels and types of carbohydrate have been shown to induce variable effects on the stress response of fish. A study of *L. rohita* showed that a combination of gelatinized and non-gelatinized starch at a ratio of 20:80 provided better protection of immunity in fish than a diet containing only gelatinized starch (Kumar et al. 2008). On the other hand, impairment of fish performance due to inclusion of carbohydrate may be due to an interference with utilization of other nutrients, such as lipid digestion, or interaction with minerals, which can also affect stress resistance in fish. In order to obtain a comprehensive evaluation of carbohydrate utilization and its effect on fish, it is recommended that a long-term feeding study with integrated biomarkers based on response from cellular, tissue, and whole organism responses is conducted.

Carbohydrates and Immune Responses in Fish

In fish, both non-specific (innate) and specific immune system help to protect against damage caused by infectious agents. The specific immune system includes antigen and cell-mediated responses (Waagbø 1994). Increasing knowledge on the role of carbohydrates in the general metabolism and immunity of fish may contribute to a reduction of losses from diseases by hindering the progress of diseases and/or improving recovery from diseases. Modern diets for all farmed fish contain high levels of plant ingredients, and there is a constant search for new ingredients to support growth and health. In particular, plant feedstuffs rich in proteins have been extensively studied (Torstensen et al. 2008; Glencross et al. 2010). These protein-rich plants often contain high levels of carbohydrates, including indigestible fiber, anti-nutrients, and available sugars and starches. Evidence of inflammatory responses to components of plants suggests their involvement in the enteric immune system (Krogdahl et al. 2005). The responses will depend on whether the fish species is carnivorous, omnivorous, or herbivorous; it has been demonstrated that diets high in plant ingredients may be effective for raising omnivorous and herbivorous species, but have limitations in rearing carnivorous fish (Krogdahl et al. 2005).

Handling, changes in water temperature and pH, hypoxia, and other factors may result in stress responses in fish that lead to increased cortisol release. Cortisol has important anti-inflammatory and immunosuppressive properties, resulting in secondary suppression of the immune system (Balm 1997). A suboptimal diet, for example carnivorous fish given high starch feed, may further strengthen the stress response, and result in higher cortisol peaks in stressed fish given high-carbohydrate diets compared to fish fed on low-carbohydrate diets (Hemre et al. 2002). Suppression of the immune system may result in higher death rates when subjected to pathogenic organisms (Waagbø 1994; Sangio-Alvarellos et al. 2005). Handling of fish fed a suboptimal diet may result in further decrease of immune functions. As a result, the fish might be more susceptible to pathogens during those periods of production. Efforts should be made to find diet compositions that could be used during periods of stress, which would stimulate the immune system to respond and hinder disease.

The mechanisms whereby changes in immune parameters are altered by dietary carbohydrates have been studied by means of biomarkers describing parts of the immune system. Commonly used biomarkers, besides the already-mentioned stress enzymes, include C-reactive protein (CRP), interleukin-6 (IL-6), IL-10, IL-18, lyzosyme, tumor necrosis factor-alpha (TNF-alpha), and more.

In southern catfish, lowered lyzosyme activity as a measure of immunity was found when given high dietary carbohydrate, without any variations in fish growth (Qiang et al. 2007). For that reason, these authors recommended that immunity should always be included, besides growth, in studies to determine optimum levels of dietary nutrients. Similar results on lyzosyme were not found in European white fish (*Coregonus lavaretus*) given dietary levels of increased corn starch (Vielma et al. 2003). The increase in corn starch resulted in increased liver glycogen, increased plasma glucose, and decreased plasma immunoglobulin M (IgM). The lack of response of lyzosyme to dietary carbohydrates was also found in studies

with Atlantic salmon (Hemre et al. 2002). A set of immune parameters should therefore be included to evaluate the effects of the diet on fish immune function, as these have different functions and will respond differently depending on the species but also the size of the fish, environmental parameters, and other factors. In the carnivorous *Dentex dentex*, the various stress/antioxidant enzymes only moderately responded to a huge variation in dietary protein: lipid: carbohydrate ratio (Pérez-Jiménez et al. 2009). The only enzyme induced with *Dentex dentex* in the study was CuZn-SOD (superoxide dismutase) II in white muscle at high protein levels, while dietary carbohydrate ranging from 4 to 28% did not result in any changes in the measured stress enzymes or in lyzosyme activity.

Feeding Atlantic salmon diets high in plant ingredients resulted in several changes in macronutrient metabolism (Torstensen et al. 2008). The plant diets held close to equal levels of lipid and protein but varied in starch, fiber, and composition of fatty acids and amino acids; however, all known requirements were covered (NRC 2011). The increased inclusion of plants resulted in changed transcription of glucose transporters and enzymes related to carbohydrate turnover (Sissener et al. 2013). In addition to alterations in glucose turnover, several of the stress enzymes (anti-oxidant enzymes) responded to the high plant diets (Olsvik et al. 2011). The transcription factors affected by plant diets were involved in pathways related to cell division and differentiation, immunology, cytokine expression, inflammatory responses, and more. Alternative feed ingredients potentially altering growth and intermediary metabolism may be expected to affect the oxidative homoeostasis, and thereby parts of the immune system in fish (Olsvik et al. 2011). The determination of which components in the plants are responsible for those alterations is not fully understood, but they are likely caused by several dietary factors and not only the carbohydrates.

In mammals, the liver is the main source of complement proteins; hepatic diseases can disturb complement and increase susceptibility to diseases (Ellison et al. 1986). Liver damage caused by excess dietary carbohydrates might therefore negatively affect the liver complement, but limited knowledge exists on this matter in fish (Holland and Lambris 2002). In both carnivorous and omnivorous fish, excess dietary carbohydrate may cause metabolic stress and cellular changes. For example, both *Catla catla* (omnivorous) and rainbow trout (carnivorous) responded with changes in liver histology due to large glycogen stores when given high dietary carbohydrate levels (Krogdahl et al. 2005). However, no evidence of a carbohydrate-mediated effect on the immune system was found in rainbow trout by Page et al. (1999), indicating that large glycogen stores in rainbow trout liver did not necessarily result in altered immune function. On the contrary, the omnivorous Indian major carp, *Labea rohita*, responded without any liver histology or hematology changes, and with improved respiratory burst activity of phagocytes when fed a diet containing 46% dietary starch along with 50 mg kg^{-1} amylase (Kumar et al. 2005). Increasing amylase further to 100 mg kg^{-1} and/or gelatinizing the starch did, however, resulted in increased starch availability along with negative effects on immunity. This indicates that, even in omnivorous fish, the amount of available starch needs to be balanced to have positive and prevent negative effects on fish immunology. The variable results reported on the importance of dietary carbohydrate on immunity both within and between species may be due to variable rearing conditions (water temperature, pH, dissolved oxygen, etc.), fish size, fish species, as well as the response parameters measured to evaluate the status of fish health in the various investigations. In Atlantic salmon, a reduced tolerance to dietary carbohydrate was registered at low water temperatures (Hemre et al. 2002). A focus should therefore be placed on carbohydrate metabolism not only due to changes in raw materials in diets (marine versus plants), but also due to expected changes in climate. The non-digestible carbohydrates such as ß-glucans have been found to greatly affect fish immune system, especially the enteric system. This product is being promoted as an additive (prebiotic) in fish diets to enhance certain immune parameters, thus improving the resistance to infectious diseases.

Carbohydrates and Disease Resistance

Fishes are exposed to a complex array of infectious agents such as viruses, bacteria, fungi, and parasites (Argayosa and Lee 2009; Argayosa et al. 2011).

The presence of mannose moieties on the cell surfaces of the test microorganisms confirms a lectin–carbohydrate interaction involved in agglutination activity of African catfish mannose binding protein (MBP) and serum against the microbes. Lectins bind carbohydrate moieties on viral and cell surfaces, which restricts bound and agglutinated pathogens from spreading, multiplying, and infecting other tissues and organs. Mannose-binding lectin has been proposed as a biomarker for disease resistance in vertebrates, including several teleosts (Jensen et al. 1997; Argayosa and Lee 2009; Argayosa et al. 2011). Any link between carbohydrate moieties (mannose) on the cell surface and the type of carbohydrate that dominates in the fish feed is uncertain at this time. If metabolism is normal there will probably be no interconnection, but if there is a constant elevated plasma glucose in the fish (e.g., being metabolically stressed and in a "diabetic-like" condition as found in both Atlantic salmon and Atlantic cod given high carbohydrate diets), there might be disturbances in the immune system (Hemre et al. 2002). A suboptimal feed may disturb the fine balance between cells and immune systems functioning as related to fish disease resistance. Research conducted at the University of Barcelona, Spain shows that red blood cells in fish have a complex function including resistance towards pathogenic bacteria and virus. If so, this means that any dietary damage to the red blood cells may reduce disease resistance in the fish. In Atlantic salmon fed a high starch (30%) diet however, glycosilation of the hemoglobin in red blood cells was not detected; glycosilation is the first indication of cell damage caused by high dietary starch levels (Hemre et al. 2002).

Few studies link feed carbohydrate to disease resistance after a pathogen challenge. Atlantic salmon averaging 5 kg and given diets with starch levels ranging from 0 to 30% did not show any alterations in humoral immunity after vaccination by intraperitoneal injection or dip immersion with *Vibrio salmonicida* (Waagbø 1994). Challenging the same fish population with *Aeromonas salmonicida* by intraperitoneal injection did show a slight but significantly lowered mortality in fish fed a diet containing 10% starch when compared to fish given lower or higher starch levels. Similar results could not be found in smaller fish (3 g) exposed to immersion challenge with *Vibrio anguillarum*; no difference in mortality due to diet was registered. The authors concluded that dietary carbohydrate affected the resistance to bacterial infections in Atlantic salmon but not always, and only to a minor degree.

Conclusions

Fish species differ greatly in their ability to digest, absorb, and metabolize carbohydrates. This variability reflects anatomical and functional differences of the gastrointestinal tract and associated organs. All species investigated so far have the full enzymatic apparatus necessary to hydrolyze and absorb carbohydrates, but the magnitude varies, especially between herbivorous and omnivorous as compared to carnivorous fish. However, existing evidence suggests that glucose sensing in fish brain affects feed intake in a similar manner to mammals, and that the glucose transporters necessary for uptake of glucose into organs exist with characteristics similar to those found in mammals, for example the glucose transporter 2 (low affinity transporter) dominating in liver and brain of rainbow trout.

Carbohydrate is the cheapest and most readily available energy source in fish feed. It plays important roles in determining physical quality of feed pellets, such as binding and water stability, leakage of water-soluble nutrients and floatability of fish feeds. Carbohydrates also function as prebiotics that have been shown to enhance fish growth and health. Some polysaccharides or oligosaccharides are found to serve as components of biologically active compounds (glycoproteins and glycolipids). Metabolism of carbohydrate in fish involves similar pathways as in mammals. Metabolic regulation, however, is different between mammals and fish, and even varies among fish species. All enzymes for carbohydrate digestion are present in fish but the quality and quantity of these enzymes may differ. Prolonged hyperglycemia in fish fed a carbohydrate-rich diet may be due to less regulatory capacity of metabolisms for endogenous production of glucose, but not utilization or oxidation of exogenous glucose. Glucose intolerance in fish is not due to low levels of insulin, but is instead a result of other actions that this hormone involves. Other hormones, such as growth hormone, IGF-1, T3, and T4, are also involved in the regulation of carbohydrate metabolism, but the exact mechanism of their actions

is still not well understood. Utilization of carbohydrate in fish is affected by several factors, including biological, dietary, and environmental factors. These factors should be taken into consideration when results between different studies are compared.

Inclusion of carbohydrate has indicated stress at the metabolic level especially in carnivorous fish, even though no adverse effect on growth has been observed. In fish, both a non-specific and specific immune system helps to protect against damage caused by infectious agents. Handling, changes in water temperature, hypoxia, water pH, and other factors cause stress responses in fish that lead to cortisol release. Cortisol has important anti-inflammatory and immunosuppressive properties, resulting in secondary suppression of the immune system. A suboptimal diet, for example one high in starch given to a carnivorous fish species, may further strengthen the stress response, and result in higher cortisol peaks in stressed fish given high-carbohydrate diets compared to fish fed low-carbohydrate diets. Parameters important for immune function are also found to respond differently in fish depending on the carbohydrate content of the diet and the subjected species. This may explain why Atlantic salmon withstood a challenge to the bacterial disease caused by *Aeromonas salmonicida*, with higher survival when held on a moderate- to low-carbohydrate diet compared to a high-carbohydrate diet.

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Chapter 5
β-Glucans

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Introduction

One of the most commonly used immunostimulants is β-glucan. Beta-glucans are known as "biological response modifiers" because of their ability to activate the immune system. Research efforts to improve fish survival by increasing disease resistance have identified glucans, polysaccharide polymer components of cell walls, as substances capable of increasing survival in fish exposed to pathogenic bacteria. Stimulation of non-specific immune responses in fish has been attributed to derivatives from yeast, fungus, and some bacterial preparations (Chen and Ainsworth 1992; Engstad et al. 1992; Ainsworth 1994; Chen et al. 1998; Cain et al. 2003; Bridle et al. 2005). Several β-glucan products prepared from different organisms are available commercially for use as feed additives (VitaStim-Taito (VST), MacroGard®, M-glucan, Zymosan, and EcoActiva™). Although they have been widely tested and used, researchers continue to evaluate the glucan products by examining the fishes' innate immune responses to glucans administered in feed, by injection, or in bath treatments. Reviews as recent as that by Ringø et al. (2012) still present questions about the action of glucans. In addition, more research is needed as new species of fish are farmed for human consumption or conservation purposes, and the response of these fish to immunostimulants is important to know. Approved disease treatments for fish are limited, and it will be beneficial to have alternative means to prevent or minimize disease outbreaks.

Glucans derived from fungus – lentinan from *Lentinus edodes*, schizophyllan from *Schizophyllum commune* Fries, scleroglucan from *Sclerotium glucanum*, and yeast glucan from *Saccharomyces cerevisiae* – are among the most-studied immunostimulatory glucans in mammals. These glucan preparations have also been tested in fish to increase their non-specific immune response and resistance to infections. Glucans have been given to fish by injection, bath, or orally, and all means of administration have provided some protection against opportunistic pathogens in disease challenges. However, in some instances where contradictory results have been obtained, the variable results may be due to the dosage level when injections/baths are used, or the compound's digestibility/length of the feeding period when the glucan is administered in the feed.

The use of immunostimulants in fish diets has accelerated in recent years as more production-grade diets are fortified with a variety of natural substances to heighten innate immunity. Promotions of the use of such diets cite increased health and survival of fish fed immunostimulant diets compared to fish fed standard production diets. Laboratory research continues to focus on injection, immersion, intra-gastric, and intestinal administration of immunostimulants, methods that are not practical for large-scale production applications (Raa 1996; Sakai 1999; Ringø et al.

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2012). Investigations of the effects of orally administered immunostimulants have only occurred in the past decade, and caution must be used in drawing conclusions from the studies. Production-level studies have not been conducted to any great extent and, beyond anecdotal evidence, few published reports support claims of uniform benefits from immunostimulants in large-scale aquaculture production settings. Fish farmers have experienced variable results, although laboratory trials have produced positive immunostimulatory effects at the molecular and cellular level. The effects measured *in vitro* or *in vivo* in controlled laboratory experiments cannot be assured under less-uniform production conditions.

Glucans as feed additives have met with some uncertainty and skepticism because the results of various feeding trials have not shown consistently positive effects (Ramberg et al. 2010). The exact mechanism of glucan stimulation of the innate immune response is not completely known, although there have been advancements in that area. Toll-like receptors found on neutrophils, macrophages, and dendritic cells (Armant and Fenton 2002; Dalmo and Bøgwald 2008), and other pattern recognition receptors found on macrophages and dendritic cells, dermal endothelial, and mucosal epithelial cells (Engstad and Robertsen 1993; Gordon 2002; Dalmo and Bøgwald 2008) are thought to recognize pathogen-associated molecular patterns that glucans can simulate. Novak and Vetvicka (2008) reported on the discovery of a receptor on the surface of innate immune cells called Complement Receptor 3 (CR3 or CD11b/CD18) that is responsible for binding to β-glucans, allowing the immune cells to recognize them as "non-self."

Immunostimulants are promoted in aquaculture as a means to overcome the immunosuppressive effects that occur in normal aquaculture operations due to stressors (Thompson et al. 1993; Jeney et al. 1997), or unavoidable consequences of high-density culture (Vadstein 1997). Immunostimulants might be used as a prophylactic treatment in anticipation of expected seasonal outbreaks of known endemic diseases (Nikl et al. 1993), or as a suppressive treatment for latent or sub-lethal pathogens. Culture conditions promoting chronic, subacute, or acute disease outbreaks include crowding, handling, accumulation of biologic wastes, ambient flora and fauna, low oxygen levels, exposure to sunlight, and sub-optimal water temperatures. Unlike vaccines, immunostimulants simultaneously elevate the overall resistance of animals to many infectious agents by stimulating the non-specific immune responses.

An immunostimulant can be defined as a substance that enhances the immune system by interacting directly with cells of the system and activating them. Some responses that are routinely reported are: macrophage activation, increased phagocytosis by neutrophils and monocytes, increased lymphocyte numbers, increased serum immunoglobulin, and increased lysozyme (Raa 1996; Sakai 1999). Immunostimulants that are effective in fish diets in a laboratory setting act within the non-specific immune system at several levels. The first immune system defenses are substances found in mucus secreted by endothelial cells and macrophages directly attacking pathogens; these include many lytic and agglutinating factors. Proteins and enzymes act directly with molecules on the microbe's surface to inhibit bacterial growth or facilitate phagocytosis. The most common immunostimulants are the compounds that are recognized by the cellular components of the non-specific immune system and that initiate the same humoral and cellular responses as pathogenic organisms.

The evolutionary history of each aquatic species determines the individual immune factors that are present and the magnitude and success of their response against immunomodulatory agents or pathogens. Biological rationale for immunostimulants in the fish's diet is based on the evolutionary history of immune system development in aquatic organisms (Manning et al. 1982; Chevassus and Dorson 1990; Wiegertjes et al. 1996). Survival in the aquatic environment requires an immune system that can combat the constant challenges of waterborne pathogens. Immunomodulators present in the diet stimulate the non-specific immune system while antigenic substances, such as bacterins or vaccines, initiate the more prolonged process of antibody production and acquired immunity (specific immune response) (Anderson 1993). Aquatic organisms evolved immediate, generalized responses to compensate for the continual exposure to pathogens and developed delayed specific responses that require time for acquired immunity to develop. Fish immunologists have concentrated their investigations of immunostimulation in laboratory research designed to explain the

actions of individual immune response components to immunomodulation. Responses have generally been associated with the non-specific immune system, although antigen-antibody based enhancement has been reported (Raa 1996; Sakai 1999). The difficulty in deciding whether glucans will help in a specific situation arises because not much consistency is found in the literature concerning form of the glucan, dose, length, and frequency of treatment or application of the treatment, that is, in the feed, injected, or in a bath. Additionally, the effects of the glucans on the non-specific immune responses are varied. A reported lack of response may be due to the fact that the immune response affected was not the one tested. Prophylactic and therapeutic administration of immunomodulators will need to be adapted to each cultured species in anticipation of recognized pathogens, under known environmental conditions.

This chapter will cover the sources and biochemistry of glucans. In addition, the beneficial effects of glucans on stress, immune functions, and disease resistance, as well as the effectiveness of glucans in vaccines, is discussed.

Sources and Biochemistry

Immunostimulants for aquaculture use may be chemically or biologically produced (Nikl et al. 1991; Raa 1996; Sakai 1999). However, the biologically produced compounds dominate in both research and feeding studies (Robertsen et al. 1990, 1994; Raa 1996; Sakai 1999; Bricknell and Dalmo 2005), and glucans are the predominant immunostimulants used. The biological sources of most glucans added as immunostimulants to commercial fish diets include mycelial fungus and yeasts, and a few bacterial preparations (Robertsen et al. 1990; Rodriguez et al. 2003; LaFrentz et al. 2012). Non-specific immunity in higher animals, such as fish, has developed over evolutionary time in an environment containing large amounts of the yeast, fungi, and bacterial cells and their by-products. Fish and other aquatic organisms are constantly exposed to the waterborne pathogens and substances that trigger an immune response. Through the process of selection, substances that promote immune stimulation, including pathogens, have produced different levels of immune response in fishes occupying vastly different environments.

Most of the immunostimulants in fish diets are polysaccharides derived from bacteria, fungi, or yeasts, and plants. These substances may be the cells themselves, or preparations from the cell walls containing the β-1, 3 and β-1, 6-glucan molecules that initiate the non-specific immune response. An emphasis in aquaculture research has been placed on β-glucans (Robertsen et al. 1994; Ringø et al. 2012); these are preferred because they occur naturally and are less likely to cause concerns related to water quality or residue in food fish. Structure of the glucans is very important for activity; β-glucans without any or few side chains are of limited activity in fish (Ringø et al. 2012). Figure 5.1 shows part of a generic glucan. Note the β-1, 3 and β-1, 6-glucan branches. Yeast glucans are the most commonly used immunomodulators in aquaculture. In processing, a layer of protein and lipids has to be removed to expose the active β-glucans found on the inner wall of yeast cells (Goodridge et al. 2009).

The proportion of β-1, 6 side chains to the number of glucose molecules in the β-1, 3 backbone are important because fewer and shorter side chains may reduce the activity of the glucan in fish. Physical treatment and processing of the glucans can affect length of the side chains and thus, the potency of glucan products (Saito et al. 1991; Vetvicka and Vetvickova 2007; Tiwari and Cummings 2009). The proportion of β-1, 6 side chains to the number of glucose molecules in the β-1, 3 backbone may also affect glucan potency. Several yeast species are used consistently in the production of many commercial glucan products. The most commonly used sources of glucan products include *Saccharomyces cerevisiae* (Baker's yeast), the source of MacroGard® and M-glucan, and the fungal preparations (*Schizophyllum commune* and *Sclerotium glucanicum*) (Robertsen et al. 1990; Nikl et al. 1991; Chen and Ainsworth 1992; Engstad et al. 1992; Matsuyama at al. 1992; Jeney and Anderson 1993; Jørgensen et al. 1993a, b; Jørgensen and Robertsen 1995; Siwicki et al. 1994; Sung et al. 1994; Santarém et al. 1997). The molecular structure of MacroGard® has been examined in some detail. It is a branched molecule with 83% of the glucose units bound in β-1, 3 linkages, 5% β-1, 6 branching points, 6% β-1, 6 linkages, and 5% of the glucose molecules in the non-reducing terminal positions. MacroGard® could possibly have greater efficacy if the β-1,6 polyglucose

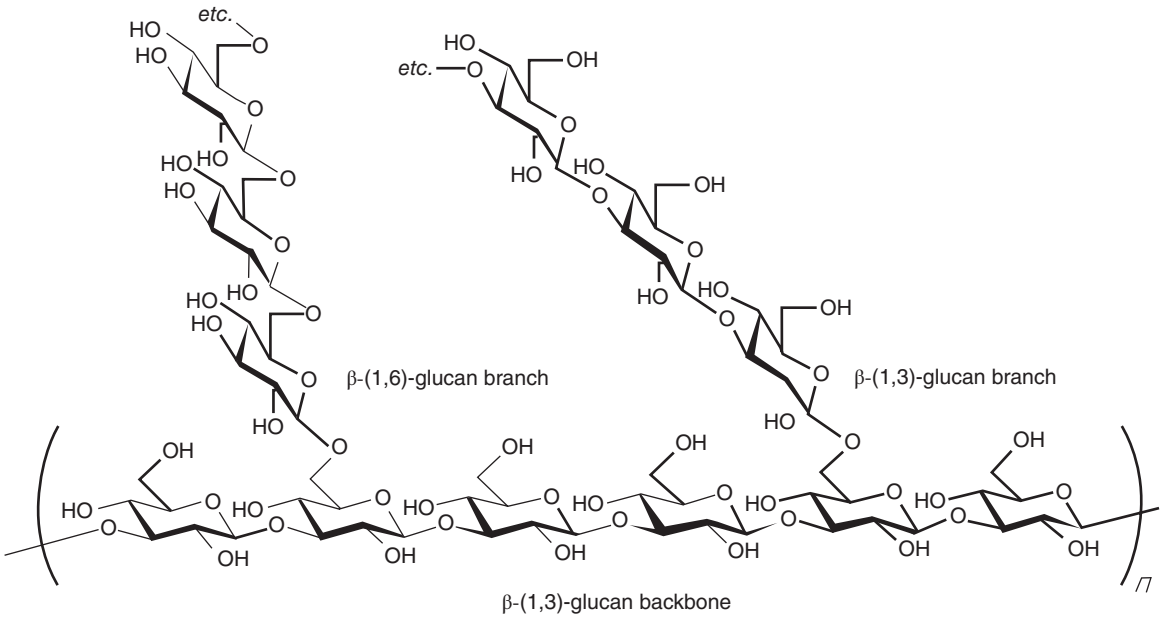

Figure 5.1 Generic glucan structure. Included are the β-(1, 3)-glucan backbone, a β-(1, 6)-glucan branch, and a β-(1,3)-glucan branch. Source: Gannam, A. L. and R. M. Schrock. Immunostimulants in fish diets. Journal of Applied Aquaculture 9: 53–89. Copyright © 1999, Taylor and Francis.

side chains were cleaved, leaving only the β-1,3 side chains on the β-1,3-glucose backbone structure (Robertsen et al. 1994).

The importance of the glucan structure is illustrated by the patent, "Method, or use of a solubilized glucan product to increase immunostimulation in animals" (Engsted et al. 2012), in which β-1,6 glucanase is used to treat *S. cerevisiae* to produce a glucan that is essentially free of β-1,6 linked glucose chains, enhancing its activity. Lentinan from *Lentinus edodes* (shiitake mushrooms) has two glucose branches for every five β-1,3 glucose units. Schizophyllan from *S. commune* contains one glucose branch for every third glucose structure in the β-1, 3 backbone. VitaStim Taito (VST) is produced from a *S. commune* preparation. Scleroglucan, an excretion product of *S. glucanum*, is similar in form to schizophyllan. Several studies have compared the immunostimulatory effect of other types of substances to β-glucans (Nikl et al. 1991; Siwicki et al. 1994; Refstie et al. 2010), but in many trials the yeast β-glucans prevail. An earlier study investigated ten polysaccharides to compare the effects of their chemical structures on the ability to activate the alternative complement pathway and macrophages (Yano et al. 1991). The β-1,6-glucosidic side chains of the β-1, 3-glucan branched side chains found in lentinan, schizophyllan, and scleroglucan preparations were identified as potentiators of the alternative complement pathway in fish. A membrane receptor protein was found for β-1,3-D-glucan in crustacean blood (Cerenius et al. 1994) and, when activated with glucan or other lipopolysaccharides, initiated phagocytosis and encapsulation of microorganisms by blood cells.

Saito et al. (1991) found that high molecular weight of β-1, 3-D-glucans in the single helix form were the most potent in activating Factor G from *Limulus* amebocyte lysate. In a later study, Aketagawa et al. (1993) also found the single helix was more active. Using curdlan, β-1,3-glucan from the culture medium of the bacterium *Alcaligenes faecalis*, purified paramylon from *Euglena gracilis*, and schizophyllan from *S. commune*, the authors determined that the glucan with the single helical conformation had a greater ability to activate *Limulus* coagulation factor G. In fish, the triple helical conformation found in VitaStim Taito (Nikl et al. 1993) appears to have greater activity than the single helix.

Other compounds that may have an immunostimulatory effect include polysaccharides containing sugars other than glucose (glycans). Atlantic salmon (*Salmo salar*) (5 g) have been immunized with an injection of a polysaccharide from the broth culture supernatant of *A. salmonicida*, which had mannose as the major constituent (Bricknell et al. 1997). Different immunostimulants may prove effective for different life stages based on solubility. Laminaran, an algal extract that is more soluble than the fungal and yeast glucans, has also proved to activate macrophages (Dalmo and Seljelid 1995) and increase respiratory burst activity in anterior kidney leucocytes of Atlantic salmon (Dalmo et al. 1996a) and cod (*Gadus morhua*) (Dalmo et al. 1996b). The authors promoted laminaran for its superior solubility compared with other β-1, 3-glucans, a factor that should be considered in candidate substances for diet applications. Absorption of laminaran from water by yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus*) through the skin and intestinal epithelium (Strand and Dalmo 1997) suggests that it may have the potential to enhance immunity in early life stages prior to the development of acquired immunity.

Products of bacteria may also stimulate non-specific immune responses in fish. However, research has predominantly focused on the effects of individual components of immune response at the cellular level rather than considering the whole aquatic organism. Bacterins (antigenic compounds prepared from bacteria) are prepared in a variety of ways for immunizations to provide protection against diseases (Jeney and Anderson 1993), even when antibodies are not detected following immunization (Anderson 1993). The bacterin may act as an immunostimulant on a non-specific level, providing the broad spectrum responses offered by other bacterial products (Jeney and Anderson 1993; Raa 1996).

How the yeast glucans are administered to the organism can, in some cases, affect the effectiveness of the glucan as an immunostimulant. Yeast glucans enhanced disease resistance in channel catfish (*Ictalurus punctatus*) against *E. ictaluri* when administered intraperitoneally (Chen and Ainsworth 1992), but not when administered orally (Duncan and Klesius 1996). The tissue distribution of radioactive-labeled, aminated β-1,3-glucan (immediately, and up to 24 hours after intravenous, intraperitoneal, and peroral administration in Atlantic salmon) suggests that regular daily administration of glucans, even orally, would maintain the presence of glucans in the identified tissues (Ingebrigtsen et al. 1993).

Effects on Stress

The use of glucans or other immunostimulants are often recommended before a stress event to enhance immune function and counteract the possible immunosuppression that can be associated with stressful events (Robertsen et al. 1994; Raa 1996; Vadstein 1997; Bagni et al. 2000; Bricknell and Dalmo 2005; Ringø et al. 2012). Stress events can be commonplace in aquaculture. Factors that can trigger stress in fish include handling (Cain et al. 2003; Kirchhoff et al. 2011), high density (Torrecillas et al. 2012), transportation (Jeney et al. 1997; Volpatti et al. 1998), impaired water quality (e.g., ammonia, nitrite, low oxygen, contaminants) (Boyd 1990; Schwaiger et al. 1997; Gupta et al. 2014), inadequate feed formulation (Bagni et al. 2000; Sitjà-Bobadilla et al. 2005; Refstie et al. 2010) and suboptimal feeding rate (Alcorn et al. 2003). Glucocorticoids secreted in response to the stress episode suppress lymphocyte, macrophage, and neutrophil function (Jeney et al. 1997), thereby suppressing the immune response. Also, in low ration-fed fish, there is an increase in phagocytosis by the macrophages, but a decrease in superoxide anion production (Alcorn et al. 2003). The feed formulation may also be a factor. Considering nutritional stress, Sitjà-Bobadilla et al. (2005) found that a diet containing 50% replacement of fish meal with plant proteins enhanced the plasma complement; however, when the level of replacement was 75% and above, plasma complement was reduced.

To address gut integrity issues often associated with the use of plant proteins, especially soybean meal, Refstie et al. (2010) tested two experimental diets with portions of fish meal (FM) replaced with 32% soybean meal (S; FM+S), or with 14% soybean meal+14% sunflower meal (FM+SS). Beta-glucans at 500 or 1000 mg kg^{-1} or a mannan oligosaccharide-rich product (MOS) at 1000 or 2000 mg kg^{-1} were added to the FM+S diet. Additionally, 1000 mg β-glucans or 2000 mg MOS kg^{-1} were added to the FM+SS diet. The β-glucans were found to improve Atlantic salmon resistance to sea lice, and adding MOS improved gut

integrity and health. Approximately two-thirds of the immune system defense is in the mucous membrane of the gut, so maintaining that integrity is important for the organism's health. Overall, the acknowledged stress of aquaculture is an important consideration when using research results to predict production-level performance.

Effects on Immune Functions

Innate (Non-specific) Immunity

Several factors can affect the activity of the innate immune parameters. Temperature changes and the aforementioned stressful events can suppress innate parameters, while immunostimulants enhance innate immune system factors (Magnadóttir 2006). Investigations of immunostimulants in fish diets have focused primarily on the humoral and cellular components of non-specific responses at the stage after absorption into the blood. Measurement of immune factors in the serum show that humoral changes occur when immunostimulants are administered, including a selective increase in lysozyme (Magnadóttir 2006; Saurabh and Sahoo 2008) and an increase in complement components in Atlantic salmon (Engstad et al. 1992). Increases in lysozyme have been measured in trout (Jørgensen et al. 1993b), salmon (Engstad et al. 1992), and yellowtail (*Seriola quinqueradiata*) (Matsuyama et al. 1992) after intraperitoneal injection or oral administration of yeast glucans. High blood leucocyte numbers were accompanied by increases in oxidative radical production and phagocytic activity in yellowtail (Matsuyama et al. 1992) and rainbow trout (*Oncorhynchus mykiss*), (Jeney and Anderson 1993; Siwicki et al. 1994). Glucan administration in Atlantic salmon increased serum protease and lysozyme activity after 4 days, and there was a peak in activity at day 10 during an *A. salmonicida* infection (Møyner et al. 1993). Lysozyme was found to increase after 2 weeks in 30 g turbot (*Scophthalmus maximus*) fed yeast glucan (de Baulny et al. 1996), an effect that persisted in a group fed the glucan plus vaccine. Further investigations should include tests for immediate increases of humoral substances found in the external mucus where pathogens are first encountered. If the immunostimulant is water-soluble, direct incidental exposure to immunostimulants leaching from the feed would be expected externally in addition to the response initiated after the diet was first ingested. The consequences of pre-existing high lysozyme levels before an infection, or the presence of increased lysozyme due to latent infection before immunostimulation, must be considered when determining the timing, dosage, and measurement of the effects of glucan-enhanced diets.

Studies of bacterial infections reveal that differences in how bacteria infect the organism will require different prophylactic immunostimulants. *V. anguillarum* is transported across the epithelium of the anterior intestine to the *lamina propria* to the liver, blood, and other tissues (Grisez et al. 1996). Yeast glucans are effective oral immunostimulants against *V. anguillarum* in salmon and as an adjuvant in a vaccine for *Vibrio* spp. The complete mechanism of action of most immunostimulants must still be defined based on experiments using intraperitoneal administration due to the limited number of oral trials to examine immune factors (Sakai 1999).

Intraperitoneal injections of glucans have both systemic and local effects on the non-specific defense system of fish (Anderson and Siwicki 1994). Injections of fungal preparations, schizophyllan, scleroglucan, and lentinan in common carp (*Cyprinus carpio*) at $2-10\,mg\,kg^{-1}$ fish enhanced the phagocytic activity of the pronephros phagocytic cells (Yano et al. 1989). Survival of the fish treated with the glucans improved. If yeast glucans are injected in Atlantic salmon (20–30 g) at a high dose ($100\,mg\,kg^{-1}$ fish), there is no protection for 1 week, but maximum protection occurs after 3–4 weeks. If a low dose ($2-10\,mg\,kg^{-1}$) is used, protection occurs at day 7 then declines (Robertsen et al. 1990). The prophylactic use of β-1,3-glucan for channel catfish culture has been proposed based on positive laboratory trials of injected glucan (Chen and Ainsworth 1992). A pulsed feeding of glucans was found to be more effective in stimulating a respiratory burst of polymorphonuclear leukocytes than continuous feeding in Nile tilapia (*Oreochromis niloticus*) (Welker et al. 2012). Bagni et al. (2000) also found that when glucans are fed in a pulsed manner, the innate immune response is stimulated. The authors fed a diet containing 2% glucan (Macrogard®) to sea bass (*Dicentrarchus labrax*) over a 2-week period every 3 months. The study lasted for 40 weeks. Both lysozyme and alternative complement activity were

elevated in the sea bass. Several factors affect the efficacy of glucans because each source acts differently.

Mode of delivery should be examined. Protection is lower when glucans are fed and higher when they are injected. One reason for the lower protection could be feeding glucans at a certain percent body weight, in which case all of the fish may not be getting a full dose and a positive affect may not be observed. In addition, the high molecular weight of some glucans raises the question of whether they are absorbed by the fish, directly affecting the gastric associated lymphoid tissue (GALT), or if they need further digestion to be absorbed.

Acquired (Adaptive or Specific) Immunity

Very few studies have documented the stimulation or enhancement of the specific immune response with the use of glucans. Dalmo and Bøgwald (2008) discussed the discovery of toll-like receptors on cell membranes that bind molecules, including pathogens and molecular structures, with pathogen-associated molecular patterns. These molecular structures can consist of repeating molecular functional groups, which form high-molecular-weight compounds such as β-glucans. Ultimately, helper T-cells are activated. Verlhac et al. (1998) found that specific immune antibody response was enhanced after vaccination when a yeast glucan was included in the diet of rainbow trout. Sahoo and Mukherjee (2001) determined that glucan in the feed at 0.1% increased both the non-specific and specific immune responses in an Indian major carp (*Labeo rohita*). In another study using common carp, Selvaraj et al. (2005) obtained increased non-specific and specific immune responses 7 days after injection of 100, 500, or 1000 μg of glucan. Immune responses and relative percent survival were better when the glucan was administered by injection as opposed to a bath or orally. In their review paper, Harikrishnan et al. (2011) indicated that immunostimulants, including glucans, have some specific immune responses in fish and shellfish.

Effects (Adjuvants) on Vaccines

Anderson (1992) compiled a review of the literature concerning immunostimulants, adjuvants, and vaccine carriers used in fish. He emphasized the need for care in using these compounds, as each substance has special issues concerning timing and method of administration, dosage adjustments for size and fish species, storage stability, and cost. In addition, non-specific immune responses in fish can be highly variable so well-controlled experiments and appropriate sample sizes are required in order to obtain valid results when testing these compounds.

Kawakami et al. (1998) found that M-glucan had no positive effects as an adjuvant for a vaccine used against *Pasteurella piscicida* infection in 8-g yellowtail. When only the immunostimulant was injected, there was a non-significant increase in protection against *P. piscicida*. Nikl et al. (1993) also found that the commercial β-glucan, VST, was not effective as an immunopotentiator when administered with the vaccine for *Aeromonas salmonicida* as a bath treatment. When VST was given in the feed at 0.1 or 1.0%, significant protection against *A. salmonicida* occurred. Generally, immunostimulants in vaccine formulations, especially the β-1, 3, β-1, 6-glucans (experimentally at least), have elicited very good antibody responses when used either to replace oil-based adjuvants or in addition to them, without the adverse side effects that have been reported for these types of adjuvants (Bricknell and Dalmo 2005).

Chen et al. (1998) used extracellular products of *Mycobacterium* spp mixed with Freund's adjuvant to stimulate non-specific response in Nile tilapia through injection. In that study, neutrophil activity, oxygen radical production, and serum lysozyme activity increased. The phasing of the peaks of activities of these immune factors emphasized the importance of the time of sampling on the analysis of effects. Glucans have been promoted as adjuvants for bacterins and vaccines; however, contradictory evidence of enhanced immunization effects and non-specific immune stimulation has been found with glucans of different origins. Jeney and Anderson (1993) found that glucans alone, in an injection or as a bath, may promote similar non-specific responses to a *Yersinia ruckeri* O-antigen in rainbow trout. They may also increase non-specific immune response as an adjuvant, compared with a bacterin alone, as Aakre et al. (1994) determined using *A. salmonicida* with Atlantic salmon. In 30-g turbot (*Scophthalmus maximus*), β-glucan did not increase survival when used as an adjuvant with a vibriosis vaccine over the vaccine

alone (de Baulny et al. 1996). Both the vaccine and the β-glucan (Macrogard®) were given to fish via the feed as a top-coating. The turbot white blood cell count did increase with the administration of only the β-glucan on the feed. Toranzo et al. (1995) used a glucan-fortified feed in conjunction with a vaccine against an *Enterococcus* sp. in turbot (45 and 150 g fish). The vaccine had a positive effect on the survival of the fish, more so for the smaller fish (89–100 relative percent survival) than the larger fish (67–86 relative percent survival), independent of the feed used. The authors also found that the feed with added glucans did enhance the non-specific immune response, but was not responsible for increased survival of the fish. Ali and Tamuli (2010) administered β-glucan by injection to an Indian major carp (*Labeo rohita*), then challenged the fish with *Aeromonas hydrophila* and reported that glucan alone enhanced the non-specific agglutinin, respiratory burst activity, and protective immunity of the fish. When given in combination with the *A. hydrophilla* vaccine, a stronger immune response was observed. Figueras et al. (1998) used β-1, 3-glucans from *S. cerevisae* as an adjuvant in a *Vibrio damsel* vaccine. The researchers injected turbot with the adjuvant either prior to, at the same time, or after the vaccine. The immune responses that were examined were index and rate of phagocytosis, passive hemolytic plaque numbers, and agglutinating antibody titers. All of the immune responses tested gave the best response when glucans were injected after the vaccine was given. In this study as well as in others, it is indicated that the sequence of glucans administration is critical when they are used as a vaccine adjuvant.

Effects on Disease Resistance

Throughout this chapter, the abilities of glucans to stimulate the immune response have been discussed. Increased activities of various components of the innate immune system have been demonstrated. Responses commonly reported are macrophage activation, increased phagocytosis by neutrophils and monocytes, increase in lymphocyte numbers, increase in serum immunoglobulins, and increase in lysozyme. However, enhanced immune responses do not guarantee disease resistance.

Many sites of entry are available to pathogens, with invasions occurring at all external sites and through gastrointestinal routes. Knowledge of the localization of macrophages in absorptive tissues and their mobilization after immune stimulation by immunomodulating substances or specific pathogens is crucial for the assessment of enhanced diets. An opportunistic pathogen, such as *A. salmonicida*, occurs commonly in cultured fish due to abrasions caused by crowding, and is inhibited in salmon and trout by immunostimulants of several biological origins (Nikl et al. 1991, 1993; Raa et al. 1992; Siwicki et al. 1994). When a pathogen first encounters the fish's external surface, mucus forms the first physical barrier and the epithelium is the second. Macrophages, one of the effector cells of immunostimulants, are first encountered in the gills and gut, and then in the internal organs such as the kidney and spleen. Macrophage stimulation is cited as the most frequently observed change in fish fed dietary immunostimulants.

It might be expected that pathogens that are effectively phagocytized by macrophages would be those most effectively combated by prophylactic feeding. Conversely, pathogens that have to be stopped by mucus or skin would be poor candidates for attack with immunostimulants. Rombout and van den Berg (1989) and Rombout et al. (1989b) showed that both soluble and particulate antigens are taken up by epithelial cells of the second gut segment of the hindgut and transported to intraepithelial macrophages. In addition, immunological defense cells, such as lymphocytes, granulocytes, macrophages, and monocyte-like cells, were identified in the intestinal mucosa of the common carp. Pathogens that can pass the physical barriers of skin, mucus, and the secreted non-specific humoral factors encounter the cells of the mucosa lining. The mucus-producing cells are found in the mucosal lining with the epidermal phagocytic cells that produce the non-specific humoral factors. Macrophages, phagocytes, and granulocytes are the effector cells of the immune systems that are enhanced by immunostimulants in fish diets.

Through absorption, the immune function and disease resistance of the entire organism is stimulated. After introduction to the fish, glucan cleared more quickly from the blood than from the kidney and liver (Rombout et al. 1989a). An important finding was that glucan absorbed in the stomach persisted in the blood much longer than glucan administered rectally, thus

the possibility of immediate stomach absorption followed by continued, slower absorption in the intestine, should be considered in designing diet studies. Daily feeding should maintain measurable levels of glucan in the organs investigated, but adequate dosages need to be determined.

The possibility of bioaccumulation and immune system inhibition also should be evaluated. Gopalakannan and Arul (2010) found that dietary glucan increased neutrophil activity, enhanced serum lysozyme activity, and increased white blood cell count in common carp. Disease resistance was improved in the carp when checked at days 30 and 60 of the feeding trial. In another diet trial, Misra et al. (2006) found that β-glucan in the feed at 250 mg glucan kg^{-1} feed gave the best results in an Indian major carp (*Labeo rohita*). The fish were fed for 56 days and their immune parameters were checked every 2 weeks; results determined that these fish had the best feed conversion, specific growth rates and best protection in a disease challenge. Couso et al. (2003) determined that gilthead sea bream (*Sparus aurata*) fed 1 or 10 g glucan kg^{-1} of diet for 2 weeks gained good protection from *Photobacterium damselae* subsp. *piscicida*. However, when the same doses were given 2 weeks on, 1 week off, and then 2 weeks on again, the fish fed the 1 g glucan kg^{-1} feed performed better than the fish on the higher dose. This study illustrates the importance of the concentration and the period of administration of glucan to obtain optimal disease protection.

Studies are needed to determine the proper time to administer immunostimulants to fish. *Vibrio anguillarum*, for example, can invade the host within 30 minutes of contact; therefore, immunostimulants would need to be fed in anticipation of a potential challenge. Outbreaks of *V. anguillarum*, a normal microflora of brackish and saltwater, often occur when fish are transferred to net pens, thus prophylactic treatment would need to be administered before transfer. Knowing the correct dosage and duration of feeding would allow the immunostimulant to reach the target organ and prime the macrophages to resist the pathogen. Studies also need to be conducted to determine if immunostimulants are useful when fed to fish that face a long emigration before reaching the estuary or ocean. For example, does the immunostimulatory effect persist long enough to warrant the use of immunostimulants as a method to enhance seawater transfer of hatchery fish that need to travel hundreds of miles to the ocean? Based on the currently available literature, in which persistence of effects was no longer than several weeks, it is questionable to assume that immunostimulators in aquaculture diets can be applied universally. Sakai (1999) noted that long-term administration can cause immunosuppression. Harikrishnan et al. (2011) also showed evidence that overdoses and long-term administration of immunostimulants may reduce their efficacy.

The mode of infection and spread of fish pathogens within individual fish has not been well documented. The spread of *Vibrio* administered in live feed to larval turbot was detailed by Grisez et al. (1996), and it appears that the bacteria are endocytosed in the anterior intestine. This information may explain a potential problem with immunostimulant therapy. Glucans administered in the diet have been shown to be absorbed in the posterior intestine and transported to the liver, spleen, and kidney (Sveinbjørnsson et al. 1995). Timing of immunostimulant administration would need to be early enough to ensure adequate absorption to tissues other than the posterior intestine. Because the two locations of absorption of *Vibrio* spp and the immunostimulant are not necessarily the same, it is suggested that the selected immunostimulant will need to address this difference. There are some factors in *Vibrio* spp that may explain the variation in disease resistance achieved by different immunostimulants. The bacteria, *V. anguillarum*, may infect the blood, kidney, spleen, gills, and intestinal tracts, which are sites of increased non-specific response to immunostimulants. On the other hand, *V. ordalii* forms colonies in tissues with low response to immunostimulants and therefore might not be exposed to engulfment and processing by macrophages.

Aeromonas salmonicida is very common in cultured salmonids. Unlike *Vibrio,* virulence is associated with the A-layer and a macromolecular refractive protein barrier of *A. salmonicida*, which protects the bacteria from bacteriophages. Phage receptors may be blocked by the protein macromolecules, and resistance to serum complement has been reported. However, another serum component, hemolysin, has been found to protect salmonid erythrocytes from *A. salmonicida* (Rockey et al. 1989). *A. hydrophila* infects warmwater species such as catfish; latent, chronic, and acute infections that occur affect different tissues (Grizzle and Kiryu 1993). The selected immunostimulant

would therefore need to act on all of these sites to elicit disease resistance. If immunostimulants are to be considered a prophylactic measure, feeding would need to be continuous to combat exposure to this disease, which is ubiquitous in natural waters and transmitted by resistant carriers. Outbreaks are generally associated with stress or compromised hosts. However, caution should be taken when using immunostimulants because immune inhibition has been documented when compounds are administered at high levels or for extended periods of time (Robertsen et al. 1990; de Baulny et al. 1996; Sakai 1999).

Varied susceptibility among different salmonid species to *Ceratomyxa shasta*, a parasite encountered during seaward emigration, suggests another possible application of immunostimulants in hatchery fish. In regions where infection is anticipated, prophylactic administration of the immunostimulant might prove effective before hatchery release. *C. shasta* invades the host through the intestine in the mucosal epithelial cells. After the skin, these cells are the second site of non-specific response and should be investigated for the mechanism of defense to the parasite in fish resistant to the pathogen. Efthimiou (1996) used β-glucans to combat parasites in juvenile dentex (*Dentex dentex*).

It is commonly accepted that specific immunostimulating substances can increase different components of the non-specific and specific immune systems, and that disease resistance against some common fish pathogens is conferred via control of dosage, duration of feeding, and timing of pathogen challenge (Robertsen et al. 1994; Raa 1996; Sakai 1999). The degree of disease resistance, as measured by survival after challenge, differs considerably among studies. The innate resistance of fish species to common bacteria may explain, in part, some of the differences. Just as basal diet formulations need to be tailored to fish and their culture environment, other specific adjustments may also be needed. It is however apparent that considerable efforts will be needed to evaluate immunostimulant-enhanced diets for production applications.

Conclusions

The effectiveness of immunostimulants is influenced by many variables including the fish species, age and size, solubility of the compound, type and chemical composition of an immunostimulant, route of treatment, virulence of pathogen(s), duration, and amount of immunostimulant used. Klesius et al. (2007) also discussed the fact that results from the use of immunostimulants have high variability.

Properly designed field trials and data analysis are often too inadequate to be accurately interpreted, particularly concerning the numbers of fish, treated replicates, untreated controls, and known cause of mortality. A lack of common standards makes it difficult to compare results across studies, and the risk/economic benefit factors are often unknown. It is not surprising that there is uncertainty regarding the use of β-glucans in aquaculture. Diverse levels of disease resistance to the same pathogen exist among related fish species. The complex sequence of non-specific immune stimulation interacting with the specific immune system when activated by pathogen invasion is not yet completely understood. The evolutionary genetics of the development of the non-specific immune system in fishes in an environment crowded with a variety of bacteria, yeasts, and fungi helps explain the complexity of the interactions that must be considered. Interpretation of results reported for individual immune factors has been complicated by different types of immunostimulants and mode of administration used. Few studies have been repeated in the laboratory or applied to fish production in aquaculture facilities. Predictions of levels of response as measured by disease resistance will need to be based on much more complex investigations than those provided by current research. Future research should attempt to separate non-specific immune factor stimulation from pre-existing, conferred disease resistance against specific pathogens. Genetics of the species, life stage, and the rearing environment all interact with the type and dosage of immunostimulant to contribute to the efficacy of the immunostimulatory compound.

Disclaimer

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the views of the US Fish and Wildlife Service. The use of trade names does not imply endorsement by the Federal government.

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Chapter 6

Vitamins (Excluding C and E)

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Introduction

Vitamins are organic compounds distinct from amino acids, carbohydrates, and lipids. They are required in trace amounts from an exogenous source, usually the diet, for normal growth, reproduction, and health with few exceptions, for example vitamin B_{12} and *myo*-inositol are not dietary essential for tilapia and hybrid striped bass. Vitamins are classified as either water soluble or fat soluble. Eight of the water-soluble vitamins, known as the vitamin B complex, are required in relatively small amounts and have primarily coenzyme functions; these are thiamin, riboflavin, niacin, biotin, folic acid, pantothenic acid and vitamins B_6 and B_{12}. The water-soluble vitamins choline, *myo*-inositol, and vitamin C, are required in larger quantities and have functions other than coenzymes. Vitamins A, D, E, and K are the fat-soluble vitamins that function independently of enzymes or, in some cases such as vitamin K, may have coenzyme roles (NRC 2011). All of these 15 vitamins have been demonstrated to be metabolically essential for most fish species.

The vitamin requirements of fish are affected by size, age, and growth rate, as well as by various environmental factors and nutrient interrelationships. Different researchers have therefore reported fairly wide ranges in requirement values for growth in the same fish species. Growth performance may not be the only parameter for determining vitamin requirements in fish. Other parameters, such as survival, lipid accumulation, skeleton deformities, activity of specific enzymes, tissue vitamin storage, absence of deficiency sign, liver lipid content, hepatosomatic index, lipid oxidation degree, and heat shock protein, have also been used to quantify vitamin requirements of fish. Estimates of the dietary requirements of the 13 vitamins (excluding vitamins C and E) for various fish species are summarized in Table 6.1.

Fish nutrition research studies often quantify nutrient requirements to maximize the animal's growth but largely ignore the roles of nutrients in disease prevention. The nutritional aspects of immune function are very important because diet can have a great impact on the immune response of the animal (Landolt 1989; Waagbø 1994).

Vitamins have been observed to enhance resistance to infection by increasing migration and proliferation of phagocytic cell. Excluding vitamins C and E, seven vitamins have been demonstrated to affect immune

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Table 6.1 Vitamin requirements estimates for growing fish determined with chemically defined diets in a controlled environment. Abbreviations: ADS: absence of deficiency signs; AASLP: ascorbic acid stimulated lipid peroxidation; BCT: blood coagulation time; CP: crude protein; ED: enzyme data; FE: feed efficiency; HSI: hepatosomatic index; LLC: liver lipid content; MBS: maximum body storage; MLS: maximum liver storage; NHV: normal hematocrit values; NR: no requirement determined; OSS: optimum swimming stamina; R: required but no value determined; SGR: specific growth rate; STC: serum total cholesterol; STG: serum total triglyceride; and WG: weight gain.

Vitamin and fish	Requirement (units kg^{-1} diet)	Response criteria	Reference
Vitamin A[a]			
Pacific salmon, *Oncorhynchus* spp.	R		Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	0.75 mg	WG, ADS	Kitamura et al. (1967a)
Channel catfish, *Ictalurus punctatus*	0.3–0.6 mg	WG	Dupree (1970)
Common carp, *Cyprinus carpio*	1.2–6 mg	WG, MLS	Aoe et al. (1968)
Yellowtail, *Seriola lalandi*	5.68 mg	WG, MLS	Shimeno (1991)
Grouper, *Epinephelus tauvina*	0.93 mg	WG	Shaik Mohamed et al. (2003)
Atlantic halibut, *Hippoglossus hippoglossus*	2.5 mg	MLS, ADS	Moren et al. (2004)
Hybrid striped bass, *Morone chrysops* × *M. saxatilis*	0.51–40.52 mg	WG	Hemre et al. (2004)
Japanese flounder, *Paralichthys olivaceus*	2.7 mg	WG	Hernandez et al. (2005)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	1.76–2.09 mg	WG, MLS	Hu et al. (2006)
European sea bass, *Dicentrarchus labrax*	31 mg	WG	Villeneuve et al. (2005a,b)
Vitamin D[b]			
Pacific salmon, *Oncorhynchus* spp.	NR		Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	40–60 μg	WG, FE	Barnett et al. (1982a)
Channel catfish, *Ictalurus punctatus*	12.5 μg	WG	Lovell and Li (1978)
	25 μg	WG	Andrews et al. (1980)
	6.25 μg	WG	Brown (1988)
Yellowtail, *Seriola lalandi*	NR		Shimeno (1991)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	9.35 μg	WG	Shiau and Hwang (1993)
Vitamin K			
Pacific salmon, *Oncorhynchus* spp.	R		Halver (1972)
Lake trout, *Salvelinus namaycush*	0.5–1 mg	NHV	Poston (1976a)
Channel catfish, *Ictalurus punctatus*	R		Dupree (1966)
	NR		Murai and Andrews (1977)
Yellowtail, *Seriola lalandi*	NR		Shimeno (1991)
Grass carp, *Ctenopharyngodon idella*	1.9 mg	BCT	Jiang et al. (2007)
Atlantic cod, *Gadus morhua*	0.2 mg	WG	Grahl-Madsen and Lie (1997)
Atlantic salmon, *Salmo salar*	< 10 mg	WG	Krossøy et al. (2009)
Thiamin			
Pacific salmon, *Oncorhynchus* spp.	10–15 mg	MLS	Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	1–10 mg	WG, ADS	McLaren et al. (1947)
	1 mg	WG, ED	Morito et al. (1986)
Channel catfish, *Ictalurus punctatus*	1 mg	WG, ADS	Murai and Andrews (1978b)
Common carp, *Cyprinus carpio*	0.5 mg	WG, ADS	Aoe et al. (1969)
Yellowtail, *Seriola lalandi*	11.2 mg	MLS	Shimeno (1991)
Jian carp, *Cyprinus carpio* var. Jian	1.02 mg	WG	Huang et al. (2011)

Table 6.1 (*Continued*)

Vitamin and fish	Requirement (units kg^{-1} diet)	Response criteria	Reference
Riboflavin			
Pacific salmon, *Oncorhynchus* spp.	20–25 mg	MLS	Halver (1972)
	7 mg	WG, ADS	Leith et al. (1990)
Rainbow trout, *Oncorhynchus mykiss*	5–15 mg	WG, ADS	McLaren et al. (1947)
	6 mg	MLS	Takeuchi et al. (1980)
	3 mg	ED	Hughes et al. (1981a)
	2.7 mg	MLS, ED	Amezaga and Knox (1990)
Channel catfish, *Ictalurus punctatus*	9 mg	WG, ADS	Murai and Andrews (1978a)
	6 mg	ED	Serrini et al. (1996)
Common carp, *Cyprinus carpio*	4 mg	WG, ADS	Aoe et al. (1967a)
	6.2 mg	MLS	Aoe et al. (1967a)
	7 mg	MLS	Takeuchi et al. (1980)
Yellowtail, *Seriola lalandi*	11 mg	MLS	Shimeno (1991)
Blue tilapia, *Oreochromis aureus*	6 mg	WG, ADS	Soliman and Wilson (1992a)
Red hybrid tilapia, *Oreochromis mossambicus* × *O. niloticus*	5 mg	WG	Lim et al. (1993)
Hybrid striped bass, *Morone chrysops* × *Morone saxatilis*	4.1–5.0 mg	WG, MLS	Deng and Wilson (2003)
Jian carp, *Cyprinus carpio* var. Jian	5.0 mg	WG	Li et al. (2010b)
Vitamin B_6			
Atlantic salmon, *Salmo salar*	5 mg	WG, ADS	Lall and Weerakoon (1990)
Pacific salmon, *Oncorhynchus* spp.	10–20 mg	MLS	Halver (1972)
	6 mg	WG, ADS	Leith et al. (1990)
Rainbow trout, *Oncorhynchus mykiss*	1–10 mg	WG, ADS	McLaren et al. (1947)
	2 mg	WG, ADS	Woodward (1990)
	3–6 mg	ED	Woodward (1990)
Channel catfish, *Ictalurus punctatus*	3 mg	WG, ADS	Andrews and Murai (1979)
Common carp, *Cyprinus carpio*	5–6 mg	WG, ADS	Ogino (1965)
Yellowtail, *Seriola lalandi*	11.7 mg	MLS	Shimeno (1991)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	1.7–9.5 mg (28% CP)	WG, ED	Shiau and Hsieh (1997)
	15–16.5 mg (36% CP)	WG, ED	
Red hybrid tilapia, *Oreochromis mossambicus* × *O. aureus*	3 mg	WG	Lim et al. (1995)
Nile tilapia, *Oreochromis niloticus*	10 mg	WG	Teixeira et al. (2012)
Indian catfish, *Heteropneustes fossilis*	3.21 mg	WG	Shaik Mohamed (2001a)
Jian carp, *Cyprinus carpio* var. Jian	6.07 mg	WG	He et al. (2009)
Vitamin B_{12}			
Pacific salmon, *Oncorhynchus* spp.	0.015–0.02 mg	MLS	Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	R		Phillips et al. (1964)
Channel catfish, *Ictalurus punctatus*	R		Limsuwan and Lovell (1981)
Common carp, *Cyprinus carpio*	NR		Kashiwada et al. (1970)
Yellowtail, *Seriola lalandi*	0.053 mg	MLS	Shimeno (1991)
Nile tilapia, *Oreochromis niloticus*	NR		Lovell and Limsuwan (1982)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	NR		Shiau and Lung (1993)
Grass carp, *Ctenopharyngodon idella*	0.094 mg	SGR	Wu et al. (2007b)
Grouper, *Epinephelus malabaricus*	NR		Lin et al. (2010)

(*continued*)

Table 6.1 (*Continued*)

Vitamin and fish	Requirement (units kg^{-1} diet)	Response criteria	Reference
Niacin			
Pacific salmon, *Oncorhynchus* spp.	150–200 mg	MLS	Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	1–5 mg	WG, ADS	McLaren et al. (1947)
	10 mg	WG, ADS	Poston and Wolfe (1985)
Channel catfish, *Ictalurus punctatus*	14 mg	WG, ADS	Andrews and Murai (1978)
	7.4 mg	WG, ED	Ng et al. (1997)
Common carp, *Cyprinus carpio*	28 mg	WG, ADS	Aoe et al. (1967b)
Yellowtail, *Seriola lalandi*	12 mg	MLS	Shimeno (1991)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	26 mg (glucose)	WG	Shiau and Suen (1992)
	121 mg (dextrin)	WG	
Grass carp, *Ctenopharyngodon idella*	25.5 mg	SGR	Wu et al. (2007a)
Mrigal, *Cirrhinus mrigala*	20 mg	WG	Shaik Mohamed and Ibrahim (2001)
African catfish, *Heterobranchus longifilis*	33.1 mg	FE	Morris et al. (1998)
Biotin			
Pacific salmon, *Oncorhynchus* spp.	1–1.5 mg	MLS	Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	0.05–0.25 mg	WG, ADS	McLaren et al. (1947)
	0.08 mg	WG, ADS	Woodward and Frigg (1989)
	0.14 mg	ED	Woodward and Frigg (1989)
Lake trout, *Salvelinus namaycush*	0.1 mg	WG, ADS	Poston (1976b)
	0.5–1 mg	OSS	Poston (1976b)
Channel catfish, *Ictalurus punctatus*	R		Robinson and Lovell (1978)
Common carp, *Cyprinus carpio*	1 mg	WG, ADS	Ogino et al. (1970b)
Yellowtail, *Seriola lalandi*	0.67 mg	MLS	Shimeno (1991)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	0.06 mg	WG, ED	Shiau and Chin (1999)
Asian catfish, *Clarias batrachus*	2.49 mg	WG	Shaik Mohamed et al. (2000)
Indian catfish, *Heteropneustes fossilis*	0.25 mg	WG	Shaik Mohamed (2001b)
Goldspot mullet, *Liza parsia*	1.6–3.2 mg	WG	Chavan et al. (2003)
Japanese sea bass, *Lateolabrax japonicus*	0.046 mg	WG	Li et al. (2010a)
Jian carp, *Cyprinus carpio* var. Jian	0.15	WG	Zhao et al. (2012)
Folic acid			
Pacific salmon, *Oncorhynchus* spp.	6–10 mg	MLS	Halver (1972)
	2 mg	WG, ADS	Leith et al. (1990)
Rainbow trout, *Oncorhynchus mykiss*	1.0 mg	WG, ADS	Cowey and Woodward (1993)
Channel catfish, *Ictalurus punctatus*	1.5 mg	WG, NHV	Duncan and Lovell (1991)
	1 mg	WG, NHV	Duncan et al. (1993)
Common carp, *Cyprinus carpio*	NR		Aoe et al. (1967c)
Yellowtail, *Seriola lalandi*	1.2 mg	MLS	Shimeno (1991)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	0.82	WG, MLS, HSI	Shiau and Huang (2001)
Grass carp, *Ctenopharyngodon idella*	3.6–4.3 mg	WG, STC, STG, NHV	Zhao et al. (2008)
Grouper, *Epinephelus malabaricus*	0.8	WG, HSI, MLS, AASLP	Lin et al. (2011)

Table 6.1 (*Continued*)

Vitamin and fish	Requirement (units kg^{-1} diet)	Response criteria	Reference
Pantothenic acid			
Pacific salmon, *Oncorhynchus* spp.	40–50 mg	MLS	Halver (1972)
	17 mg	WG, ADS	Leith et al. (1990)
Rainbow trout, *Oncorhynchus mykiss*	10–20 mg	WG, ADS	McLaren et al. (1947)
	20 mg	WG, ADS	Cho and Woodward (1990)
Channel catfish, *Ictalurus punctatus*	10 mg	WG, ADS	Murai and Andrews (1979)
	15 mg	WG, ADS	Wilson et al. (1983)
Common carp, *Cyprinus carpio*	30–50 mg	WG, ADS	Ogino (1967)
Yellowtail, *Seriola lalandi*	35.9 mg	MLS	Shimeno (1991)
Blue tilapia, *Oreochromis aureus*	10 mg	WG, ADS	Soliman and Wilson (1992b)
Grass carp, *Ctenopharyngodon idella*	25 mg	WG	Liu et al. (2007)
Jian carp, *Cyprinus carpio* var. Jian	23 mg	WG	Wen et al. (2009)
Choline			
Pacific salmon, *Oncorhynchus* spp.	600–800 mg	MLS	Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	50–100 mg	WG, ADS	McLaren et al. (1947)
	714–813 mg	WG, LLC	Rumsey (1991)
Lake trout, *Salvelinus namaycush*	1000 mg	WG	Ketola (1976)
Channel catfish, *Ictalurus punctatus*	400 mg	WG, LLC	Wilson and Poe (1988)
Common carp, *Cyprinus carpio*	1500 mg	WG, LLC	Ogino et al. (1970b)
Yellowtail, *Seriola lalandi*	2920 mg	MLS	Shimeno (1991)
White sturgeon, *Acipenser transmontanus*	1700–3200 mg	WG	Hung (1989)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	1000 mg	WG, MBS	Shiau and Lo (2000)
Hybrid striped bass, *Morone chrysops* × *Morone saxatilis*	500 mg	WG	Griffin et al. (1994)
Cobia, *Rachycentron canadum*	696 mg	WG	Mai et al. (2009)
Yellow perch, *Perca flavescens*	598–634 mg	WG	Twibell and Brown (2000)
Red drum, *Sciaenops ocellatus*	588 mg	WG	Craig and Gatlin (1996)
Grass carp, *Ctenopharyngodon idella*	3000 mg	WG, FE, LLC	Wang et al. (1995)
Gibel carp, *Carassius auratus gibelio*	2500 mg	WG	Duan et al. (2012)
***Myo*-inositol**			
Pacific salmon, *Oncorhynchus* spp.	300–400 mg	MLS	Halver (1972)
Rainbow trout, *Oncorhynchus mykiss*	250–500 mg	WG, ADS	McLaren et al. (1947)
Channel catfish, *Ictalurus punctatus*	NR		Burtle and Lovell (1989)
Common carp, *Cyprinus carpio*	440 mg	WG, ADS	Aoe and Masuda (1967)
Yellowtail, *Seriola lalandi*	423 mg	MLS	Shimeno (1991)
Hybrid striped bass, *Morone chrysops* × *Morone saxatilis*	NR		Deng et al. (2002)
Nile tilapia, *Oreochromis niloticus*	NR		Peres et al. (2004)
Hybrid tilapia, *Oreochromis niloticus* × *O. aureus*	400 mg	WG, MLS	Shiau and Su (2005)
Grouper, *Epinephelus malabaricus*	335–365 mg	WG, MLS	Su and Shiau (2004)
Olive flounder, *Paralichthys olivaceus*	617 mg	WG	Lee et al. (2009)
Grass carp, *Ctenopharyngodon idella*	166–214 mg	WG, FE	Wen et al. (2007)
Jian carp, *Cyprinus carpio* var. Jian	518 mg	WG	Jiang et al. (2010)

[a] 10,000 IU ≈ 3,000 μg vitamin A (retinol);
[b] 1 IU = 0.025 μg cholecalciferol

responses and/or disease resistance in fish: vitamin A, D, thiamin, B_6, folic acid, pantothenic acid, and *myo*-inositol.

In this chapter, all 13 vitamins, their biochemistry and metabolic function, deficiency symptoms, requirements, and effects on immune responses and/or disease resistance (if any) are reviewed.

Lipid-Soluble Vitamins

The fat-soluble vitamins A, D, E, and K are absorbed in the intestine along with dietary fats; conditions favorable for fat absorption therefore also enhance the absorption of fat-soluble vitamins. As fish seem to lack the lymphatic system found in mammals, lipid and fat-soluble vitamins are most likely transported to the peripheral tissues via the portal vein and the liver. If dietary intake of fat-soluble vitamins exceeds metabolic needs, animals will store the excess actively in specific cell compartments or by simple accumulation in the lipid compartment. Animals can therefore accumulate enough fat-soluble vitamins in their tissues to produce a toxic condition (hypervitaminosis). This has been demonstrated in the laboratory with rainbow trout, *Oncorhynchus mykiss*, but it is unlikely to occur under practical conditions (Poston et al. 1966; Poston 1969; Poston and Livingston 1969).

Since fat-soluble vitamins can be stored in the body, it is critical that the nutritional history of experimental fish is known prior to conducting requirement studies. The time required to deplete fish of their stored fat-soluble vitamins is highly variable. Differences in vitamin intake prior to an experiment may be responsible for some of the conflicting findings on the induction and severity of deficiency signs (NRC 2011).

Vitamin A

Biochemistry and Metabolic Function

Vitamin A occurs in three forms: an alcohol (retinol), an aldehyde (retinal), and an acid (retinoic acid). Vitamin A_1 (retinol) is found in mammals and marine fish, whereas both vitamins A_1 and vitamin A_2 (3-dehydroretinol) are found in freshwater fish (Braekkan et al., 1969). In freshwater fish, the oxidative conversion of retinol to 3-dehydroretinol occurs (Goswami 1984), as well as the reversible oxidation and reduction reactions of retinol to retinal and of 3-dehydroretinol to 3-dehydroretinal (Wald 1945–1946). For example, Nile tilapia, *Oreochromis niloticus*, have been shown to convert dietary retinol into 3-dehydroretinol and retinal into 3-dehydroretinal (Katsuyama and Matsuno 1988).

Vitamin A is crucial in a number of physiological processes necessary for optimal function of an animal. It is involved in cell differentiation and is therefore vital for the following processes: reproduction as a key factor in embryo development; development of epithelial cells from stem cells to fully functional layers, including mucus-producing cells; and proper differentiation of immune cells in response to exposure to pathogens or foreign proteins. The function of vitamin A in vision is well established, although its mechanisms of action are not well established for other functions. However, retionoic acid, bound to retionoic acid receptors in the nucleus, is a key factor in expression of genes involved in cell differentiation (NRC 2011).

Deficiency Symptoms

Vitamin A deficiency in rainbow trout causes anemia, twisted gill opercula, and hemorrhages in the eyes and base of fins (Kitamura et al. 1967a). When fed a vitamin A-deficient purified diet from first feeding, brook trout, *Salvelinus fontinalis*, exhibited poor growth, high mortality, and eye lesions such as edematous eyes, displaced lens, and degeneration of the retina (Poston et al. 1977). Anorexia, pale body color, hemorrhagic skin and fins, exophthalmia, and twisted gill opercula occurred in common carp fed a vitamin A-deficient diet after 8–11 weeks (Aoe et al. 1968). Vitamin A deficiency caused hemorrhages in the fins and the area surrounding the eyes in Atlantic halibut, *Hippoglossus hippoglossus* (Moren et al. 2004), and hemorrhages on the skin overlaying the base of the fins and erosion on the caudal peduncle in grouper, *Epinephelus tauvina* (Shaik Mohamed et al. 2003). Furuita et al. (2003) reported that low vitamin A content in the diet of Japanese flounder, *Paralichthys olivaceus*, caused negative effects on reproduction, such as buoyant egg rate and percentage of normal larvae.

Requirements

Coldwater fish can use β-carotene as a vitamin A precursor (Poston et al. 1977). Dupree (1970) found

that channel catfish, *Ictalurus punctatus*, could use β-carotene as a vitamin A source only if the dietary concentration exceeded 2000 international units per kilogram (IU kg^{-1}). It has been shown that β-carotene and canthaxanthin can be biotransformed into vitamin A_1 in the liver of Nile tilapia, and that dihydroxy-carotenoids such as astaxanthin, zeaxanthin, lutein, and tunaxanthin can be directly bioconverted into vitamin A_2 (Katsuyama and Matsuno 1988). The conversion ratio of β-carotene to vitamin A has been established to be 19:1 in hybrid tilapia (*O. niloticus* × *O. aureus*) (Hu et al. 2006).

Takeuchi et al. (1998) reported that Japanese flounder larvae fed either *Artemia* enriched with 30 mg kg^{-1} diet of retinol, retinyl palmitate or retinyl acetate, or 100 mg kg^{-1} diet of all-trans-retinoic acid, had compressed vertebrae. Signs of vitamin A toxicity, such as increased mortality, abnormal vertebral growth, and reduced growth, were found in Atlantic salmon, *Salmo salar*, that were fed a diet containing 938 mg retinol kg^{-1} (Ørnsrud et al. 2002). Villeneuve et al. (2005a) reported that a diet containing 31 mg vitamin A kg^{-1} is required for European sea bass, *Dicentrarchus labrax*, larvae, and excess levels of dietary retinyl acetate resulted in an alternation of head organization characterized by the abnormal development of both the splanchnocranium and neurocranium, as well as scoliotic fish. Of the larvae fed 1000 mg retinyl acetate kg^{-1} diet, 78.8% exhibited skeletal abnormalities. The authors indicated that a linear correlation between the level of vitamin A in larvae and malformation percentage was linked to a modification in the relative retinoic acid receptor gene expression. This demonstrates the influence of nutrition on the retinoid pathway, which plays an important role in body morphology and the induction of skeletal malformations during post-hatching development in European sea bass larvae. Retinoid pathway can also be influenced by dietary lipid, leading to skeletal malformation during sea bass larvae development (Villeneuve et al. 2005b).

Effect on Immune Responses and Disease Resistance

Rainbow trout fed a diet supplemented with vitamin A resulted in higher total serum antiprotease, classical complement activity, and leukocyte migration, but not the serum immunoglobulin level, lysozyme activity, and phagocyte respiratory burst activity when compared with fish fed a vitamin A-free diet (Thompson et al. 1995). However, it was suggested that vitamin A has only limited potential as an immunostimulatory agent in practical rainbow trout diets (Thompson et al. 1995).

Hernandez et al. (2007) examined the effects of dietary vitamin A deficiency and excess on serum antibacterial activity of juvenile Japanese flounder. They observed that serum antibacterial activity was significantly higher in the groups fed diets containing 10,000 and 25,000 IU A kg^{-1} than that of the fish fed diets containing 0 IU A kg^{-1}. The data suggested that dietary vitamin A significantly improved antibacterial activity of juvenile Japanese flounder.

Yang et al. (2008) found that Jian carp fed a diet containing 3969 IU vitamin A kg^{-1} had higher lysozyme activity than fish fed a diet not supplemented with vitamin A.

Vitamin D

Biochemistry and Metabolic Function

The two major natural sources of vitamin D are ergocalciferol (vitamin D_2; occurs predominantly in plants) and cholecalciferol (vitamin D_3; occurs in animals). Both forms of vitamin D are hydroxylated in the liver to the 25-hydroxy forms. The 25-hydroxy-D_3 is further hydroxylated in the kidney to 1,25-dihydroxyvitamin D_3, which is the biologically active form of vitamin D that is responsible for facilitating mobilization, transport, absorption, and use of calcium and phosphorus in concert with the actions of parathyroid hormone and calcitonin.

A primary function of Vitamin D is to maintain calcium homeostasis together with two peptide hormones, calcitonin and parathyroid hormone. It is critically important for the development, growth, and maintenance of a healthy skeleton from birth until death. Vitamin D is also involved in alkaline phosphatase activity, promotes intestinal absorption of calcium, and influences the action of parathyroid hormone on bone. Aside from these functions, it has been found to play important roles in differentiation of bone, skin, and blood cells; in secretion of insulin and prolactin; muscle function; immune and stress responses; and melanin synthesis. Excessive intake of

vitamin D may cause irreversible calcification of the heart, kidneys, and other soft tissue (NRC 2011).

Deficiency Symptoms

Rainbow trout fed a vitamin D-deficient diet exhibited poor growth, elevated liver lipid content, impaired calcium homeostasis manifested by tetany of white skeletal muscles, and ultrastructural changes in the white muscle fibers of the epaxial musculature (George et al. 1981). However, in a similar study also with rainbow trout, no hypocalcemia or changes in bone ash were observed (Barnett et al. 1982a). A lordosis-like droopy tail syndrome observed in vitamin D-deficient trout (Barnett et al. 1982b) was suggested to be related to an epaxial muscle weakness. Channel catfish fed a vitamin D-deficient diet for 16 weeks showed poor growth, lowered body calcium and phosphorus levels, and lowered total body ash (Lovell and Li 1978). Andrews et al. (1980) reported that vertebral ash level in channel catfish was not significantly affected by vitamin D deficiency.

Requirements

Cholecalciferol has been shown to be at least three times more effective than ergocalciferol in meeting the vitamin D requirement of rainbow trout (Barnett et al., 1982a). Andrews et al. (1980) found that vitamin D_3 was used more effectively by channel catfish than vitamin D_2 at a dietary concentration of 50 μg kg^{-1} diet (1 IU = 0.025 μg cholecalciferol), and that high concentrations of vitamin D_3 (500–1250 μg kg^{-1} diet) reduced weight gain. However, Brown (1988) found that vitamin D_2 was utilized as well as vitamin D_3 up to 37.5 μg kg^{-1} of diet, but higher concentrations of vitamin D_2 depressed weight gain and feed efficiency in channel catfish reared in calcium-free water.

Fingerling brook trout fed a diet containing 93.75 mg vitamin D_3 kg^{-1} for 40 weeks had hypercalcemia and increased hematocrit levels, but no differences in rates of growth and survival (Poston 1969). However, Hilton and Ferguson (1982) did not detect any incidence of renal calcinosis in rainbow trout fed a diet containing up to 25 mg vitamin D_3 kg^{-1}. Supplementation of 1.25 mg vitamin D_3 kg^{-1} diet significantly depressed the growth rate of channel catfish (Andrews et al. 1980). In contrast, a diet of 25 mg vitamin D_3 kg^{-1} has been reported to show no toxic effects in channel catfish reared in calcium-free water for 14 weeks (Brown 1988). Atlantic salmon fry seemed to be highly tolerant of megadoses of vitamin D_3 over a period of time. When the fry were fed diets supplemented with three levels of vitamin D_3 (0.2, 5, and 57 mg kg^{-1} diet) for 14 weeks, no differences in weight, length, specific growth rate, mortality, or kidney calcium concentration or any skeleton malformation or histopathological changes were observed among the three dietary groups (Graff et al. 2002).

Effect on Immune Responses and Disease Resistance

Cerezuela et al. (2009) conducted a study to assess the *in vivo* effect of vitamin D_3 on some innate immune parameters of the gilthead sea bream, *Sparus aurata* L. The immunostimulant effect was greater in gilthead sea bream on the cellular immune parameters (leucocyte peroxidase content, phagocytic, respiratory burst, and natural cytotoxic activities) compared to the humoral immune parameters (peroxidase and complement activity), suggesting that similar receptors to those present in mammals are involved in the action of this vitamin in the immune systems of fish.

Vitamin K

Biochemistry and Metabolic Function

The term "vitamin K" is used as a generic descriptor for both 2-methyl-1,4-naphthoquinone and all 3-substituted derivatives of this compound. The three major forms of vitamin K are: vitamin K_1 or phylloquinone, which can be isolated from plants; vitamin K_2 or the menaquinones, which are synthesized by bacteria; and vitamin K_3 or menadione, which is a synthetic product.

Vitamin K is typically associated with its role in coagulation of blood, and it also has an important role in calcium transport. In vertebrates, the action of osteocalcin, the major bone matrix protein, is vitamin-K-dependent. Vitamin K is also required for the post-translational carboxylation of specific glutamate residues to gamma-carboxyglutamate residues. These residues interact with calcium, allowing osteocalcin to regulate the incorporation of calcium phosphates into bone tissue (NRC 2011).

Vitamin K is required for the stimulation of prothrombin activity in plasma and synthesis of blood- clotting factors VII, IX, and X. The metabolic role of vitamin K involves the vitamin-K-dependent carboxylase, which carries out the post-translational conversion of specific glutamyl residues in the vitamin-K-dependent plasma proteins to γ-carboxy-glutamyl residues. These residues are essential for the normal Ca^{2+}-dependent interaction of the vitamin-K-dependent clotting factors with phospholipid surfaces (Suttie 1985).

Deficiency Symptoms

The prothrombin time was increased 3–5 times in salmon fed diets devoid of vitamin K and, during prolonged deficiency states, anemia and hemorrhagic areas appeared in the gills, eyes, and vascular tissues. Increased blood-clotting time has also been reported for other fish reared on diets containing low levels of vitamin K (Poston 1964; Dupree 1966). Interrelationships with other vitamins have not been documented in fish experiments, and the primary deficiency signs in wounded fish remain slow blood clotting, hemorrhage, severe anemia, and death. Hemorrhagic areas often appear in fragile tissues such as the gills. Intake of vitamin K at a level of 2000–3000 mg kg^{-1} of feed can be tolerated by trout (Poston 1971), but higher levels may cause liver toxicity and death.

Requirements

Many animals do not require vitamin K in their diet because of bacterial synthesis in the intestinal tract; however, intestinal vitamin-K-synthesizing microflora have not been described in fish. Supplementation of sulfaguanidine to a vitamin-K-deficient diet in combination with low water temperature caused prolonged blood coagulation time and low hematocrit values without affecting growth performance of trout (Poston 1964). The addition of an antibiotic (nifurazolidon) to a vitamin-K-deficient diet resulted in reduced growth, but did not provoke external signs of vitamin K deficiency in Atlantic cod (Grahl-Madsen and Lie 1997). Dupree (1966) reported hemorrhages in channel catfish fed a vitamin-K-deficient diet. However, Murai and Andrews (1977) failed to detect any deficiency signs in channel catfish fed a diet devoid of vitamin K and supplemented with sulfaguanidine. The addition of dicumarol, a vitamin K antagonist, did not increase prothrombin time in catfish. The addition of pivalyl, a stronger vitamin K antagonist than dicumarol by a factor of 20, completely blocked the blood coagulation of channel catfish (Murai and Andrews 1977). High dietary concentrations of menadione sodium bisulfite (2400 mg kg^{-1} of diet) had no adverse affect on growth, survival, blood coagulation, or the number of erythrocytes of young brook trout (Poston 1971). The amount of vitamin K found naturally in the practical diet ingredients (approximately 0.1 mg kg^{-1} diet) may be enough to maintain optimal growth, health, and bone strength in Atlantic salmon fry from the start of feeding (Krossøy et al. 2009).

Water-Soluble Vitamins

The water-soluble vitamins include eight well-recognized members of the vitamin B complex and the water-soluble essential nutritional factors *myo*-choline, inositol, and ascorbic acid. The vitamin B complex members are required in small amounts in the diet but play major roles in growth, physiology, and cellular metabolism. For some warmwater fish, intestinal synthesis by microorganisms supplies the requirement for certain vitamins. In those cases, deficiency signs result only when antibiotics are fed along with a deficient diet. A constant supply of essential water-soluble vitamins is required to prevent deficiency signs in fish, since these vitamins are not stored in body tissues.

Thiamin

Biochemistry and Metabolic Function

Thiamin hydrochloride is a water-soluble, colorless, monoclinic, crystalline compound that is comparatively stable to dry heat, yet rapidly broken down in neutral or alkaline solutions, and is split by sulfites into constituent pyrimidine and thiazole moieties. It has a characteristic yeast-like odor. The pyrimidine ring is relatively stable, but the thiazole ring is easily opened by hydrolysis. Several derivatives are stabile to heat, appear to be more completely soluble in weak alkaline solutions than thiamin itself, and still show biological activity in animals; these derivatives include thiamin

propyl disulfide, benzoylthiamin disulfide, dibenzoylthiamin, and benzoylthiamin monophosphate. Both thiamin hydrochloride and thiamin mononitrate have been successfully used as the active vitamin in test diets for fish nutrition studies and for fish diet formulations (Halver 2002).

Thiamin was the first vitamin to be recognized. In animal tissue, thiamin occurs predominantly in a di-phosphate form known as thiamin pyrophosphate (TPP). TPP is an essential cofactor for a number of important enzymatic steps in energy production, including both decarboxylations and transketolase reactions. The coenzyme form of thiamin is thiamin pyrophosphate. Thiamin pyrophosphate functions in the oxidative decarboxylation of α-keto acids, such as pyruvate and α-ketoglutarate, and in the transketolase reaction in the pentose shunt (NRC 2011).

Deficiency Symptoms

Dietary thiamin deficiency has been shown to result in neurological disorders such as hyperirritability in salmonids (Halver 1957; Coates and Halver 1958; Kitamura et al. 1967b; Lehmitz and Spannhof 1977), channel catfish (Dupree 1966), Japanese eel, *Anguilla japonica* (Hashimoto et al. 1970), and Japanese parrotfish, *Scarus coeruleus* (Ikeda et al. 1988). Arai et al. (1972) found only subcutaneous hemorrhages and congested fins in subadult Japanese eels, and Hashimoto et al. (1970) observed neurological disorders in small Japanese eels. Similar deficiency signs with varying degrees of mortality have been reported in common carp (Aoe et al. 1969), red sea bream (Yone and Fujii 1974), and turbot (Cowey et al. 1975). Thiamin deficiency has been observed in wild stocks of fish both in fresh and salt waters. The deficiency is induced in fish that eat food containing high thiaminase activity originating from certain algae.

Requirements

Erythrocyte transketolase activity has been used as a specific indicator of thiamin status in turbot (Cowey et al. 1975). Kidney or liver transketolase activity in rainbow trout (Lehmitz and Spannhof 1977; Masumoto et al. 1987) and grouper (Huang et al. 2007) have also been shown to decrease much earlier than the appearance of external deficiency signs.

Effect on Immune Responses and Disease Resistance

Feng et al. (2011) reported that the survival rate, leucocyte phagocytic activity, lectin potency, acid phosphatase activity, lysozyme activity, total iron-binding capacity, and immunoglobulin M content of Jian carp injected with *Aeromonas hydrophila* were all improved with an increase in dietary thiamin levels up to 0.8–1.1 mg kg^{-1} diet, respectively. This level is close to the thiamin requirement (1.02 mg kg^{-1} diet) for maximal growth of the species (Huang et al. 2011).

Riboflavin

Biochemistry and Metabolic Function

Riboflavin is a yellow-brown crystalline pigment. It is slightly soluble in water, soluble in alkali, and insoluble in most organic solvents except alcohol. Riboflavin is stable to oxidizing agents in strong mineral acids and in neutral aqueous solutions, and is heat stable in dry form. It is irreversibly decomposed on irradiation with ultraviolet rays or visible light, breaking down to lumiflavin (Halver 2002).

Riboflavin functions in the intermediary transfer of electrons in metabolic oxidation-reduction reactions as a component of two coenzymes: flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These coenzymes serve as prosthetic groups of oxidation-reduction enzymes involved in the metabolism of keto-acids, fatty acids, and amino acids in the mitochondrial electron transport system (NRC 2011).

Deficiency Symptoms

Riboflavin deficiency signs are dependent upon the species of fish. The only common signs are anorexia and poor growth. The first sign of riboflavin deficiency observed in salmonids (McLaren et al. 1947; Halver 1957; Steffens 1970; Takeuchi et al. 1980; Hughes et al. 1981a, b) appeared in the eyes and included photophobia, cataracts, corneal vascularization, and hemorrhages. A lack of coordinated swimming and dark skin coloration have also been reported in

riboflavin-deficient Chinook salmon (Halver 1957) and rainbow trout (Kitamura et al. 1967b; Steffens 1970). In contrast, Woodward (1984) did not observe cataracts or corneal occlusion in riboflavin-deficient rainbow trout fry and fingerlings; however, severe fin erosion and light skin coloration, accompanied by high mortality, were observed. Monolateral or bilateral cataracts have been reported in riboflavin-deficient channel catfish (Dupree 1966); two independent feeding trials also found poor growth and short-body dwarfism. Riboflavin deficiency caused lethargy and high mortality in Japanese parrotfish (Ikeda et al. 1988); hemorrhages in various parts of the body, nervousness, and photophobia in common carp (Aoe et al. 1967a; Ogino 1967; Takeuchi et al. 1980) and Japanese eel (Arai et al. 1972); lethargy, fin erosion, anorexia, loss of normal body color, short-body dwarfism, and cataracts in blue tilapia, *Oreochromis aureus* (Soliman and Wilson 1992a); short-body dwarfism and cataracts in red hybrid tilapia, *O. mossambicus* × *O. niloticus* (Lim et al. 1993); short-body dwarfism, anorexia, and poor growth in channel catfish (Serrini et al. 1996); and anorexia, dark body color, and cataracts in hybrid striped bass (Deng and Wilson 2003). Huang et al. (2010) reported that riboflavin-deficient grouper showed high oxidative stress and low antioxiative enzyme activity.

Requirements

Hughes et al. (1981a) used the activation coefficient (ratio of activity following pre-incubation with FAD: basal activity) of erythrocyte glutathione reductase to measure the riboflavin status of rainbow trout. However, Woodward (1983) found the activity of D-amino acid oxidase to be a more sensitive indicator of the riboflavin status in rainbow trout, since the low activity of erythrocyte glutathione reductase made its quantification difficult. Hepatic D-amino acid oxidase was also found to be a reliable indicator of riboflavin status in rainbow trout (Amezaga and Knox 1990), channel catfish (Serrini et al. 1996), and hybrid striped bass (Deng and Wilson 2003). However, Amezaga and Knox (1990) pointed out that an assay for glutathione reductase activity in erythrocytes would be advantageous since it could be used on live fish. Woodward (1985) reported that the riboflavin requirement was not affected by temperature or by genetic differences in growth rate; this might be one reason why the riboflavin requirement values agree fairly well, even among different species.

Hughes (1984) found that feeding high concentrations of riboflavin (up to 600 mg kg^{-1} diet) had no adverse effects on growth of rainbow trout. These results were expected since riboflavin has not been shown to cause hypervitaminosis in other animals. However, two studies (McLaren et al. 1947; Woodward 1982) reported depressed growth in rainbow trout fed moderate concentrations of riboflavin. It was concluded that the growth depression observed in the earlier studies must have resulted from some factor other than riboflavin.

Vitamin B_6

Biochemistry and Metabolic Function

Compounds that have vitamin B_6 activity include pyridoxine, pyridoxal, and pyridoxamine. Pyridoxine hydrochloride is readily soluble in water and heat stable in either acid or alkaline solution. Pryridoxal phosphate acts as a coenzyme in a number of enzyme systems, and pyridoxic acid, deoxypyridoxine, and methoxypyridoxine are closely related compounds with varying degrees of activity. Pyridoxine is sensitive to ultraviolet light in neutral or alkaline solutions. Pyridoxamine and pyridoxal in dilute solutions are labile compounds that are rapidly destroyed on exposure to air, heat, or light. Most vitamin supplementation is therefore in the form of pyridoxine hydrochloride, and analysis for pyridoxine activity by microbiological assay of diet ingredients likely measures pyridoxal phosphate and other intermediates as well (Halver 2002).

All three forms of vitamin B_6 (pyridoxine, pyridoxal, and pyridoxamine) are readily converted in animal tissue to the coenzyme forms, pyridoxal phosphate and pyridoxamine phosphate. Pyridoxal phosphate is required for many enzymatic reactions involving amino acids including transamination, decarboxylation, and dehydration. Pyridoxal phosphate also functions in the biosynthesis of porphyrins and in the catabolism of glycogen (NRC 2011).

Deficiency Symptoms

Pyridoxal phosphate is required for the synthesis of the neurotransmitters (5-hydroxytryptamine and

serotonin) from tryptophan. Consequently, signs of pyridoxine deficiency include nervous disorders (erratic swimming, hyperirritability, and convulsions), which have been observed in salmonids (Halver 1957; Coates and Halver 1958), gilthead sea bream (*Sparus auratus*) (Kissil et al. 1981), channel catfish (Andrews and Murai 1979), common carp (Ogino 1965), yellowtail, *Seriola lalandi* (Sakaguchi et al. 1969), Japanese eel (Arai et al. 1972), and red hybrid tilapia (Lim et al. 1995). Other deficiency signs, such as anorexia and poor growth, were reported in hybrid tilapia (Shiau and Hsieh 1997), and usually appeared in the fish within 3–8 weeks after being fed a pyridoxine-deficient diet. Pyridoxine deficiency has been reported to cause various histopathological changes in rainbow trout liver (Jurss and Jonas 1981) and kidney (Smith et al. 1974), in the intestinal tissue of both rainbow trout (Smith et al. 1974) and gilthead sea bream (Kissil et al., 1981), and in the intestinal tissue and kidney of Indian catfish, *Heteropneustes fossilis* (Shaik Mohamed 2001a).

Requirements

The activity of certain aminotransferase enzymes that require pyridoxal phosphate as a coenzyme has been used as an index of pyridoxine status in fish. Serum or tissue alanine and/or aspartate aminotransferase activities have been used to evaluate pyridoxine status in common carp (Ogino 1965), rainbow trout (Smith et al. 1974; Jurss 1978), Chinook salmon (Hardy et al. 1979), turbot (Adron et al. 1978), gilthead sea bream (Kissil et al. 1981), and hybrid tilapia (Shiau and Hsieh 1997).

Vitamin B_6 requirements of hybrid tilapia have been reported to vary with dietary protein levels, with 1.7–9.5 and 15–16.5 mg B_6 kg^{-1} diet required in diets with 28% and 36% protein, respectively (Shiau and Hsieh 1997). Vitamin B_6 supplementation increased the docosahexaenoic acid concentration of muscle lipids of rainbow trout (Maranesi et al. 2005).

Effect on Immune Responses and Disease Resistance

Atlantic salmon (14 g) were fed a practical fishmeal-based diet supplemented with 0, 10, 20, 40, 80, and 160 mg vitamin B_6 kg^{-1} for 20 weeks. Serum hemolytic complement activity and head kidney lysozyme activity, and the specific antibody response following immunization with *Vibrio salmonicida*, were not influenced by the dietary regimes (Albrektsen et al. 1995). Challenge with *Aeromonas salmonicida* showed that increasing the dietary levels of vitamin B_6 for Atlantic salmon did not improve resistance to furunculosis.

Feng et al. (2010) reported that Jian carp fed diets with $\geq$5 mg kg^{-1} of vitamin B_6 had higher white blood cell (WBC) count, hemagglutination titre, activities of lysozyme, acid phosphatase, and total iron-binding capacity than fish fed vitamin-B_6-unsupplemented diet (1.7 mg B_6 kg^{-1} in basal diet). One study found that rohu, *Labeo rohita*, fed diets containing pyridoxine had higher erythrocytes count, hemoglobin content, total serum protein, albumin, globulin, nitroblue tetrazolium, and lysozyme activity, and lower cortisol, blood glucose, and survival after challenge with *Aeromonas hydrophila* compared to the pyridoxine-deficient group (Akhtar et al. 2010). Akhtar et al. (2012) reported that dietary B_6 supplementation at a level of 100 mg kg^{-1} diet enhanced immune responses and resistance to high-temperature stress in rohu.

Vitamin B_{12}

Biochemistry and Metabolic Function

The term "vitamin B_{12}" should be used as the generic descriptor for all corrinoids qualitatively exhibiting the biological activity of cyanocobalamin. Vitamin B_{12} is a large molecule (molecular weight 1355) that contains a cobalt (Co) atom. Neither higher plants nor animals can synthesize vitamin B_{12}, so both depend on certain microorganisms for the trace amounts required.

In animals, vitamin B_{12} is known to be involved in two separate enzyme systems: (1) methylmalonyl CoA mutase, by which propionic acid is converted to succinate, and (2) methyltetrahydrofolate-homocysteine methyltransferase, a catalyst in methionine, methane, and acetate synthetase. Vitamin B_{12} is required for normal maturation and development of erythrocytes, for the metabolism of fatty acids, in the methylation of homocysteine to methionine, and for the normal recycling of tetrahydrofolic acid. A deficiency of

vitamin B_{12} can therefore result in symptoms similar to those of folate deficiency (NRC 2011).

Deficiency Symptoms

Salmon (Halver 1957) and trout (Phillips et al. 1964) fed low amounts of dietary vitamin B_{12} showed a high variability in numbers of fragmented erythrocytes and hemoglobin values, with a tendency for a microcytic, hypochromic anemia. Channel catfish fed a vitamin-B_{12}-deficient diet for 36 weeks exhibited reduced growth but no other clinical deficiency signs (Dupree 1966). John and Mahajan (1979) observed reduced growth and lower hematocrit in rohu fed a vitamin-B_{12}-deficient diet. Japanese eel were found to require vitamin B_{12} for normal appetite and growth (Arai et al. 1972).

Requirements

Intestinal microfloral synthesis appeared to satisfy the B_{12} requirement of common carp (Kashiwada et al. 1970), Nile tilapia (Lovell and Limsuwan 1982), and hybrid tilapia (Shiau and Lung 1993), but channel catfish (Limsuwan and Lovell 1981) required dietary supplementation of B_{12} to prevent anemia. Intestinal microfloral synthesis of vitamin B_{12} has been demonstrated in common carp (Kashiwada et al. 1970; Sugita et al. 1991a), channel catfish (Limsuwan and Lovell 1981; Sugita et al. 1990, 1991a), Nile tilapia (Lovell and Limsuwan 1982; Sugita et al. 1990, 1991a), rainbow trout (Sugita et al. 1991b), ayu, *Plecoglossus altivelis*, and goldfish, *Carassius auratus* (Sugita et al. 1991a). Sugita et al. (1991a) found a close relationship between the amount of vitamin B_{12} and the viable counts of *Bacteroides* type A in the intestinal contents of the various fish studied. They found that this bacterium was present in the intestinal contents of fish that do not require vitamin B_{12} and absent from fish that do require vitamin B_{12}. Grouper has been reported to require 10 mg Co kg^{-1} diet for optimal growth. This amount of Co can promote gastrointestinal bacterial production of vitamin B_{12} in sufficient amounts to supply growth requirements of grouper, so no additional dietary vitamin B_{12} supplementation is needed (Lin et al. 2010). Dietary vitamin B_{12} requirements have been quantified in three fish species: Pacific salmon (Halver 1972), yellowtail (Shimeno 1991), and grass carp (Wu et al. 2007b). The requirements are 0.015–0.02, 0.053, and 0.094 mg kg^{-1} diet, respectively.

Niacin

Biochemistry and Metabolic Function

Niacin is used as the generic descriptor of pyridine 3-carboxylic acids and their derivatives, which exhibit the biological activity of nicotinamide (the amide of nicotinic acid). Of the compounds with niacin activity, nicotinic acid and nicotinamide have the greatest biological activity.

Niacin is a component of two coenzymes: nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). These coenzymes are essential for several oxidation-reduction reactions involving the transfer of hydrogen and electrons in carbohydrate, lipid, and amino acid metabolism. They are also involved in various energy-yielding and biosynthetic pathways, including the mitochondrial electron transport system (NRC 2011).

Deficiency Symptoms

Trout and salmon fed niacin-deficient diets exhibited anorexia, poor growth, poor feed conversion, photosensitivity or sunburn, intestinal lesions, abdominal edema, muscular weakness, spasms, and increased mortality (McLaren et al. 1947; Phillips and Brockway 1947; Halver 1957). Channel catfish (Andrews and Murai 1978) and common carp (Aoe et al. 1967b) showed skin and fin lesions, high mortality, skin hemorrhages, anemia, and deformed jaws when fed niacin-deficient diets for 2–6 weeks. Skin hemorrhages, dermatitis, anemia, abnormal swimming, and ataxia were observed in Japanese eels fed a niacin-deficient diet for 14 weeks (Arai et al. 1972). Two weeks after exposure to ultraviolet radiation, niacin-deficient rainbow trout showed a total loss of mucus-producing cells in histopathological sections of the epidermis. Signs of niacin-deficiency include dermal lesions in rainbow trout (Poston and Wolfe 1985); skin, fin, and mouth lesions and hemorrhages, as well as deformed snout and gill edema in hybrid tilapia (Shiau and Suen 1992); and anemia, anorexia, lethargy, and skin hemorrhage in Indian catfish (Shaik

Mohamed and Ibrahim 2001) were observed in niacin-deficient fish.

Requirements

Tryptophan can be metabolically converted to niacin in many animals, but not in certain salmonid fish (Poston and DiLorenzo 1973; Poston and Combs 1980). Ng et al. (1997) reported that excess tryptophan in niacin-deficient diets did not improve growth, hematocrits, or hepatic NAD concentrations in channel catfish. The fact that niacin deficiency can be readily induced in various species indicates that most, if not all, fish lack the capacity for niacin synthesis. Niacin is widely distributed in both plant and animal tissue. It is generally considered that much of the niacin in plant material is bound, and available to fish in limited quantities. Bioavailability of niacin from ingredients commonly used in feeds for channel catfish (i.e., menhaden fishmeal, meat and bone/blood meal, wheat middlings, cooked corn, uncooked corn, cottonseed meal, and soybean meal) were estimated to be 100, 100, 60, 44, 28, 58, and 57%, respectively (Ng et al. 1998). These authors concluded that endogenous total niacin present in the ingredients used in commercial channel catfish diets were more than sufficient to meet the niacin requirements of this fish without a need for niacin supplementation.

Complex carbohydrates in the diet may increase the requirement for niacin. Shiau and Suen (1992) reported that 26 and 121 mg niacin kg^{-1} of feed was required for hybrid tilapia fed diets containing glucose and dextrin as the carbohydrate source, respectively.

Biotin

Biochemistry and Metabolic Function

Biotin is a monocarboxylic acid that is slightly soluble in water and alcohol, and insoluble in organic solvents; salts of the acid are soluble in water. Aqueous solutions and the dry material are stable at 100°C and to light. The vitamin is destroyed by acids and alkalis, and by oxidizing agents such as peroxides or permanganate. Biocytin is a bound form of biotin isolated from yeast, plant, and animal tissues. Other bound forms of the vitamin can generally be liberated by peptic digestion. Oxybiotin has partial vitamin activity, but oxybiotin sulphonic acid and other analogues are antimetabolites that inhibit the growth of bacteria. This inhibition can be overcome by additional biotin and must therefore be due to inhibition of incorporation of the biotin into coenzymes. Avidin, a protein found in raw egg white, binds biotin and makes it unavailable to fish and other animals. This binding is irreversible in raw material, but heating to denature the protein makes the bound biotin available again to the fish. Biocytin or å-biotinyl lysine (the epsilon amino group of lysine and the carboxyl of biotin being combined in a peptide bond) is hydrolyzed by the enzyme biotinase, making the protein-bound biotin available (Halver 2002).

Biotin acts in certain metabolic reactions as an intermediate carrier of carbon dioxide during carboxylation and decarboxylation reactions. Specific enzymes that require biotin include acetyl-CoA carboxylase, pyruvate carboxylase, and propionyl-CoA carboxylase. Metabolic pathways requiring biotin include the biosynthesis of long-chain fatty acids and the synthesis of purines (NRC 2011).

Deficiency Symptoms

In many animals, a biotin deficiency can only be induced by feeding avidin, a glycoprotein found in raw chicken egg white that binds biotin and which prevents absorption of the vitamin from the intestine. Robinson and Lovell (1978) fed channel catfish avidin in a biotin-free chemically defined diet to channel catfish and noted a growth suppression which led them to suggest some biotin synthesis by intestinal microflora in this species. Common carp required 8–12 weeks (Ogino et al. 1970a) to show growth depression when fed biotin-deficient diets, while channel catfish required 11 weeks (Lovell and Buston 1984). A similar effect in rainbow trout took only 4–8 weeks in water temperatures of 15°C (Woodward and Frigg 1989). Anorexia, reduced weight gain, and higher feed conversion were more noticeable in smaller rainbow trout fed biotin-deficient diets than in larger fish (Walton et al. 1984). Biotin-deficient channel catfish exhibited skin depigmentation (Robinson and Lovell 1978), whereas biotin-deficient Japanese eels had darker skin coloration (Arai et al. 1972). Histological signs of biotin deficiency were not detected after 12 weeks in rainbow trout having an initial weight of 25 g (Walton et al. 1984). However, severe deficiency signs were produced in rainbow trout and

lake trout, *Salvelinus namaycush*, with initial weights of 1.3 and 6.7 g, respectively (Poston and Page 1982; Woodward and Frigg 1989). Rainbow trout and lake trout developed biotin-related histopathological signs in the gills (Castledine et al. 1978; Poston and Page 1982), liver (Poston 1976b; Poston and Page 1982), and kidney (Poston and Page 1982). Biotin-deficient diets caused anorexia, dark skin color, convulsions, and high mortality in Asian catfish, *Clarias batrachus* (Shaik Mohamed et al. 2000), and convulsions, heavy mortality, listlessness, poor feed conversion and feed intake, dark skin color, tetanus, and weight loss in Indian catfish (Shaik Mohamed 2001b).

Requirements

Signs of biotin deficiency were not detected in rainbow trout (Castledine et al. 1978) or channel catfish (Lovell and Buston 1984) fed natural ingredient diets without supplemented biotin for 24 and 17 weeks, respectively. Mæland et al. (1998) reported no need for supplemental biotin in practical fishmeal diets for Atlantic salmon fry to achieve optimum growth, survival, and maximal hepatic pyruvate carboxylase activity. These studies concluded that adequate biotin was available in the natural ingredient diets used to meet the requirements of the fish.

Folic acid

Biochemistry and Metabolic Function

The term "folate" is used as the generic descriptor for folic acid and related compounds qualitatively exhibiting the biological activity of folic acid. Folic acid is composed of a pteridine ring linked through a methylene bridge to p-aminobenzoic acid to form pteroic acid, which is in turn linked to glutamic acid as an amide. It deteriorates when exposed to sunlight or during prolonged storage. Several analogs have biological activity, including pteroic acid, rhizopterin, folinic acid, xanthopterin, and several formyltetrahydropteroyl-glutamic acid derivatives. These have closely allied ring structures, and many have been isolated as derivatives in various animals or microbiological preparations.

Folic acid undergoes enzymatic reduction in the tissues to its active coenzyme form, tetrahydrofolic acid. It functions as a pivoting intermediate carrier of one-carbon groups in a number of complex enzymatic reactions also involving vitamin B_{12}, biotin, niacin, methionine, choline, betaine, and homocysteine. In these reactions, methyl, methylene, and other one-carbon groups are transferred from one molecule to another. The C-3 of serine is the major source of one-carbon units for folate metabolism. Other sources include formate, much of which is derived from serine metabolism in the mitochondria and the C-2 of histidine. The folate-dependent reactions are found in the metabolism of certain amino acids and the biosynthesis of purines and pyrimidines, along with the nucleotides found in DNA and RNA (NRC 2011).

Deficiency Symptoms

Trout and salmon fed folate-deficient diets exhibited anorexia, reduced growth, poor feed conversion, and macrocytic normochromic megaloblastic anemia (Smith 1968; Smith and Halver 1969), characterized by pale gills, anisocytosis, and poikilocytosis. The erythrocytes were large with abnormally segmented and constricted nuclei, and a large number of megaloblastic proerythrocytes were present in the erythropoietic tissue of the anterior kidney. Production of erythrocytes decreased with time in fish fed the folate-deficient diet. Some of these signs have also been observed in the rohu (John and Mahajan 1979).

Poor growth and dark skin coloration were noted in Japanese eels fed a folate-deficient diet for 10 weeks (Arai et al. 1972). Rainbow trout fed a folic-acid-deficient diet showed a blood pathology, that is, megaloblastosis (Cowey and Woodward 1993). Folate deficiency signs in channel catfish included reduced growth, anemia, and increased sensitivity to bacterial infection (Duncan and Lovell 1991). Lin et al. (2011) found poor growth and high hepatic oxidative stress in folate-deficient grouper.

Requirements

Deficiency signs were not observed in common carp (Aoe et al. 1967c) and hybrid tilapia (Shiau and Huang 2001) fed a folate (FA)-free diet. Deficiency signs were not observed in fish fed a FA-free diet, presumably due to bacterial synthesis of FA in the intestine (Kashiwada et al. 1971; Duncan et al. 1993). It is generally believed that intestinal microorganisms may contribute a considerable quantity of folic acid

to the host. Duncan et al. (1993) demonstrated that intestinal microorganisms are a significant source of folic acid for channel catfish. Kashiwada et al. (1971) isolated folic-acid-synthesizing bacteria from the intestine of common carp and concluded that this explained why common carp do not require a dietary source of FA.

The hepatosomatic index (HSI) has been reported to be a good parameter, other than growth and FA tissue concentrations, for estimating folate requirements in hybrid tilapia (Shiau and Huang 2001) and grouper (Lin et al. 2011).

Effect on Immune Responses and Disease Resistance

Lim and Klesius (2001) reported that dietary folic supplementation levels do not alter the agglutinating antibody titers and cumulative mortality of Nile tilapia infected with *Streptococcus iniae*. A dietary folic acid level of 0.8 mg kg^{-1} diet is required for maximizing the growth of grouper; this amount is also adequate for non-specific immune responses, including superoxide anion production and plasma lysozyme activity of the fish (Lin et al. 2011).

Pantothenic acid

Biochemistry and Metabolic Function

Pantothenic acid may be considered as a dihydroxydimethylbutyric acid bonded to â-alanine. The free acid is a yellow, viscous oil; the compound generally used in fish nutrition is the calcium salt. This salt is a white crystalline powder readily soluble in water and mild acid, and almost insoluble in organic solvents. It is stable to oxidizing and reducing agents, as well as autoclaving, but is labile to dry heat, hot alkali, or hot acid (Halver 2002).

Pantothenic acid is a component of coenzyme A (CoA), acyl-CoA synthetase, and acyl carrier protein. The coenzyme form of the vitamin is therefore responsible for acyl group transfer reactions. Coenzyme A is required in reactions in which the carbon skeletons of glucose, fatty acids, and amino acids enter into the energy-yielding tricarboxylic acid cycle. Acyl carrier protein is required for fatty acid synthesis (NRC 2011).

Deficiency Symptoms

A deficiency of pantothenic acid impairs the metabolism of mitochondria-rich cells, which undergo rapid mitosis and high-energy expenditure. Gill lamellar hyperplasia, or clubbed gills, is a characteristic sign of pantothenic acid deficiency in most fish. In addition to clubbed gills, anemia and high mortality have been observed in pantothenic acid-deficient salmonids (Phillips et al. 1945; McLaren et al. 1947; Coates and Halver 1958; Kitamura et al. 1967b; Poston and Page 1982; Karges and Woodward 1984) and channel catfish (Dupree 1966; Murai and Andrews 1979; Brunson et al. 1983; Wilson et al. 1983). Pantothenic acid-deficient Japanese parrotfish exhibited anorexia, convulsions, and cessation of growth, followed by high mortality (Ikeda et al. 1988). Similar deficiency signs were observed in red sea bream (Yone and Fujii 1974). Slow growth, anorexia, lethargy, and anemia were observed in common carp (Ogino 1967). Poor growth, hemorrhage, skin lesions, and abnormal swimming were found in Japanese eel fed pantothenic acid-deficient diets (Arai et al. 1972). Pantothenic acid deficiency caused poor growth, hemorrhage, sluggishness, high mortality, anemia, and severe hyperplasia of the epithelial cells of gill lamellae in blue tilapia (Soliman and Wilson 1992b), and anorexia, poor growth, exophthalmus, and hemorrhage of body surface and fin in Jian carp (Wen et al. 2009). Fatty liver were found in pantothenic-acid-deficient lake trout, *Salvelinus namaycush* (Poston and Page 1982), Mexican cichlid, *Cichlasoma urophthalmus* (Martinez et al. 1990), and blue tilapia (Roem et al. 1991).

Requirements

Good sources for pantothenic acid are cereal bran, yeast, liver, kidney, heart, spleen, and lung (Halver 2002). No differences in growth rate, feed efficiency, or survival were observed in Asian sea bass, *Lates calcarifer*, fingerlings fed practical diets without supplemental pantothenic acid (Boonyaratpalin 1997).

Effect on Immune Responses and Disease Resistance

Wen et al. (2010) indicated that survival rate after *A. hydrophila* challenge was higher with the increasing

dietary pantothenic acid (PA) levels. Immunoglobin M (Ig M) content, agglutination antibody titre to *A. hydrophila*, and serum lysozyme activity were positively affected by the dietary PA levels. Correlation analysis showed survival rate after bacterial challenge was positively related to IgM and agglutination antibody titre to *A. hydrophila*. By the broken-line analysis, the dietary PA optimal level for the phagocytic activity of leucocytes and serum IgM content were 42.2 and 47.2 mg kg^{-1} respectively, which is twice the requirement (23.0 mg kg^{-1}) for optimal growth of juvenile Jian carp (Wen et al. 2009).

Choline

Biochemistry and Metabolic Function

Choline is a very strong organic base that forms many derivatives widely distributed in animal and vegetable tissue. The derivative acetylcholine is involved in the transmission of nerve impulses across synapses. Choline is very hydroscopic, very soluble in water, and is stable to heat in acid, but decomposes in alkaline solutions. Since it is a strong base, choline reacts with many chemical compounds (Halver 2002).

Unlike the other water-soluble vitamins, choline has no known coenzyme function. Choline has three major metabolic functions: as a component of phosphatidylcholine, which has structural functions in biological membranes and in tissue lipid utilization; as a precursor of the neurotransmitter acetylcholine; and as a precursor of betaine, which serves as a source of labile methyl groups for methylation reactions, such as the formation of methionine from homocysteine and creatine from guanidoacetic acid (NRC 2011).

Deficiency Symptoms

Deficiency signs include poor growth, poor feed conversion, and impaired fat metabolism. Hemorrhagic kidneys and intestines have been reported in rainbow trout (McLaren et al. 1947), and increased gastric emptying time has been observed in Chinook salmon (Halver 1957, 1972). Similar deficiency signs were observed in catfish (Dupree 1966), common carp (Ogino et al. 1970a), Japanese eel (Arai et al. 1972), and lake trout (Ketola 1976). Hepatic lipid content increased in choline-deficient channel catfish (Wilson and Poe 1988), common carp (Ogino et al. 1970b), and hybrid striped bass (Griffin et al. 1994), but decreased in hybrid tilapia (Shiau and Lo 2000) and red drum (Craig and Gatlin 1996).

Requirements

Channel catfish fed casein-gelatin diets containing excess methionine did not develop signs of choline deficiency; however, catfish fed diets adequate but not excessive in methionine did develop deficiency signs (Wilson and Poe 1988). Rumsey (1991) has suggested that 50% of the choline requirement of rainbow trout can be met from betaine. These observations indicate that certain fish can meet part of their choline needs through the synthesis of choline by the methylation of ethanolamine, which uses methyl groups from S-adenosyl methionine.

Myo-inositol

Biochemistry and Metabolic Function

Seven optically inactive and two optically active isomers of hexahydroxycyclohexane can exist. One of the optically active forms, *myo*-inositol, is a white crystalline powder soluble in water and insoluble in alcohol and ether. The material can be synthesized, but is easily isolated from biological material in free or combined forms. The mixed calcium-magnesium salt of the hexophosphate is phytin. Isomers have little biological activity but will compete in chemical reactions. *Myo*-inositol is a highly stable compound (Halver 2002).

Myo-inositol is a biologically active cyclohexitol that occurs as a structural component in biological membranes as phosphatidylinositol. Phosphatidylinositol has been shown to be involved in signal transduction of several metabolic processes. Although similar in many respects to the adenylate cyclase transduction system, the phosphoinositide system is distinctive in that the hormonal stimulus activates a reaction that generates two second messengers. Membrane-bound phosphatidylinositol 4,5-bisphosphate is cleaved to release *sn*-1,2-diacylglycerol and inositol 1,4,5-triphosphate, following the interaction of a hormone or agonist with the receptor on the cell membrane. Inositol 1,4,5-triphosphate stimulates the release of calcium from its intracellular stores in the endoplasmic

reticulum, and *sn*-1,2-diacylglycerol activates protein kinase C to phosphorylate specific target proteins. Examples of cellular processes controlled by the phosphoinositide second messenger system include amylase secretion, insulin release, smooth muscle contraction, liver glycogenolysis, platelet aggregation, histamine secretion, and DNA synthesis in fibroblasts and lymphoblasts (NRC 2011).

Deficiency Symptoms

Signs of *myo*-inositol deficiency have been reported to include poor appetite, anemia, poor growth, fin erosion, dark skin coloration, slow gastric emptying, and decreased cholinesterase and certain aminotransferase activities in rainbow trout (McLaren et al. 1947; Kitamura et al. 1967b), red sea bream (Yone et al. 1971), Japanese eel (Arai et al. 1972), and Japanese parrotfish (Ikeda et al. 1988). Rainbow trout fed a diet devoid of *myo*-inositol had large accumulations of neutral lipids in the liver and increased levels of cholesterol and triglycerides, but decreased amounts of total phospholipid, phosphotidylcholine, phosphotidylethanolamine, and phosphotidylinositol (Holub et al. 1982). Total lipid content was increased in hybrid tilapia (Shiau and Su 2005) and grouper (Su and Shiau, 2004) fed *myo*-inositol-deficient diets. Lee et al. (2009) reported that olive flounder, *Paralichthys olivaceus*, fed a *myo*-inositol-free diet had abnormal lipid metabolism and a decreased amount of polyunsaturated fatty acids.

Requirements

Myo-inositol appears to be synthesized in common carp intestine (Aoe and Masuda 1967), but not in an amount sufficient to sustain normal growth of young fish without an exogenous source of this vitamin; this is because younger carp require a higher level of *myo*-inositol than older fish. High concentrations of dietary glucose may increase the need for *myo*-inositol in some fish (Yone et al. 1971). Burtle and Lovell (1989) demonstrated *de novo* synthesis of *myo*-inositol in the liver of channel catfish, as well as intestinal synthesis. Deng et al. (2002) demonstrated that *de novo* synthesis of *myo*-inositol was sufficient for normal growth and tissue storage of hybrid striped bass. Intestinal microbial synthesis was not a significant source of *myo*-inositol for hybrid tilapia (Shiau and Su 2005) and olive flounder (Lee et al. 2009), since addition of an antibiotic to a inositol-free diet had similar growth and tissue *myo*-inositol levels with fish fed the *myo*-inositol- and antibiotic-free diet. Peres et al. (2004) reported that Nile tilapia did not require an exogenous source of *myo*-inositol for normal growth, feed utilization, and erythropoiesis. In addition, supplementation of dietary *myo*-inositol did not improve disease resistance of the fish. There is no need for *myo*-inositol supplementation to fishmeal-based diets (containing 296 mg kg^{-1} diet of *myo*-inositol) for young Atlantic salmon (Waagbø et al. 1998).

Effect on Immune Responses and Disease Resistance

Red blood cell count, white blood cell count, phagocytosis activity, hemagglutination titre, lysozyme activity, and anti-*Aeromonas hydrophila* antibody titre of Jian carp were all improved with an increase in the *myo*-inositol levels from 232.7 mg kg^{-1} diet to 687.3 mg kg^{-1} diet (Jiang et al. 2010).

Conclusions

Both qualitative and quantitative vitamin requirements for fish growth have been well documented. Dietary immunomodulation has the potential to greatly aid aquaculture production through prevention and/or improvement of disease resistance. However, information on the influence of dietary vitamins on fish health, including immune response and disease resistance, is scarce. Future study in this aspect is needed.

The effects of diet composition with regard to interaction between vitamins and other nutrient components in diet on immunostimulatory properties in fish should be investigated. The effects of feeding rates and feed efficiencies at different rearing conditions, such as temperature, also need to be considered when evaluating diet-borne immunostimulants.

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Chapter 7

The Effect of Vitamin C on Fish Health

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Introduction

The goals of the aquaculture industry are to optimize growth and to produce high-quality fish. As in all farming, the outbreak of diseases in fish and shrimp farming is of major concern. The high susceptibility of fish to stress and the rapid spread of diseases in water have forced fish and shrimp farmers to concentrate their efforts on health maintenance in order to achieve sustainable economic performances. Growing healthy fish requires them to be able to develop strong defense mechanisms, either innate or specific immune responses, against pathogen invasion. The innate immune responses play a major role in fish and shrimp. Improving the immune response also leads to better vaccination efficiency. Vaccines induce a specific immune response and an increased capacity to kill the pathogens by innate defense mechanisms.

During the early development of the salmon industry, antibiotics were commonly used in the treatment of diseases. However, the consumption of drugs has been progressively reduced due in part to environmental and regulatory concerns and in part to increased resistance of pathogens. Furthermore, the curative effect of oral drugs is minimized by the fact that diseased fish frequently do not feed. The eradication of major diseases by improved husbandry and vaccination has reduced mortality levels considerably. Although some efficient vaccines against major bacterial diseases of finfish exist, others need to be improved, in terms of efficiency and duration of protection, or be developed. Consumption of drugs has to be reduced to alleviate environmental and health concerns; more potent immune modulators, disease-resistant strains, and anti-microbial substances therefore have to be developed to contribute to the reduction.

Intensively raised fish may also be exposed to stressful situations that often result in a depressed immune status. Good management practices reduce stress and therefore help to maintain healthy animals. However, since not all stressing situations can be avoided, fish with enhanced defense mechanisms will be better prepared to combat the negative effects of stress.

The nutritional quality of the feed represents a major factor in sustaining healthy fish and shrimp. It has been shown that the immune system can be enhanced by the use of immunomodulators such as antioxidant vitamins, carotenoids, and other feed additives. The combination of good management, vaccination, and nutritional prophylaxis will ensure higher survival rates and improve growth in intensive

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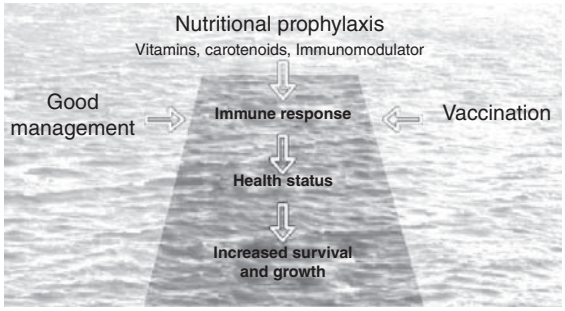

Figure 7.1 Benefits of nutritional prophylaxis in aquaculture. For color details, please see color plate section.

farming systems. This chapter highlights the importance of vitamin C in modern aqua-farming as an immunomodulator and a key nutritional element in promoting optimal survival and performance (Fig. 7.1).

The Immune Response of Fish

Fish are primitive vertebrates and are an important link between invertebrates and higher vertebrates. They possess the innate defense mechanisms of invertebrates, such as the phagocytic mechanisms developed by macrophages and granular leukocytes, but were also the first animals to develop both cellular and humoral immune responses mediated by lymphocytes. The main lymphoid organs of fish are the anterior kidney, the thymus, and the spleen. In fish, innate immunity is considered the first line of defense and represents a considerable part of the immune response, in contrast to mammals. When a pathogen penetrates the body of fish, the innate immune mechanisms may be sufficient to stop the infection. If not, the disease will develop and the specific immune mechanisms will also be involved. If the animal survives, it will be protected against re-infection by the same pathogen due to the development of a specific immunological memory. However, the immune memory is less developed in fish than in mammals, and even weaker in crustaceans.

Innate immunity consists of several host-defense mechanisms that do not require a specific recognition of the antigen, and which occur in humoral and cell-mediated responses of the specific immunity, also called adaptive immunity. Table 7.1 gives an overview

Table 7.1 Overview of fish defenses.

Innate immunity		Specific immunity
First line of defense	Second line of defense	Third line of defense
Skin	Phagocytes, NCC	Lymphocytes
Scales	Anti-microbial proteins	Antibodies
Mucus	Inflammatory response	Cytokines
Gut-associated lymphoid tissue (GALT)		
Mucosal and systemic immune system		

of the different lines of fish defenses and Figure 7.2 illustrates the different elements involved in the innate and specific immune responses. While the innate response is developed in hours to days after infection, the development of an adaptive response can take weeks.

The Innate Immune Response

As shown in Table 7.1, the primary line of defense consists mainly of the skin and mucus. When pathogens enter the body, cellular and humoral innate defense mechanisms are initiated. The most important cells involved in this defense are the phagocytes that are able to perform many functions such as chemotaxis, phagocytosis, pinocytosis, and intracellular killing. Extracellular killing is performed by, among others, natural cytotoxic cells (NCC). All these cells are helped by several soluble factors such as complement and lysozyme, but also a battery of other soluble factors such as cytokines.

Fish have adapted to their aquatic environment by developing efficient physical and chemical barriers, such as skin and mucus. The skin represents an important innate defense mechanism to prevent microorganisms from entering the body. The integrity of the skin is of great importance and wound healing is therefore much faster than in mammals. Another important barrier is the mucus, which helps to prevent microorganisms entering the body through the skin, gills, and gastrointestinal mucosa. The mucus prevents bacteria from adhering to epithelial cells. Furthermore, several components of the innate immune

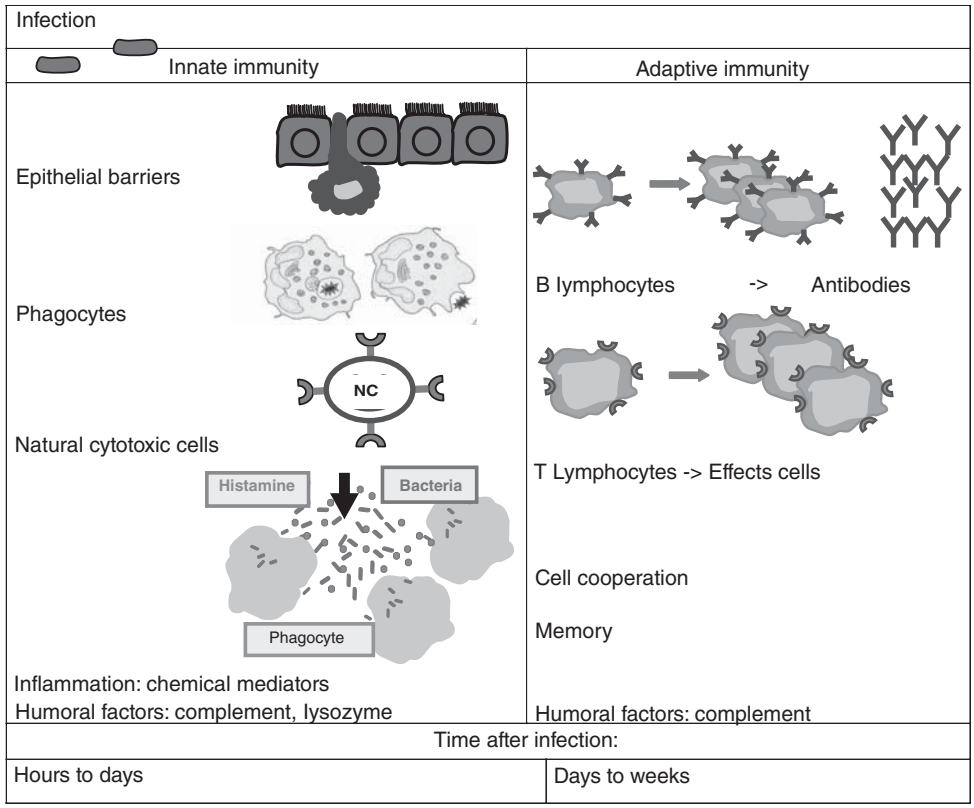

Figure 7.2 Distinction between innate and specific immunity. Source: V. Verlhac Trichet 2010. Nutrition and immunity: an update. Aquaculture Research 41(3): 356–372. For color details, please see color plate section.

response are found in the mucus, emphasizing its importance as a first defense mechanism (i.e., natural antibodies, lysozyme, lysins, and complement). When microorganisms penetrate the body, the innate immune system reacts with cellular mechanisms and soluble factors.

Cellular Mechanisms

Cells involved in innate immunity are mainly phagocytes (monocytes/macrophages and granular leukocytes such as neutrophils). Phagocytosis is the most primitive defense mechanism. Originally a nutritive function in lower life forms, phagocytosis has evolved to become solely a protective function in vertebrates. Natural cytotoxic cells and eosinophils are also involved and act via an extracellular killing mechanism.

The macrophage and neutrophil functions performed by phagocytes are as follows:

- *Chemotaxis* is the process by which phagocytic cells are attracted by various molecules and migrate to the sites of inflammation, tissue damage, or immune reactions. Molecules known as chemotactic inducers are either produced by the bacteria or are components of the immune system, for example, complement factors. Migration-inhibiting factors block the further migration of phagocytes when they are active at a site of infection.
- *Phagocytosis* occurs when bacteria have adhered to the surface of the phagocyte. It involves recognition and attachment, and then engulfment and digestion of a foreign particle which initiates the ingestion phase by activating an actin-myosin contractile system that extends pseudopods around it. As adjacent receptors attach to the surface of the microbe, the plasma membrane is pulled around the particle until it is completely enclosed in a phagosome. Cytoplasmic granules then fuse

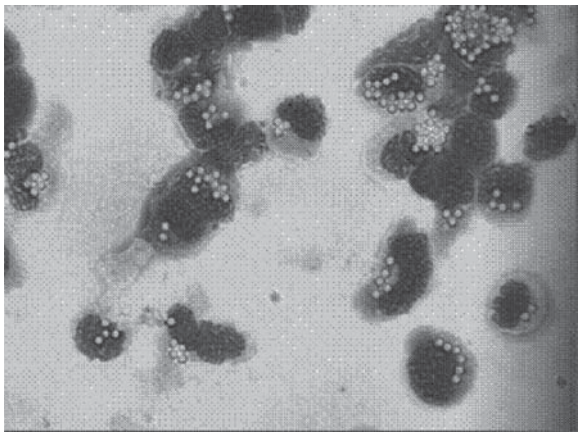

Figure 7.3 Phagocytosis of latex beads by trout macrophages. For color details, please see color plate section.

with the phagosome and discharge their contents around the microorganism, which is subjected to a range of microbicidal mechanisms. An example of phagocytosis is given in Figure 7.3.

- *Pinocytosis* is a mechanism comparable to phagocytosis that is used in the internalization of very small particles. This phenomenon is characterized by the invagination of the membrane to form a small vacuole by endocytosis.
- Fish granulocyte *kills extracellularly* through the discharge of their hydrolytic and oxidizing enzymes rather than intracellularly via phagosome-lysosome fusion, as occurs in mammals.
- In some fish species, *thrombocytes* have also been demonstrated to have phagocytic properties.

Killing

The killing of bacteria is characterized by oxygen-dependent and oxygen-independent mechanisms. Figure 7.4 presents an overview of the microbicidal mechanisms of phagocytosis. The oxygen-dependent mechanisms occur when phagocytosis is initiated in case of bacterial invasion. In such case, there is a dramatic increase in the oxygen consumption by the phagocytes called the oxidative burst. The NADPH-oxidase system located in the membranes of these cells is activated in response to the penetration of the pathogen in the organism and releases superoxide anions and hydrogen peroxides.

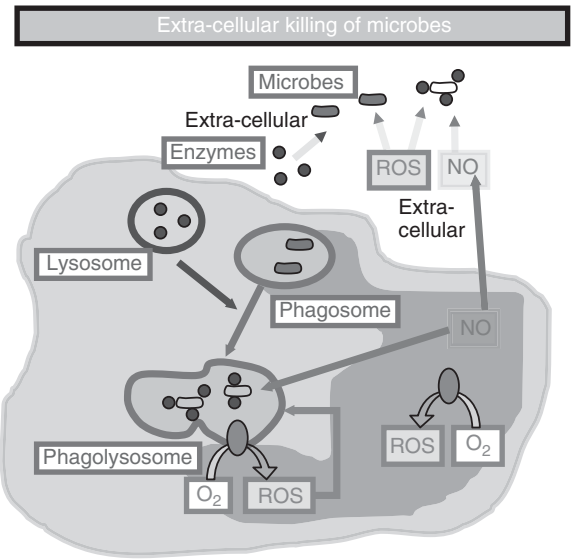

Figure 7.4 Microbicidal mechanisms of phagocytosis. ROS: reactive oxygen species. Source: V. Verlhac Trichet 2010. Nutrition and immunity: an update. Aquaculture Research 41(3): 356–372. For color details, please see color plate section.

Furthermore, the combination of peroxides, myeloperoxidase released by granular leukocytes, and halide anions constitute a potent halogenating system capable of killing both bacteria and viruses.

The oxygen-independent mechanisms such as low pH, lysozyme, and lactoferrin constitute bactericidal and bacteriostatic factors that are oxygen-independent and can function under anaerobic circumstances. Finally, proteolytic enzymes and a variety of other hydrolytic enzymes digest the killed organisms, and degradation products are released to the extracellular compartment.

Extracellular killing by natural cytotoxic cells and eosinophilic granulocytes are minor cellular mechanisms compared to phagocytosis. Natural cytotoxic cells are involved in the killing of virus-infected cells and they also have anti-tumoral function. They act through the release of perforin granules, which will disrupt the membrane of the infected cells and inhibit the replication of the virus. Eosinophils are required for the killing of large pathogens such as some parasites that are too large to be eliminated through a phagocytic process.

Soluble Factors

Complement

The complement system consists of a group of protein and non-protein components that are involved in both innate and specific defense mechanisms. The complement system can be activated along two different pathways: the alternative pathway, which is initiated by contact with certain microbial cell wall polysaccharides and is related to innate immunity; and the classical pathway, related to specific immunity which will be detailed in section entitled 'The Specific Immune Response'. Both pathways of complement activation involve a cascade of enzymatic reactions resulting in the opsonization and/or lysis of foreign cells by disruption of cell membranes.

The alternative pathway allows the elimination of invading pathogens without the presence of antibodies. The functions of the complement system related to innate defense mechanisms are mainly the chemotaxis and the opsonization. The complement allows the lysis of cell membranes from numerous bacterial species. Some components released following activation of the alternative pathway influence the migration of phagocytic cells towards the site of infection. This function is called chemotaxis. Other components cover the bacteria and facilitate their adherence to phagocytes and their subsequent killing by these cells. This phenomenon is called opsonization.

Lysozyme is an enzyme that is able to split mucopolysaccharides from bacterial cell walls, leading to the destruction of pathogens. Lysozyme is mainly produced by phagocytic cells. It is found in the bloodstream and is produced mainly in relation to the development of an infection. The destruction of bacterial cell walls by lysozyme facilitates their attack by the complement system.

Cytokines and Interferons

Cytokines are small proteins that regulate inflammation, immunity and hematopoiesis. They are produced by most of the cell types involved in the immune responses and secreted *de novo* in response to immune activation. Most of them act locally at low concentrations, usually through receptor binding and subsequent cell activation. Interferons are proteins produced during viral infections that increase the resistance of cells to viral invasion.

Other soluble factors

These include C-reactive protein, transferrin, lactoferrin, ceruloplasmin, lectins, natural agglutinins, cytokines, and interferons. They are involved in the innate defense mechanisms of fish. C-reactive protein (CRP) is an acute-phase protein found in the serum that increases rapidly upon exposure to bacterial pathogens. CRP reacts with molecules at the cell surface of microorganisms and acts as an opsonization factor to facilitate phagocytosis or activate the complement system. Transferrin has a protective role in fish. It is an iron-binding protein that limits the amount of free iron in the bloodstream, thus making it unavailable for bacteria during infection. Lactoferrin, produced by neutrophils, has a similar role to transferrin. Ceruloplasmin is an acute-phase protein in mammal inflammatory processes that is also described in fish. Lectins or natural agglutinins are important in neutralizing bacterial components released by pathogens, such as exotoxins or in immobilizing microorganisms; they therefore facilitate phagocytosis.

The Specific Immune Response

When an infectious agent penetrates the organism, innate defense mechanisms are stimulated. Their sole activation might be sufficient to stop the infection. If not, the disease will develop, leading to the induction of specific defense mechanisms. These will then lead to the cure of the disease and the establishment of an immunological memory, blocking the development of a new infection caused by the same pathogen.

One example of the development of specific immune response is vaccination. The pathogen is either introduced to the organism in an attenuated way or killed in order to avoid the outbreak of the disease, but still with the capacity to initiate a specific immune response. This will protect the organism for a certain period of time. The cells involved in the specific immune response leading to the production of antibodies are macrophages serving as antigen-presenting cells and lymphocytes composed of two populations: T- and B- lymphocytes. T-lymphocytes have a role in cellular cooperation (cell-mediated immunity) and B-lymphocytes are the antibody producers.

At the start of the antibody response, there is a certain delay before the first specific antibodies appear in the bloodstream. During this lag phase, the antigens

are processed and cellular cooperation between antigen-presenting cells and lymphocytes occurs. The macrophages act as antigen-presenting cells in the specific immune response. Their role is to process the antigen and to present the processed antigenic determinants in association with recognition molecules to the lymphocytes. Subsequently, T-lymphocytes are activated by interaction with the antigenic determinants and interleukins secreted by the macrophages. The activated T-lymphocytes, also called helper cells, stimulate the differentiation and proliferation of B-lymphocytes via the secretion of interleukins.

Depending on the circumstances, B-lymphocytes will develop into long-lived B-memory cells or short-lived plasmocytes. These plasmocytes secrete huge amounts of specific antibodies (immunoglobulins) of M type. These antibodies will bind or kill invading microorganisms presenting the corresponding determinants (Fig. 7.5).

The complement is involved in the specific immune response through its classical pathway of activation. The antibodies will stick to the membrane of the pathogen, and the activation of the complement system is required in order to process the destruction of the pathogen. This classical pathway of activation of the complement system requires that the antibody contact the membrane of the antigen in order to be initiated.

Factors Influencing the Immune Response

Many factors can influence the immune response of fish (Fig. 7.6). Among them are stressors and environmental factors of natural origin. Nutrients, micronutrients, and substances of no nutritional value can also modulate the immune response. Depending on type, amount, and duration of exposure, their effect can be either negative or positive. Substances with immunostimulating properties can compensate the immune depression caused by other factors, for example, the immune depression caused by a stressor

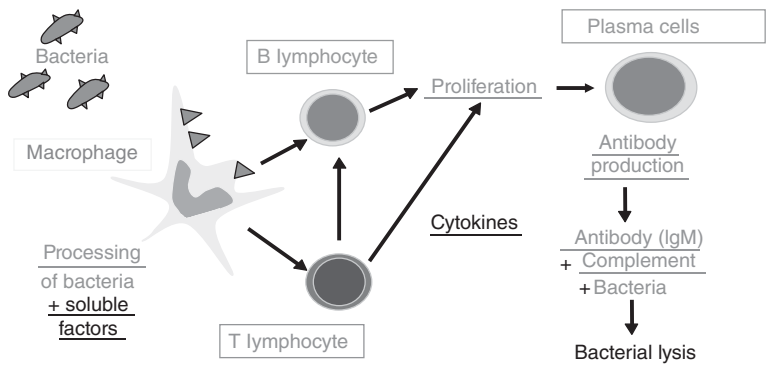

Figure 7.5 Specific immune response: cellular cooperation and antibody production. For color details, please see color plate section.

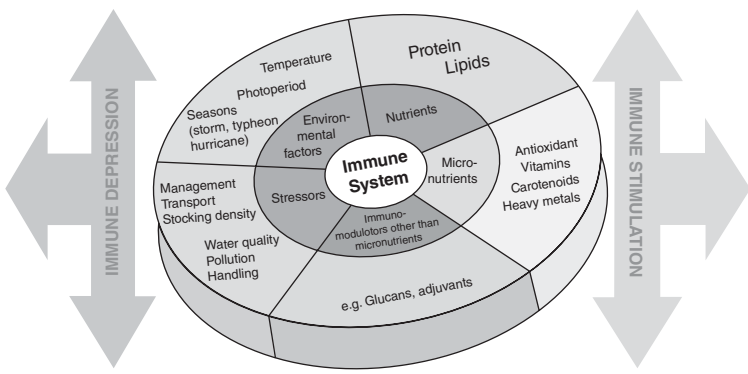

Figure 7.6 Factors influencing the immune response. For color details, please see color plate section.

can be compensated by an increased intake of vitamin C before a predictable stress event, such as grading.

Physiological processes of fish are influenced by temperature. Major defense mechanisms are temperature dependent and develop faster at the optimal temperature of the fish species concerned. Low temperatures are known to slow down all the metabolic processes, including the immune ones. However, high temperatures can also depress the immune functions. Indeed, it would appear in fish that antigen processing and cellular cooperation between macrophages and lymphocytes are sensitive to temperature in fish. The normal function of fish lymphocytes is highly dependent on homoviscous adaptation of membrane lipids. Fatty acid composition and environmental temperature are factors determining the fluidity and permeability of membranes, as well as the activity of membrane-associated receptors and enzymes.

Stress conditions influence the health status of fish. Immunodepression is known to be a major secondary effect in the response of an organism to stress. Many situations such as transport, crowding, handling, and poor water quality can cause a stress response in fish. The fish will react by secreting high levels of corticosteroids, which are known to be immunosuppressive. The stress response is accompanied by lymphocyte depletion in blood and in lymphoid organs. Pollutants (water quality and heavy metals) are also known to have detrimental effects on the immune response, causing various effects depending on the nature of the substance. Drugs such as antibiotics can also be immunosuppressive.

A well-balanced diet is essential for adequate host defense mechanisms as well as to optimize growth and the eating quality of fish for human consumption. Micronutrients such as antioxidant vitamins (vitamins C and E) have been demonstrated to have immunomodulatory properties when fed at elevated doses. The presence of carotenoids in the diet has also been demonstrated to improve the health status of fish and shrimp.

There are immune modulators other than micronutrients such as adjuvants, which are substances that when combined with antigens enhance fish-specific immune response in addition to innate defenses. Generally, adjuvants slow down the rate of antigen elimination, thereby prolonging antigen contact with macrophages and lymphocytes and augmenting the fish specific immune response. An example of a feed additive with limited nutritional value that is able to enhance immune response is β-1,3-1,6 glucan from yeast *Saccharomyces cerevisiae*. Nucleotides, as well as lactoferrin, have also shown immunomodulatory properties in fish.

Vitamin C and Health Status

Vitamin C as Nutritional Factor

With the exception of perhaps two or three species, vitamin C biosynthesis does not occur in fish due to the lack of the last enzyme of the biosynthetic pathway: L-gulonolactone oxidase. Vitamin C is an essential micronutrient and must therefore be supplied via the feed. Major signs of ascorbate deficiency include reduced growth, scoliosis, lordosis, internal and fin hemorrhage, distorted gill filaments, fin erosion, anorexia, and increased mortality (Fig. 7.7).

Because of its modes of actions, vitamin C is involved in several physiological functions including growth, development, reproduction, wound healing, response to stressors and possibly lipid metabolism through its action on carnitine synthesis. Vitamin C also plays a significant role in the immune response and resistance to infectious diseases of fish, probably through its antioxidant properties.

Vitamin C has no coenzyme functions, unlike other water-soluble vitamins, but acts as a cofactor in many reactions involving hydroxylating enzymes. Collagen synthesis (the hydroxylation of specific prolyl and lysyl residues of procollagen) is catalyzed by hydroxylases dependent upon ascorbic acid. Hydroxyproline residues contribute to the stiffness of the collagen triple helix and bind carbohydrates to form intramolecular cross-links which give the structural integrity of the collagen. These tissues will therefore be damaged if the formation of collagen is impaired by insufficient vitamin C levels in the body. Ascorbate deficiency also reduces complement activity in fish (the complement component C1q is rich in hydroxyproline and hydroxylysine).

A second function of vitamin C is catecholamine biosynthesis in fish. Stress response is primarily controlled by the endocrine system via cortisol and catecholamines, whose synthesis depends upon ascorbic-acid-dependent hydroxylases. Ascorbic acid requirement is increased in stressful situations. It can

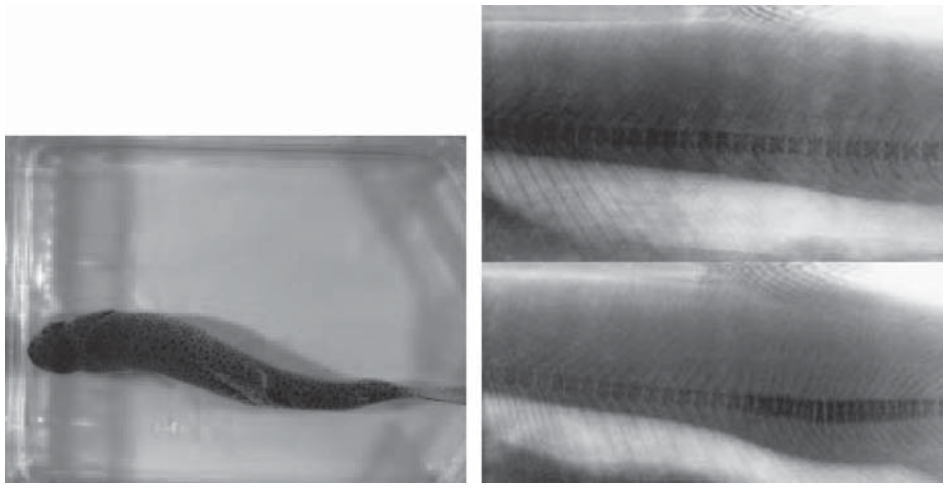

Figure 7.7 Left: vitamin-C-deficient rainbow trout showing broken back syndrome. Right: X-ray of a healthy fish (top) and a vitamin-C-deficient fish showing deformed vertebrae (bottom).

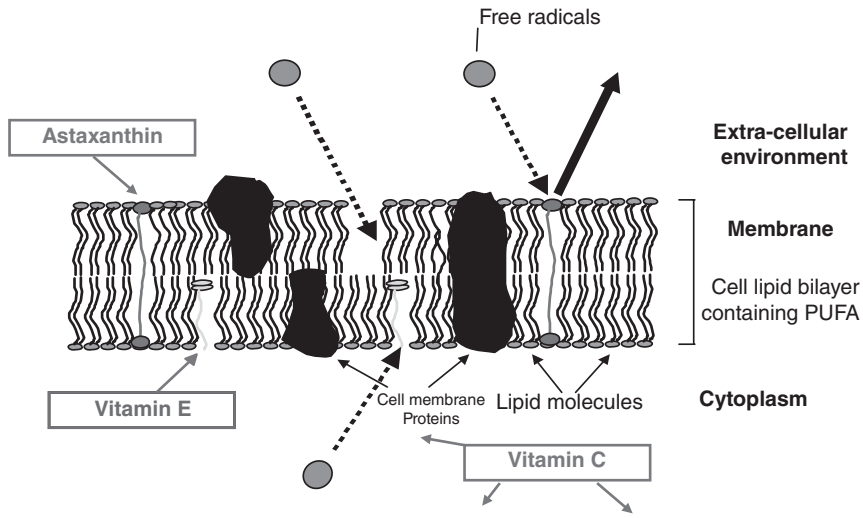

Figure 7.8 Antioxidant vitamins at cellular levels. For color details, please see color plate section.

compensate for the stress-induced down-regulation of the immune system.

Vitamin C is also involved in other physiological processes such as tyrosine metabolism (the active degradation of tyrosine is made via two oxidases that are vitamin C dependent. In turbot, vitamin C deficiency causes hypertyrosinemia and the excretion of tyrosine metabolites and metal ion metabolism. Vitamin C interacts with several metallic elements of nutritional significance such as selenium and reduces the toxicity of metals such as cadmium, nickel, lead (the elements are transformed into their reduced forms, which are less absorbed and excreted more rapidly).

Vitamin C is involved in the protection of cells from oxidative damage and the regeneration of vitamin E in its metabolically active form. Figure 7.8 illustrates the localization of vitamin C and vitamin E at the cellular level and therefore their potential as antioxidants when combined.

Vitamin C affects immune functions in protecting cells from auto-oxidation, especially in the case of the initiation of the oxidative burst of macrophages.

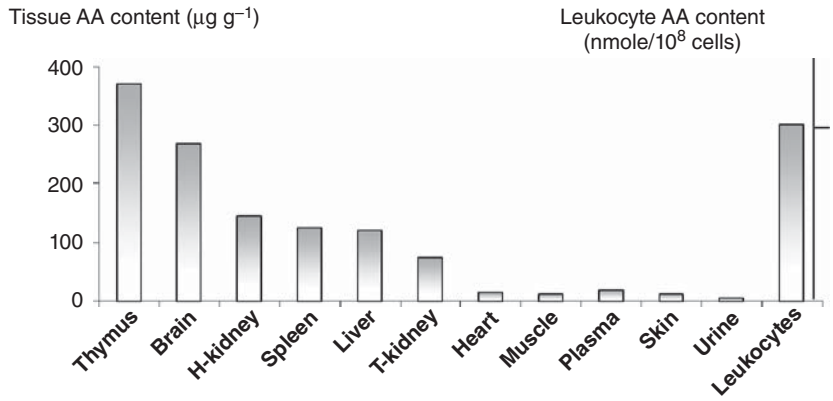

Figure 7.9 Ascorbic acid (AA) concentration in various tissues of rainbow trout fed a diet supplemented with 200 mg ascorbic acid (AA) per kilogram feed (Gabaudan and Verlhac, unpub. data, 1992). For color details, please see color plate section.

Indeed, vitamin C helps to maintain the integrity of the immune cells through their protection from oxidation and within the cells (high amount of vitamin C stored in the immune cells).

Species that cannot synthesize vitamin C instead absorb ascorbic acid by an active transport mechanism which is Na^+-dependent. This active uptake of vitamin C seems to be very important at low doses; at high doses, uptake by passive diffusion also occurs. The uptake of vitamin C in cells such as lymphocytes, neutrophils, and leucocytes involves dehydroascorbic acid because ascorbic acid cannot cross their membrane. Once dehydroascorbic acid is taken up by the cells, it is rapidly reduced to ascorbic acid by an intracellular dehydroascorbic acid reductase.

Vitamin C is concentrated in many vital organs with active metabolism. The tissue concentration of vitamin C is related to its dietary intake. Moreover, in some tissues such as brain, thymus, and leukocytes, ascorbic acid accumulates at high concentrations and seems to be retained longer in the case of dietary vitamin C depletion, compared to storage organs such as liver. An example of tissue distribution of vitamin C is given in Figure 7.9, where rainbow trout were fed vitamin C as ascorbate phosphate at the dose of 200 mg ascorbic acid equivalents per kilogram of feed. The very high levels found in thymus, brain, and leukocytes confirm the hypothesis of the importance of ascorbic acid in preserving vital tissues from oxidation processes. Liver and head kidney are important storage organs for vitamin C in fish. The high level found in the head kidney is likely to be related to the presence of lymphopoietic tissues. Trunk kidney and spleen are also able to store a large amount of vitamin C. Trunk kidney is the site of chromaffin cells that are responsible for catecholamine biosynthesis. Ascorbic acid is concentrated at the site of catecholamin formation and is released with newly synthesized corticosteroids in response to stressors. There is a relationship between the ascorbic acid concentration (nmoles/10^8 cells) in leukocytes and the dietary intake of vitamin C (Fig. 7.10).

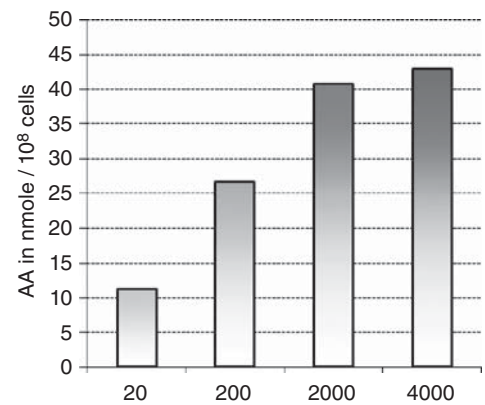

Figure 7.10 Influence of dietary intake of vitamin C on leucocyte ascorbic acid (AA) concentration in rainbow trout fed graded doses of vitamin C as ascorbate phosphate for four weeks (Verlhac, unpub. data). For color details, please see color plate section.

Vitamin C and Immune Response

Tables 7.2–7.14 summarize the results of the studies related to the evaluation of the effect of vitamin C on the immune response in fish. The order of the tables follows the organization of the chapter related to the description of the fish immune response. When a positive effect has been demonstrated, the related study is formatted in bold. Treatments without vitamin C supplementation are normally not quoted in the tables. Where indicated (in the 'Reference column'), results from Tables 7.7 and 7.10 are depicted by Figures 7.11–7.13.

Vitamin C and Resistance to Disease

Tables 7.15 and 7.16 summarize the results of the studies related to the evaluation of the effect of vitamin C

Table 7.2 Vitamin C and innate immunity: wound healing. Notes relevant to Tables 7.2–7.15: When a positive effect has been demonstrated, the related study is formatted in bold. The doses at which the effect has been observed are formatted in bold.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**24**	50, 100, 200**, 400, 1000**	**Halver (1972)**
Rainbow trout	**4**	20, **150, 1000**	**Wahli et al. (2003)**
Coho salmon	**24**	50, 100, 200**, 400, 1000**	**Halver (1972)**
Channel catfish	**16**	30**, 60**	**Lim and Lovell (1978)**
African catfish	2	600, 1000, 3000, 7000	Erazo-Pagador and Din (2001)
Sea bream	?	0, 3200; >**600**	**Alexis et al. (1997)**

Table 7.3 Vitamin C and cellular mechanisms of innate immunity: phagocytosis.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**12**	120**, 1200**	**Blazer (1982)**
Rainbow trout	**10**	200**, 1000**	**Verlhac et al. (1993)**
Rainbow trout	9	0, MRL for A, C and E	Amar et al. (2001)
Rainbow trout	**4**	150, **1000**	**Verlhac et al. (unpub. data)**
Turbot	10	400, 800, 1200	Roberts et al. (1995)
Turbot	**18**	**400, 800,** 1200	**Roberts et al. (1995)**
Yellow croaker	**8**	23.8, **489**	**Ai et al. (2006)**
Atlantic salmon	26	50, 310, 2750	Hardie et al. (1991)
Bagrid catfish	**9**	10**, 100**	**Anbarasu and Chandran (2001)**
Channel catfish	20	30, 60, 150, 300, 3000	Li and Lovell (1985)
Sea bream	**2, 4**, 6, 10	500, **3000**	**Ortuño et al. (1999)**
Sea bream	*In vitro*	**1,** 10, 100 μM	**Mulero et al. (1998)**
Sea bream	2, **4**, 6	100, 3000, **3000 (+ 1200 vit. E)**	**Ortuño et al. (2001)**
Rohu	**9**	0, 500, **1000, 1500**	**Tewary and Patra (2008)**
Rohu	**2**	100, 200, **500**	**Misra et al. (2007)**
Rohu	**4, 8**	100, **200**, 500	**Misra et al. (2007)**
Rohu	**6**	100, 200, 500	**Misra et al. (2007)**
Asian catfish	**4**	0, **500**	**Kumari and Sahoo (2006)**

Table 7.4 Vitamin C and cellular mechanisms of innate immunity: pinocytosis.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**2**	150, **1000**	**Verlhac et al. (1998)**
Rainbow trout	**4**	150, **1000**	**Verlhac et al. (unpub. data)**

Table 7.5 Vitamin C and cellular mechanisms of innate immunity: chemotaxis, adherence, migration.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Channel catfish	**14**	0, 50, **3000** (+0/300 ppm iron)	**Lim et al. (2000)**
Bagrid catfish	9	10, **100**	**Anbarasu and Chandran (2001)**
Sea bream	2, 4, 6, 8, 10	500, 3000	Ortuño et al. (1999)
Sea bream	*in vitro*	**1, 10, 100 μM**	**Mulero et al. (1998)**

Table 7.6 Vitamin C and cellular mechanisms of innate immunity: killing, oxidative burst.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**2**	150, **1000**	**Verlhac et al. (1998)**
Rainbow trout	**8**	60, **1000**	**Verlhac and Gabaudan (1994)**
Rainbow trout	**9**	0, **MRL, for A, C and E**	**Amar et al. (2001)**
Rainbow trout	16	30, 1000	Verlhac & Gabaudan (1994)
Rainbow trout	20	60, 1000	Verlhac & Gabaudan (1994)
Rainbow trout	**32**	30**, 2000 (+ vit. E)**	**Wahli et al. (1998)**
Atlantic salmon	8	60, 1000	Verlhac and Gabaudan (1994)
Atlantic salmon	26	50, 310, 2750	Hardie et al. (1991)
Bagrid catfish	**9**	10, **100**	**Anbarasu and Chandran (2001)**
Hybrid striped bass	*In vitro*	**Increase**	**Sealey and Gatlin (2002a)**
Hybrid striped bass	10	25, 2500	Sealey and Gatlin (2002b)
Rohu	**9**	0, 500, **1000**, **1500**	**Tewary and Patra (2008)**
Rohu	**2**	100, 200**, 500**	**Misra et al. (2007)**
Rohu	**4, 6, 8**	**100, 200, 500**	**Misra et al. (2007)**
Yellow croaker	**8**	23.8, **489**	**Ai et al. (2006)**
Sturgeon	**2**	0, **500**	**Xie et al. (2006)**
Tilapia	**4**,8	0, **500**	**Ibrahem et al. (2010)**
Asian catfish	**4**	0, **500**	**Kumari and Sahoo (2006)**

Table 7.7 Vitamin C and cellular mechanisms of innate immunity: killing, oxidative burst (H_2O_2, O_2, OH production).

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**2**	150, **1000**	**Verlhac et al. (1998)**
Rainbow trout	**2**	150, 1000, **4000**	**Verlhac et al. (1996)**
Rainbow trout	**3**	20, 200, **2000, 4000** (+ glucan)	**Verlhac et al. (1996)** See Figure 7.12
Rainbow trout	**4**	60, **2000**	**Dunier et al. (1995)**
Rainbow trout	**8**	60, **1000**	**Verlhac and Gabaudan (1994)**
Rainbow trout	16	30, 1000	Verlhac and Gabaudan (1994)
Rainbow trout	20	60, 1000	Verlhac and Gabaudan (1994)
Rainbow trout	**32**	30, **2000**	**Wahli et al. (1998)**
Atlantic salmon	**3**	150, 1000 (+ lactoferrin)	**Lygren et al. (1999a)**
Atlantic salmon	**8**	60, **1000**	**Verlhac and Gabaudan (1994)**
Sea bream	2, 4, 6, **8**, 10	500, **3000**	**Ortuño et al. (1999)**
Sea bream	*In vitro*	1, 10, 100 μM	Mulero et al. (1998)
Sea bream	*In vitro*	**1**, 10, 100 μM (+ vit. E)	**Mulero et al. (1998)**
Sea bream	2, **4**, 6	100, **3000, 3000 (+ vit. E)**	**Ortuño et al. (2001)**
Hybrid striped bass	*In vitro*	No effect	Sealey and Gatlin (2002a)
Hybrid striped bass	10	25, 2500 (+ vit. E)	Sealey and Gatlin (2002b)
Grouper	8	0, 3, 14, 27, 46, 76, 135, **288**	**Lin and Shiau (2005a)**
Trout	*In vitro*	0, **50** μg mL^{-1}	**Verlhac et al. (unpub. data)** See Figure 7.11

Table 7.8 Vitamin C and cellular mechanisms of innate immunity: natural cytotoxicity.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**9**	0–**MRL for A, C, and E**	**Amar et al. (2001)**
Sea bream	*In vitro*	**0–2 mg/mL**	**Cuesta et al. (2002)**
Sea bream	2, 4, **6**	100, 3000, **3000 (+ vit. E)**	**Cuesta et al. (2002)**

Table 7.9 Vitamin C and cellular mechanisms of innate immunity: soluble factor complement alternative pathway.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**2**	150, **1000, 4000**	**Verlhac et al. (1996)**
Rainbow trout	2	150, 1000	Verlhac et al. (1998)
Rainbow trout	4	150, 1000	Verlhac et al. (unpub. data)
Rainbow trout	**9**	150, **MRL for A, C and E**	**Amar et al. (2001)**
Sea bream	2, **4, 6**, 8, 10	500, **3000**	**Ortuño et al. (1999)**
Sea bream	2, 4, **6**	100, 3000, **3000 (+ vit. E)**	**Ortuño et al. (2001)**
Sea bream	**2, 4, 6**	0, **3000**	**Ortuño et al. (2003)**
Gold shiner	**10, 16**	0, **219**	**Chen et al. (2003)**
Gold shiner	**14, 19**	23, 43, **98, 222**	**Chen et al. (2004)**
Grouper	8	7, 18, 31, 51, **93, 145**	**Lin and Shiau (2004)**
Grouper	8	0, 3, 14, 27, 46, 76, 135**, 288**	**Lin and Shiau (2005a)**
Grouper	8	4, 9, **15, 31, 49, 75**	**Lin and Shiau (2005b)**
Rohu	2, 4, 6, 8	0, 100, 200, 500	Misra et al. (2007)
Sea bass	**2 (every 3 months)**	100, **500 (+ vit. E and glucan)**	**Bagni et al. (2000)**
Yellow croaker	**8**	0, 23.8**, 489**	**Ai et al. (2006)**
Sea bass	**8**	0, graded 12.2–188.5, **489**	**Ai et al. (2004)**

Table 7.10 Vitamin C and cellular mechanisms of innate immunity: soluble factor lysozyme.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	**2**	150, 1000	**Verlhac et al. (1998)**
Rainbow trout	2	150, 1000, 4000	Verlhac et al. (1996)
Rainbow trout	3	20, 200, 2000, 4000	Verlhac et al. (1995)
Rainbow trout	**9**	0, MRL for A, C and E	**Amar et al. (2001)**
Atlantic salmon	3	150, 1000 (+ vit. E)	Lygren et al. (1999b)
Atlantic salmon	**36**	40, 400, 2000, 4000	**Waagbø et al. (1993)** See Figure 7.13
Turbot	**10**	400, 800, 1200	**Roberts et al. (1995)**
Turbot	18	400, 800, 1200	Roberts et al. (1995)
Sea bream	**2, 4**, 6	100, 3000, **3000 (+ vit. E)**	**Cuesta et al. (2002)**
Hybrid striped bass	10	25, 2500 (+ vit. E)	Sealey and Gatlin (2002b)
Grouper	8	7, 18, 31, **51, 93, 145**	**Lin and Shiau (2004)**
Grouper	8	0, 3, 14, 27, 46, 76, 135**, 288**	Lin and Shiau (2005a)
Grouper	8	4, 9, **15, 31, 49, 75**	Lin and Shiau (2005b)
Rohu	2, **4, 6, 8**	0, **100, 200, 500**	**Misra et al. (2007)**
Sea bass	**2** (every 3 months)	100, **500 (+ vit. E and glucan)**	**Bagni et al. (2000)**
Tilapia	**12**	**0, 100,** 2000	**Lim et al. (2010)**
Tilapia	**4, 8**	0**, 500**	**Ibrahem et al. (2010)**
Japanese eel	**3**	32**, 762** (with lactoferrin)	**Ren et al. (2007)**
Sturgeon	**2**	0, **500**	**Xie et al. (2006)**
Tilapia	**16**	30, **300, 1000**, 3000, 5000	**Hung et al. (2007)**
Yellow croaker	**8**	0, 23.8**, 489**	**Ai et al. (2006)**
Sea bass	**8**	0, graded 12.2–188.5, **489**	**Ai et al. (2004)**
Red sea bream	3	0, 107, 325	Ren et al. (2008)

Table 7.11 Vitamin C and cellular mechanisms of innate immunity: soluble factor cytokines (macrophage activating factor).

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rohu	**2, 4**	100, 200, **500**	**Misra et al. (2007)**
Rohu	**6, 8**	**100, 200**, 500	**Misra et al. (2007)**

Table 7.12 Vitamin C and cellular mechanisms of specific immunity: lymphocyte proliferation.

Species	Feeding (weeks)	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Rainbow trout	2	150, 1000	Verlhac et al. (1998)
Rainbow trout	**3**	20, 200, **2000, 4000**	**Verlhac et al. (1995)**
Rainbow trout	**10**	200, **1000**	**Verlhac et al. (1993)**
Rainbow trout	**16**	30**, 1000**	**Verlhac and Gabaudan (1994)**
Rainbow trout	**20**	60, **1000**	**Verlhac and Gabaudan (1994)**
Rainbow trout	**32**	30, **2000** (+ vit. E)	**Wahli et al. (1998)**
Rainbow trout	**In vitro**	**Increase**	**Hardie et al. (1993)**
Rainbow trout	**Parenteral**	**Increase**	**Hardie et al. (1993)**
Atlantic salmon	**8**	60, **1000**	**Verlhac and Gabaudan (1994)**
Rainbow trout	2	150, 1000	Verlhac et al. (1998)
Rainbow trout	**3**	20, 200, 2000, **4000**	**Verlhac et al. (1995)**
Atlantic salmon	8	60, 1000	Verlhac and Gabaudan (1994)
Rainbow trout	**In vitro**	**Increase**	**Hardie et al. (1993)**

Table 7.13 Vitamin C and cellular mechanisms of specific immunity: antibody response.

Antibodies	Sampling time after vaccination	Species	Feeding (weeks) before/after vaccination	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Total Ig		Tilapia	**12**	0, 100, **2000 (+ vit. E)**	**Lim et al. (2010)**
Y. *ruckeri*	Kinetics	Trout	2	150, 1000, 4000	Verlhac et al. (1996)
	8 weeks	Trout	**2**	150**, 1000**	**Verlhac et al. (1998)**
	Kinetics	Trout	2	150, 1000	Verlhac et al. (1998)
	Kinetics	Trout	3/end	20, 200, 2000, 4000	Verlhac et al. (1995)
	5,7 weeks	Trout	**4/end**	60, **2000**	**Dunier et al. (1995)**
Vibrio	**11,17 weeks**	Trout	**10/end**	40, 400, 2000, **4000**	**Waagbø et al. (1993)**
L. Anguillarum	4 weeks	A. salmon	12/end	50 to 2000 (+ vit. E)	Lall et al. (1989)
	Kinetics	Trout	**28/end**	100, **500, 1000, 2000**	**Navarre and Halver (1989)**
V. vulnificus	5 weeks	Grouper	20/end	0, **500, 1000, 1500, 2000**	**Qin et al. (2000)**
A. salmonicida	4 weeks	Trout	12/end	50 to 2000 (+ vit. E)	Lall et al. (1989)
E. ictaluri	**3 weeks**	C. catfish	**9/end**	30, 60, 150, 300**, 3000**	**Li and Lovell (1985)**
	13 weeks	C. catfish	9/end	100, 500, 1000, 4000	Liu et al. (1989)
E. tarda	4	Rohu	4/4	0, 500	Sahoo and Mukherjee (2002)
E. tarda	4	Rohu	4/4	0, **500**	**Sahoo et al. (1999)**
		Hybrid s. bass	10	25, 2500 (+ vit. E)	Sealey and Gatlin (2002b)
IHN virus	3 weeks	Trout	6/end	20, 80, 320	Anggawati-Satyabudhy et al. (1989)

Table 7.14 Complement (classical pathway of activation).

Species	Feeding (weeks)	Vitamin C doses	Reference
Rainbow trout (vaccinated)	**2**	150, **1000**	**Verlhac et al. (1998)**
Rainbow trout	2	150, 1000, 4000 (+ glucan)	Verlhac et al. (1996)
Rainbow trout	3	20, 200, 2000, 4000	Verlhac et al. (1995)
Rainbow trout	8	60, 1000	Verlhac and Gabaudan (1994)
Rainbow trout	**10**	200, **1000**	**Verlhac et al. (1993)**
Rainbow trout	20	60, 1000	Verlhac and Gabaudan (1994)
Rainbow trout	32	30, 2000 (+ vit. E)	Wahli et al. (1998)
Atlantic salmon	3	150, 1000 (+ lactoferrin)	Lygren et al. (1999a)
Atlantic salmon	8	60, 1000	Verlhac and Gabaudan (1994)
Atlantic salmon (after infection)	20	50 to 2000 (+ vit. E 350)	Lall et al. (1989)
Atlantic salmon	**26**	50, 310, **2750**	**Hardie et al. (1991)**
A salmon (before infection)	27	40, 400, 2000, 4000	Waagbø et al. (1993)
A salmon (surviving infection)	36	40, 400, 2000, 4000	Waagbø et al. (1993)
Channel catfish	9	100, 500, 1000, 4000	Liu et al. (1989)
Channel catfish	**20**	30, 60, 150, 300, **3000**	**Li and Lovell (1985)**

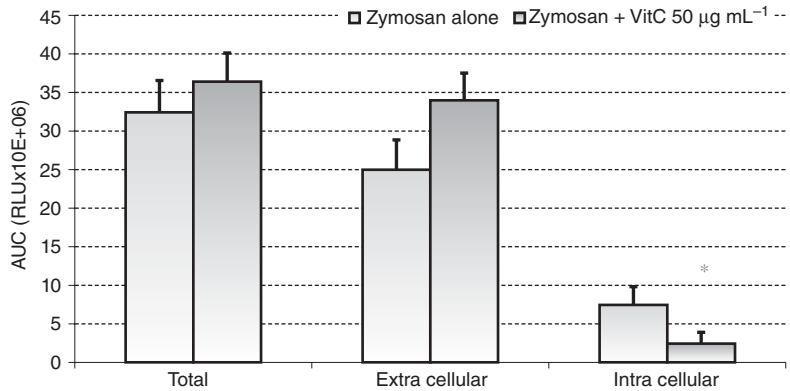

Figure 7.11 *In vitro* influence of ascorbic acid on total, extra- and intracellular oxidative burst of from rainbow trout phagocytes stimulated by zymosan particles (Verlhac et al., unpub. data). For color details, please see color plate section.

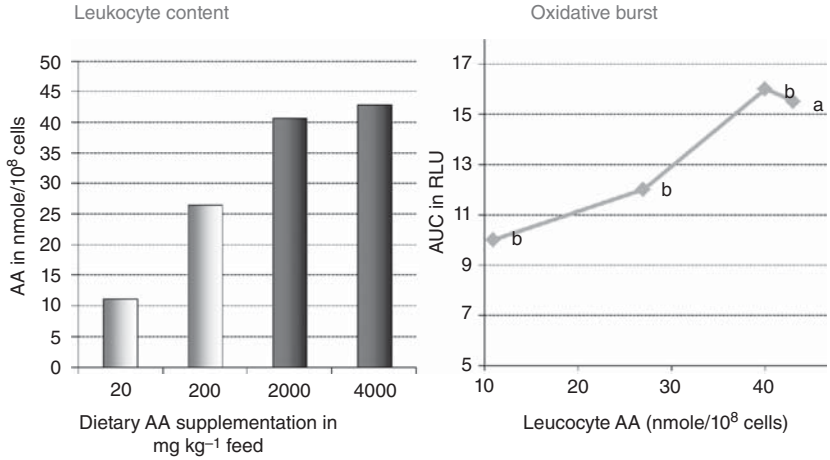

Figure 7.12 *In vivo* influence of vitamin C on total oxidative burst from rainbow trout phagocytes stimulated by zymosan particles, after 4 weeks of feeding an elevated dose of vitamin C as ascorbate phosphate (Verlhac et al., unpub. data).

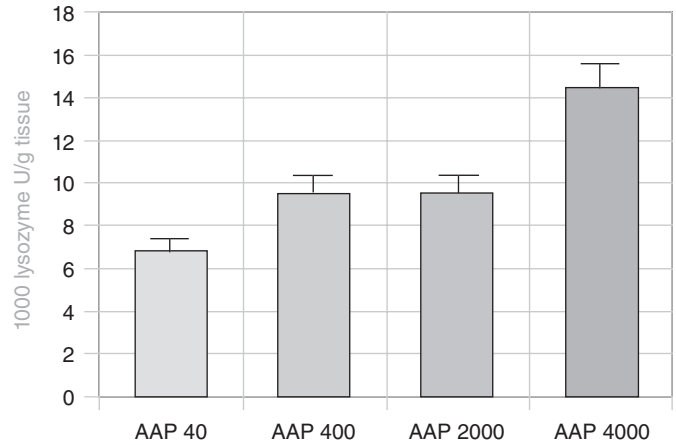

Figure 7.13 *In vivo* influence of vitamin C on head kidney lysozyme activity in Atlantic salmon fed elevated doses of vitamin C as ascorbate phosphate for 36 weeks R. WaagbØ, J. Glette, E. Raa-Nilsen and K. Sandnes. Dietary vitamin C, immunity and disease resistance in Atlantic salmon (Salmo salar). Fish Physiology and Biochemistry. 12(1): 61–73. Copyright © 1993, Springer Business+Science Media.

Table 7.15 Vitamin C and disease resistance in non-vaccinated fish. (IP: intra-peritoneal, ERD: enteric redmouth disease; VHS: Viral hemorrhagic septicemia; Ich: ichthyophthiriosis; IHN: Infectious hepatic necrosis; Fur: Furunculosis; Cohab: cohabitant; Infectious salmon anemia: ISA; Enteric septicemia: ES; Hemorrhagic septicemia: HS)

Disease	Mode of infection	Species	Feeding (weeks) before/after infection	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Vibriosis	Ip	Trout	**28/end**	100, 500, **1000, 2000**	**Navarre and Halver (1989)** See Figure 7.14
	Bath	Trout	**28/end**	100**, 500, 1000, 2000**	**Navarre and Halver (1989)**
ERD	Bath	Trout	**32**	30, **2000 (**+ **vit. E)**	**Wahli et al. (1998)**
VHS	Bath	Trout	32	30, 2000 (+ vit. E)	Wahli et al. (1998) See Figure 7.15
ICH	Bath	Trout	**8**	0, 50**, 2000** (+ vit. E)	**Wahli et al. (1995)**
		Trout	**32**	30, **2000 (**+ **vit. E)**	**Wahli et al. (1998)**
Infectious hepatic necrosis	Bath	Trout	**6/end**	20, **80, 320**	**Anggawati-Satyabudhy et al. (1989)**
Vibriosis	IP/bath	A. salmon	22	50 to 2000 (vit. E 350)	Lall et al. (1989)
Fur	IP/bath	A. salmon	22	50 to 2000 (vit. E 350)	Lall et al. (1989)
	Bath	A. salmon	26	50, 310, 2750	Hardie et al. (1991)
	Cohab +IP	A. salmon	**27/end**	40, 400, 2000, **4000**	**Waagbø et al. (1993)**
	Cohab	A. salmon	3 + 1	150, 1000 (+ lactoferrin)	Lygren et al. (1999a)

(*continued*)

Table 7.15 (*Continued*)

Disease	Mode of infection	Species	Feeding (weeks) before/after infection	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
ISA	IP	A. salmon	3 + 1	150, 1000 (+ lactoferrin)	Lygren et al. (1999a)
ES	Bath	Channel catfish	8/end	25, 50, 100, 1000, 2000	Li et al. (1993)
	Bath	Channel catfish	8/end	100, 250, 500, 1000, 2000	Li et al. (1993)
	Bath	Channel catfish	**13/end**	30, 60, 150, 300, **3000**	**Li and Lovell (1985)**
	Bath	Channel catfish	**14/end**	60**, 150**	**Durve and Lovell (1982)**
	Bath	Channel catfish	**14**	0, 50, **3000 (+ lactoferrin)**	**Lim et al. (2000)**
HS	IP	Bagrid catfish	**9**	10**, 100**	**Anbarasu and Chandran (2001)**
	IP	Tilapia	–	458 (pond conditions)	Nitzan et al. (1996)
E. tarda	IP	Rohu	**4/end**	0, **500**	**Sahoo and Mukherjee (2002)**
E. tarda	IP	Carp	**4**	0, 250, **400, 250 (+ vit. E)**	**Nair and Prasad (1999)**
E. tarda	IP	Rohu	8/4	0, 100, 200, 500	Misra et al. (2007)
S. iniae	–	Hybrid striped bass	10	25, **2500** (with vit. E)	**Sealey and Gatlin (2002b)**
V. carchariae	–	Grouper	8	0, 3, **14, 27, 46, 76, 135, 288**	**Lin and Shiau (2005a)**
A. hydrophila	Bath	Rohu	**9/end**	0, **500, 1000**, **1500**	**Tewary and Patra (2008)**
A. hydrophila	IP	Tilapia	**4/end**	0, **500**	**Ibrahem et al. (2010)**
S. agalactica	Bath	Tilapia (larvae)	**3/1**	0, 1000, **2000, 3000**	**Areechon et al. (unpub. data)**
S. agalactica	Bath	Tilapia (larvae)	**3/2**	0, **1000, 2000, 3000**	**Areechon et al. (unpub. data)**
S. agalactica	Bath	Tilapia (larvae)	**3/3**	0, **1000, 2000, 3000**	**Areechon et al. (unpub. data)**
V. vulnificus	IP/bath	Grouper	**20/end**	0, 500, **1000, 1500, 2000**	**Qin et al. (2000)**
V. harveyi	IP	Yellow croaker	**8**	23.8, **489**	**Ai et al. (2006)**

Table 7.16 Vitamin C and disease resistance in vaccinated fish.

Disease	Mode of infection	Species	Feeding (weeks) before/after infection	Vitamin C doses (mg vitamin C kg^{-1} of feed)	Reference
Infectious hepatic necrosis	Bath	Trout	**6/end**	20, **80, 320**	**Anggawati-Sayabudhy et al. (1989)**
Enteric Septicemia	Bath	Channel catfish	13/end	30, 60, 150, 300, 3000 (100% vaccine efficiency)	Li and Lovell (1985)
	IP	Channel catfish	**13/end**	100, 500, 1000, 4000	**Liu et al. (1989)**
Hemorrhagic septicemia	IP	Bagrid catfish	**9**	10**, 100**	**Anbarasu and Chandran (2001)**
E. tarda	IP	Rohu	4/end	0, 500	Sahoo and Mukherjee (2002)
V. vulnificus	IP	milkfish	6/end	90, 500, 1500	Azad et al. (2007)
V. vulnificus	IP	Grouper	**20/end**	0, **500, 1000, 1500, 2000**	**Azad et al. (2007)**

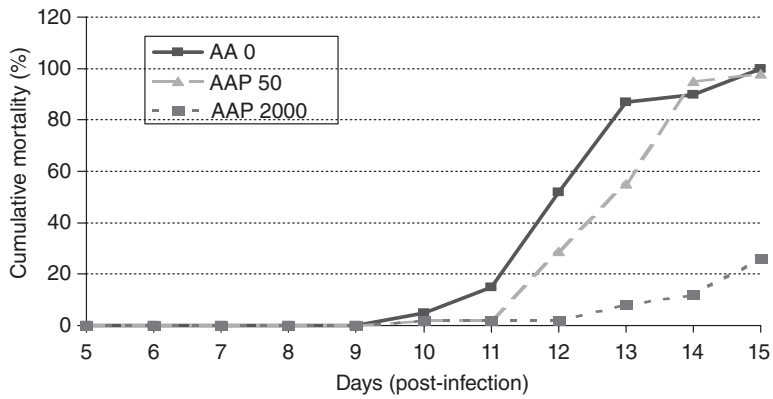

Figure 7.14 Resistance of non-vaccinated rainbow trout to Ichthyophthiriosis infection in relation to dietary vitamin C. Source: T. Wahli, V. Verlhac, J. Gabaudan, W. Schüep and W. Meier 1998. Influence of combined vitamins C and E on non-specific immunity and disease resistance of rainbow trout (Oncorhynchus mykiss). Journal of Fish Diseases 21: 127–137.

on disease resistance of non-vaccinated and vaccinated fish, respectively. As above, bold formatting is used to indicate a positive effect. Where indicated (in the 'Reference column'), results from Table 7.15 are depicted by Figures 7.14 and 7.15.

Conclusion

Vitamin C is one of the strongest antioxidant defence systems in fish. It acts against intracellular and extracellular reactive oxygen species. Phagocytes, major actors of the innate immune response of fish, contain a high concentration of vitamin C in their cytoplasm; this represents strong protection against the huge production of reactive oxygen species in fighting against pathogens. Vitamin C does not act alone: it collaborates with other antioxidants (vitamin E, antioxidant enzymes) to further strengthen the body antioxidant defense system.

Based on the combination of experimental results obtained in several controlled studies and field

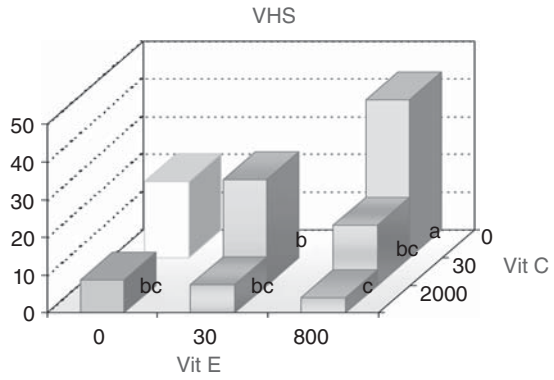

Figure 7.15 Influence of dietary combinations of vitamins C and E on resistance of rainbow trout to VHS virus infection. Source: T. Wahli, V. Verlhac, J. Gabaudan, W. Meier and W. Schüep 1997. Influence of combined vitamins C and E on immunity and disease resistance of rainbow trout (Oncorhynchus mykiss). Journal of Fish Diseases 21:127137. For colour details, please see colour plate section.

experience, the beneficial effect of a dietary supplementation of vitamin C above the recommended level for optimal growth has been demonstrated whenever the immune system is challenged (stressful situations, e.g., handling and grading, vaccination, winter wounds, disease outbreak, and sea transfer) and also after reduced intake during winter.

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Chapter 8

Vitamin E

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Introduction

Vitamin E is a group fat-soluble factor required for normal growth, reproduction, and maintainenance of normal physiological functions and health of vertebrates (Evans and Bishop 1922). Deficiency of this vitamin produces a wide variety of pathological conditions depending on animal species (Wasserman and Taylor 1972). The most characteristic lesion is a chronic necrotizing myopathy known as muscular dystrophy; another pathological sign of deficiency includes ceroid deposition in several tissues (Blanc et al. 1958; Machlin 1980; Lovell et al. 1984; Gatlin et al. 1986; Frischknecht et al. 1994; Bowater and Burren 2007; Betancor et al. 2011). In general, vitamin E is recognized as the most significant fat-soluble secondary antioxidant (Burton et al. 1983; Sargent et al. 1997). It is essential for maintaining the integrity of immune responses (Waagbø 1994; Kiron 2012), the normal resistance of red blood corpuscles to hemolysis, and the permeability of capillaries and heart muscle (Halver 2002). In mammals, vitamin E also plays an important physiological role in preserving normal neurological structure and function (Mangialasche et al. 2010).

Numerous studies have demonstrated that vitamin E is a dietary essential for different fish species and has a marked effect on fish physiology (Watanabe et al. 1970; Murai and Andrews 1974; González et al. 1995; Peng et al. 2009; Betancor et al. 2011, 2012a, b, 2013a, b; Atalah et al. 2012). Various reports describe how the predominant role of vitamin E in antioxidant defense mechanisms is improving the stability of tissue lipids to oxidation in many fish species such as rainbow trout, *Oncorhynchus mykiss* (Frigg et al. 1990); Atlantic salmon, *Salmo salar* (Waagbø et al. 1993); turbot, *Psetta maxima* (Stéphan et al. 1995); European sea bass, *Dicentrarchus labrax* (Messager et al. 1992; Betancor et al. 2011); and gilthead sea bream, *Sparus aurata* (Atalah et al. 2012). Dietary vitamin E is also essential for fish reproduction, improving egg viability, and reducing the occurrence of malformations (Fernández-Palacios et al. 1998). In fish, as in other animals, vitamin E also affects disease resistance and health-modulating immune responses (Waagbø 1994, 2006; Verlach Trichet 2010), including the boosting of the non-specific immune system (Montero et al. 1998), and prevents erythrocyte fragility (Halver 1995). Moreover, dietary vitamin E has a synergistic effect with n-3 highly unsaturated fatty acids (HUFA) and vitamin C, therby enhancing the non-specific immune responses and disease resistance (Wang et al. 2006).

Vitamin E is particularly vital under situations of stress or when fish are fed rancid feeds. For instance, under chronic and acute stress conditions, juvenile

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gilthead sea bream fed a vitamin-E-deficient diet not only had reduced growth and survival, but also lower stress resistance (Montero et al. 2001). Similarly, supplementing vitamin E to diets containing oxidized oil improves growth and reduces the lipid peroxidation products in gilthead sea bream and turbot (Tocher et al. 2003).

Molecular Forms and Bioavailability

Vitamin E is a generic term that includes ten homologous derivatives of a 6-chromanol ring with a phytyl side chain: α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, α-tocotrienol, β-tocotrienol, γ-tocotrienol, δ-tocotrienol, α-tocomonoenol, and the tocomonoenol named MDT (marine-derived tocopherol; Fig. 8.1). Tocopherols have a saturated side chain, tocotrienols have an unsaturated side chain with three double bonds, and tocomonoenols have only one double bond in the phytyl side chain. The tocopherol and tocotrienol homologs are named α, β, γ, and δ depending on the position and number of the methyl substitutions on the aromatic side of the chromanol ring. MDT was originally found in salmon roe, but is also present in a broad range of marine organisms (Yamamoto et al. 1999). Despite all the homologs being able to inhibit lipid oxidation *in vitro*, α-tocopherol has the highest *in vivo* antioxidant activity in vertebrates (Traber and Kayden 1989). This predominance as a fat-soluble antioxidant in biological systems could be related to the selective uptake and transport of α-tocopherol, suggesting the evolutionary selection of a molecule with unique functions not shared by other antioxidants (Azzi 2004).

Since these types of compounds are rapidly oxidized in the presence of peroxides or other oxidizing agents (Dam and Sondergaard 1964) and are sensitive to ultraviolet light, they are included in animal diets as the corresponding derived ester forms, which are more stable. The most common dietary form of vitamin E used in fish feeds is α-tocopheryl acetate, which is hydrolyzed in the digestive tract and absorbed in association with fat molecules. As has been proposed in mammals, vitamin E absorption could be protein mediated through the transmembrane protein SR-BI, which expresses its gene in the enterocyte (Rigotti 2007). The transport of vitamin E to the liver requires the synthesis of chylomicrons

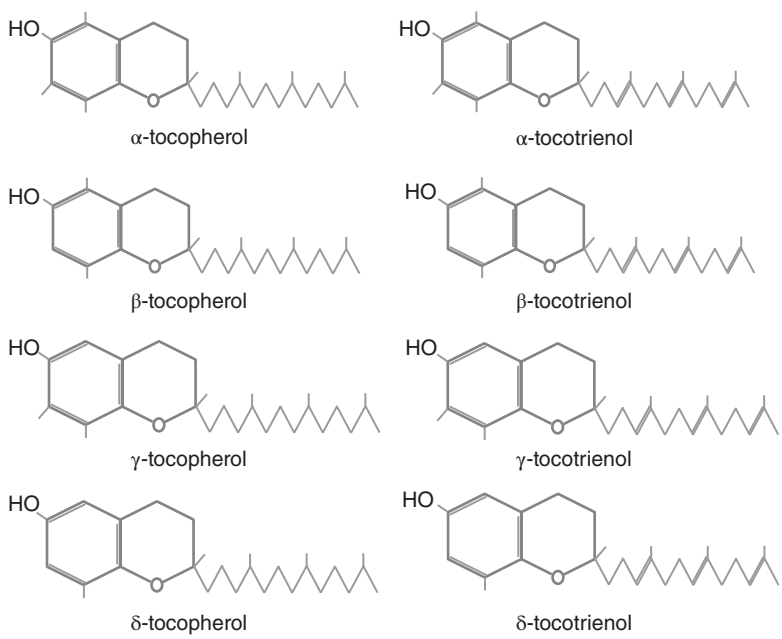

Figure 8.1 Derivatives of tocol and trienol.

or very low-density lipoproteins (VLDL) in the enterocyte, and impairment in chylomicron assembly may lead to vitamin E deficiency (Hamre 2011). In the liver, tocopherols are bound by tocopherol transfer protein (TTP), which also facilitates the release of α-tocopherol from hepatocytes into lipoproteins (Manor and Morley 2007). TTP gene has been characterized in Atlantic salmon and zebrafish, *Danio rerio*; it was expressed from early larval development in the later species (Usenko et al. 2008). In mammals, TTP binds α-tocopherols with higher affinity than other vitamin E homologs and stereoisomers (Yoshida et al. 1992; Kayden and Traber 1993), contributing to the higher *in vivo* antioxidant activity of α-tocopherol. In agreement with this preferential binder affinity of TTP in the liver, γ- and δ-tocopherol are more rapidly depleted than α-tocopherol in plasma, liver, and bile of starved Atlantic salmon, whereas the loss from the fillet is similar for all the tocopherols (Hamre et al. 1998). Low-density lipoproteins (LDL) and high-density lipoproteins (HDL) seem to be the main lipoproteins responsible for the transport of α-tocopherol to peripheral tissues (Hung et al. 1982; Lie et al. 1994). A linear relationship is found between dietary α-tocopherol and body levels except in the liver, where α-tocopherol increases exponentially (Hung and Slinger 1980; Cowey et al. 1983; Satoh et al. 1987; Frigg et al. 1990; Hardie et al. 1990; Hamre and Lie 1995a, b; González 1997; Hamre et al. 1997; Puangkaew et al. 2005).

Metabolic Functions

Vitamin E is a structural component of cell membranes (Putnam and Comben 1987), which acts as an inter- and intracellular chain-breaking antioxidant and plays a chief role in several biological processes (Burton et al. 1982; Burton and Ingold 1989; Sies and Murphy 1991).

The most widely documented function of vitamin E is its antioxidant activity. In general, the antioxidant activity of tocopherols and tocotrienols are due to their abilities to donate their phenolic hydrogen to lipid free radicals, thereby retarding the autocatalytic lipid peroxidation processes. Lipid-free radicals have an unpaired electron that makes them highly reactive towards unsaturated lipids, where they capture a hydrogen atom of a double bond, create a new free radical, and initiate the chain of reactions that rapidly lead to lipid autoxidation. These reactions include the formation of primary products such as hydroperoxides and conjugated dienes that may be cleaved to secondary products including aldehydes, alkanes, alkenes, alcohols, or acids. The resulting secondary products may damage membrane structure and function, or even disperse into different cell organelles and intercellular spaces, altering and inactivating protein compounds and being highly deleterious for fish (Kawatsu 1969; Watanabe et al. 1970; Murai and Andrews 1974; Sakai et al. 1989; Kanazawa 1991, 1993).

The specific location as a structural component of cell membranes allows vitamin E to play a role in the control of peroxidation of polyunsaturated fatty acids (PUFA) (Putnam and Comben 1987). The saturated side-chain of α-tocopherol is buried in the hydrophobic inner part of the membrane, whereas the 6-chromanol ring with the reactive hydrophy (OH) group is near the membrane surface and donates a hydrogen atom to the lipid free radical to neutralize it. This breaks the chain of reactions involved in autocatalytic lipid peroxidation and preventing PUFA oxidation (Quinn 2004). Tocopherols can also act as quenchers of singlet oxygen. Consequetly, a stable tocopheroxyl radical is formed, which is removed from the cycle when it reacts with another peroxyl radical to form inactive non-radical products. In turn, this tocopheroxyl radical can also be reduced by ascorbate, thereby regenerating α-tocopherol. However, unless it is regenerated, vitamin E will need to be replenished through the diet or from reserves elsewhere (Burton and Traber 1990). This vitamin is recognized as the major hydrophobic chain-breaking antioxidant that prevents the propagation of free radical reactions in membranes and lipoproteins, acting as an inter- and intracellular antioxidant to maintain homeostasis of labile metabolites in the cell and tissue plasma (Blazer 1982). Moreover, α-tocopherol acts as an effective antioxidant, even post-mortem, being responsible for maintaining membrane stability of fish fillet throughout its shelf life (Baker 1997), delaying color deterioration and rancid flavor. For instance, fillet quality can be markedly improved by supplementing vitamin E in diets for rainbow trout (Frigg et al. 1990; Chaiyapechara et al. 2003; Yildiz 2004), Atlantic salmon (Hamre et al. 1998; Scaife et al. 2000), turbot (Ruff et al. 2003, 2004), or European sea bass (Gatta et al. 2000; Pirini et al. 2000).

In addition to its antioxidant function, vitamin E influences membrane fluidity and maintenance, and is neccessary in capillaries and heart muscle (Zhang et al. 2009; Atkinson et al. 2010). Vitamin E, particularly α-tocopherol, also plays a relevant role in cell signaling and proliferation. For instance, α-tocopherol inhibits protein kinase C (PKC) activity in different cell types including vascular smooth muscle, monocytes, macrophages, neutrophils, fibroblasts, and mesangial cells (Devaraj et al. 1996, 1997; Freedman et al. 1996; Tada et al. 1997). α-Tocopherol inhibits PKC activity by dephosphorylating the enzyme at the cellular level with phosphatase PP2A stimulation (Freedman et al. 1996). Consequently, α-tocopherol inhibits PKC-dependent phosphorylation and translocation of the cytosolic factor p47 (phox) in monocytes, thereby impairing the NADPH-oxidase assembly and production of superoxide (Azzi 2004). α-Tocopherol also inhibits the activity of other enzymes such as phospholipase A2, cyclooxygenases, lipoxygenases, or mitogen-activated protein kinase, but activates other enzymes such as protein phosphatase 2A, diacylglycerol phosphatase, and protein tyrosine phosphatase (Zingg and Azzi 2004; Zingg 2007). Hence, many of the proteins modulated by α-tocopherol are associated with cell membranes or the synthesis of eicosanoids, which affects the immune system.

In mammals, α-tocopherol regulates genes related to lipid uptake, as well as degradation and expression of extracellular proteins (Li et al. 2010; Rimbach et al. 2010). In fish, the increase in α-tocopherol in diets high in docosahexaenoic acid (DHA) reduced the expression of specific antioxidant genes, such as superoxide dismutase (SOD) and catalase (CAT) in gilthead sea bream (Izquierdo et al. 2013) and European sea bass (Betancor et al. 2012a, b, 2013a, b; Fig. 8.2). Superoxide dismutase (SOD) prevents the initialization of the radical chain reaction that is produced by the superoxide anion through the conversion of superoxide into hydrogen peroxide, which is broken down by CAT in the peroxisomes. Since superoxide anions are efficiently scavenged by α-tocopherol, the reduced expression of SOD and CAT could be a reflection of the protective effect of α-tocopherol against oxidative stress. In those studies, an increase in dietary α-tocopherol also lead to a significant reduction in the expression of insulin-like growth factor I (IGF-1) gene in larvae of gilthead sea bream (Izquierdo et al. 2013) and European sea bass, and an increase in expression of myosin heavy chain (MyHC) and ghrelin (GRH) genes in the latter species (Betancor et al. 2012a, b, 2013a, b; Fig. 8.3). The regulation of these genes could simply reflect the role of oxidative stress in the expression of these types of growth factors, rather than a direct effect of α-tocopherol in gene expression. Similarly, the down-regulation of gulonolactone oxidase, an enzyme implied in vitamin C synthesis, by α-tocopherol (Moreau and Dabrowski 2003) could also be due to an improved oxidative status. In a recent study, an

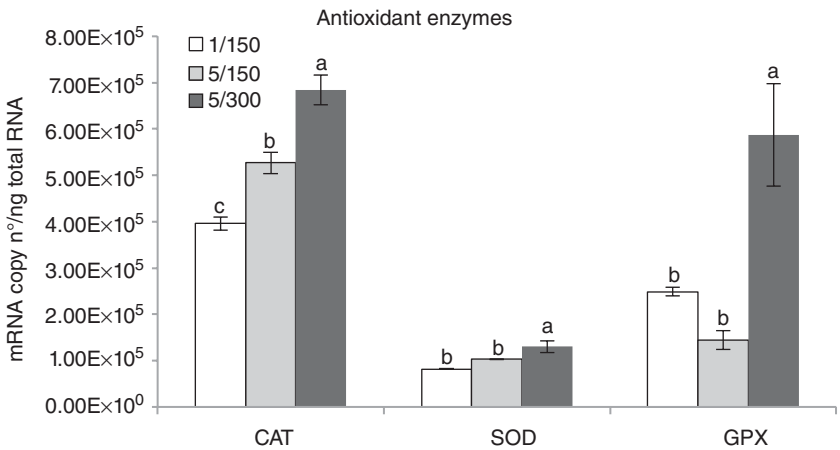

Figure 8.2 CAT, SOD, and GPX expression levels measured by real-time PCR in sea bass larvae when fed diets containing 1% or 5% of DHA in combination with 150 or 300 mg α-tocopherol per 100 g of diet.

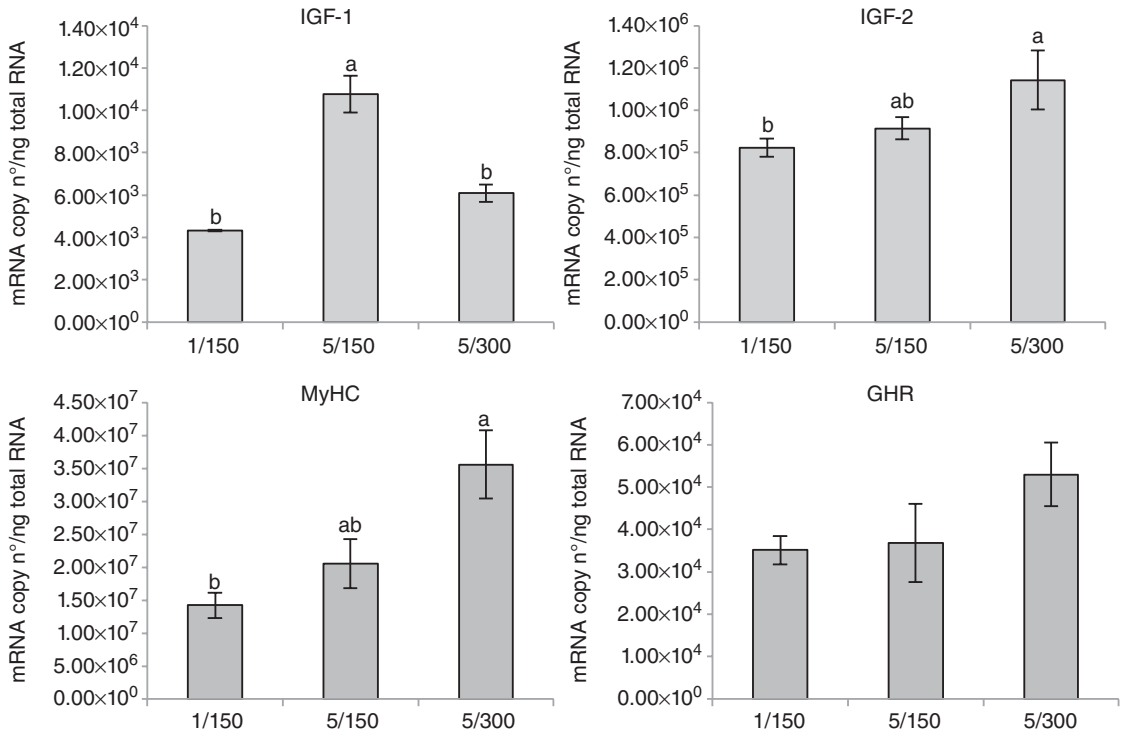

Figure 8.3 IGF-1, IGF-2, and GHR expression levels measured by real-time PCR in sea bass larvae when fed diets containing 1% or 5% of DHA in combination with 150 or 300 mg α-tocopherol per 100 g of diet.

increase of dietary α-tocopherol from 150 to 300 mg per 100 g in microdiets with krill phospholipids for larval gilthead sea bream significantly reduced SOD and CAT gene expression, as well as TBARs content, wherea, 300 mg α-tocopherol per 100 g in microdiets with soybean lecithin did not reduce larval TBARs content, nor SOD or CAT gene expression (Saleh et al. 2014). This data suggests that these genes are regulated by oxidative stress and not directly by the dietary level of α-tocopherol. Vitamin E deficiency also increased transcript levels of ApoE, NR4A, CREBBP, PGC1A, and PGC1B in juvenile zebrafish (Miller et al. 2013).

Deficiency Signs

Growth and Survival

Deficiency in α-tocopherol did not affect growth of red sea bream, *Pagrus major* (Sakaguchi and Hamaguchi 1979); rainbow trout (Blazer and Wolke 1984); Coho salmon, *Oncorhynchus kisutch* (Forster et al. 1988; Huang et al. 2004); channel catfish, *Ictalurus punctatus* (Gatlin et al. 1986; Jarboe et al. 1989; Bai and Gatlin 1993); Atlantic salmon (Hardie et al. 1990; Raynard et al. 1991; Dantagnan et al. 2012); European sea bass (Stéphan et al. 1993); common carp, *Cyprinus carpio* (Takeuchi 1996); Atlantic halibut, *Hippoglossus hippoglossus* (Tocher et al. 2002); and white sturgeon, *Acipenser transmontanus* (Moreau and Dabrowski 2003). Growth in rainbow trout was not affected after feeding a diet without added α-tocopherol for 4 months (Blazer 1982), or very high dietary levels of this vitamin (Puangkaew et al. 2005). However, increase in α-tocopherol has been found to improve growth rates in very young juveniles of Atlantic salmon (Poston et al. 1976; Hamre et al. 1994); rainbow trout (Cowey et al. 1984; Frischknecht et al. 1994); Chinook salmon, *Oncorhynchus tshawytscha* (Thorarinsson et al. 1994); gilthead sea bream (Montero et al. 2001; Tocher et al. 2002); Adriatic sturgeon, *Acipenser naccarii* (Agradi et al. 1993); hybrid striped bass, *Morone chrysops*

female, *Morone saxatilis* male (Kocabas and Gatlin 1999); rohu, *Labeo rohita* (Sau et al. 2004); mrigal, *Cirrhinus mrigala* (Paul et al. 2004); juvenile beluga, *Huso huso* (Falahatkar et al. 2012); and red sea bream (Gao et al. 2013).

In juvenile gilthead sea bream, growth was reduced in fish fed α-tocopherol-deficient diets, particularly in those animals challenged by chronic stress (Montero et al. 2001) or when oxidized oils were present in diets (Tocher et al. 2003). Marine fish larvae were also subjected to a high oxidative risk due to their high metabolic rate, high PUFA requirements, elevated water content, and the significant water reabsorption processes during metamorphosis. In marine fish larvae of European sea bass (Betancor et al. 2011) and gilthead sea bream (Atalah et al. 2012; Saleh et al. 2014), it has been shown that growth markedly improved with the elevation of α-tocopherol in microdiets. Dietary α-tocopherol therefore seems to affect growth only in fish under situations of high oxidative risk, such as stress (Montero et al. 2001), oxidized diets (Tocher et al. 2003), larvae fed high PUFA levels (Betancor et al. 2011; Atalah et al. 2012), or insufficient dietary antioxidants (Atalah et al. 2012).

Dietary levels of α-tocopherol did not affect survival rate of gilthead sea bream larvae (González et al. 1995; Atalah et al. 2012; Saleh et al. 2014) or European sea bass (Betancor et al. 2011). In addition, no mortality was registered when juvenile gilthead sea bream were fed diets without α-tocopherol supplementation (Montero et al. 1998); however, mortality increased up to 5% when fish were reared under high densities (Montero et al. 1999b, 2001).

Muscular Dystrophy

Muscular dystrophy has been described as one of the most representative symptoms of α-tocopherol deficiency (Lovell et al. 1984; Gatlin et al. 1986; Frischknecht et al. 1994; Bowater and Burren 2007). Muscular dystrophy is characterized by lesions in the axial musculature (Betancor et al. 2011, 2013a). In general, a marked edema can be observed between muscular fibers and the normal myotome architecture is lost (Betancor et al. 2013a). The lesions are formed by swelling of the affected fibers, which display hyaline degeneration, eosinophilic and pale cytoplasm without striations, and hydropic degeneration caused by the distension of the sarcoplasmic reticulum (Betancor et al. 2013a; Fig. 8.4). Hydropic degeneration is due to the failure of ion pumps and Na^+, and water influx to the endoplasmic reticulum; it is a common response to free radical injury in mammals (Cotran et al. 2004). The process continues by fragmentation of the myofibrils, disintegration of

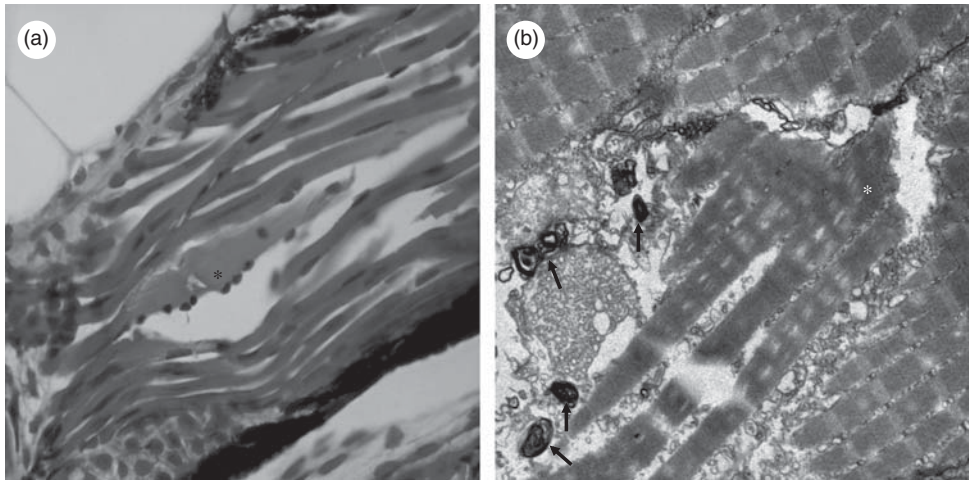

Figure 8.4 (a) Longitudinal optical microscopy and (b) transmission electron microscopy sections of sea bass larvae displaying mutirional muscular dystrophy. (a) Affected muscular swollen fiber (*) displacing the adjacent fibers and showing a marked eosinophilia. (b) Fragmentation of an affected muscle fiber (*) surrounded by myelin figures (arrow). For color details, please see color plate section.

myofilaments, necrosis, and infiltration of lymphocytes and macrophages to clear cytoplasmic debris (Betancor et al. 2013a). Hyper-contracted myofilaments can also be observed, indicating the attack of free radicals on muscle proteins. Despite both red and white fibers possibly being affected, the highest incidence of muscular lesions is found in white fibers in European sea bass larvae (Betancor et al. 2013a), which is in agreement with vitamin E deficiency myopathy described in mammals (Tomasi 1979; Lazaro et al. 1986). Red fibers, with a primarily oxidative metabolism, seem to have a higher free radical scavenging capacity and oxidative repair mechanisms than white fibers, as in mammals (Salminen and Vihko 1983; Asayama et al. 1986).

Muscular dystrophy has been attributed to vitamin E deficiencies in common carp (Hashimoto et al. 1966); yellowtail, *Seriola quinqueradiata* (Sakaguchi and Hamaguchi 1969); rainbow trout (Cowey et al. 1984; Frischknecht et al. 1994); Atlantic salmon (Poston et al. 1976); and channel catfish (Gatlin et al. 1986). The type and degree of incidence of muscular lesions may differ among fish species, nutritional status, age, size, diet quality, and feeding period (Moccia et al. 1984). For instance, α-tocopherol stored in the liver can prevent the development of these muscular lesions in rainbow trout (Cowey et al. 1981). Muscular dystrophy was not found in other reports for rainbow trout (Bell et al. 1985) or Atlantic salmon (Hamre et al. 1994; Hamre 2011), a fact that has been related to the presence of sufficient dietary selenium (Gatlin et al. 1986; Hamre 2011). However, in larvae of European sea bass, the increase in dietary selenium was not able to completely avoid muscular dystrophy (Betancor et al. 2012a), which was associated with high dietary docosahexaenoic acid (DHA) levels. Moreover, increase in dietary ascorbic acid was more effective than selenium in reducing the incidence of muscular dystrophy (Betancor et al. 2012b), highlighting the importance of α-tocopherol in preventing muscular degeneration and its regeneration by vitamin C. This type of muscular dystrophy is originated by free radical injury to muscle fibers and organelle membranes in mammals (Cotran et al. 2004) and fish (Betancor et al. 2013a), and its occurrence may simply indicate the unbalance between antioxidants and pro-oxidant factors in the muscle. Fish skeletal muscle is particularly rich in phospholipids high in PUFA, and is profoundly susceptible to oxidative stress (Song et al. 2000). Moreover, some cell types may be more susceptible to oxidation than others due to a high rate of oxidative metabolism, low α-tocopherol to PUFA ratio, high turnover rate of vitamin E, or the presence of pro-oxidant factors (Hamre 2011).

Liver Damage

Due to the fact that the liver is responsible for storing major lipids in fish and α-tocopherol is the principal fat-soluble antioxidant, vitamin E deficiency frequently damages this organ, which is highly susceptible to oxidation. Moreover, according to Hamre (2011), the liver may be especially susceptible to damage by vitamin E deficiency because of its abundance of heme-containing enzymes that, like hemoglobin, may be prone to oxidation (Andersen et al. 1994). As a consequence of the lipid oxidation processes, products such as thiobarbituric reactive substances (TBARs) increase in liver of α-tocopherol-deficient fish, whereas the contents of some PUFAs are reduced in gilthead sea bream (Montero et al. 1996). In Atlantic salmon, these oxidation processes lead to increased desaturation of 18:3n-3 and 20:5n-3 in hepatocytes (Mourente et al. 2007). Deficiency symptoms related to liver damage include increased hepatosomatic index, hepatocyte hypertrophy, inflammation, ceroidosis, and necrosis (Thorarinsson et al. 1994; Montero et al. 1996). For instance, up to 37% of gilthead sea bream juveniles had liver inflammation 15 weeks after being fed a diet not supplemented with α-tocopherol (Montero et al. 1996, 1999c). Hepatic ceroidosis also seems to be a consequence of alterations in the fish oxidative balance (Miyazaki 1995; Sakai et al. 1998; Porta et al. 2002). Thus, a vacuolar intra-cytoplasmic pigment of ceroid nature is found in liver of larval European sea bass fed weaning diets high in PUFA and insufficient antioxidant nutrients, such as α-tocopherol (Betancor et al. 2013a). In acute cases, ceroid deposits are found only secondarily inside of hepatocytes and principally in the Kupffer cells that phagocytize them (Lovell et al. 1984; Lewis et al. 1985). The process is followed by liver degeneration and focal necrosis (Murai and Andrews 1974; Lovell et al. 1984; Roem et al. 1990; Hamre et al. 1994). Ceroid deposition and hemosiderosis could also appear in the spleen (Berchieri-Ronchi et al. 2011).

Anemia

One of the first signs of red blood cell deficiency is erythrocyte hemolysis (Woodall et al. 1964; Cowey et al. 1981; Moccia et al. 1984; Wilson et al. 1984; Hamre et al. 1994, 1997). Even when low dietary vitamin E levels do not affect growth or survival, erythrocyte fragility increases five times over normal values in gilthead sea bream juveniles weighing 20–80 g (Montero et al. 1999b, 2001). Erythrocyte fragility also increased in vitamin-E-deficient channel catfish (Wilson et al. 1984; Wise et al. 1993); European sea bass (Obach et al. 1993); Atlantic salmon (Hamre et al. 1994); and rainbow trout (Cowey et al. 1983; Boggio et al. 1985; Furones et al. 1992). Vitamin E deficiency increases the occurrence of erythrocytes that are immature (poikilocytosis) or have a great size variation (anisocytosis) in Atlantic salmon, yet the same symptoms were also found for selenium deficiency (Poston et al. 1976) or when fish were fed oxidized fats (Moccia et al. 1984).

Reproduction and Early Development

Early studies in the 1990s showed that deficiencies of vitamin E inhibit gonadal maturation and decrease egg hatching and larval survival rates in common carp; ayu, *Plecoglossus altivelis* (Watanabe 1990); and red sea bream (Watanabe et al. 1991a). An increase in dietary vitamin E also improves spawning quality in carpin, *Carassius auratus* (Sutjaritvongsanon 1987); red sea bream (Watanabe et al. 1985b, 1991a, b); African catfish *Heteropneustes fossilis* (Dube 1993); Nile tilapia, *Oreochromis niloticus* (Schimittou 1993); yellowtail (Mushiake et al. 1993); pearlspot, *Etroplus suratensis* (Shiranee and Natarajan 1996); gilthead sea bream (Izquierdo et al. 2001); Australian trumpeter, *Latris linneata* (Morehead et al. 2001); and the grouper, *Epinephelus coioides* (Xiao et al. 2003). In gilthead sea bream, diets deficient in vitamin E also decreased the percentage of fertilized eggs (Fernández-Palacios et al. 1998; Fernández-Palacios and Izquierdo 2010). This may have been related to the decrease in the number and motility of the spermatozoids, as has been described for other vertebrates (Donnelly et al. 1999; Danikowski et al. 2002) and fish, including ayu (Hsiao and Mak 1978) and American perch, *Perca flavescens* (Lee and Dabrowski 2004). Insufficient vitamin E also decreased the percentage of viable eggs with normal morphology in several species: rainbow trout (King 1985), Japanese sea bream (Watanabe et al. 1991a, b), gilthead sea bream (Fernández-Palacios and Izquierdo 2010), and milkfish, *Chanos chanos* (Emata et al. 2000). Vitamin E deficiencies have also been shown to cause loss of sexual coloration and decrease in reproductive activity in Nile tilapia (Schimittou 1993).

Increase in vitamin E levels in parental diets is also required to prevent larval deformities. For instance, a high percentage of deformed larvae with hypertrophy of the yolk sac appeared when gilthead sea bream broodstock were fed high levels of dietary PUFA and insufficient levels of vitamin E (Fernández-Palacios et al. 1998); however, deformities were prevented with an increase in dietary α-tocopherol (Fernández-Palacios et al. 1998, 2011). Vitamin E deficiency in parental diets also caused developmental abnormalities in zebrafish larvae (Miller et al. 2012) that were very similar to those described in sea bream. Moreover, broodstock diets deficient in vitamin E misregulate progeny genes that perturb energy metabolism and mitochondrial function, ultimately causing embryo malformations and mortality (Miller et al. 2012; Montero et al. 2014).

Skeleton Development

Despite the importance of vitamin E in mammals for the proper development of skeleton (Xu et al. 1995; Jilka et al. 1996) and its role in the lipid bilayer of bone cells as the first line of defense against free radicals (Arjmandi et al. 2002), studies in fish are very scarce. Increase in dietary α-tocopherol significantly increased bone mineralization in gilthead sea bream larvae and reduced the occurrence of chondroid bone anomalies (Izquierdo et al. 2013; Saleh et al. 2014). Lewis-McCrea and Lall (2007) found an increase in the incidence of scoliosis in Atlantic halibut, *Hippoglossus hippoglossus*, juveniles fed increasing levels of oxidized oils. This may be a result of lipid peroxidation product effects, which stimulate osteoclastic differentiation and inhibit osteoblastic activity; this potentially causes bone resorption, and may lead to bone abnormalities (Tintut et al. 2002; Parhami 2003; Kruger et al. 2010).

Other symptoms, although more rarely associated with vitamin E deficiency in fish, include xerophthalmia (Hashimoto et al. 1966; Sakaguchi

and Hamaguchi 1969), altered startle response, and neurologic deficits (Miller et al. 2012).

Requirements

Despite the abundant information on the pathological consequences of α-tocopherol deficiency in fish, studies to determine the quantitative requirements of vitamin E for various fish species are insufficient (NRC 1993, 2012). Published requirements fall within the range 30–50 mg kg^{-1} diet for channel catfish (Murai and Andrews 1974; Wilson et al. 1984); 30 mg kg^{-1} for hybrid striped bass (Kocabas and Gatlin 1999); over 100 mg kg^{-1} for rohu (Sau et al. 2004) and the grouper *Epinephelus malabaricus* (Lin and Shiau 2005); 120 mg kg^{-1} for Atlantic salmon (Hamre and Lie 1995a); 200–300 mg kg^{-1} for common carp (Watanabe et al. 1977); 210 mg kg^{-1} for mrigal fry (Paul et al. 2004); 437 mg kg^{-1} for Australian trumpeter (Brown et al. 2005); and 1200 mg kg^{-1} for gilthead sea bream (Ortuño et al. 2000; Tocher et al. 2002). Vitamin E requirements also seem to depend on the fish life cycle. For instance, a level of 250 mg kg^{-1} dietary α-tocopherol is sufficient to meet the requirements for successful reproduction in gilthead sea bream (Fernández-Palacios et al. 1998), but increasing dietary levels to 540–3000 mg kg^{-1} during larval stages significantly improved fish growth (Atalah et al. 2012).

Excessively high levels of vitamin E may also have negative consequences for fish. For instance, high dietary levels of α-tocopherol caused poor growth, toxic liver reaction, and death in rainbow trout (Watanabe et al. 1970). In brook trout, *Salvelinus fontinalis*, increasing dietary α-tocopherol levels from 500 mg kg^{-1} to 5000 mg kg^{-1} caused reduced growth and hematocrit value (Poston and Livingston 1971). Indeed, vitamin E is pro-oxidant when is accumulated in the tissues at high concentrations (Porter et al. 1995).

Interactions with Other Nutrients

The high vitamin E requirements of fish larvae have been associated with high PUFA needs during larval stages (Atalah et al. 2012). Dietary vitamin E levels must therefore be increased when PUFA are high, as found in carp (Watanabe et al. 1981; Schwarz et al. 1988); Nile tilapia (Satoh et al. 1987); Atlantic salmon (Hamre and Lie 1995b); grouper (Lin and Shiau 2005); gilthead sea bream (Atalah et al. 2012); and European sea bass (Betancor et al. 2010). PUFA presence accelerates the autocatalytic peroxidation of vitamin E to prevent PUFA oxidation, potentially increasing the need for vitamin E (Watanabe 1982; Sargent et al. 1997; Izquierdo et al. 2001). For instance, increasing the dietary PUFA level diminishes vitamin E concentration in the liver of Atlantic salmon (Waagbø et al. 1993) and African catfish, *Clarias gariepinus* (Lim et al. 2001). Furthermore, dietary levels of lipid oxidation affect the vitamin E requirements of fish (Mourente et al. 2002; Lewis-McCrea and Lall 2007; Zhong et al. 2008).

Dietary levels of other pro-oxidant or antioxidant nutrients, such as selenium or vitamin C, can also affect vitamin E requirements (Gatlin et al. 1986; Chávez de Martínez 1990; Roem et al. 1990; Hamre et al. 1994; Thorarinsson et al. 1994; Sealey and Gatlin 2002; Shiau and Hsu 2002; Lim et al. 2001). For instance, elevating dietary vitamin E up to 1500 mg kg^{-1} in diets for larval sea bream (containing ascorbic acid) significantly reduced larval survival, whereas the same diets supplemented with vitamin C as ascorbyl acid polyphosphate prevented larval mortality and significantly improved larval growth (González 1997). Increasing dietary vitamin C levels prevents the development of pathological signs of vitamin E deficiency in several species, such as rainbow trout (Frischknecht et al. 1994); Atlantic salmon (Hamre et al. 1997); and golden shiner, *Notemigonus crysoleucas* (Chen et al. 2004).

An increase in vitamin C from 1800 to 3600 mg kg^{-1} markedly increases tissue contents of α-tocopherol and reduces the occurrence of muscular dystrophy and tissue TBARs, highlighting its sparing effect over dietary vitamin E (Betancor et al. 2012b). The concentration of α-tocopherol in the liver was also increased by dietary vitamin C in yellow perch (Lee and Dabrowski 2003) and channel catfish (Yildirim-Aksoy et al. 2008). Ascorbic acid seems to play a significant role in α-tocopherol metabolism, reducing α-tocopheroxyl radicals and regenerating α-tocopherol (Niki et al. 1985). Moreover, dietary requirements of both vitamin E and vitamin C increased when either vitamin C or vitamin E was

deficient in the diets of juvenile hybrid striped bass (Sealey and Gatlin 2002). Ascorbic acid concentrations also increased in liver of red sea bream as levels of dietary α-tocopherol increased (Gao et al. 2013).

Selenium is another potent antioxidant nutrient that interacts with α-tocopherol in fish (Poston et al. 1976; Bell et al. 1985). In particular, marine fish diets with elevated PUFA contents require an adequate combination of dietary α-tocopherol and Se to prevent oxidative stress and improve fish performance.

Vitamin E and Immune System

Vitamin E has been considered as a key immune stimulating factor in fish (Sakai 1999; Waagbø 2006). Inadequate amounts of vitamin E have negative effects on both non-specific and specific immune responses in several fish species, whereas increase in vitamin E concentrations slightly above the general recommended levels lead to optimum health benefits. For instance, immune response parameters were enhanced in darkbarbel catfish, *Pelteobragus vachelli*, subjected to high ammonia levels when fish were fed 400 mg kg^{-1} vitamin E compared to those fed 50 mg kg^{-1} (Li et al. 2013).

Diets deficient in α-tocopherol reduced serum hemolytic activity in rainbow trout sensitized with intra-peritoneally injected sheep red blood cells (Blazer and Wolke 1984). Because serum from these fish was not heat inactivated, the vitamin E effect on serum hemolytic activity has been related to serum complement activity (Landolt 1989; Montero et al. 1996, 1998). Complement activation in serum is one of the main responses of the fish immune system, and is characterized either by the triggering of the first component of the complement cascade by formation of antigen-antibody compounds (classical pathway) or by a non-specific response (alternative pathway). Increasing dietary vitamin E enhances the activity of the complement system in several fish species (Blazer and Wolke 1984; Hardie et al. 1990; Obach et al. 1993; Wise et al. 1993; Montero et al. 1998, 1999a, 2001). Diets without α-tocopherol supplementation reduce the serum alternative complement pathway activity in gilthead sea bream in comparison to fish fed 150 mg kg^{-1} α-tocopherol supplementation (Montero et al. 1996). In the same species, 1200 mg kg^{-1} of dietary vitamin E also stimulates serum hemolytic activity (Ortuño et al. 2000). Increasing dietary vitamin E up to 1000 mg kg^{-1} in trout (Pearce et al. 2003) and from 7 mg kg^{-1} to 326 or 800 mg kg^{-1} in Atlantic salmon (Hardie et al. 1990) raised serum complement. In fish, vitamin E specifically affects complement activity independent of general protein depletion (Hardie et al. 1990). Lysozyme activity is also reduced in fish fed low dietary levels of vitamin E (Montero et al. 1999a; Kiron et al. 2004).

Increase in dietary vitamin E from 0, 100, or 450 mg kg^{-1} promoted macrophage recruitment and giant cell formation in pacu, *Piaractus mesopotamicus* (Belo et al. 2005). In several species, dietary vitamin E also boosts granulocyte activity (Blazer 1982; Blazer and Wolke 1984; Wise et al. 1993; Pulsford et al. 1995). In rainbow trout for instance, vitamin-E-deficient diets diminished the phagocytic activity of intestinal and head kidney cell suspension (Clerton et al. 2001), and reduced peritoneal macrophage function (Blazer 1982). Vitamin E deficiency in Atlantic salmon reduced the ability of serum to opsonize bacteria (Hardie et al. 1990) and the bacterial killing activity of isolated head-kidney macrophages (Waagbø et al. 1993), whereas impaired peritoneal macrophage function was observed in channel catfish (Wise et al. 1993). Finally, 1200 mg kg^{-1} of dietary vitamin E stimulated serum hemolytic activity and phagocytosis of head kidney leucocytes in gilthead sea bream (Ortuño et al. 2000).

The lack of an immune modulatory effect by dietary vitamin E as seen in certain studies has been related to the dietary contents of other antioxidant nutrients (Sealey and Gatlin 2002). The combination of dietary vitamin E with vitamin C seems to further promote the immune stimulant effects of these nutrients. For instance, feeding sea bream with diets supplemented with both vitamins E and C prevented an increase in lysozyme activity when fish were subjected to high stocking density (Montero et al. 1999c). Similarly, the combination of dietary vitamin E and C improved all immune-related parameters affected by the single addition of each of these vitamins (Ortuño et al. 2003). Later, other studies showed a marked interaction between vitamin C and vitamin E on immune system (Wahli et al. 1998; Yildirim-Aksoy et al. 2008).

Vitamin-E-depleted diets also impaired antibody response in fish (Verlhac et al. 1993). Dietary supplementation with vitamin E therefore enhanced

lymphocyte T and B function (Blazer and Wolke 1984) and antibody production (Ndoye et al. 1990) in rainbow trout sensitized against *Yersinia ruckeri*. The molecular mechanisms implied in the enhancement of immune function are not well understood in fish. In mammals, on the other hand, α-tocopherol has been reported to enhance leukocyte trans-endothelial migration and leukocyte recruitment (McCary 2011), and stimulate the production of cyclic adenosine monophosphate (cAMP; Salinthone et al. 2013).

Despite its importance, excessive levels of this nutrient do not seem to improve the fish immune response. For instance, neither low (100 mg kg^{-1}) nor high (1800 mg kg^{-1}) levels of vitamin E induced immune stimulation in gilthead sea bream (Ortuño et al. 1999). In rainbow trout, 1000 mg kg^{-1} of dietary vitamin E did not have any effect on the oxidative burst and pinocytosis of head kidney macrophages (Clerton et al. 2001). This level of vitamin E seems to be excessive for rainbow trout, even when high contents of PUFA are included in the diet (Puangkaew et al. 2004). Despite the fact that both humoral and cellular immune functions were deteriorated in fish fed vitamin-E-deficient diets, increasing vitamin E levels to 1000 mg kg^{-1} did not substantially enhance the alternative complement activity, total immunoglobulin, phagocytosis, or non-specific cytotoxicity in comparison to the 100 mg kg^{-1} dose (Puangkaew et al. 2004).

Vitamin E and Resistance to Infectious Diseases

Early studies in rainbow trout suggested the potential immune stimulatory value of vitamin E in enhancing disease resistance of fish (Blazer and Wolke 1984). It was reported that rainbow trout challenged with *Yersinia ruckeri* showed decreased immune response when vitamin E was missing from the diet (Blazer and Wolke 1984). In agreement, deficient levels of vitamin E lead to increased mortality in European sea bass infected with *Aeromonas salmonicida* (Obach et al. 1993). In contrast, an increase in dietary vitamin E improved antibody production against *Edwarsiella ictaluri* when darkbarbel catfish was subjected to high ammonia levels (Li et al. 2013).

Lall et al. (1988) and Hardie et al. (1990) reported that vitamin E supplementation had no effect on humoral antibody responses in Atlantic salmon. Likewise, dietary levels of vitamin E did not affect immune system or resistance to the causative agent of bacterial kidney disease (BKD), *Renibacterium salmoninarum*, in Chinook salmon (Leith and Kaattari 1989). Moreover, Chinook salmon challenged with *Renibacterium salmoninarum* did not show enhanced growth or hematocrit values with dietary vitamin E supplementation (Thorarinsson et al. 1994). Similarly, increasing levels of vitamin E in diets of pacu did not improve hematocrit values after challenge with *Aeromonas hydrophila* (Garcia et al. 2007). Vitamin E levels higher than 250 mg kg^{-1} can lead to altered hematocrit and hemoglobin values (Garcia et al. 2007). Both vitamin E and vitamin C have been shown to have a synergistic effect on non-specific immune responses and disease resistance in Japanese flounder, *Paralychthis olivaceous* (Wang et al. 2006).

Vitamin E and Stress Resistance

Cortisol is the major corticosteroid in teleost and rapidly increases in plasma as a primary stress response (Pickering and Pottinger 1989). Insufficient amounts of dietary vitamin E have been found to raise plasma cortisol levels in gilthead sea bream (Montero et al. 1998, 1999b, 2001) and cause severe nutritional oxidative stress in rainbow trout (Puangkaew et al. 2004). Moreover, increase in dietary vitamin E levels promotes resistance to both chronic and acute repetitive stress, and increases fish survival and serum alternative complement pathway activity (Montero et al. 1998, 1999b, 2001). For instance, feeding a vitamin-E-deficient diet reduced fish growth and survival and altered hematological indicators in sea bream cultured at high stocking densities (Montero et al. 2001), but vitamin E supplementation reduced the severity of glomerulonephritis (increase in number of macrophage aggregates in kidney; Montero et al. 1999c). Under repetitive chasing stress conditions, gilthead sea bream fed a vitamin-E-deficient diet had a faster elevation of plasma cortisol levels and lower survival (Montero et al. 2001). Vitamin E has also been found to increase survival of larvae subjected to handling stress. Betancor et al. (2010) showed that an increase in dietary α-tocopherol from 1500 to 3000 mg kg^{-1} in both medium and high n-3 HUFA levels diets markedly increased the resistance

to handling stress in European sea bass larvae. In agreement, vitamin E increased the resistance of white leg shrimp, *Litopenaeus vannamei*, to salinity stress (Liu et al. 2007) and the resistance of golden shiner, *Notemigonus crysoleucas*, to heat stress (Chen et al. 2004).

Nevertheless, inclusion of dietary vitamin E at 250 mg kg^{-1} does not completely protect sea bream from the adverse effects of high stocking density, with fish growth being lower than in low-density production (Montero et al. 2001). In agreement, extra-supplementation of vitamin E did not prevent elevation of plasma cortisol levels in gilthead sea bream exposed to several stressors, although it allowed reduction in plasma glucose levels (Ortuño et al. 2003). Similarly, in rainbow trout subjected to crowding stress, increasing vitamin E levels enhanced growth and survival, but did not prevent elevation of plasma cortisol levels (Trenzado et al. 2007, 2008). In agreement, dietary vitamin E did not affect cortisol levels in the sterlet, *Acipenser ruthenus* (Tatina et al. 2010) or beluga sturgeon, Huso huso, before or after stress exposure (Falahatkar et al. 2012). However, in the latter study, the cortisol response of beluga sturgeon to the stressor was very low, regardless of diet (Falahatkar et al. 2012).

Conclusions

Vitamin E, particularly α-tocopherol, is an essential nutrient that plays different physiological roles including protection of fat-soluble compounds from oxidation, membrane mantainance, cell signaling, gene expression, and eicosanoids synthesis. Many of these functions have a relevant impact on immune system, so inclusion of adequate amounts of α-tocopherol in fish diets markedly enhances fish health and promotes resistance to stress and, potentially, infectious diseases. On the contrary, vitamin E deficiency has negative consequences on fish health including the occurrence of anemia, muscular dystrophy, liver damage, skeleton anomalies, and mortalities. However, more research is needed to define the specific dietary requirements for this vitamin, particularly in relation to the presence of different lipid sources and pro- and antioxidant nutrients to take full advantage of the beneficial effects of vitamin E on fish health.

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Chapter 9
Minerals

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Introduction

Over the past two decades the aquaculture industry has expanded rapidly throughout the world; it is expected to continue to grow in the years to come due to the high cost of harvesting fish from the oceans, the un-sustainability of ocean fishing methods, and the increased demand for fish. Several factors including rapid population growth, increased disposable income, and preferences for fish over other animal protein for personal, cultural, geographic, ethnic, and health reasons have led to the increased demand. There has therefore been an increased effort to produce higher yields per unit area. However, under intensive production systems, fish are exposed to numerous potential stressors which could include poor water quality, crowding, transport and handling, and poor nutrition. These factors may directly or indirectly affect the health of the fish.

It has generally been recognized that under intensive culture operations, good nutrition plays a key role in promoting good growth and maintaining the health and well-being of cultured animals. Essential nutrients should be provided at adequate levels in the diets to sustain health and maintain the ability of fish to withstand stress and resist disease-causing agents. Evidence has indicated that most, if not all, essential dietary nutrients, as well as feeding practices, influence disease resistance. Dietary deficiencies of essential nutrients such as minerals could adversely affect the immune system. Further, addition of some minerals may increase immuno-competence of fish, making them less susceptible to diseases. This chapter reports on the general functions of minerals in fish and their effects, when added as a dietary supplement, on immune response and disease resistance of aquacultured organisms. The effects minerals present in the water as an environmental pollutant are not discussed.

Minerals are inorganic elements that are found in ash when a food or body tissue is burned. Based on relative dietary requirements, minerals are classified into two groups: macro or major minerals, and micro or trace minerals. Macrominerals are elements that are required in large amounts (from a few tenths of a gram to over a gram per day). Microminerals are minerals that are required in very small amounts (from micrograms to milligrams per day). The general functions of minerals include: adding strength to the skeletal system, serving as components of organic compounds (such as proteins and lipids), enzyme-system activators, and maintaining acid-base and osmotic balances.

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Fish can absorb dissolved minerals in the aquatic environment through the skin, gill membrane, and the digestive tract (marine fish) to satisfy part of their metabolic requirements. Some minerals have been shown to enhance immunity of various animals including some fish species. However, less research has been conducted with minerals in this regard as compared to vitamins. Many minerals have biological functions that affect the defense mechanism and immuno-competence of the fish.

Macrominerals

Calcium

Calcium gives strength to bones and is also important for the control of the heartbeat, the transmission of nerve impulses, and muscle contraction; it is also necessary for blood clotting, maintenance of cell membrane integrity, and activation of various enzymes. Calcium ions are absorbed at the proximal end of the intestine, and several factors enhance uptake by the body including vitamin D, dietary protein intake, and an acid medium. Conversely, vitamin D deficiency reduces the absorption of calcium, as does an excess of dietary phosphorus, excessive lipid or fiber in the diet, and the presence of phytic acid. Phytic acid, which is found in many cereal grains and oilseed meals, prevents the absorption of calcium by forming an insoluble salt (calcium phytate).

Chloride

Chloride is an essential mineral because it is required to form hydrochloric acid present in gastric juice. Most chloride is found in extracellular fluids in the body, and the chloride content of the blood is higher than any other mineral. Chlorine, in the form of the negatively charged chloride ion, plays a major role in the regulation of osmotic pressure and acid-base balance.

Magnesium

Magnesium (Mg) is the second-most abundant cellular cation; it plays a role in osmoregulation of fish, which is necessary for ATPase activity and subsequent release of stored energy from adenosine triphosphate (ATP). This energy is used to transport salts across the gill membranes and concentration gradients. Nearly 60% of magnesium is located in bones in the form of phosphates, while approximately 30% is found in soft tissues (such as the liver and muscle). Magnesium is also a constituent of bones and teeth, and is involved in certain peptidase activations for protein digestion. If a diet is deficient in magnesium, calcium could be deposited in soft tissues and result in calcified lesions; an excess of magnesium may disrupt calcium and phosphorus metabolism.

Mg has been reported to be involved in protein synthesis of immune factors and in the alternate complement pathway via properdin activity, as Mg and C3 (complement factor 3) are required to activate properdin. Atlantic salmon, *Salmo salar*, which had either been vaccinated against *Vibrio anguillarum* or not, were fed diets containing various levels of Mg (0, 300, and 500 mg kg^{-1} of diet) and no differences in serum antibodies were observed among vaccinated fish fed the three Mg levels. There were also no differences among unvaccinated fish; however, antibody levels were higher in vaccinated fish (El-Mowafi et al. 1997). This pattern was also observed for serum lysozyme activity and serum hemolytic activity after 8 weeks where there were no differences among the various levels of Mg inclusion, but vaccinated fish had significantly higher values compared to unvaccinated fish. Hemoglobin (Hb) and total serum protein concentration of vaccinated or unvaccinated fish were likewise unaffected by dietary levels of Mg, yet unvaccinated fish had higher Hb and total serum protein concentrations compared to vaccinated fish (El-Mowafi et al. 1997). However, the Mg that was added to the culture water during the experimental period may have been absorbed at a level sufficient to prevent a deficiency.

No significant differences in total cell count, red blood cell count, hematocrit, and hemoglobin were found when channel catfish, *Ictalurus punctatus*, were fed diets containing various levels (0, 200, 400, 600, 800, and 1000 mg kg^{-1} of diet) of Mg (Lim and Klesius 2003). However, mean macrophage migration and macrophage chemotaxis were significantly higher in fish fed a diet containing 400 mg Mg kg^{-1} of feed compared to fish fed diets containing 0, 800, and 1000 mg Mg kg^{-1} of feed in one experiment, but this was not found in the second experiment. When fish were challenged with *Edwardsiella ictaluri*, those fed a

diet containing 0 mg Mg kg^{-1} of feed had numerically lower (78.8%) mortality compared to fish fed diets containing Mg, though there was no clear statistical differences among treatments (Lim and Klesius 2003).

When grass carp, *Ctenopharyngodon idella*, were fed diets containing various levels (76.9, 247.7, 382.9, 692.0, 1298.1, and 2481.2 mg kg^{-1} of diet) of Mg, activities of superoxide dismutase, glutathione peroxidase, and lysozyme were higher in fish fed a diet containing 692.0 mg Mg kg^{-1} of feed compared to fish fed all other diets; however, activities of these variables among the other fish were similar (Wang et al. 2011). Magnesium can act as an antioxidant possibly by three modes of action: it could protect cell membranes from oxidative damage by reducing the influx of calcium into the cell; it could reduce the production of free radicals by preventing NADPH oxidase activity; and/or it could eliminate free radicals by increasing the activities of antioxidase enzymes such as glutathione peroxidase and superoxide dismutase. Wang et al. concluded that their data supported the third possible mechanism, since it indicated that activities of antioxidases were similar in grass carp fed various levels of Mg, except for fish fed 692.0 mg Mg kg^{-1} of diet.

Phosphorus

Phosphorus (P) is an essential mineral for fish and is important as a constituent of bones and scales. Phosphorus has many functions in an organism. In vertebrates, approximately 78% of the body phosphorus is in bones as calcium phosphate and hydroxyapatite. Phosphorus is also important in energy metabolism as a component of adenosine mono-, di- and triphosphate (AMP, ADP, and ATP, respectively) and as a component of nucleic acids, phospholipids, buffer systems and enzymes, and is important in antibody formation. Dietary P must be provided, as there is usually very little present in natural aquatic environments. Excessive supplementation of P can result in adverse water quality, while low dietary P can result in insufficient bone mineralization and reduced growth.

Fish absorb phosphorus from the water via their gills so, in theory, a dietary requirement for phosphorus should not be needed. However, since plants and animals take up phosphorus and leave a limited supply, a dietary source is needed.

Phosphorus can be either a lethal (white phosphorus is used in incendiary bombs, and organic phosphate can be used in insecticides) or an essential mineral to living organisms. In living organisms, it is a constituent in every cell and body fluid. Diets that were deficient in P produced lower growth and severe malformations in Japanese flounder, *Paralichthys olivaceus* (Uyan et al. 2007). Deformations of the operculum and bone ultra-structure is evidence of P deficiency in fish, characterized by a failure in mineral deposition in hard tissues such as bone, scales, and cartilage. Uyan et al. (2007) stated that P was utilized in small Japanese flounder to maintain growth, but vertebral bone formation was adversely affected. Fish fed P-deficient diets had more perforated spongy areas compared to fish fed P-sufficient diets.

Phosphorus is involved in several chemical relationships. If the diet contains an excess of calcium over phosphorus, free calcium will be present and form insoluble tricalcium phosphate. An excess of dietary phosphorus over calcium will decrease the absorption of both calcium and phosphorus. In addition, excesses of iron, aluminum, and magnesium may bind phosphorus as insoluble salts and therefore inhibit phosphorus absorption.

Much of the phosphorus in plant-protein sources (e.g., soybean meal) and plant ingredients is in the form of phytate, which is poorly utilized and may depress the absorption of iron, zinc, and calcium. Highly digestible sources of phosphorus, such as monocalcium- and dicalcium-phosphate, are often added to fish diets to ensure that the phosphorus requirement for growth, bone mineralization, and physiological and immunological function is met.

In guinea pigs, *Cavia corbaya*, increasing P levels in diets resulted in improved resistance to challenge with *Salmonella typhimurium*, and adding excess P to the diets (2–3 times the requirement) gave the highest percentage survival when exposed to the pathogen (Nabb and O'Dell 1964; O'Dell 1969). In chickens (Gaafar and Ackert 1958) and dogs (Craddock et al. 1974), impaired immune function was observed when animals were fed diets deficient in P.

Phosphorus (P) and magnesium (Mg) are the only two macrominerals whose effects on immune responses and disease resistance in fish have been investigated. When channel catfish were fed diets containing various (0.0, 0.05, 0.10, 0.25, 0.40, 0.55,

and 0.85%) levels of P (monosodium phosphate), weight gain, serum alkaline phosphatase activity, percentage survival after challenge with *E. ictaluri*, and antibody titer against the same pathogen were all significantly higher in fish fed diets containing 0.40% P and higher compared to fish fed diets containing 0.25% and lower (Eya and Lovell 1998). It is not known how P affects immune function in fish, but a lack of P may impair antibody production due to a lack of sufficient energy for synthesis. It could also be that a lack of sufficient P could result in reduced phagocyte activity, as reported in mammals (Craddock et al. 1974; Kiersztjn et al. 1992). Eya and Lovell (1998) suggested, however, that a dietary P level to meet the growth requirement (0.4%) is sufficient to improve the resistance of fish against *E. ictaluri* infection.

Jokinen et al. (2003) studied the influence of low-P and high-P supplementation on non-specific and specific immune response of European whitefish, *Coregonus lavaretus*. They found that fish fed the low-P diet were significantly smaller than fish fed diets containing adequate P, and had lower plasma immunoglobulin M (IgM) levels than those of fish fed the high-P diet. However, serum lysozyme and antibody production did not differ between fish fed low-P or high-P diets.

Potassium

Potassium is the third-most abundant element in the body, after calcium and phosphorus. Potassium and sodium are closely interrelated in the maintenance of proper osmotic pressure within cells and, along with other minerals, are involved in the maintenance of proper acid-base balance. Potassium ions relax muscles and are used in enzyme reactions. Excessive levels of potassium could interfere with magnesium absorption.

Sodium

Sodium is the major positively charged ion (cation) in the fluid outside the cell (extracellular fluid) where it assists in the maintenance of osmotic balance and acid-base balance. Sodium is also associated with muscle contraction, nerve function, and carbohydrate absorption. Sodium, potassium, and chloride are all central components to maintain osmotic pressure and acid-base equilibrium.

Microminerals

Except for iron (Fe), little is known about the effects of dietary trace elements (manganese, zinc, selenium, chromium, iodine, and copper) in fish immunity and disease resistance. Many trace minerals have biological functions that affect the host immune system and defense mechanisms.

Chromium

Chromium (Cr) is an important micronutrient that is essential for humans and animals. It has been shown to have a beneficial influence on growth, reproductive performance, and carcass composition, as well as enhance immune response in cows (Burton et al. 1996; Kegley et al. 1996); however, no beneficial effects of chromium on immunity were found in pigs (van Heugten and Spears 1997) or cattle (Kegley et al. 1997). In water, Cr has two ionic forms: hexavalent Cr (Cr-6) and trivalent Cr (Cr-3). Hexavalent Cr is used in industrial processes and is much more toxic than Cr-3. Cr-6 rapidly enters the cell and becomes reduced to Cr-3; however, during this process it forms reactive intermediates which can cause DNA damage, alter gene expression, and damage cellular membranes. Cr-3 is an essential nutrient and is required in energy metabolism. Exposure of Mossambique tilapia, *Oreochromis mossambicus*, to Cr-6 in water at 5 mg L^{-1} resulted in reduced antibody titer, serum lysozyme level, and plaque-forming cells and increased mortality to *Aeromonas hydrophila* challenge than fish exposed to 0–0.05 mg L^{-1} of Cr-6 (Prabakaran et al. 2006). This corroborates the findings of Arunkumar et al. (2000) who reported that injection of Cr-3 had little effect on immune parameters of Mossambique tilapia, but that Cr-6 suppressed immune function.

Chromium is an essential element that does not work alone, but instead acts with other substances to control metabolism. Chromium, in the form of glucose tolerance factor (GTF), is released into the blood whenever there is a dramatic increase in glucose and/or insulin levels. GTF and insulin both act to allow for easier passage of amino acids, fatty acids, and sugars from the blood into the cells of tissues. Chromium, as well as other metals, can also activate the digestive protease trypsin.

Feeding juvenile (56 g) rainbow trout, *Onchorynchus mykiss*, for 6 weeks on diets containing Cr in the form of a commercial chromium yeast at levels of either 1540 ppb Cr (control), 2340 ppb Cr (supplemented), and 4040 ppb Cr (enhanced) resulted in significant, but minor, changes to various immune system parameters (Gatta et al. 2001). White blood cell count and serum hemolysis were not different among treatments, but fish fed the diet containing 2340 ppb Cr (supplemented) had significantly lower red blood cell count and hematocrit compared to fish fed the control and enhanced diets. Further, fish fed the supplemented diet had significantly higher serum lysozyme activity after 6 weeks compared to fish fed the other two diets. Fish fed the supplemented and enhanced diets both had significantly higher percentages of phagocytic activity of head kidney macrophages compared to fish fed the control diet (Gatta et al. 2001).

Results from Gatta et al. (2001) are in contrast to those of Ng and Wilson (1997) who reported that they were unable to determine any differences in hematocrit in channel catfish fed diets containing various levels of Cr. However, in that study, chromium oxide was added instead of chromium yeast. Since chromium yeast was used in the rainbow trout study by Gatta et al. (2001), it could be that the results observed were due to the increase in Cr bioavailability and/or to the yeast subcomponents. Serum lysozyme levels and serum hemolytic complement activity in rainbow trout were positively affected by increasing dietary Cr levels.

Copper

Copper (Cu) is a co-factor for enzyme systems that utilize antioxidants, such as superoxide dismutase, and for enzymes used in the electron transport chain; it is also required for normal functioning of the brain, skeleton, and spinal cord. In addition, copper is involved with iron metabolism, as it facilitates the absorption of iron from the intestine and releases iron from storage in the liver. However, excess amounts of cadmium, iron, lead, and zinc reduce the utilization of copper. Copper is relatively non-toxic to most monogastric species; however, fish can be very susceptible to Cu toxicity if exposed to copper in the water. High levels of dietary Cu can reduce growth, increase mortality, increase oxidative stress, and reduce immune response (Krox et al. 1982; Baker et al. 1998; Berntssen et al. 1999; Shiau and Ning 2003). Fish can obtain Cu from water as well as through dietary means, but it is difficult to meet Cu requirements from the culture water alone so a dietary source is needed.

It has been reported that grouper, *Epinephelus malabaricus*, fed a diet containing Cu and selenium (Se) at levels that met requirements (5 mg Cu kg^{-1} of diet and 0.77 mg Se kg^{-1} of diet) had higher weight gain, leukocyte superoxide anion production, plasma total immunoglobulin (Ig) concentration, and plasma lysozyme activity compared to fish that were fed a diet containing a higher level of Cu (21 mg Cu kg^{-1} of diet) and a low level of Se (0.16 mg Se kg^{-1} of diet) (Lin and Shiau 2007); this indicates that levels of Cu that exceed the requirement in grouper impair immune function unless dietary levels of Se are also increased.

In a feeding study with blunt snout bream, *Megalobrama amblycephala*, plasma ceruloplasmin, alkaline phosphatase, acid phosphatase, and nitric oxide had variable responses when fish were fed diets containing different (0, 3, 6, 9, 25, 50, 100, and 150 mg Cu kg^{-1} of diet) levels of Cu (as tribasic copper chloride) for 56 days (Shao et al. 2012). At the conclusion of the feeding trial, there were no clear trends on the effects of Cu on the various immune parameters measured. It was reported that ceruloplasmin was significantly higher in fish fed the 150 mg Cu kg^{-1} diet compared to all other treatments. Alkaline phosphatase levels, which have been reported to be a good indicator of a strong immune system (Xing et al. 2002), were significantly higher in fish fed the 150 mg kg^{-1} of feed compared to fish fed a diet containing 0 mg kg^{-1} of feed; however, there were no differences among any other dietary treatment. Acid phosphatase, which has also been associated with a properly functioning immune system (Cheng 1989), was significantly higher in fish fed a diet containing 100 mg Cu kg^{-1} of feed compared to all other treatments. Nitric oxide, a component of the innate immune system that participates in the elimination of pathogens, was significantly higher in fish fed a diet containing 25 mg Cu kg^{-1} of feed compared to all other dietary treatments, except fish fed a diet containing 9 mg Cu kg^{-1} of feed. No disease challenge was conducted to determine the effects of dietary copper levels on disease resistance.

Iron

Iron (Fe) is an essential mineral in the formation of red blood cells. Iron combines with protein to make hemoglobin, the iron-containing compound of red blood cells. Iron is involved in transporting oxygen via red blood cells and is also a component of enzymes that are utilized in energy metabolism. A deficiency of iron may cause nutritional anemia, which is characterized by small, pale-red blood cells (hypochromic microcytic anemia). An excess of iron in the diet can bind with phosphorus to form an insoluble iron-phosphate complex that could lead to a phosphorus deficiency. Since free iron is toxic, the iron molecule is transported along with a protein. Two atoms of ferric iron are bound to one molecule of transferrin, a beta-globulin protein. If the level of iron ions exceeds the binding capacity of transferrin, iron toxemia may occur.

Iron is a trace mineral of particular interest because of its effect on immune system function and host defense against infection (Beisel 1982; Bhaskaram 1988). It is an essential element due to its function in oxygen transport and cellular respiration. Iron has been shown to be an essential dietary nutrient for several fish species that can have profound effects on immune response and resistance of fish to infectious diseases. A deficiency or excess of Fe could compromise the immune system and therefore resistance to infection (Lim et al. 2001).

Diets supplemented with an inorganic form of Fe (up to 1200 mg Fe kg^{-1}) or heme Fe (up to 300 mg Fe kg^{-1}) had no effect on either non-specific immune responses, such as serum bactericidal activity, phagocytic activity, and differential leukocyte count, or disease resistance of Atlantic salmon against *V. anguillarum*. Diets containing 100 mg heme Fe kg^{-1} and 300 mg Fe kg^{-1} from inorganic Fe showed the highest respiratory burst activity. During the vibrio infection, fish showed rapid depletion of Fe from spleen, liver, and kidney, particularly those fed low-Fe diets (Naser et al. 1998). In another study with Atlantic salmon, supplementation of 400 mg Fe kg^{-1} to a basal diet containing 160 mg Fe kg^{-1} had no effect on serum total protein, antibody level, hemolytic complement activity or lysozyme activity (Anderson et al. 1998). Nakai et al. (1987) reported that availability of free Fe following intramuscular injection of ferric ammonium citrate increased the virulence of *V. anguillarium* infection in eels and ayu. Ravndal et al. (1994) suggested a positive association between a high concentration of serum Fe and mortality of Atlantic salmon infected with *V. anguillarum*.

Lim and Klesius (1997) fed channel catfish diets that were either deficient or replete in Fe for 13 weeks. After this period of time, they switched the diets in half of the treatments, while the remaining fish were fed their original diet. They reported that fish fed a diet replete with Fe had significantly higher final weight, serum iron, hematocrit, and total blood cell count than fish fed a diet deficient in iron. Further, fish fed a diet replete with Fe had higher macrophage chemotaxis ratio than fish fed a diet deficient in iron. When challenged with *E. ictaluri*, channel catfish fed an iron-deficient diet started to die on day-1 post-challenge, while the fish fed a diet replete in Fe started to die on day-5 post-challenge (Lim and Klesius 1997). However, after 7 days post-challenge, both treatments had 100% mortality.

While it is generally believed that anemic organisms are more susceptible to infection than those with adequate iron, the results from Lim and Klesius (1997) appear to contradict this notion, as fish fed either an iron-deficient diet or an iron-sufficient diet had similar mortality when challenged with a bacterial pathogen. This is in agreement with Barros et al. (2002) who found no differences in cumulative mortality to *E. ictaluri* challenge of channel catfish fed three levels (40, 336, and 671 mg kg^{-1} of feed) of iron added to diets containing either cottonseed meal, soybean meal, or a mixture of the two ingredients. Similarly, the amount of Fe did not affect hematocrit, hemoglobin, mean macrophage migration, macrophage chemotaxis ratio, and antibody titer. This is in agreement with Ye et al. (2007) who reported no differences in red blood cell, hemoglobin, and hematocrit in grouper, *Epinephelus coioides*, fed various levels of iron (in the form of ferrous sulfate). Growth of grouper was very low however, possibly due to suboptimum water temperature, which could make the data unreliable.

In contrast, Ravndal et al. (1994) reported that Atlantic salmon fed diets deficient in iron had higher percentage survival when challenged with *Vibrio anguillarum* compared to fish fed diets with sufficient iron; however, Sealey et al. (1997) observed increased mortality of Fe-deficient channel catfish following challenge by bath immersion with *E. ictaluri*. More

recent studies showed that dietary Fe does not protect against mortality of channel catfish from *E. ictaluri*, but the average number of days at which the first mortality occurred after *E. ictaluri* challenge was significantly earlier for fish fed Fe-deficient diets (Lim and Klesius 1997; Lim et al. 2000). Sealey et al. (1997) observed that supplementation of 180 mg Fe kg^{-1} of feed from iron sulfate, but not from iron methionine, resulted in increased mortality of channel catfish challenged with *E. ictaluri*. They suggested that the total dietary Fe level of 30 mg kg^{-1} required for optimum growth and prevention of anemia in channel catfish may be sufficient for optimum immune response and resistance to *E. ictaluri*.

It may be that once the amount of dietary Fe is near the requirement for the organism, adding excess Fe will have no benefit regarding immunological effectiveness. This appears to be possible as it has been reported that when the amount of dietary iron is between 50% and 76% of the requirement (120–198 mg kg^{-1} of feed) in the soft-shell turtle, *Pelodiscus sinensis*, all hematological parameters measured (red blood cell count, hemoglobin, hematocrit, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration) were similar for turtles fed 92–483 mg Fe kg^{-1} of feed (Chu et al. 2009).

Sealey et al. (1997) reported that Fe deficiency did not significantly suppress antibody titer of channel catfish in response to formalin-killed *E. ictaluri*. Engulfment of bacteria by macrophages as measured by chemi-luminescence was not affected by Fe deficiency. It appeared, however, that maximum phagocytic engulfment of opsonized *E. ictaluri* by macrophages was observed in fish fed the 60 mg supplemental Fe per kilogram of feed from either iron sulfate or iron methionine. They also suggested that antibody titer and engulfment of bacteria by macrophages were generally unaffected by the source of dietary Fe. Macrophage chemotaxis, on the other hand, was significantly suppressed for fish fed the diet with no Fe supplementation (Lim and Klesius 1997; Sealey et al. 1997). The chemotactic ratio also decreased when the group fed the Fe-replete diet (30 mg total Fe kg^{-1} of feed) was switched to a Fe-deficient diet for 4 weeks. However, macrophage chemotaxis was reversed when the Fe-deficient catfish were fed a Fe-replete diet (Lim and Klesius 1997). In another study with channel catfish, Lim et al. (2000) obtained higher macrophage chemotaxis in fish fed a diet containing 30 mg Fe kg^{-1} as compared to those fed an Fe-unsupplemented diet or the diet supplemented with 300 mg Fe kg^{-1} of feed. The suppression of macrophage chemotactic response was however more pronounced in fish fed the Fe-deficient diet than in those fed Fe-excessive diets.

Two reports investigated possible synergistic effects of Fe and vitamin C. Andersen et al. (1998) fed Atlantic salmon diets containing Fe at either 160 mg kg^{-1} of feed or 600 mg kg^{-1} of feed and 150 mg kg^{-1} of ascorbic acid (AA) as either ethyl cellulose-coated AA or AA polyphosphate. After 20 weeks of feeding, there were no significant differences in the final weight of fish in any treatment, nor were there any correlations between hepatic Fe and hepatic AA concentrations. Serum total antibody, specific hemolytic complement activity, spontaneous hemolytic activity, serum lysozyme activity, serum total protein, lysozyme activity of head kidney, and lysozyme activity of spleen were not significantly different among treatments (Andersen et al. 1998). In agreement, no interactions between dietary levels of Fe and ascorbic acid on weight gain were reported by Lim et al. (2000) in channel catfish. Fish fed diets without Fe supplementation had significantly lower total cell count, red blood cell count, hematocrit, and hemoglobin compared to fish fed iron-sufficient diets (30 and 300 mg kg^{-1} of diet). When channel catfish were challenged with *E. ictaluri*, fish fed iron-deficient diets died earlier but cumulative mortality after 14 days was not different among treatments.

Iodine

Iodine (I) is an essential component of the thyroid hormones thyroxin and tri-iodo-thryronine, which regulate the rate of oxidation within cells. In so doing, the thyroid influences physical growth, nerves, and muscle tissue functions, circulatory activity, and metabolism. Some aquaculture diet ingredients, such as mustard seed, can contain goitrogens which interfere with the use of thyroxin and may produce goiter. Thyroid hormones are essential for flatfish metamorphosis (Schreiber and Specker 1998) and are required for synthesis of pepsinogen in summer flounder, *Paralichthys dentatus*, larvae (Huang et al. 1998).

Woodall and LaRoche (1964) found that in Chinook salmon, *Oncorhynchus tshawytscha*, the incidence of bacteria kidney disease was related to the level of I in the diet. Lall et al. (1985) reported that diets containing high levels of I and fluorine (Fl) reduced prevalence of bacterial kidney disease in Atlantic salmon. Increasing dietary levels of other minerals (Mg, Mn, Ca, Zn, Cu, Co, and Fe) reduced prevalence of bacterial kidney disease, but to a lesser degree than the diet with additional I and Fl. Atlantic cod, *Gadus morhua*, larvae that were fed rotifers enriched with iodine and selenium had higher survival than larvae fed un-enriched rotifers, but did not have different final body weights (Hamre et al. 2008); however, it was not possible to discern which mineral was responsible.

Manganese

Manganese (Mn) is involved in the formation of bone, blood-clotting, insulin function, and cholesterol synthesis, and is an activator in several enzymes in the metabolism of proteins, lipids, carbohydrates, and nucleic acids. Manganese acts through its ability to sustain proper enzyme activity as a metal co-factor for a number of enzymes that form metal-enzyme complexes. Deficiency of Mn and Zn reduced leukocyte natural killer activity of rainbow trout, and supplementation of these minerals restored that activity (Inoue et al. 1998). However, supplementation of both Mn and Zn at over 100 mg kg^{-1} diet did not enhance resistance of sockeye salmon, *Oncorhynchus nerka*, to bacterial kidney disease from *Renibacterium salmoninarum* (Bell et al. 1984). Dietary Mn levels up to 24 mg kg^{-1} for 12 weeks did not affect the mortality after bath challenge of Atlantic salmon with a virulent strain of *V. anguillarium* (Maage et al. 2000).

Selenium

Selenium (Se) is a rare, non-metallic element that is important to maintain health of animals under stressful conditions. Vitamin E and the amino acids cystine and methionine may act as partial substitutes for Se in some of their functions. Biological availability from various ingredients for Se varies. For instance, only about 30–50% is available in fish meal, possibly due to Se being bound to mercury and other heavy metals.

Fish diets with high percentages of polyunsaturated fatty acids (PUFA) but reduced levels of vitamin E might increase the requirement for Se because PUFA may be converted to toxic peroxides unless there is sufficient vitamin E to prevent this process; selenium is required to activate the enzyme glutathione peroxidase. Glutathione peroxidase reduces hydrogen peroxide or organic hydroperoxides and oxidizes glutathione to glutathione disulfide; it may also mediate hydrogen peroxide concentrations. Selenium is the center of glutathione peroxidase in the form of selenocysteine. The enzyme catalyzes reactions required for the conversion of hydrogen peroxide and fatty acid hydroperoxides into water and fatty acid alcohol by using a reduced glutathione. This protects cell membranes from oxidative damage from the effects of heavy metals by formation of selenium-metal protein and selenide-metal complexes (Levander 1986; Rana and Verma 1997). However, Se can also be toxic to fish. High levels of Se can react with sulphydryls, which can decrease amounts of glutathione and increase lipid peroxidation. It has been reported that a deficiency or excess of dietary Se resulted in reduced growth, increased lipid peroxidation, tissue degeneration, higher mortality, and decreased glutathione peroxidase activity in fish (Poston et al. 1976; Hilton and Hodson 1983; Gatlin and Wilson 1984; Deng et al. 2007; Wang et al. 2007).

Se acts as an antioxidant, like vitamin E, to protect the cell against oxidative damage and to protect polyunsaturated phospholipids from oxidative damage. However, the antioxidants act differently. Selenium functions throughout the cytoplasm to destroy peroxides, while vitamin E is present in the membrane constituents of the cell and prevents peroxide formation. The requirements for each nutrient can therefore be partially offset by addition of the other. Further, when diets are deficient in the amino acids cystine and methionine, selenium requirements may increase. This is due to the two sulfur amino acids being converted to glutathione, which has a limited ability to carry out the functions of glutathione peroxidase.

Because of its roles in protecting cells and cell membranes against oxidative damage, selenium plays an important role in maintaining normal immune response. Selenium deficiency in animals decreased antibody responses especially if associated with vitamin E deficiency, while Se supplementation has been shown to positively affect the resistance of animals against infections (Combs and Combs 1986;

Dhur et al. 1990). In fish, Thorarinsson et al. (1994) reported that chinook salmon fed a diet deficient in Se had higher mortalities when exposed to *R. salmoninarum* compared to fish fed a diet in which Se was at required levels. Selenium may positively affect immune function in fish through its role in antibody production through proliferation and influence of glutathione peroxidase activity (GSH-Px) on B-lymphocytes. Further, GSH-Px may protect cell membranes against damage during phagocytosis. Hydrogen peroxide is produced during phagocytosis as peroxide kills pathogenic organisms; however, cell membranes are susceptible to damage due to peroxide if it enters the cytoplasm.

When channel catfish were fed diets containing either 0.0 mg Se kg^{-1} of feed or 0.2 mg Se kg^{-1} of feed at two levels of vitamin E (0 or 60 mg vitamin E from alpha-tocopherol acetate per kilogram of diet), it was found that red blood cell peroxidation was significantly higher in fish fed diets without vitamin E, but was similar if vitamin E was present (Wise et al. 1993). Red blood cell peroxidation in fish fed a diet that contained four times the recommended level of vitamin E was not different from fish fed the recommended (60 mg kg^{-1} of feed) level of the vitamin. Glutathione peroxidase activity was significantly higher in livers of fish fed a diet containing four times the recommended level of Se (0.8 mg Se kg^{-1} of feed) compared to fish fed diets deficient in Se, but was not different compared to fish fed the recommended level of Se (0.2 mg Se kg^{-1} of feed), although the value was numerically higher (twice as high). The importance of the presence of Se in diets for adequate glutathione peroxidase activity has also been reported in Atlantic salmon (Poston et al. 1976) and rainbow trout (Hilton et al. 1980).

Wise et al. (1993) evaluated extracellular and intracellular superoxide anion production of kidney macrophages from channel catfish fed diets containing various levels of vitamin E and selenium from sodium selenite. They reported that extracellular superoxide anion production was not affected by dietary treatments. However, intracellular superoxide anion production was higher in the fish fed four times the normal requirements of Se and vitamin E, but not in fish fed the normal levels required for growth (0.2 mg Se kg^{-1} and 60 mg vitamin E kg^{-1}).

This is consistent with the report of another study that showed that channel catfish fed a diet deficient in Se had reduced hepatic glutathione peroxidase activity and superoxide anion production in macrophages compared to fish fed a diet containing Se. Wang et al. (1997) fed small (1.7 g) channel catfish diets containing various levels of Se from three different sources (sodium selenite, DL-selenomethionine, and selenoyeast) for 9 weeks. At the conclusion of the feeding trial, the fish were challenged with *E. ictaluri*. Fish fed a diet not supplemented with Se had more than 90% mortality after 12 days post-challenge. Mortality did not decrease with supplementation of 0.02 mg Se kg^{-1} of feed regardless of source, but did with higher levels of supplementation. Further, the source of Se determined the concentration that corresponded with minimum mortality: 0.20 mg kg^{-1} of feed for fish fed a diet with selenomethionine and 0.40 mg kg^{-1} of feed for fish fed diets supplemented with sodium selenite and selenoyeast.

Serum antibody titers were also influenced by amount and source of Se used (Wang et al. 1997). Antibody titer did not increase in fish fed a diet with sodium selenite until the level reached 0.40 mg kg^{-1}, while antibody titers of fish fed selenomethionine and selenoyeast showed an increase at 0.06 mg kg^{-1}, but had the highest level in fish fed a diet containing 0.40 mg kg^{-1} as selenoyeast. Macrophage chemotaxis was highest in fish fed selenomethionine followed by fish fed selenoyeast, while fish fed sodium selenite had the same level as fish fed a diet deficient in Se (Wang et al. 1997).

In general, it was found that organic sources of Se were more effective than inorganic Se for immune response (Wang et al. 1997). This is in agreement with an earlier report (Wang 1996) that found that availability of Se from selenomethionine (organic source) was 336% of that from sodium selenite (inorganic source). This could be due to the fact that chelated sources of Se may be absorbed and transported to tissues more rapidly and completely than inorganic sources. Whole-body retention of Se in rainbow trout was greater for fish fed an organic (Se-yeast) source compared to fish fed sodium selenite, an inorganic source (Rider et al. 2009). In unstressed fish, hepatic glutathione peroxidase and hepatic thioredoxin reductase activities were similar among fish, regardless of the source or level of Se added, indicating that Se requirement was met with feeding the unsupplemented diet which had 0.73 mg Se kg^{-1}

of feed. This is in agreement with a previous report in Atlantic salmon (Lorentzen et al. 1994). However, when rainbow trout were handled and confined, whole-body Se decreased and glutathione peroxidase activity increased (Rider et al. 2009), indicating an increase in Se requirement that is consistent with data from Halver et al. (2004) for chinook salmon. While Se supplementation enhanced glutathione peroxidase activity in stressed rainbow trout, it lacked an effect on immune function and health, which had no differences in extracellular superoxide production, hematocrit, lysozyme activity, and respiratory burst activity among treatments, indicating that when the Se requirement is met, no enhancement effect on immune function is observed.

Chinook salmon sub-clinically infected with *R. salmoninarum*, the etiological agent of bacterial kidney disease, and reared in seawater were sensitive to Se and vitamin E deficiency (Thorarinsson et al. 1994); mortality significantly increased among fish fed the Se and vitamin E unsupplemented diets. Wang et al. (1997) showed that channel catfish challenged with *E. ictaluri* were also sensitive to Se deficiency, and that the source of Se affected the rate of mortality. Mortality significantly decreased when Se was increased to meet the growth requirement. At this supplemental level, fish fed the selenomethionine diet exhibited significantly lower mortality than fish fed the sodium selenite diet. Mortality value was intermediate for fish fed the selenoyeast diet. They suggested that dietary Se concentrations for maximum survival from *E. ictaluri* challenge was 0.2 mg kg^{-1} for fish fed selenomethionine and 0.4 mg kg^{-1} for fish fed selenoyeast and sodium selenite.

Use of Se, ascorbic acid, and alpha-tocopheryl acetate to enhance growth and increase immune response in Nile tilapia, *Oreochromis niloticus*, has been investigated. Diets were formulated to contain either recommended levels of the three nutrients (control), an excessive level of one of the nutrients while the other two had levels of the control diet; and one diet had excessive levels of all three nutrients. After 10 weeks, fish fed excessive levels of ascorbic acid, alpha-tocopheryl acetate, and all three nutrients had significantly higher weight gain, feed efficiency ratio, protein efficiency ratios, and specific growth rate compared to fish fed the control diet and the diet with excessive levels of Se (Kim et al. 2003). It therefore appears that feeding diets with greater than 0.2 mg Se kg^{-1} of diet does not increase growth. Further, there were no significant differences in cumulative mortality when the fish were challenged with *E. tarda*, indicating that Se does not enhance immune response at levels greater than 0.2 mg Se kg^{-1} of diet.

Lin and Shiau (2007) conducted a feeding trial to determine the effects of dietary Se on the oxidative stress of Malabar grouper, *Epinephelus malabraricus*, fed diets containing various levels of copper (Cu). High levels of Cu caused growth reduction, increased mortality, oxidative stress, and reduced immune response in fish. In their study, Lin and Shiau (2007) fed Malabar grouper a basal diet with a high level (20 mg kg^{-1}) of Cu supplemented with various levels of Se (0.0, 0.8, and 1.6 mg Se kg^{-1}). Weight gain and feed efficiency were significantly higher in fish fed the diet containing 0.8 mg Se kg^{-1} compared to fish fed the diet containing 0.0 mg Se kg^{-1}. Addition of high levels of Se (1.6 mg Se kg^{-1}) did not offer additional benefit to grouper in terms of weight gain, feed efficiency, and survival. However, addition of increased levels of Se did offer protection against oxidative damage to tissues caused by high Cu ingestion as indicated by increased glutathione peroxidase activity and lower thiobarbituric acid reactive substances (TBARS) values. Glutathione peroxidase protects cell membranes against oxidative damage. These data also suggest that dietary Se supplementation prevents tissue Cu accumulation and oxidative damage in grouper fed high-Cu diets. Diets with 0.8 and 1.6 mg added Se per kilogram feed increased leukocyte respiratory burst activity and plasma lysozyme activity in grouper compared to fish fed the control diet with high Cu levels, suggesting that immunity was impaired due to oxidative stress induced by high Cu ingestion.

When cod larvae were fed rotifers, *Brachionus plicatilis*, enriched with Se, glutathione peroxidase activity was similar to larvae fed un-enhanced rotifers at 7 and 29 days post-hatch, but was significantly higher at day 17 post-hatch (Penglase et al. 2010). Further, mRNA expression of glutathione peroxidase 1 (cytosolic) and glutathione peroxidase 3 (plasma) was significantly higher in cod larvae fed the Se-enriched rotifers, but mRNA activity of glutathione peroxidase 4 (phospholipid) was similar between both treatments. The differences observed may be due to rotifers' deficiency in Se for larval cod; results may not have

been confirmed if a live food item contained Se at a level that met the requirement for the larvae.

Zhu et al. (2011) fed largemouth bass, *Micropterus salmoides*, diets containing various (0.97, 1.17, 1.42, 1.60, 1.85, and 2.06 mg Se kg^{-1} of diet) levels of Se (as sodium selenite) and found that weight gain was not significantly different in fish fed diets containing 1.17 mg Se kg^{-1} of feed or higher, but that fish fed a diet containing 1.60 mg Se kg^{-1} of diet had higher weight gain than fish fed 0.97 mg Se kg^{-1} of feed. Glutathione peroxidase activity increased as dietary level of Se was increased, while glutathione reductase generally decreased with increasing Se levels.

Wang et al. (2012) reported that glutathione peroxidase activity in serum of juvenile abalone, *Haliotis discus hannai*, significantly increased linearly as dietary selenium increased, while phenoloxidase was higher in abalone fed diets containing 0.15–0.88 mg Se kg^{-1} of diet compared to abalone fed diets containing 2.63 and 9.16 mg Se kg^{-1} of diet. Lysozyme activity was significantly higher in abalone fed a diet containing 0.88 mg Se kg^{-1} of feed compared to organisms fed diets containing 2.63 and 9.16 mg Se kg^{-1} of feed, but was not different compared to abalone fed diets containing 0.15, 0.53, and 1.55 mg Se kg^{-1} of feed. The data for glutathione peroxidase activity are similar to those reported in previous studies (Wan et al. 2004; Wu et al. 2010).

The effects of different sources and amounts of Se on the immune response and disease resistance of giant freshwater prawn, *Macrobrachium rosenbergii*, were evaluated by Chiu et al. (2010). In that study, prawns were fed a diet without Se added (control); a diet with 0.5 mg kg^{-1} of diet of seleno-L-methionine; 1.0 mg kg^{-1} of diet of seleno-L-methionine; 0.5 mg kg^{-1} of diet of sodium selenate; and 1.0 mg kg^{-1} of diet of sodium selenate for 75 days in indoor tanks. After 75 days, prawns were randomly selected for challenge with *Debaromyces hansenii* by injection into the ventral sinus of the cephalothorax. Cumulative mortality of prawns in the control group (no Se added) was significantly higher ($P < 0.05$) (80%) than prawns fed the diets with added Se. Prawns fed diets with 0.5 mg kg^{-1} of feed (either form of Se) had numerically lower mortalities (30%) compared to all treatments, but were not significantly different ($P > 0.05$) from prawns fed 1.0 mg kg^{-1} of feed seleno-L-methionine (57%) or 1.0 mg kg^{-1} of feed sodium selenate (43%). Measurement of various immune parameters showed inconsistent and variable results without clear patterns of the effects of Se amount or form. Parameters measured were total hemocyte count, phenoloxidase activity, respiratory burst, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-s-transferase (GST), and phagocytic activity.

Zinc

Zinc (Zn) is present in the tissues of most living organisms and is an important micronutrient in fish. It is a specific cofactor in several enzymes; an important constituent of metalloenzymes, such as alcohol dehydrogenase and alkaline phosphatase; associated with prostaglandin metabolism; is vital for blood coagulation; required for erythrocyte membrane integrity; protects tissues from auto-oxidation; and may have a role in nucleoproteins. Fish can obtain Zn either through their diet or directly through water. Zinc deficiency signs in fish include reduced growth, lower digestibility of protein, cataracts, fin erosion, reduced appetite, and delayed wound healing.

In a study with channel catfish, *Ictalurus punctatus*, Scarpa et al. (1992) fed immunized and non-immunized fish diets that were deficient in Zn (2 mg kg^{-1} of feed), met Zn requirement (20 mg kg^{-1} of feed), or had an excess of Zn (200 mg kg^{-1} of feed) for 10 weeks. Fish were also challenged with *Aeromonas hydrophila* at the conclusion of the feeding experiment. It was reported that serum immunoglobulin (IgM) levels of non-immunized fish were not affected by dietary Zn, but that immunized fish showed significant differences among treatments, with fish fed a Zn-deficient diet having the highest IgM levels compared to the other two treatments. When challenged with *A. hydrophila*, mortality in non-immunized fish was lowest in fish fed Zn-deficient diet (0%) and highest in fish fed a diet that met requirement (41%). For immunized fish, supplementation of Zn had little effect on mortality, as all treatments experienced little (<10%) mortality.

Lim et al. (1996) showed that supplementation of Zn enhanced chemotactic response of channel catfish peritoneal macrophages to *E. ictaluri* exoantigen, and that zinc methionine was more effective than zinc sulfate in stimulating macrophage chemotaxis. A level of

60 mg Zn kg^{-1} of feed (as zinc sulfate) was required to attain a chemotactic response similar to that obtained with 5 mg Zn kg^{-1} of feed (as zinc methionine). However, the phagocytic activity of phagocytes to zymosan was suppressed by supplementation of Zn in the diets. Although Zn has been found to increased macrophage chemotaxis, it may have an inhibitory effect on phagocytosis.

Bell et al. (1984) reported no differences in the serum agglutinating antibody titer of immunized (with formalin-killed *A. salmonicida*) sockeye salmon, *Oncorhynchus nerka*, fed diets deficient in Zn or manganese. However, Paripatananont and Lovell (1995) found that low dietary Zn significantly reduced the agglutinating antibody response of channel catfish 14 days after challenge with *E. ictaluri*; maximum antibody titer was obtained with fish fed diets containing 15 mg Zn kg^{-1} as zinc methionine or at least 30 mg Zn kg^{-1} as zinc sulfate.

Paripatananont and Lovell (1995) found that dietary Zn influenced the resistance of channel catfish challenged with *E. ictaluri* and that zinc methionine was three to four times more potent than zinc sulfate in improving survival of channel catfish against this bacterium. However, Lim et al. (1996) observed that dietary Zn did not protect channel catfish against mortality from *E. ictaluri*. The intensity of the *E. ictaluri* infection, based on numbers of colony-forming units per gram of trunk kidney in fish three days post-challenge was not affected by dietary Zn. Also, there was no evident trend of the effect of dietary Zn on the percentage of fish infected with *E. ictaluri* 15 days post-challenge. However, examination of the data indicates that fish fed diets containing 0 mg Zn kg^{-1} of feed and 20 mg Zn kg^{-1} of feed (as zinc methionine) had mortality near 100%, while fish fed diets containing 20 mg kg^{-1} of feed as zinc sulfate and 60 mg kg^{-1} of feed as zinc methionine had less than 70% mortality.

Fountoulaki et al. (2010) evaluated diets containing various (0, 30, 70, 110, and 150 mg kg^{-1} of feed) levels of an organic Zn and one level (150 mg kg^{-1} of feed) of an inorganic Zn (Zn oxide) on European sea bass, *Dicentrachus labrax*. They found no significant differences in final weight of fish fed the different diets, nor were there any differences in wound healing among treatments. Respiratory burst activity also demonstrated very little difference among treatments or form of Zn supplementation. This lack of difference in growth and immune function is in agreement with Hu et al. (2001) who reported that juvenile grouper, *Epinephelus malabaricus*, fed Zn-supplemented diets showed no differences in phagocytosis, complement activity, agglutination titer, and lysozyme. However, Hung et al. (2007) found a decrease in superoxide anion production of hybrid tilapia fed diets containing 150 mg Zn kg^{-1} of feed or greater, although the authors reported an increase in lysozyme in fish fed a diet containing 150 mg kg^{-1} of feed.

Conclusions

Fish require the same minerals or inorganic elements as terrestrial animals for tissue formation, osmoregulation, and various metabolic functions. Those required in large quantities are termed macro- or major minerals and those required in small quantities are called micro- or trace minerals. Fish can absorb dissolved minerals from the water to satisfy part of their metabolic requirements. The dietary mineral requirements for growth and maintenance of normal physiological functions and health have been established (NRC 1993, 2011). However, little research has been carried out on the influence of dietary minerals on immune response and resistance of aquacultured fish to infectious microorganisms. Published information, which is limited to a few minerals (such as phosphorus, magnesium, chromium, copper, iron, iodine, selenium, and zinc), is rather inconsistent and contradictory. This discrepancy among the research results could be related to several factors such as differences in fish species, strain and size, composition and nutrient content of experimental diets, nutrient interrelationships, soluble minerals present in the water, experimental conditions, feeding management and duration, pathogenicity of infected organisms, and method and dose of challenge. Moreover, because most of this research was conducted under laboratory conditions, the applicability of the results to large-scale commercial settings is questionable. However, published information generally indicates that diets containing inadequate or excessive levels of dietary essential minerals can have marked effects on fish performance, immune system function, and health. In the absence of clear-cut information on the beneficial effects of dietary minerals on immune

response and resistance to infectious agents, essential dietary minerals should therefore be provided at levels sufficient to meet the requirements for growth and prevention of deficiency.

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Chapter 10
Antinutrients

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Introduction

Antinutrients are endogenous components in plants that may disturb digestion and/or alter biochemical, physiological, and immunological responses in organisms using the plants as nutrient sources. In the context of animal nutrition, they are considered negative because they may reduce feed efficiency, cause depression of growth performance, and degrade overall health. However, in the context of human nutrition, antinutrients may be beneficial due to their potential to prevent some lifestyle diseases and increase life expectancy (Pusztai et al. 2008). Their functions in plants may be to reduce the chance of being eaten, or they may play active roles in the metabolism of the plant; the functions may be the same in animals eating the plants or parts of the plant. Some antinutrients can be removed or inactivated through processing. Most plant feedstuffs used in the aquaculture feed industry need some processing to reduce the impact of the antinutrients; grinding, pressing, heating, fermentation, sprouting, and extraction are commonly used methods.

Research on the effects of antinutrients in animals, including domesticated land animals, is limited; there is a particular lack of information on fish. A main obstacle hindering our further understanding of antinutrient effects is the limited availability and high cost of purified preparations. Thus, semi-purified products or plant parts known to contain high levels of certain antinutrients are often used as an alternative. However, most antinutrients are not present alone and observed effects in such feeding experiments may be the result of interactions between two or more antinutrients. In any case, designing feeding trials to study antinutrient effects is challenging due to the possibility that the balance of macronutrients, micronutrients, and non-nutrients in a diet may modulate the antinutrient effects.

This chapter discusses the accumulated knowledge of selected antinutrients in the context of fish nutrition. Focus is on their sources (see Table 10.1 for overview) and chemical structure, and on effects on fish performance, physiological processes including immune functions. Compounds that may be present in feedstuffs as a result of suboptimal storage, such as mycotoxins and biogenic amines, as well as contaminants from the environment, including pollutants, are not discussed. Experiments with purified or semi-purified preparations of the antinutrients form the main basis for the presented information. When scientific information in fish is limited, this is stated and a selection of information from other animals is provided with the intention of shedding at least some light on possible effects and mechanisms of action in fish. Reviews published by Francis et al. (2001), Krogdahl et al. (2010) and by NRC (2011) have been

Dietary Nutrients, Additives and Fish Health, First Edition. Edited by Cheng-Sheng Lee, Chhorn Lim, Delbert Gatlin III, and Carl D. Webster.

Table 10.1 Presence of antinutrients in some common plant feedstuff meals, where x indicates the presence of an inhibitor at a level potentially affecting animal production and health. Adapted from the reviews of Tacon (1995) and Francis et al. (2001).

	Soybean	Kidney bean	Pea	Lupin	Sunflower	Cotton	Rape	Mustard	Sesame	Linseed	Alfalfa leaf
Protease inhibitors	x	x	x	x	x		x				x
Lectins	x	x	x								
Phytoestrogens	x	x		x		x				x	x
Phytic acid	x	x	x			x	x	x	x	x	
Gossypol						x					
Cyclopropenoic acid						x		x			
Glucosinolates	x						x	x			
Erucic acid							x	x			
Saponins	x	x	x	x	x						x
Alkaloids			x	x							

useful sources of information for the content of the present chapter.

Thiaminase

Structure, Sources, and Mechanism of Action

Thiaminases are enzymes produced by organisms that are able to synthesize thiamine, such as bacteria and some protozoa. They catalyze the reaction connecting or breaking thiamine's two ring structures (Fig. 10.1), and are necessary catalysts in the metabolism of thiamine. There seem to be two types of thiaminases: type I, which is quite ubiquitous; and type II (also named TenA), which is of mostly bacterial origin (Begum et al. 2011). The two types differ structurally but have similar mechanisms of action and lead to the same result (Bos and Kozik 2000; Benach et al. 2005; Toms et al. 2005).

Thiaminases are associated with intestinal organs in several fish, for example, herring species and carp, as well as in some crustaceans and molluscs (NRC 1982). Salmonids, cod, and flatfishes are among several species that do not usually contain thiaminases (NRC 1982). The origin of the thiaminases found in fish is not well understood. However, microorganisms and plankton inhabiting the intestines or consumed as

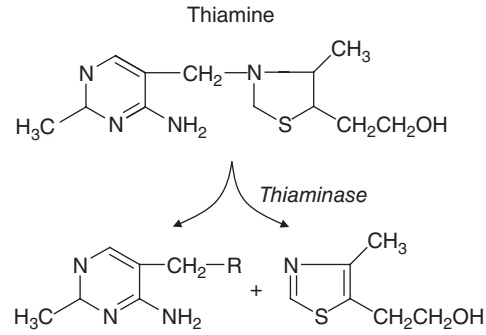

Figure 10.1 Thiamine and products of thiaminase activity.

nutrient sources may be the source (Tillitt et al. 2005; Riley et al. 2008; Riley and Evans 2008). Accordingly, fish offal has the highest thiaminase activity, whereas the carcass has very little or no activity. Storage of moist diets containing ingredients with thiaminase activity will reduce the thiamine content to very low levels within a few hours.

Thiaminases are efficiently inactivated by heat, whereas freezing has only a minor inactivating effect. The activity of the enzyme is dependent on pH; optimum pH levels, which have been studied for some thiaminases, have been observed to be in the range 5–7, indicating that acid preservation may also be a means of reducing activity (Zajicek et al. 2009).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Fish eating diets with high thiaminase activity have a high risk of developing thiamine deficiency with symptoms typical for this condition. A summary of present knowledge on thiamine deficiency is provided in NRC's Nutrient Requirements of Fish and Shrimp (NRC 2011). Deficiency signs vary among fish species. However, all species show reduced feed intake and performance, as well as increased mortality. Neurological symptoms such as loss of equilibrium, swimming in a spiral pattern, lethargy, and hyperexcitability have been observed in many but not all species. Thiamine deficiency due to ingestion of thiaminase-containing feed was recognized in the early years of the salmon industry when wet and moist feeds were common. Thiamine deficiency has also been observed in wild fish, particularly predators, and is a situation of concern for salmonids in the Baltic Sea (Wistbacka and Bylund 2008) and the Great Lakes (Tillitt et al. 2005; Riley et al. 2008), for example.

While investigating consequences of low thiamine supply on disease susceptibility in Nile tilapia (*Oreochronis niloticus)*, Lim et al. (2011) observed increased mortality upon challenge with *Streptococcus iniae*, but no apparent effects on the post-challenge antibody titre, complement, or lysozyme activities. Impaired disease susceptibility in a situation of thiamine deficiency has also been observed in juvenile Jian carp (*Cyprinus carpio* var. *Jian*) after injection with *Aeromonas hydrophila* (Feng et al. 2011). The following functions, which are necessary for disease resistance, were found to be impaired with decreasing dietary thiamine levels in the fish: leukocyte phagocytic activity, lectin potency, acid phosphatase activity, lysozyme activity, total iron-binding capacity, and immunoglobulin M levels. Observations during episodic outbreaks of bacterial diseases in wild lake trout populations support these conclusions that suboptimal thiamine status adversely affects disease resistance and physiological responses in fish challenged by disease. The most recent results indicate that T-cell dysfunction is a mechanism that underlies the symptoms of the immunological impairment that occurs following consumption of thiaminase (Ottinger et al. 2012).

Avidin

Structure, Sources, and Mechanism of Action

Avidin is a dimeric or tetrameric protein that strongly binds biotin, one molecule per monomer. Its affinity for biotin is among the highest recorded. The dissociation constant for the complex is about 10^{-15} M, making it one of the strongest-known non-covalent bonds (Green and Toms 1972; Livnah et al. 1993). In its tetrameric form with a carbohydrate content of about 10%, avidin's molecular weight is estimated to be between 66 and 69 kDa (Korpela 1984). Avidin is produced in the oviduct of birds, reptiles, and amphibians and deposited in the egg white (Helppolainen et al. 2007). The functions of the protein in the oviduct of these animals are not well understood, but it will inhibit growth of biotin-dependent bacteria and, as such, may serve as an antibacterial agent. An insecticidal function is also possible, as indicated by studies of transgenic maize expressing avidin that conferred resistance to insect attacks during storage (Kramer et al. 2000). Avidin has great heat stability compared to other proteins, but heating above 70°C efficiently eliminates its biotin-binding ability (Durance and Wong 1992). Avidin is also quite resistant to proteolytic enzymes present in the gastrointestinal tract, and it prevents absorption of biotin from the intestinal content; biotin deficiency is therefore the likely and main effect of avidin consumption. In order to prevent development of biotin deficiency, byproducts from the egg industry need to be treated with heat. The need to heat these potentially highly nutritious byproducts is well-known, for example to the fur animal industry (Wehr et al. 1980). Biotin deficiency in reptiles and dogs as a result of consumption of raw eggs is also an issue for owners and veterinarians.

Biotin is one of the B-vitamins that functions as a cofactor of four important enzymes: propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC), methylcrotonyl-CoA carboxylase, and acetyl-CoA carboxylase. These participate in the metabolism of proteins, lipids, and carbohydrates. A biotin deficiency caused by avidin therefore results in impaired function of these enzymes. The common symptoms in mammals include anorexia, hair loss, dermatitis, anemia, reproductive failure, increased susceptibility

to microbial infections, and death, which are quite unspecific and often observed as results of deficiencies of numerous water-soluble vitamins.

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

The primary signs of biotin deficiency and biotin requirements have been investigated in several fish species (NRC 2011). They seem to differ between developmental stages within and between species. However, a few non-specific symptoms, such as anorexia and growth retardation, affect all. Alterations in hepatic pyruvate carboxylase and acetyl-CoA carboxylase activities have been used to evaluate the biotin status in hybrid tilapia (Shiau and Chin 1999); Asian catfish (*Clarias batrachus*) (Mohamed et al. 2000); and Indian catfish (*Heteropneustes fossilis* Bloch) (Mohamed 2001). Younger fish may show more severe symptoms than older, more developed fish. Alterations in skin coloration, such as depigmentation and darkening, have been observed in channel catfish (*Ictalurus punctatus*) and Japanese eel (*Angulilla japonica*), respectively (Arai et al. 1972; Robinson and Lovell 1978). Histological signs of biotin deficiency have been observed in gills, liver, and kidney of rainbow trout (*Oncorhyncus mykiss*) (Castledine et al. 1977, 1978), and in gills of lake trout (*Salvelinus namaycush*) (Poston and Page 1982). Convulsions and high mortality were observed in Asian catfish and Indian catfish (Mohamed et al. 2000; Mohamed 2001).

The effects of ingested avidin have been investigated in various fish species, and the symptoms that commonly result from longer-term feeding are typical of primary biotin deficiency. A dose response trial with zebra fish (*Danio rerio*) showed that avidin levels at or above a molar ratio of 60:1 (avidin: biotin) caused biotin deficiency with anorexia, growth retardation, and decreased feed utilization efficiency (Kuroishi et al. 2009; Yossa et al. 2011). Gene expression studies showed elevated levels of biotin-dependent enzymes, that is, acetyl-CoA carboxylase-A, methylcrotonyl-CoA carboxylase, and propionyl-CoA carboxylase-A. A similar study has been conducted with Nile tilapia with very similar results, that is, lethargy, anorexia, circular swimming, and convulsions, which ultimately led to death. Effects on expression of genetic coding for enzymes involved in pathways dependent on biotin were also observed (Sarker et al. 2012). Dietary inclusion of avidin has also been found to cause biotin deficiency in channel catfish with symptoms such as growth retardation, anemia, and depigmentation (Robinson and Lovell 1978).

No data have been found regarding the effects of biotin deficiency on immune responses and disease susceptibility in fish. However, studies on the involvement of biotin in immune functions and inflammatory responses in other animals have led to a good understanding of the process. In biotin-deficient rats, decreased antibody response against diphtheria toxoid has been observed, as well as lower numbers of antibody-forming cells in the spleen of animals challenged by injection of sheep erythrocytes (Pruzansky and Axelrod 1955). Moreover, serum tumor necrosis factor (TNF)-concentration was elevated in biotin-deficient mice after intravenous administration of lipopolysaccharide (LPS). A study of biotin deficiency in a murine macrophage cell line has shown transcriptional upregulation of TNFα (Kuroishi et al. 2009). In human peripheral blood mononuclear cells, biotin deficiency was observed to decrease expression of genes encoding interferon-γ, interleukin-1β, and 3-methylcrotonyl-CoA carboxylase, and increase expression of interleukin-4 (Wiedmann et al. 2003). It is therefore likely that fish will display alterations in the immune system as a result of biotin deficiency and/or exposure to avidin.

In conclusion, ingredients containing avidin may be useful nutrient sources for fish and other animals, but inactivation is necessary to avoid biotin deficiency and subsequent negative metabolic and immunological consequences. The high heat stability should be kept in mind and sufficient heating should be applied (Wehr et al. 1980; Quigley 2002).

Protease Inhibitors

Structure, Sources, and Mechanism of Action

The so-called Kunitz trypsin inhibitor in soybeans was the first protease inhibitor discovered (Kunitz 1947). It has since become clear that many plants used as sources of nutrients contain proteins that have the ability to inhibit activity of a whole range

of proteases. As a group, they are therefore called protease inhibitors. They are found in the protein-rich parts of the plants, comprising up to 10% of the protein, as well as in leaves and other parts, where they serve as protection against insects (Ryan 1990). Legumes are particularly rich in protease inhibitors. The affected enzymes are the endo-peptidases trypsin, chymotrypsin, elastase and enterokinase, as well as the exo-peptidases carboxypeptidase A and B (reviewed by Habib and Fazili 2007). Microorganisms and higher animals may also produce protease inhibitors, allegedly as protection against auto-digestion. De Leo et al. (2002) suggested clustering the many protease inhibitors found in plants into ten groups based on their structure and specificity: Bowman–Birk serine protease inhibitors (BBI), cereal trypsin/α-amylase inhibitors, cysteine protease inhibitors, metallocarboxypeptidase inhibitors, mustard trypsin inhibitors, potato type I and type II protease inhibitors, serpin, Kunitz soybean trypsin inhibitors (KTI), and squash protease inhibitors. Recently, Bateman and James (2011) presented a more refined classification.

Most of the plant inhibitors affecting the activity of endo-peptidases in animals are found in two of the groups: the KTI (Kunitz 1947), with one cystin-bridge and molecular weight of above 20 kDa, and the smaller BBI (Birk 1985; Clemente and Domoney 2006), with seven cystin-bridges and weight less than 10 kDa. Their mechanism of enzyme inhibition is competitive. The BBI type inhibitors show greater stability towards heat treatment than the KTI inhibitors, apparently due to the stabilizing effect of the numerous cystin-bridges in the BBI inhibitors (Dipietro and Liener 1989a,b). The active site of KTI binds trypsin strongly, whereas the binding of chymotrypsin is much weaker. The BBI type inhibitors, on the other hand, have two active sites and may bind two enzyme molecules at one time. Depending on the specificity of the active sites, the BBI type inhibitors bind either two molecules of the same enzyme or two different enzymes.

The *in vivo* mechanisms of the action of protease inhibitors have been revealed in studies with mammals and birds (reviewed by Liener 1980). They bind to and inhibit the activity of pancreatic proteolytic enzymes in the intestinal chyme by forming complexes that greatly resist digestion. The resulting reduction in proteolytic capacity in the chyme is monitored by receptors in the intestinal wall, triggering release of cholecystokinin (CCK), which in turn stimulates the pancreas to increase production and secretion of proteolytic enzymes (Liener 1980). However, full compensation may not be reached, and protein digestibility can be reduced to the extent of causing protein deficiency. As all proteolytic enzymes are rich in cysteine, the cysteine requirement increases as the level of protease inhibitors in the diet increases. A deficiency of sulfur-containing amino acids may develop as a secondary effect (Yokogoshi et al. 1986; Hara et al. 1997; Peace et al. 1991; Moundras et al. 1995).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Investigations into the effects of purified preparations of protease inhibitors have been the goal of only a limited number of fish studies, all of which were short-term and mostly with salmonids. Those studies confirmed the mechanisms of action described above. Inhibitor preparations from soybeans, containing a mixture of BBI and KTI inhibitors, have been found to cause a dose-dependent reduction in apparent protein digestibility in rainbow trout (Berg-Lea et al. 1989; Krogdahl et al. 1994). The trypsin inhibitors reduced proteolytic activity in the chyme of the mid-intestine. Apparent lipid digestibility was also affected, particularly digestibility of saturated fatty acids. A study of Atlantic salmon (*Salmo salar* L.) by Olli et al. (1994), with observations of intestinal effects after 12 and 31 days of feeding, indicates that protease inhibitors stimulate pancreatic enzyme secretion, causing the enzyme level in the intestinal content to rise without increasing trypsin activity. The enzyme activity seemed unaffected when fish were fed diets with the lower inhibitor levels for a short time (12 days), but fish fed higher levels showed decreased trypsin activity. The results also suggest that after longer-term feeding, the pancreas was no longer able to compensate for decreased enzyme activity by increasing secretion, even with lower inclusion levels of the inhibitors.

As observed in studies with other animals, methionine supplementation in diets with high soybean inclusion improves performance in fish, including southern catfish (*Silurus meridionalis*; Ai and Xie 2005), Indian major carp, rohu (*Labeo rohita* H.; Sardar et al. 2009), rainbow trout (Cheng et al. 2003), and red sea bream

(*Pagrus major*; Takagi et al. 2001). It is therefore likely that protease inhibitors, when present in diets in active form, may increase excretion of cysteine, resulting in a need to increase dietary levels of sulfur-containing amino acids to meet nutritional requirements.

No information on the effects of protease inhibitors on immune responses and disease susceptibility in fish or in other production animals was found in the literature. As discussed, longer-term feeding of diets containing high levels of protease inhibitors may result in general protein deficiency and/or deficiency of sulfur-containing amino acids. From studies performed on other animals, including humans, it is evident that severe protein deficiency will compromise immune functions, and increase susceptibility to infection and severity of contracted infectious diseases (Franca et al. 2009). It is likely that fish immune functions will also be affected by protein and/or sulfur-containing amino acid deficiency (Rombout et al. 2011; Boehm et al. 2012). In addition to their role in protein synthesis, sulfur-containing amino acids supply elements to metabolically important compounds with a wide range of metabolic functions including glutathione, taurine, several phospholipids, and S-adenosylmethionine. The latter is involved in the synthesis of creatine, epinephrine, melatonin, and the polyamines spermine and spermidine, along with several other substances. Among the many functions of methionine, several link to elements of the immune system (see reviews of Brosnan and Brosnan 2006; Yoneda et al. 2009).

Under practical conditions, effects of residual protease inhibitor activity in fish feed apparently result only in reduced protein and amino acid digestibility, which can be compensated with an increase in dietary protein and/or enrichment with sulfur-containing amino acids. However, the possibility that protease inhibitors can alter the intestinal proteolytic processes and, subsequently, the immune stimulatory activity of the resulting peptide profile in the digesta should not be excluded (Hajos et al. 1996).

Lectins (Hemagglutinins)

Structure, Sources, and Mechanism of Action

Lectins, also known as agglutinins or hemagglutinins due to their ability to bind and agglutinate red blood cells, are glycoproteins with at least one domain binding reversibly to a specific mono- or oligosaccharide (van Damme et al. 1998, 2008). Their effects in animals are secondary responses following lectin-binding to carbohydrate moieties of cell receptors. Lectins were first discovered in plants (Stillmark 1888), but later lectins were also found in microorganisms and animals. Most plants seem to contain lectins, although the majority of them do not bind to cells of higher animals (van Damme et al 1998, 2008). As demonstrated in Table 10.1, legumes in particular can contain lectins that may affect animal production.

Lectins are commonly divided into four major groups based on their structure: (1) merolectins, which are small proteins with a single carbohydrate-binding site with the ability to bind to one cell at a time; (2) hololectins, which are proteins with two or more identical or homologous carbohydrate-binding domains, binding to more than one cell and therefore having cell agglutinating ability; (3) chimerolectins, which are fusion proteins composed of a carbohydrate-binding domain and an unrelated domain that may act independently of the carbohydrate-binding domain; and (4) superlectins, which are a special type of chimerolectin with two carbohydrate-binding domains recognizing structurally unrelated sugars. Another classification of plant lectins is based on sequence data and categorizes the lectins into four groups of evolutionarily-related proteins: (1) legume lectins; (2) chitin-binding lectins; (3) Type 2 RIP (ribosome-inactivating proteins); and (4) monocot mannose-binding lectins (van Damme et al. 1998).

The legume lectin group is a large family of homologous proteins (Sharon and Lis 1990). Most are hololectins, although chimerolectins (e.g. Type 2 RIP) are also described in some species (van Damme et al. 1998). One plant species may contain different forms of lectins in different tissues. Most legume seeds contain 1–5 g lectins kg^{-1}, but the level in some seeds may reach 20 g kg^{-1} on a dry matter (DM) basis. Lectin levels vary between cultivars within a species, as well as with stress factors and other ecological and climatological conditions that the plants are exposed to during growth (van Damme et al. 1998). Their resistance to heat denaturation varies, as does their resistance to proteolytic enzyme degradation during passage through the digestive tract. The latter also appears to depend on the animal species ingesting

them (Hara et al. 1984; Pusztai et al. 1990; Bardocz et al. 1995).

Since many cellular receptors and other components of the intestinal tract's epithelium are equipped with carbohydrate-rich side chains, lectins may bind to the cells depending on their carbohydrate specificity, and may elicit responses in an animal that are related to the function of the receptors/components. Plant lectins have been described as "one of the most important physiologically active components and potent exogenous biological signals in the diet" (Pusztai and Bardocz 1996), for better or for worse (Kjaer and Frokiaer 2005; Pusztai et al. 2008). Some are able to interrupt the mucosal barrier and cause rapid death of the animal due to septicaemia, whereas others may secure beneficial microflora, have anti-inflammatory properties, or even prevent cancer (Pusztai et al. 2008).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Present knowledge on the effects of plant lectins in fish is very limited. Soybean lectin, which has specificity for cellular receptors/components with N-acetyl-d-galactosamine side chains, binds to the intestinal brush border membrane of Atlantic salmon and rainbow trout (Hendriks et al. 1990; Buttle et al. 2001). Tissue from the distal intestine shows higher maximum binding and lower dissociation constants compared to tissues from the more proximal regions. Buttle et al. (2001) observed morphological changes in the distal intestine of Atlantic salmon, such as cellular infiltration in the submucosa and lamina propria and alterations in the villous tips, when purified soybean lectin was added to a fishmeal-based diet at a level of 3.5%. Iwashita et al. (2008, 2009) also observed morphological alterations in the wall of the distal intestine of rainbow trout fed a diet containing soybean lectin, but only when the diet also contained soybean saponins. The work seems to demonstrate that, when given alone, some may not be as harmful as they are when given together with other plant components.

There seem to be only two studies reporting effects of soybean lectins on growth in fish, and both were conducted with rainbow trout. In a six-week feeding trial (Iwashita et al. 2008), no significant effects on growth were observed in fish fed a dietary inclusion level of 75 mg kg^{-1}, a level considered equivalent to the level in diets containing about 40% standard soybean meal. On the other hand, an eight-week feeding trial with a diet containing 80 mg kg^{-1} revealed a significant growth reduction of 14% (Hart et al. 2010). Neither of these studies supplies information regarding immune functions or disease susceptibility. However, effects of lectins on immune and allergic responses in laboratory animals may provide some relevant information. Several *in vitro* and *in vivo* studies clearly indicate that plant lectins modulate immune functions in experimental animals, as well as in humans. In sensitive individuals, lectins may aggravate allergic responses (Kjaer and Frokiaer 2005; Pusztai et al. 2008). It is likely that lectins can also have various immunological effects in fish, despite some differences in their immune apparatus when compared to other farmed animals and humans (Rauta et al. 2012).

Phytoestrogens and Phytosterols

Structure, Sources, and Mechanism of Action

Phytoestrogens are non-steroidal compounds with structures resembling estradiol (17-β-estradiol) that have the ability to bind to estrogen and other hormone receptors. Phytosterols, on the other hand, have a structure similar to that of cholesterol, varying only in sterol side chains and/or differences in double bonds. Both phytoestrogens and phytosterols are bioactive compounds and considered antinutrients due to their effects on glucose, lipid, and cholesterol metabolism when ingested with the diet, as well as their ability to modulate estrogen receptor responses, a well-documented effect for the phytoestrogens that has also been suggested for phytosterols (Dinsdale and Ward 2010; Nieminen et al. 2008). Due to the similarity in these respects, these two classes of compounds are discussed together in this section.

The list of phytoestrogen-containing plants exceeds 300 species (Farnsworth et al. 1975). These compounds are found in fruits and vegetables, but are most abundant in leguminosae. The most common phytoestrogens found in feed ingredients are isoflavones, coumestans, and lignans (Fig. 10.2). Soybean isoflavones include genistein, daidzein, glycitein, formononetin, and biochanin A, as well as

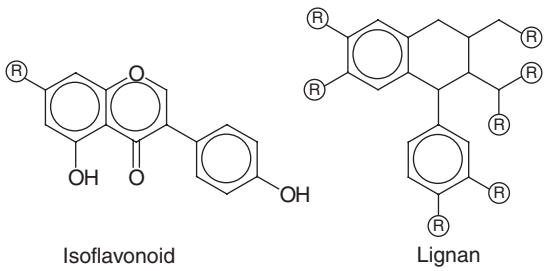

Figure 10.2 Structure of isoflavonoids and lignans.

equol, a metabolite of daidzein (Wang and Murphy 1994). Total isoflavone content of soybeans can reach levels above $4\,g\,kg^{-1}$ (Wang and Murphy 1994), but considerable variation exists; levels are influenced by variety, location, and variation in environmental conditions. Some species, such as alfalfa, may produce phytoestrogens when infested by insects (Cederroth et al. 2012). The primary phytosterols, mostly found in plant oils and oil-rich plant meals, include sitosterol, stigmasterol, and campesterol. In mammals and man, the antinutrient or metabolic effects of phytoestrogens have been attributed to their ability to bind to estrogen receptors and other nuclear hormone receptors present in cells of various metabolically important tissues, including intestine and liver. Phytoestrogen binding to estrogen receptors possess the ability to act as synergists and antagonists to estrogens, depending on the function in focus and physiological status of the animal, that is, the circulating level of estrogen in the organism. Phytoestrogens are therefore classified among the endocrine disruptors (Bennetts et al. 1946) that interfere with regulation of reproduction and the involvement of estrogens in feed intake, lipid metabolism, regulatory action on peroxisome proliferator-activated receptor γ (PPARγ), and insulin sensitivity. Consequenty, phytoestrogens may affect energy utilization (reviewed by Mauvais-Jarvis 2011).

The binding of both phytoestrogens and phytosterols to farnesoid X receptor (FXR), liver X receptor (LXR), and/or PPARs explains effects seen on bile acid synthesis, metabolism, and on lipid, glucose, and cholesterol homeostasis, resulting in the hypoglycaemic, hypolipemic, and hypocholestrolemic effects often observed with the dietary intake of these antinutrients (see review by Caiozzi et al. 2012). In the case of soybean isoflavones, their PPARα-mediated stimulating effect on ß-oxidation is apparently independent of their ability to bind to estrogen receptors (Kim et al. 2004; Setchell et al. 2005). The hypocholestrolemic effect of phytosterols has also been attributed to their ability to reduce cholesterol absorption from the intestine. Due to the slight differences in chemical structure, phytosterols are not efficiently absorbed but may instead block cholesterol absorption by an unidentified mechanism (Jones et al. 2009).

Estrogen receptors and PPARs are also expressed in cells of the immune system (Rubtsov et al. 2010; Varga et al. 1996, 2011), but the effects of phytoestrogens on the immune system are apparently an under-investigated area of research (Caiozzi et al. 2012). However, changes in immune responses observed between genders, as well as those caused by various endocrine disruptors, suggest that phytoestrogens may also modulate immune responses (see review by Rogers et al. 2013).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Few reports exist from experiments conducted to assess effects of phytoestrogens or phytosterols on fish performance. Depending on the dosage inclusion of phytosterol in Atlantic salmon diets (5 and $10\,g\,kg^{-1}$) appears to decrease lipid digestibility, especially digestibility of saturated fatty acids; this is allegedly related to the parallel decrease in bile salt concentrations in the chyme (Chikwati 2007). The recent work of Mai et al. (2012) showed significant reductions in feed intake, feed efficiency, and nutrient digestibility in Japanese flounder (*Paralichthys olivaceus*) fed diets containing soybean isoflavones, but only when included at levels above $4\,g\,kg^{-1}$, that is, equivalent to total isoflavone levels reported in soybeans. The results are in line with results reported for striped bass fed genistein-containing diets, which exhibited no significant effects on growth performance (Ardia and Clotfelter 2006). In the latter study, vitellogenesis was altered at inclusion levels of $2\,g\,kg^{-1}$ and above. As reported in mammals (Adams 1995; Cederroth et al. 2012), numerous studies on various fish species confirm that exposure to phytoestrogens can have considerable effects on reproduction and gender phenotype, but species differences in sensitivity apparently exist (Latonnelle et al. 2002; Green and Kelly 2008, 2009).

Phytoestrogens can also potentially interfere with smoltification in salmonids, even when circulating

levels of estrogens are normally very low. In this situation, exposure of fish to compounds with estrogen mimicking effects may disturb smoltification, possibly by reducing presence of growth hormone receptors and impairing osmoregulatory functions (Lerner et al. 2012).

Presence of estrogen receptors in immune organs, such as spleen and head kidney, has been shown by molecular studies of samples from rainbow trout. The expression of immune- related genes were down-regulated when the trout were fed a diet supplemented with 20 mg kg^{-1} 17β-estradiol (Casanova-Nakayama et al. 2011). The same study presents results of a challenge test with the bacterium *Yersinia ruckeri*, comparing mortality in fish fed diets supplemented with various levels of 17β-estradiol. A dose-dependent increase in mortality was observed. Similar effects of estrogen supplementation have been observed in goldfish (*Carassius auratus*; Wang and Belosevic 1994) and gilthead sea bream (*Sparus aurata* L.; Liarte et al. 2011a, b, c). These findings clearly indicate immune-modulating effects of estrogens in fish, suggesting that phytoestrogens, whether present in the diet or in the surrounding waters, may also affect the immune responses. Support for this is found in a study of kidney bean phytohemagglutinin (PHA)-challenged (locally under the skin) Siamese fighting fish (*Betta splendens*; Ardia and Clotfelter 2006). The study indicated that the isoflavones genistein and equol and the phytosterol ß-sitosterol in the fishes' surrounding water reduced the PHA-induced swelling and mitogenic T-cell proliferation, suggesting immunosuppressive effects of these phytoestrogens/-sterols. Even though present information on immunological effects from experimental studies is very limited and no challenge tests seem to have been conducted, data from field observations of disease outbreaks among fish in polluted waters suggest the likelihood of important immune-modulating effects of phytoestrogens and other endocrine disruptors (reviewed by Milla et al. 2011).

Phytic Acid

Structure, Sources, and Mechanism of Action

Phytic acid is the hexaphosphoric ester of the hexahydric cyclic alcohol meso-inositol with molecular formula $C_6H_{18}O_{24}P_6$ (Fig. 10.3), in some contexts abbreviated to IP6. Phytic acid therefore contains six phosphate groups and is the principal storage form of phosphorus and inositol in many plant tissues. Phytate occurs as a highly negatively charged ion at a broad pH range, and therefore chelates positively charged minerals such as iron, zinc, magnesium, manganese, and copper (Bretti et al. 2012). These chelates are insoluble, rendering all of their components unavailable to animals (Lonnerdal 2002). In addition, phytate forms strong complexes with proteins at pH levels lower than the iso-electric point of the proteins, as the negatively charged phosphoric groups of phytate bind the positively charged groups of basic amino acids such as arginine, histidine, and lysine. Such complex formations may decrease protein solubility and, in this manner, may inhibit the function of digestive enzymes (Cosgrove 1966; Deshpande and Damodaran 1989).

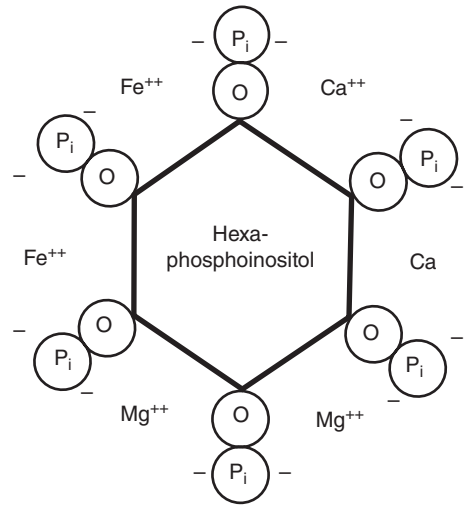

Figure 10.3 Phytic acid.

Around 50–80% of the total P content in plant seeds is stored as phytate (Ravindran et al. 1995). The level of phytic acid in ingredients that are commonly used in feeds for monogastric animals ranges from 1 to more than 10 g kg^{-1}. Grain seeds contain lower concentrations, while typical oilseeds from legumes, cotton, rape/canola, and sunflower contain higher levels (reviewed by Kumar et al. 2012). Protein concentrates and beans generally have higher phytic acid content than their unprocessed counterparts.

The binding characteristics of phytic acid, combined with the inability of digestive enzymes of

monogastric and agastric animals to hydrolyze phytic acid, equate to a relatively low availability of phosphorus and any divalent ions bound to phytate for diets based on plant ingredients, particularly high-protein diets. A large number of reports indicate that phytic acid has negative consequences on mammal health. The most-observed health effects stem from the reduced availability of amino acids and divalent minerals, which induces nutrient deficiencies. Whether or not a deficiency occurs depends on the nutrient profile of the diet, particularly levels of limiting nutrients. Correspondingly, the symptoms of excess phytic acid in the diet will reflect which nutrient is most limiting. Mineral deficiencies have been considered the most likely result of high phytic acid content in diets, and many cases of phosphorus, zinc, and iron deficiencies have been reported (Oatway et al. 2001). It is beyond the scope of this chapter to review present knowledge regarding signs and consequences of specific mineral deficiencies, however. Effects of high dietary phytate levels may be overcome by treatment or supplementation of the diet with phytase (Oatway et al. 2001).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Experiments with several fish species (gastric and agastric fish) have confirmed their inability to utilize phosphorus in phytic acid, as well as the minerals associated with this molecule (Storebakken et al. 1998). However, low but detectable phytase activity has been observed in the intestine of some fish species, such as hybrid striped bass (*Morone chrysops x M. saxatilis*), hybrid tilapia (*Oreochromis niloticus x O. aureus*), and koi (*Cyprinus carpio*; Ellestad et al. 2002a, b). Whether the organism itself, intestinal microorganisms, or feed is the source of this enzyme activity is not clear.

A recent experiment with Atlantic salmon (Denstadli et al. 2006) confirmed the antinutrient characteristics of phytic acid. Negative, dose-dependent relationships between dietary phytic acid intake (0, 1.0, 2.1, 4.7, 10.0, and 20.7 g kg^{-1} feed intake) and growth, digestibility of zinc and magnesium, and retention of phosphorus, magnesium, and lipid were observed. Trypsin activity in intestinal chyme and concentration of bile acids in the pyloric intestine were also significantly reduced in fish fed the diet with the highest phytic acid level. Moreover, whole-body calcium and magnesium levels, Ca: P ratios, and vertebral zinc levels were negatively affected. Increasing levels of phytate did not reduce apparent digestibility of protein or starch. Regarding apparent lipid digestibility, the results indicated a reduction at the highest phytate inclusion level. No morphological changes in the distal intestine of the fish were detected. In other studies, however, negative effects on apparent protein digestibility have been observed in Atlantic salmon (Sajjadi and Carter 2004), rainbow trout (Spinelli et al. 1983), and chinook salmon (*Oncorhynchus tshawytscha*; Richardson et al. 1985). Reduced availability of nutrients in the presence of phytic acid may be due to reduced activity of digestive enzymes. However, *in vitro* studies have given variable results (reviewed by Denstadli et al. 2006).

No studies have reported phytic acid effects on immune responses or disease susceptibility in fish. Most minerals and amino acids have functions in the maintenance of the immune apparatus; phytic acid and ensuing nutrient deficiencies may therefore affect disease defense mechanisms. In broilers fed phytate-containing diets, an impaired response to vaccination was observed that was not seen in animals given diets supplemented with phytase (Liu et al. 2008). From the available research, it seems clear that phytic acid may affect fish nutrition, particularly mineral nutrition, and result in possible deficiencies; this may have potential implications for immune responses and disease susceptibility.

Based on the results of growth and feed intake trials, a tolerance level of phytic acid of between 4.7 and 10.0 g kg^{-1} has been suggested (Denstadli et al. 2006).

Gossypol

Structure, Sources, and Mechanism of Action

Gossypol is a yellow, lipid-soluble, polyphenolic aldehyde synthesized in cotton (genus *Gossypium*, family Malvaceae) and concentrated in discrete pigment glands in various parts of the plant (Fig. 10.4). Gossypol can be toxic for monogastric animals, and the metabolites may also be toxic. In cotton seeds, the

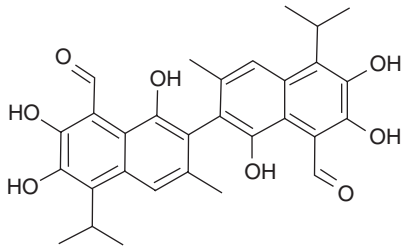

Figure 10.4 Gossypol.

concentration varies from zero in the glandless varieties to about 6%, depending on climate, soil type, and fertilization. Gossypol occurs in bound as well as free forms. In cottonseed meal it is present in bound form, and is mostly bound to lysine residues of peptides. Gossypol has high affinity for dietary iron and high oxidative capacity, that is, antinutrient characteristics that may challenge an animal's antioxidant reserves and increase the need for dietary levels of iron and vitamins E, C, and A.

Bound gossypol is not readily absorbed, but it may prevent both iron and lysine absorption and subsequently induce iron and lysine deficiency. Free gossypol, on the other hand, is readily absorbed from the gastro-intestinal (GI) tract. In animals, conjugation, metabolism, and urinary excretion of gossypol are limited and most is eliminated with the feces in the free form. The free form is found as two enantiomers (Huang et al. 1987; Gamboa et al. 2001a), and the ratios vary among cotton varieties and cottonseed meal processing methods (Gamboa et al. 2001a, b).

The mechanism of action of gossypol is not well understood. *In vitro* it has been found to bind to microsomal membranes, inhibit DNA synthesis, and cause depletion of iron and glutathione in mammalian cells (Gawai et al. 1995; Kovacic 2003).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Effects of gossypol have been studied in several species of fish, resulting in functional impairments in most but not all. Decreased feed consumption, growth retardation, reduced blood hematocrit, reduced hemoglobin, and reduced reproductive capacity have been observed (reviewed by Li and Robinson 2006). Structural lesions in liver, kidney, spleen, and gonads also indicate toxic effects. A dose–response study with up to 1500 mg kg^{-1} free gossypol in diets fed to channel catfish revealed histomorphological effects in the GI tract at levels above 600 mg kg^{-1} (Evans et al. 2010); these effects were characterized by gastric gland necrosis, mild to severe necrosis of pancreatic tissue, and elevated pigment deposition in the liver. Moreover, investigation of gossypol effects on gut tissue employing an Ussing chamber has indicated alterations in ion transport functions. Both cellular and paracellular ion transport seemed to be affected (Trischitta and Faggio 2008).

Contradictory results have been reported from a study of silver crussian carp (*Carassius auratus gibelio* female x *Cyprinus carpio* male) by Cai et al. (2011), which showed that free gossypol up to dietary levels of 642 mg kg^{-1} had no effect on growth performance, blood hemoglobin concentration, activities of various serum enzymes, or histomorphology of hepatic and gut tissues. Studies conducted to explore effects on reproduction have given conflicting results. Studies by Blom et al. (2001) indicated impairment of reproduction in rainbow trout and transfer of gossypol to the offspring. However, this was not confirmed in other experiments with rainbow trout and tilapia (Lee and Dabrowski 2002; Rinchard et al. 2003; Garcia-Abiado et al. 2004). Iron supplementation of diets with cottonseed may prevent gossypol accumulation in the liver, as has been reported for parrot fish (*Oplegnathus fasciatus*; Lim and Lee 2009).

Under certain circumstances gossypol may be beneficial, as indicated by reports on immune parameters and challenge trials. In a study by Yildirim et al. (2003), gossypol improved macrophage chemotaxis and serum lysozyme activity in channel catfish. Although this alone does not predict enhanced ability to successfully fight disease pathogens, the same group followed up with a challenge trial. Juvenile catfish fed casein-gelatin-based diets with various gossypol levels were infected with the bacterial pathogen *Edwardsiella ictaluri*; levels of 900 mg kg^{-1} and higher significantly reduced mortalities (Yildirim-Aksoy et al. 2004). Interestingly, when soybean-meal-based diets were supplemented with gossypol up to 800 mg kg^{-1}, no positive effects were observed (Yildirim-Aksoy et al. 2004). The explanation for a beneficial effect of gossypol in disease resistance may be related to its iron-binding capacity, as iron is the first limiting nutrient for growth of several bacterial pathogens.

Seemingly, contradictory results between experiments and species may be related to differences in dietary iron level and availability.

Some researchers conclude that gossypol is of no concern in aquaculture, as other characteristics of cottonseed restrict its inclusion level in fish feeds more than its content of gossypol (Li and Robinson 2006). Nevertheless, some countries have regulatory restrictions regarding the amount of free gossypol in feed materials, and maximum allowable level in complete feeds for fish is set at 20 mg kg^{-1} in the EU/EEC (European Commission 2010). Gossypol is not a concern if cottonseed meal from glandless varieties of cotton plants is utilized in feeds.

Cyclopropene Fatty Acids

Structure, Sources, and Mechanism of Action

Two fatty acids – both containing a cyclopropene ring, one characterized as 8-(2-octyl-cyclopropen-1-yl)-octanoic acid (sterculic acid) the other as 7-(2-octyl-cyclopropen-1-yl)-heptanoic acid (malvalic acid) (Fig. 10.5) – are found in many seed oils. Oils from plant families of the order *Malvales* contain especially high levels, but members of the *Fabales* and *Sapindale* families also contain high levels (reviewed by Bao et al. 2002). The most important member of the order of *Malvales* in the context of animal nutrition is cotton. The cyclopropene fatty acids are present in cottonseed oil as well as in the cake meal. However, the lipids of seeds from the sterculia tree (sometimes called wild almond) used as food and feed in some countries contain the highest level of sterculic acid.

The cyclopropene ring is highly reactive and the mechanism of action seems to be an irreversible reaction between the ring structures and thiol moieties on desaturases, which inhibits their activity (Raju and Reiser 1967). The result of ingestion of these fatty acids is alterations in lipid metabolism and increased accumulation of saturated fatty acids (Raju and Reiser 1967).

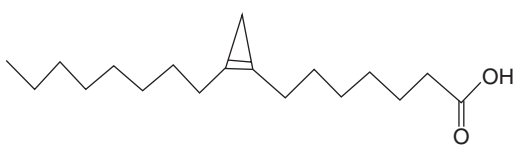

Figure 10.5 Cyclopropene fatty acid.

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Desaturases from fish have been found to be inhibited by the cyclopropene fatty acids, and effects on lipid metabolism have been observed in rainbow trout (Roehm et al. 1970, 1971; Malevski et al. 1974a, b; Struthers et al. 1975a, b; Hendricks 2002). In rainbow trout, the mixed function oxidase system (Eisele et al. 1978) and other liver enzymes are altered upon exposure to these fatty acids (Selivonchick et al. 1981). Structural effects in the liver, which are characterized by necrosis, fibrous accumulation in hepatocytes, and abnormal accumulation of glycogen, have also been observed (Selivonchick et al. 1981). When rainbow trout were fed diets with 7.5% cottonseed oil that resulted in a level of 90 mg kg^{-1} sterculic acid, one-third of the fish developed hepatic neoplasm at 12 months; two-thirds showed similar signs when fed diets with 25% glandless cottonseed meal (Sinnhuber et al. 1976; Voss et al. 1982). Interaction effects between the cyclopropene fatty acids and aflatoxin, a liver carcinogen, have been studied. Synergistic effects were observed to be dependent on the type of aflatoxin used. Aflatoxin B1 was much more potent than the aflatoxin Q1 (Sinnhuber et al. 1974; Hendricks et al. 1978; Loveland et al. 1978, 1979). The majority of information regarding effects of the cyclopropene fatty acids in fish originates from experiments with rainbow trout. However, carcinogenesis has also been observed in sockeye salmon (*Oncorhynchus nerka*), but apparently only when supplied together with aflatoxin B1 (Wales and Sinnhuber 1972). In channel catfish, sterculic acid has been observed to cause growth retardation (Hendricks 2002).

Because the chances of transferring cyclopropene fatty acids from broodstock to their offspring are high, caution should be exercised if cottonseed meal is included in broodstock diets. Moreover, as young fish are more vulnerable, care should also be taken before cottonseed meal is included in starter feeds (Hendricks 2002).

No scientific reports on the possible effects of cyclopropenic acids on immune function and disease susceptibility in fish or other animals have been found in the scientific literature.

Glucosinolates

Structure, Sources, and Mechanism of Action

Glucosinolates are secondary plant metabolites produced from amino acids and glucose. They are sulfur-containing with a varying degree of glycosylation. More than 120 glucosinolates have been identified in plants. Nearly all members of the Brassicales order, including feedstuffs such as rapeseed, synthesize this antinutrient, as do soybeans. The toxicity of glucosinolates is related to their metabolites, that is, thiocyanates, isothiocyanates, nitriles, 5-vinyl-2-oxazolidinethione, and 5-vinyl-1,3-oxyzolodine-2-thione (VOT). Hydrolysis of the glucosinolates can be catalyzed by thioglucosidases present in the feedstuffs themselves, but in the intact plant they are separated from the substrate by physical barriers. Disruption of this barrier, which can happen in the field or during processing such as grinding, will release the enzyme and initiate the hydrolysis that will eventually result in the toxic metabolites. Some microbes of the GI tract also possess thioglucosidases, which will add to the hydrolysis (Combourieu et al. 2001; Shapiro et al. 2001; Fuller et al. 2007).

The glucosinolates interfere with iodine uptake in the thyroid gland and cause iodine deficiency and goiter, with symptoms such as decreased feed intake and growth, hypertrophy of the thyroid gland, liver and kidneys, reproductive failure, and increased mortality (Tripathi and Mishra 2007). The thiocyanates interfere with iodine availability, whereas VOT is responsible for the morphological and physiological changes of the thyroid. The nitriles are known to affect liver and kidney functions.

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

In their review of the effects of glucosinolates in animals, Tripathi and Mishra (2007) conclude that the general response of fish to dietary inclusion of canola and rapeseed meals is favorable. Nevertheless, most reports recommend limiting the inclusion level depending on the glucosinolate content of the meals. Diets containing less than 2.18 mmol kg^{-1} of glucosinolates did not affect feed intake, growth, or thyroid hormone levels in red sea bream (*Pagrus auratus*; Glencross et al. 2004a, b). At higher levels however, glucosinolates reduced growth. Rainbow trout fed feeds containing 1.4–19.3 mmol kg^{-1} glucosinolate have shown significant reduction in growth and changes in thyroid histology (Burel et al. 2000). A strong growth depression was observed at glucosinolate intake of 30–47 μmol kg^{-1} fish body weight per day. Feed containing 1.4 mmol kg^{-1} did not cause growth-depressing effects, but decreased plasma thyroxin level. The authors suggested that a total glucosinolate content of 1.4 mmol kg^{-1} diet can be considered a safe upper limit.

Erucic Acid

Structure, Sources, and Mechanism of Action

Erucic acid is the common name of the fatty acid cis-13-docosenoic acid. Many native plants in the brassica family produce erucic acid, and some rapeseed and mustard oils contain as much as 60%.

Erucic acid gained considerable attention when lipid accumulation and lesions of the heart tissue were observed in rats fed oils containing high concentrations (McCutcheon et al. 1976). However, the mechanisms behind these effects are uncertain. Whether erucic acid is the main causative agent or not has been investigated (Food Standards Australia New Zealand 2003). Other biologically active compounds in some rapeseed oils, or interactions between one or more of these compounds and erucic acid, may be involved.

Erucic acid is of less concern in human nutrition today than in the 1970s, as genetic selection has changed the oil composition of rapeseed tremendously. Erucic acid has been eliminated and glucosinolate content has not been reduced to negligible levels. This successful development of rapeseed has resulted in a most attractive oil on the food market (McCutcheon et al. 1976), which is also used in animal feeds. However, "old" varieties are still on the market,

particularly varieties used for fuel production. Care must be taken in selecting the correct oil for human and animal consumption.

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Information on the effects of erucic acid in fish is very limited. Dietary inclusion of rapeseed oil rich in erucic acid was found to cause negative effects in coho salmon (*Oncorhynchus kisutch*) when included at 6% and 12% dietary levels (Hendricks 2002). The signs were growth depression, increased mortalities, and histopathological alterations in the skin, gills, kidney, and heart. No lesions were observed in the heart muscle, but accumulation of lipid in the connective tissue of the outer layer of the heart wall was observed. Whether erucic acid affects immune responses and disease susceptibility in fish is not known.

The improved strains of rape have seeds with oil high in mono-unsaturated fatty acids and low in n-6 polyunsaturated fatty acids. In recent years, rapeseed oil has become very attractive as a replacement for marine oils in diets for salmonids and other fish species. Inclusion of rapeseed oil does not affect the n-3 to n-6 ratio as much as most other plant oils, which is considered by many to be nutritionally beneficial. Recent research on the usefulness of the double (erucic acid + glucosinolates) low rapeseed oils in fish diets have not indicated any negative health implications for the fish (reviewed by Hendricks 2002). In the production of fish feed, the use of rapeseed or mustard oils with high levels of eruric acid should be avoided.

Saponins

Structure, Sources, and Mechanism of Action

Saponins are polycyclic triterpene glycosides; glycation varies considerably and may include glucose, galactose, glucuronic acid, xylose, or rhamnose. Saponins are heat-stable, alcohol-soluble amphipathic molecules. They can be degraded by acid and alkaline hydrolysis (Anderson and Wolf 1995), as well as bacterial glucosidases (Gestetne et al. 1968). Saponins have the ability to bind cholesterol to form insoluble complexes.

Saponins are found in more than 100 families of plants, especially legumes such as soybean, pea, and lupin. They are usually present in the range $1-5\,g\,kg^{-1}$, but the level in soybean is generally higher than in other common plant feedstuffs (Anderson and Wolf 1995). The cholesterol-binding and amphipathic properties of saponins explain many of their biological activities. Saponins can form micelles and can intercalate into cholesterol-containing membranes, forming holes. In mammals, this appears to increase the permeability of intestinal epithelial cells, facilitating uptake of substances that may not normally be absorbed, such as allergens (Johnson et al. 1986; Gee et al. 1996). Saponins themselves cannot normally be absorbed by the intestine (Malinow et al. 1977), and those incorporated into cell membranes will eventually be lost in the normal process of cell turnover (Sjolander and Cox 1998). On the other hand, they also exhibit antifungal, antiviral, and anticancer activities, immune-stimulating (adjuvant) and antioxidant properties, inhibitory effects on protein digestion and vitamin absorption, and glucocorticoid-like effects (reviewed by Francis et al. 2001).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

In an investigation into the fate of an orally administered soybean fraction containing high levels of saponins in Atlantic salmon, the saponins did not appear to be degraded during passage through the GI tract (Knudsen et al. 2006). This does not appear to have been investigated in other fish species. Different saponins have been shown to vary in their biological activities (Oda et al. 2000). Depending on feed intake, studies on the effects of saponins in some fish species show either positive or negative levels in the diet and feed composition. Investigations by Francis et al. (2005) indicate growth-promoting effects of *Quillaja saponaria* saponins in common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus* L.), whereas soybean saponins generally did not stimulate feed intake or growth in channel catfish ($2.6\,g\,kg^{-1}$; Twibell and Wilson 2004) or Atlantic salmon ($2.0\,g\,kg^{-1}$; Chikwati et al. 2012). In the latter study however, the response in feed intake varied depending on the main plant ingredient in the

diet. Together with pea protein concentrate, saponin supplementation reduced feed intake.

Recent studies have shed light on the involvement of soybean saponins in the development of the enteritis induced by soybeans in salmonids. There are strong indications that saponins play a role in the development of the inflammatory response in salmonids, but possibly not alone (Knudsen et al. 2008). Supplementation of semi-purified (1.7, 2.6 $g\,kg^{-1}$; Knudsen et al. 2008) or purified (2.0 $g\,kg^{-1}$; Chikwati et al. 2012) soybean saponins in a fish-meal-based diet did not seem to induce inflammatory changes in the distal intestine. However, increased gut tissue permeability, as assessed *in vitro* with an Ussing chamber, was reported (Knudsen et al. 2008). Chikwati et al. (2012) observed decreased lipid and mineral digestibilities, decreased fecal dry matter, decreased activity of brush border membrane enzymes in the distal intestine, and decreased distal intestinal fold heights and enterocyte vacuolization, but no immune cell infiltration.

Research results suggest that saponin effects may become more severe when other plant components are present. When a saponin-supplemented diet also contained lupin kernel meal (Knudsen et al. 2008) or pea protein concentrate (Penn et al., 2011; Chikwati et al. 2012), inflammatory and pathophysiological changes similar to those that occur in salmonids fed soybean meal were observed. In a more recent study, inflammatory changes were observed in Atlantic salmon fed diets with high fish meal content; these legume-free diets included supplements of 4 $g\,kg^{-1}$ or more of highly purified soybean saponins (Penn et al. 2012). Similarly, Iwashita et al. (2008) reported inflammatory changes in the distal intestine of rainbow trout fed a semi-synthetic diet supplemented with 3.8 $g\,kg^{-1}$ saponin.

Data from these studies indicate that soybean saponins play a key role in soybean-induced enteritis in salmonids, but the pure compound will not induce enteritis at levels below about 2 $g\,kg^{-1}$ unless some other plant ingredients, such as lupin kernel meal or pea protein concentrate, are also present in the diet. Our knowledge of the role and effects of saponins in fish diets requires strengthening, particularly regarding interactions with other feed components, possible growth-promoting potential of at least certain saponins, and especially their effects in species other than salmonids. Current knowledge does not allow an accurate upper safe level to be set for the various saponins from different sources; however, levels up to 1 $g\,kg^{-1}$ appear to be safe (Francis et al. 2001). Caution should be exercised in mixing saponin-containing plant ingredients, especially legumes, in diets for farmed fish.

Alkaloids (Lupinine and Mimosine)

Structure, Sources, and Mechanism of Action

Alkaloids are heterocyclic compounds, mostly with basic characteristics, that are synthesized from amino acids in plants and other organisms. They are secondary metabolites that seem to serve roles in plant defense (Li et al. 2011). The effects of certain well-known alkaloids such as cocaine, caffeine, nicotine, morphine, atropine, ephedrine, and quinine are thoroughly characterized, but they are not important in animal nutrition. More relevant are alkaloids in lupins (genus *Lupinus*) and the legume tree *Leucaena leucocephala*, often called Jumpy-bean or wild tamarind.

Lupin seeds are well-known nutrient sources in animal nutrition. Screenings of the presence of alkaloids in *Lupinus* sp. show great variation and diversity (Wink et al. 1995; El-Shazly et al. 2001), and new discoveries are made as analytical methods improve (Neto et al. 2011). The predominant antinutrients in lupins belong to the quinolizidine alkaloids, comprising compounds such as lupinine, multiflorine, sparteine, and anagirine. These are of concern for animal health. Total alkaloid content has been shown to vary between species and also between cultivars of a single species (Sujak et al. 2006). Selective breeding has produced varieties of lupins with low alkaloid content, which are commonly called "sweet lupins" (Siddique et al. 2012). Currently, low alkaloid varieties contain less than 600 $mg\,kg^{-1}$ dry matter with some cultivars consistently containing less than 100 $mg\,kg^{-1}$ (Glencross 2001). Wild, high-alkaloid varieties can contain up to 40 000 $mg\,kg^{-1}$.

The negative effects of lupin alkaloids on feed intake appear to be mediated through the neural system. The mechanisms of action of lupin alkaloids are not well known. A bitter taste may be the major effect. However, they also seem to modulate anti-muscarinic

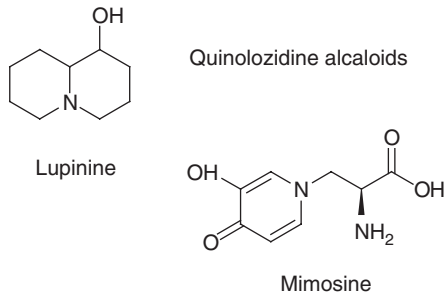

Figure 10.6 Quinolizidine alcaloids lupinine and mimosine.

acetylcholine receptor and inhibit nicotinic activity (Wink et al. 1998). Lupinine (Fig. 10.6) and sparteine may also inhibit α_2-adrenergic receptors, while sparteine also inhibits butylcholine esterase (Wink et al. 1998). Rodents have shown acute toxicity effects of lupin alkaloids (Robbins et al. 1996; Pothier et al. 1998). They may also be toxic for chickens (Olver and Jonker 1997), ducks (Olver 1997), and pigs (Godfrey et al. 1985). Pigs appear more sensitive to alkaloids than poultry.

The Jumpy-bean tree is a rapidly growing, drought-resistant tropical tree considered to be the fodder tree of highest quality and greatest palatability native to South America. The leaf quality compares favorably with alfalfa or lucerne in feed value, with 20% crude protein on a dry-matter basis. However, its content of mimosine (Fig. 10.6) renders it toxic to non-ruminants (Orwa et al. 2009). *In vitro* studies of effects of this compound have shown antimitotic activity and blocking of the cell cycle. It was found to inhibit DNA synthesis by altering deoxyribonucleotide metabolism (reviewed by Soedarjo and Borthakur 1998). Mimosine may also activate apoptosis through mitochondrial activation and formation of H_2O_2 (Hallak et al. 2008). It is a cell-specific antagonist of folate metabolism (Oppenheim et al. 2000) that binds iron in complexes, which may catalyze cleavage of DNA (Mikhailov et al. 2000).

Deleterious effects of mimosine in animals are impairment of growth, ill health, disruption of reproductive processes, and teratogenicity (D'Mello 2013). In experiments with rats and mice, one of the degradation products of mimosine, 3-hydroxy-4-pyridone (HP), has been observed to reduce growth and cause goiter and hair loss (Hegarty et al. 1979). Mimosine chelates iron strongly, a characteristic which may be important in its mechanism of action (Katoh et al. 1992).

Effects on Fish Performance, Immune Responses, and Disease Susceptibility

Only a few studies shed light on the effects of lupin alkaloids in fish. A recent dose response trial with rainbow trout showed dose-dependent negative effects on both feed intake and growth (Serrano et al. 2011). No effect on carcass composition was observed, but lupinine caused the depletion of glycogen and lipid in the hepatocytes. Histological examination of other internal organs did not reveal any dose-dependent effect of lupinine. The negative effects on feed intake, and consequently on liver glycogen and lipid stores, were suggested to be an organoleptic effect, that is, a result of the bitter taste of lupinine. Other studies have also shown negative effects of lupin alkaloids on feed intake (Glencross et al. 2006). The authors suggest that rainbow trout may tolerate higher levels of lupin alkaloids than other vertebrates, and that the low palatability of these alkaloids may prevent the ingestion of toxic doses. Lupinine levels above 100 mg kg^{-1} appeared to cause palatability problems. This indicates that palatability problems should not be a concern for the low alkaloid varieties of lupins (Glencross et al. 2004a). Serrano et al. (2012) also investigated effects of sparteine with very similar results. They concluded that sparteine alkaloid primarily reduced palatability without affecting the health of the fish. An upper limit of 100 mg kg^{-1} in trout diets should avoid growth reduction.

Reports of experiments with inclusion of purified mimosine in fish diets have not been found. *Leuceana* leaf meal has been evaluated as a protein source for some fish species with varying results. Performance data from experiments with Nile tilapia indicate that these leaves may be a valuable nutrient source (Panatastico and Baldia 1980; Ghatnekar et al. 1982). In Java tilapia (*Oreochronis mossambicus*), however, reduced performance was observed when fish were fed *leucaena* leaf meal (Jackson et al. 1982). Similar results have been reported for Nile tilapia (Santiago et al. 1988) and common carp (Mohire and Devaraj 1990). Male and female tilapia seem to react differently to *leuceana* leaf meal, with males having higher tolerance than females (Santiago et al. 1988).

At inclusion levels above 40% in the diet, *leuceana* leaf meal reduced growth of fry. The nutritive value of *leuceana* leaf meal may be improved by water extraction (Penaflorida et al. 1992) and/or fermentation (Bairagi et al. 2004); both methods reduce mimosine content.

New Ingredients May Introduce Other Antinutrients

As aquaculture expands, new plant ingredients are needed and are being investigated as sources of nutrients in fish feeds. The number of antinutrients in aquafeeds may therefore increase, and currently unknown antinutrients may be discovered. Known antinutrients that may play more important roles in the future with the introduction of novel feed ingredients include amylase, lipase, and arginase inhibitors, tannins, and various toxins and antigens/allergens.

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Chapter 11

Mycotoxin Contamination of Fish Feeds

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Introduction

Mycotoxin contamination of fish feeds that are used in commercial aquaculture is a potential threat to the health and productivity of aquaculture fish and the profitability of fish farming operations. Currently, more emphasis is placed on formulating fish diets that rely less on the inclusion of animal proteins as ingredients, such as marine fish meal and animal slaughter-house by-product meals, and more on protein and energy ingredients that originate from plant sources. Plant-source ingredients are considered to be renewable and as such are considered an important component of sustainability. As we make greater use of plant-source ingredients in fish feeds, we open the door to the possibility that aquaculture feeds will be contaminated with feed-borne mycotoxins. As commercial interests vie for the limited quantities of available commodity feed ingredients, it is important to know the effects that grain and grain by-products naturally contaminated with various levels of mycotoxins can have on fish health and productivity. Corn and wheat and their by-products are heavily used in commercial aquaculture feeds as sources of energy and protein. They also provide certain desirable manufacturing characteristics, such as binding capacity to maintain steam pellet hardness, which improves pellet endurance during shipping and handling. Additionally, the starch component of corn is needed in feeds manufactured using the cooker-extrusion process to improve the floatability of feeds that are used to feed pond-raised channel catfish (*Ictalurus punctatus*) in the USA, as well as other cultured finfish species worldwide.

Due to the current high prices of corn and wheat, by-products of these grains are being utilized to a greater extent to replace or partially replace expensive grains in fish feed formulations. The wheat by-product, wheat meddling (midds), and the corn by-product, dried distiller's grains with solubles (DDGS), are presently being used extensively in fish feeds to partially replace the energy and protein components of these feeds. As the need to use more grain by-products in aquaculture feeds increases because of higher grain commodity costs, there will be a need to consider the potential risks for mycotoxin contamination of these feed ingredients. Wheat midds is a by-product of wheat milling for flour that is used for human consumption. This by-product contains the outer portions of wheat: bran, wheat germ, and also small amounts of flour. However, because of its origin from the outer portions of the wheat grain, wheat midds may also contain *Fusarium* mycotoxins, especially deoxynivalenol (DON) and zearalenone (ZEN). Dried distiller's grain with solubles (DDGS) is a by-product of the grain fermentation industry to produce ethanol for automobile fuel. Since grains, such as corn or wheat, are used for the feedstock of this fermentation process and mycotoxins are thereby not destroyed; DDGS may contain up to three times the mycotoxin concentration of the original grain feedstock (M.H. Li, pers. comm., 2009).

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Aspergillus Mycotoxins

Aflatoxin was the first mycotoxin to be "discovered" as a contaminant of moldy peanut meal used as a feed supplement for turkeys in the UK in 1960. This single episode of mycotoxin contamination of feed resulted in the loss of over 100,000 turkeys and other agricultural animals (Blount 1961). Later that same decade, outbreaks of aflatoxicosis occurred at governmental and commercial trout hatcheries in the USA and resulted in the loss of numerous rainbow trout (*Onchorynchus mykiss*) that had been diagnosed with hepatocellular carcinoma (HCC; Halver 1969; Post 1987). The contaminated feed ingredient in those incidents was cottonseed meal used in dry feed pellets.

Since the 1960s, aflatoxin and other mold toxins have been some of the most studied natural toxic compounds in the world. Aflatoxins consist of at least four similarly structured chemical compounds designated as AFB_1, AFB_2, AFG_1, and AFG_2, with AFB_1 being the most common and also the most toxic form to animal systems. These toxins are produced by three main species of fungal organisms: *Aspergillus flavus*, *A. parasitcus*, and *A. nomius*. Because aflatoxin is classified as a carcinogen, numerous studies have been conducted to determine its effect on the health and productivity of agricultural animals. Regulations are enforced to limit its presence in animal feeds and foods designated for human consumption. Presently, the US Food and Drug Administration (FDA) enforces an action level of 20 ppb ($\mu g\,kg^{-1}$) aflatoxin in foods (except milk for human consumption: 0.5 ppb) and animal feeds (Meronek and Xie, 1999). The reason for this action level is that foods directly consumed by humans are considered safe at or below 20 ppb aflatoxin, and animal feeds that contain no more than 20 ppb aflatoxin are safe for most agricultural animals (except rainbow trout) and will not result in the accumulation of aflatoxin residues above 20 ppb in the edible portions.

Experiments with various species have demonstrated that rainbow trout are sensitive to the presence of even very small amounts of aflatoxin in feed, but warmwater fish, such as channel catfish and Nile tilapia (*Oreochromis nilotica*), tolerate much higher dietary levels of AFB_1 without exhibiting any harmful effects. Research has shown that rainbow trout fed a diet containing 20 ppb AFB_1 (a dietary level compliant with the FDA action level) for eight months had a 58% incidence of HCC, and trout fed the same diet for 12 months had an 83% incidence of HCC (Schoendhard et al. 1981). While evaluating the effect of AFB_1 on channel catfish, Jantrarotai and Lovell (1990a) found during a 10-week aquarium study that catfish fed semi-purified diets containing up to 2154 ppb purified AFB_1 experienced no reduction in growth or internal lesions; catfish fed a similar diet containing 10 000 ppb AFB_1 had reduced growth and internal lesions but no HCC. Manning et al. (2005a) found that channel catfish fed practical diets containing either 20% or 40% moldy corn (550 ppb total aflatoxins) over a 12-week period experienced no significant reductions in growth, feed efficiency, or survival. Using the same lot of moldy corn, channel catfish were fed practical diets containing either 50% moldy corn or a combination of 25% moldy corn and 25% clean corn in a 130-day pond study. The results showed no significant reductions in average body weight gain (grams per fish), feed efficiency (kilogram of gain per kilogram diet), or net pond production ($kg\,ha^{-1}$) when compared to catfish fed the control diet consisting of 50% clean corn (Manning et al. 2005a). It was also determined in this study that pond diets containing aflatoxin-contaminated corn manufactured by the cooker-extrusion method had a reduction of over 60% of total aflatoxins (Manning et al. 2005a).

Nile tilapia that were fed a diet containing 940 ppb AFB_1 for 25 days did not experience significant reduction in growth, although tilapia fed similar diets that contained at least 1880 ppb AFB_1 had reduced growth rates (Chavez-Sanchez et al. 1994). Nguyen et al. (2002) found that juvenile Nile tilapia fed semi-purified diets that contained at least 2500 ppb AFB_1 derived from an *A. parasiticus* culture material had significantly reduced body weight gains, poor feed conversion ratios (FCR), and lower hematocrit values when compared to the control-diet fed tilapia, but these deleterious effects were not observed for tilapia fed a diet containing 250 ppb AFB_1. HCC was not observed in tilapia fed any of the different levels of AFB_1 (Nguyen et al. 2002). It is apparent from the previous experimental results that warmwater fish, such as channel catfish and tilapia, are more tolerant to the deleterious effects of aflatoxin than coldwater species such as rainbow trout. Reasons for the differences in sensitivity to aflatoxin may lie in

differences of the liver enzyme detoxification systems of these two groups of fish. Jantrarotai and Lovell (1990a) speculated that channel catfish have a more efficient liver detoxification system than rainbow trout. Strength et al. (1982) reported that channel catfish have two liver enzyme systems to detoxify xenobiotics: the glucuronyltransferase and sulfotransferase systems. Rainbow trout have only one: a glucuronyltransferase liver detoxification system (Loveland et al. 1984).

Another mycotoxin produced by a fungal organism in the *Aspergillus* genus is cyclopiazonic acid (CPA) which is produced by *A. flavus*, the same species of mold organism that elaborates aflatoxin. Jantrarotai and Lovell (1990b) observed that channel catfish exposed to diets containing 100 ppb CPA or higher had lower body weights than fish fed the control diet. Because *A. flavus* produces both aflatoxin and CPA, there is reasonable concern that fish feed contaminated with both of these mycotoxins would have a greater toxic effect than feed contaminated with only one. The effects of the combination of these two mycotoxins have yet to be evaluated.

One mycotoxin that has been shown to have detrimental effects on aquaculture fish is Ochratoxin A (OA), which is produced by *Aspergillus ochraceus* and certain mold species of the *Penicillium* genus. OA is produced using corn, wheat, barley, and oilseeds such as soybeans or peanuts as substrates. Manning et al. (2003a) found that diets serially amended with *A. ochraceus*-contaminated corn culture material that contained 80 ppm OA had detrimental effects on juvenile channel catfish reared in aquaria under controlled environmental conditions. The semi-purified diets contained 0 (control), 0.5, 1.0, 2.0, 4.0, and 8.0 ppm OA. Significant reductions in body weight gains were observed for all diets containing 2.0 ppm OA or greater during the first 2 weeks, and at each subsequent 2-week weighing. After 8 weeks of feeding, all diets containing 1 ppm OA or above had significantly lower body weight gains compared to fish fed the control diet. Poorer FCR values were observed for catfish fed diets containing 4 and 8 ppm OA after 8 weeks. Hematocrit values and survival rates were significantly lower for catfish fed the highest level of OA (8 ppm) when compared to the control diet or the other treatments containing lower levels of OA.

When kidney and liver tissues were examined for histopathological changes, there was evidence of increased presence and severity of melanomacrophage centers in both tissues of catfish fed OA amended diets containing 2.0 ppm or greater. Usually, consumption of foods contaminated with OA has been associated with nephropathy in both humans and other animals. However, channel catfish used in this study did not experience nephropathy, but did demonstrate substantial reductions (and in some specimens, complete absence) in the number of cells of exocrine pancreatic tissue that are associated with the hepatic portal veins of channel catfish fed diets with levels of 1.0 ppm OA or greater (Manning et al. 2003a).

Fusarium Mycotoxins

Fusarium mycotoxins, while not as well studied as aflatoxin, have garnered considerable attention during the past decade, especially since the harvest season of 2009 when high levels of mycotoxin contamination in field corn with deoxynivalenol (DON) and zearalenone (ZEN) occurred. The weather-related conditions that provided an optimum environment for mycotoxin development that year included cooler-than-normal temperatures and higher moisture levels as a result of excessive rains coupled with delayed harvests. DON is a fairly common mycotoxin contaminant in the trichothecene mycotoxin group, and is produced by *Fusarium graminearum*. This fungal organism infects field crops such as corn and wheat, resulting in the corn disease, corn ear rot, and the wheat disease, *Fusarium* head blight. The presence of DON in grains used to manufacture agricultural animal feeds can cause disruption of normal feeding activity in swine and poultry. Swine fed diets containing more than 2–3 ppm DON have demonstrated some degree of feed refusal and eventually emesis, hence the vernacular name "vomitoxin". Chickens are sensitive to dietary DON, but not to the same extent as swine. Chickens fed diets containing 5 ppm or greater DON have exhibited feed refusal and emesis. Other animals that are sensitive to the presence of DON in their food are dogs and cats (Hughes et al. 2001).

The effect of DON on aquaculture fish appears to vary depending on the species of fish exposed to this mycotoxin. Warmwater fish, such as channel catfish, are only affected by relatively higher dietary

concentrations of DON. Juvenile channel catfish (5.0 g/fish) were fed practical diets containing 2.5, 5.0, 10.0, 15.0, and 17.5 ppm DON (prepared using wheat contaminated with 37.5 ppm DON), and only fish fed the two highest levels of DON experienced reduced weight gain and poor FCR (Manning 2005). On the other hand, coldwater fish such as rainbow trout appear to be very sensitive to dietary DON. Woodward et al. (1983) found that rainbow trout fed diets containing 1.0–13.0 ppm DON for four weeks had progressively lower body weights. More recently, Hooft et al. (2011) reported that rainbow trout fed diets containing relatively low levels of DON at 0.3 (control), 0.8, 1.4, 2.0, and 2.6 ppm (prepared from DON-contaminated corn) had significant linear reduction in weight gain, feed intake, and feed efficiency during the eight-week feeding trial.

Fumonisin is a *Fusarium* mycotoxin that was isolated and chemically defined in the late 1980s (Gelderbloom et al. 1988). Its potential presence in animal feeds was widely accepted beforehand because of the devastating condition equine leukoencephalomalacia (ELEM), which occurred in equines that had consumed feeds containing fumonisin. ELEM results in the liquefaction of brain tissue, and occurs in horses that consume feeds containing as little as 2–5 ppm fumonisin (Ross et al. 1993). In addition, consumption of fumonisin-contaminated feeds caused porcine pulmonary edema in swine (Colvin and Harrison 1992). Fumonisin actually refers to a mixture of mycotoxins that have similar chemical structures. The most abundant and most toxic fumonisin is FB_1, which accounts for approximately 75% of the mixture. The fungal organisms that produce fumonisin are *F. verticillioides* and *F. proliferatum*, usually found in field corn during its growth stage.

The effect of fumonisin on channel catfish was examined by Lumlertdacha et al. (1995). They discovered that juvenile catfish (with an initial body weight of 1.2 g/fish) had significant reduction in body weight gain after consuming a diet containing 20 ppm FB_1 for 10 weeks, while 2-year-old catfish (with initial body weight of 30.0 g/fish) required 80 ppm FB_1 before significantly reducing body weight gain. Catfish liver sampled from this experiment also had significant increases in the ratio of sphinganine to sphingosine (SA: SO; Goel et al. 1994). Fumonisin disrupts sphingosine metabolism by competitive inhibition of the liver enzyme, ceramide synthetase, which results in accumulation of sphinganine in liver and serum by inhibiting conversion of sphinganine to sphingosine. The increase in SA: SO ratio is an accepted biomarker of fumonisin toxicosis (Riley et al. 1994).

Yildirim et al. (2000) fed semi-purified diets containing the *Fusarium* mycotoxins FB_1 and moniliformin (MON) either individually or in combination to juvenile channel catfish with an initial body weight of 1.5 g/fish. Catfish fed the lowest level (20 ppm) of either mycotoxin individually for 10 weeks had significantly reduced body weight gain compared to the catfish fed the control diet. For FB_1, this reduction in body weight gain is in agreement with the results of Lumlertdacha et al. (1995) for juvenile catfish with similar initial body weight. Catfish fed semi-purified diets containing 20, 40, 60, or 120 ppm MON had significantly reduced weight gain when compared to fish fed the control diet. For catfish fed diets containing either 20 or 40 ppm FB_1, weight gains were significantly poorer than the control-fed fish. The combinations of 40 ppm MON with either 20 or 40 ppm FB_1 resulted in further significant reduction of body weight gain when compared to the control-fed fish or fish fed the diet containing 40 ppm MON only. Feeding the combination of MON and FB_1 at 40 ppm resulted in significantly greater reductions in body weight gains than catfish fed either mycotoxin individually at the same concentration.

Since MON can be produced by isolates of the same fungal organisms that synthesize FB_1, specifically *F. vertillioides* and *F. proliferatum*, there is concern that co-contamination of field corn may occur and result in greater reduction in body weight gain through the synergistic action of the combination of these two mycotoxins. Catfish fed diets containing MON had increased levels of serum pyruvate, which is the accepted biomarker for exposure to dietary MON. Pyruvate accumulates in the serum and other tissues because MON disrupts pyruvate metabolism by inhibiting pyruvate formed from glycolysis from entering the TCA cycle (Yildirim et al. 2000). Catfish fed diets containing FB_1 had elevated SA: SO ratio, which confirmed that these fish had been fed diets containing fumonisin.

The effect of feeding Nile tilapia with diets containing FB_1 from corn culture material was evaluated by Nguyen et al. (2003). Tilapia with an initial body

weight of 2.7 g/fish were fed diets containing 0 (control), 10, 40, 70, or 150 ppm FB_1 for 8 weeks. Fish that were fed diets with 40 ppm FB_1 or greater had reduced weight gain and poorer FCR than fish fed the control diet or the diet with 10 ppm FB_1. Tilapia fed 150 ppm FB_1 also had significantly lower hematocrit values than fish fed the other experimental diets. The sphingolipid ratio (SA: SO) was significantly elevated for tilapia fed the diet with 150 ppm FB_1. In the same experiment, these researchers also evaluated the effect of feeding diets containing 0 (control), 10, 40, 70, or 150 ppm MON to tilapia for eight weeks. Tilapia fed diets with 70 or 150 ppm MON had lower body weight gain and poorer FCR values than tilapia fed the control diet or the diet with 10 ppm MON. Fish fed the diet with 40 ppm MON had significantly higher weight gain than fish fed 150 ppm MON only. Hematocrit values were significantly lower for tilapia fed diets containing 70 or 150 ppm MON compared to the hematocrits of fish fed the control diet or the diet with 10 ppm MON. Serum pyruvate levels for all fish fed diets containing MON were significantly elevated above the serum pyruvate level of the control-fed tilapia, confirming that tilapia fed the treatment diets had received MON from the feed (Nguyen et al. 2003).

Gbore et al. (2010) evaluated the effects of feed-borne FB_1 on African catfish (*Clarias gariepinus*) fingerlings. African catfish (with an average initial weight of 17.35 g/fish) were fed for 6 weeks with practical diets prepared with corn that had been cultured with *F. vertillioides* and contained FB_1. The experimental diets were formulated with the cultured corn to contain 5.0, 10.0, and 15.0 ppm FB_1. A control diet was prepared with non-cultured corn that did not contain FB_1. Results showed that significant body weight gain and FCR of African catfish were not adversely affected by exposure to feed-borne FB_1 at these levels. However, there were significant reductions in weight gain when expressed as a percent of initial weight. The reasons for non-significant reductions in weight gain were probably related to feeding dietary levels of FB_1 that were not high enough for fish with an average initial body weight 17.35 g/fish.

Since African catfish is a warmwater fish, it would be expected that this species is reasonably tolerant of higher dietary levels of FB_1. In support of this concept, Lumlertdacha et al. (1995) found that channel catfish, another warmwater species, had reduced body weight gain when much smaller fish with initial body weight of 1.2 g/fish were fed diets containing at least 20 ppm FB_1 for 10 weeks. Lumlertdacha et al. (1995) also found that larger year-2 channel catfish with average initial body weight of 30.0 g/fish experienced significantly reduced body weight gain when fed a diet with a concentration of 80 ppm FB_1 for 14 weeks. These results indicate that there is a clear body weight/dose–response relationship to feeding diets containing FB_1 to warmwater fish such as the channel catfish and possibly the African catfish. If such is the case, a strategy to maintain fumonisin levels below those shown to reduce catfish growth and health status could be developed in locations where field corn used in fish feed is routinely contaminated with fumonisin.

The effect of the trichothecene mycotoxin, T-2 toxin, was assessed by Manning et al. (2003b). T-2 toxin is usually produced by *Fusarium* spp. on small grains, such as wheat. The levels of T-2 toxin contamination of field corn may be enhanced by the same environmental conditions that boost the levels of DON, such as prolonged cool and rainy environmental conditions, which impose delayed harvest of the infected grain. T-2 toxin can initiate the same effects as DON: disruption of normal feeding patterns in animals. When consumed by an animal, T-2 toxin triggers increased levels of tryptophan in the brain. Tryptophan is the amino acid precursor of serotonin, the ubiquitous neurotransmitter that influences the expression of appetite specifically to create the awareness of satiety. Aside from its influence on biochemical transmitters such as serotonin, T-2 toxin has some direct toxic effects on animals. T-2 toxin is classified as a radiomimetic compound because it mimics the effects of ionizing radiation, especially on the hematopoietic tissue found in the bone marrow (Otokawa 1983) and the head kidney (channel catfish).

Channel catfish (8.9 g/fish) were fed semi-purified diets containing various levels of purified T-2 toxin. The dietary levels of T-2 toxin were 0 (control), 0.625, 1.25, 2.5, or 5.0 ppm, and three treatments were pair-fed the control diet at the same daily weighed amounts as the catfish fed the 1.25, 2.5, and 5.0 ppm T-2 toxin diets (Manning et al. 2003b). Results showed that catfish fed any of the diets with T-2 toxin had significantly lower body weight gains than catfish fed the control diet. The FCR of the treatment fed the

5 ppm diet was significantly poorer than that of the control treatment, but FCR of the comparative (5 ppm treatment) pair-fed treatment was not significantly different from the control treatment. Weight gain of the pair-fed treatment (5 ppm) was lower than the weight gain of the control treatment because of the reduced daily allowance of diet. Hematocrit values of catfish fed the three highest concentrations of T-2 toxin were significantly lower than the hematocrits of their pair-fed comparative treatments and the control treatment. This response could be anticipated because of the potential radiomimetic effect of ingested T-2 toxin on hematopoietic tissue. This outcome was verified by the finding from histological examination of head kidney tissue, which revealed greater depletion of hematopoietic cells in catfish fed diets with the three highest concentrations of T-2 toxin.

It is apparent from these results that the adverse effects of feeding diets containing T-2 toxin to channel catfish are due to actual toxic effects rather than simply reductions in diet intake (Manning et al. 2003b). Both T-2 toxin and DON are not destroyed by the feed manufacturing processes used to produce fish feeds. Usually, the processes of steam pelleting and cooker-extrusion require the application of heat derived from steam and the friction of forcing the moistened feed mixture through restrictive openings of dies that are used to form feed particles.

Other *Fusarium* mycotoxins that have not been examined for their effects on aquaculture fish include zearalenone and fusaric acid. Zearalenone (ZEN) causes reproductive problems in terrestrial farm animals. Zearalenone is a non-steroidal compound that displays estrogenic activity in certain animals. While cattle and sheep are minimally affected by the presence of ZEN in feed, swine are particularly vulnerable to its effects (CAST, 2003). Affected gilts and sows display signs of reproductive activity including swollen vulva, mammary glands, and uterus. Young male pigs develop testicular atrophy (Coulombe 1993). Female pigs have shown the effects of consuming ZEN in their feed at concentrations as little as 1 ppm. Zearalenone appears to function as an estrogen antagonist by competing for estrogenic receptors and thus preventing endogenous estrogen from attaining normal physiological function. Because ZEN is produced by *F. graminearum*, the same mold organism that elaborates DON under similar environmental conditions, the presence of ZEN in field corn that is contaminated with DON can be expected. Zearalenone has not been evaluated in fish but should be, especially since aquaculture diets are currently being formulated with increased amounts of plant-source ingredients, which could be contaminated with ZEN.

Fusaric acid is produced by *F. vertillioides*, the same *Fusarium* fungi that produce other mycotoxins (Bacon et al. 1996) including fumonisin and fusaric acid. Fusaric acid acts to increase the levels of serotonin in animals that ingest this mycotoxin through consumption of contaminated grains. As a result, animals may exhibit feeding behavioral characteristics that are similar to those displayed by animals that consume DON-contaminated grains, that is, feed refusal and emesis.

Synergism

Even though fusaric acid is considered to be a phytotoxin, it is capable of synergizing with other *Fusarium* mycotoxins to produce toxic effects in animals that are greater than those produced by exposure to each mycotoxin individually. Bacon et al. (1995) demonstrated that injections of fusaric acid at non-toxic concentrations (5 µg/egg) into fertile chicken eggs, along with concurrent injections of FB_1 at non-toxic concentrations (5 µg/egg), increased the mortality of developing embryos to 50% compared to no mortality for injections of 5 µg FB_1/egg alone. Other synergistic responses have been reported under experimental conditions; as mentioned earlier, a synergistic response of greater reduction in weight gain of channel catfish as a result of feeding a diet containing 40 ppm MON and 40 ppm FB_1 was observed by Yildirim et al. (2000). While it has not been evaluated, there is concern that the presence of a combination of aflatoxin and CPA in feeds for fish could have negative synergistic effects on fish growth and health, since both mycotoxins are produced by *A. flavus*.

Mycotoxin Effects on Fish Disease Resistance and Immune Response

Even though literature usually suggests that the consumption of mycotoxin-contaminated feeds will result in impaired immune response, this is not always

the case. Manning et al. (2011) fed channel catfish in aquaria practical corn-soy diets containing various levels of moldy, aflatoxin-contaminated corn blended with clean corn (no aflatoxin) to achieve six dietary levels of total aflatoxins at 0, 10, 20, 40, 80, and 160 ppb. The fish were fed weighed amounts of the diets based on percent body weight for 7 weeks, at which time the fish were group weighed and counted. After weighing, the catfish were returned to their original aquaria and feeding of assigned diets resumed for another 3 weeks to restore acclimation of the fish before implementing a bacterial challenge. After 10 weeks of feeding the research diets, catfish were exposed *in situ* for 30 minutes to 30 mL of a nutrient broth suspension of the channel catfish bacterial pathogen *Edwardsiella ictaluri* containing 3.0×10^9 CFU mL^{-1} to provide a final aquarium concentration of 7.5×10^6 CFU mL^{-1}. Results showed that there were no significant differences among treatment mortalities at 21 days post-challenge, the 7-week data on weight gain, feed intake, and feed efficiency, or 10-week survival. These responses by channel catfish fed feed-borne aflatoxin could have been anticipated since Jantrarotai and Lovell (1990a) and Manning et al. (2005a) did not observe unfavorable responses to feeding diets containing aflatoxin. The two earlier experiments did not include any evaluation of channel catfish disease resistance to *Edwardsiella ictaluri*, but that study (Manning et al. 2011) underscores that channel catfish productivity, that is, growth, feed consumption, and feed efficiency, is not adversely affected by feed-borne aflatoxin. Notably, that study demonstrated that, at the concentrations tested, feeding diets containing aflatoxin derived from moldy corn did not increase the incidence of mortality among channel catfish challenged with *E. ictaluri.*

In contrast, Manning et al. (2005b) evaluated the effects of diets containing T-2 toxin or ochratoxin A (OA) on channel catfish survival after a challenge with *E. ictaluri*. Feeding a semi-purified diet amended with 1.0 or 2.0 ppm T-2 toxin and 2.0 or 4.0 ppm OA for six weeks resulted in significantly reduced body weight gains for catfish fed any of the diets containing either mycotoxin when compared to catfish fed the control diet containing no mycotoxin. Pre-challenge survival was high for all diets with no significant differences observed. For catfish challenged with the bacterial pathogen *E. ictaluri* (2.25×10^6 CFU mL^{-1} aquarium water), 21-day post-challenge mortality was significantly higher for both dietary levels of T-2 toxin; for OA however, only the post-challenge mortality of the higher level (4.0 ppm) was significantly different. Catfish fed the higher level of T-2 toxin experienced greater post-challenge mortality that was 31% higher than the control-fed catfish.

Lumlertdacha and Lovell (1995) observed that year-2 channel catfish fed practical corn-soy diets amended with *F. vertillioides* corn culture material that contained a known concentration of FB_1 (80 ppm) had reduced disease resistance when challenged with *E. ictaluri*. In the same study, antibody production was significantly decreased for catfish fed diets containing 20 or 80 ppm FB_1 when compared to the antibody titer of the control-fed catfish.

It is apparent from the abovementioned studies that feed-borne mycotoxins may contribute to decreased disease resistance and impaired antibody production to fish disease pathogens, resulting in increased levels of mortality. These studies demonstrate that there is a potential risk to feeding fish diets that are obviously contaminated with mold growth because of the possibility that the molds have produced mycotoxins that will reduce aquaculture fish productivity and impair disease resistance to bacterial epizootics, thereby reducing fish farm profitability.

Fish Feeds

Manufacture of Fish Feeds

Proper manufacturing of commercial fish feeds is a key component of fish farming. During manufacturing, a blended mixture of ingredients is integrated into a uniformly nutritious feed particle or pellet through steam pelleting or cook-extrusion processes. Both of these feed manufacturing processes include heated water and steam, which aid in the formation of the pellets or feed particles and improve their nutritional quality and handling durability. Because heated water and steam are used to facilitate these processes, the moisture content of the dry feed mixture prior to exiting pellet dies will increase from about 12% to approximately 16% for steam pelleting and up to 24% for cook-extrusion process.

It is necessary to reduce the water content of feed pellets to: (1) harden them; and (2) reduce the

development of mold organisms, thereby diminishing the likelihood of mycotoxin contamination. The feed pellets must therefore be dried to a moisture level of 10–12%. Moisture reduction of aquaculture feeds is accomplished by two different methods. Steam-pelleted feeds are dried using forced-air evaporative cooling in a vertical pellet dryer for 20–25 minutes to achieve a moisture content of approximately 10–11%. Drying of cook-extrusion processed feeds is accomplished using a more dynamic drying method, such as the horizontal dryer. This type of dryer consists of 3–4 levels of continuously moving expanded-steel belts that convey the extruded feed particles horizontally within a large metal cabinet that operates with forced air heated to 120°C by gas-fired burners. It takes about 15–20 minutes to lower the moisture to approximately 9–11%. The last part of the drying process uses forced air at ambient temperature to cool the dried feed particles. The cooled, dried feed pellets are conveyed within the feed mill to bulk bins for bagging or, in some cases, for loading into bulk trucks for farm delivery.

Storage of Fish Feeds to Prevent Mycotoxin Contamination

Proper storage of fish feeds is very important to avoid the growth of molds or fungi and maintain mycotoxin-free feeds. Bagged feeds should be stored in structurally sound buildings with leak-proof roofs that protect the feeds from rain and other forms of precipitation. Bagged feeds should be stacked on wooden pallets several centimeters from structural floors and walls that could potentially convey condensed, humid air to the bag contents, causing the feeds to become moldy. Feeds that are shipped in bulk to the fish farm should be stored in bulk bins that have been periodically cleaned of old feed that could be moldy. Fish farms are often located in tropical or semi-tropical areas that not only experience high temperatures but also high humidity, which could provide optimum conditions for development of mold on feeds. If moldy feeds are detected in storage facilities, it would be prudent to delay the use of these feeds until they can be screened for mycotoxin content. If the quantities of moldy feed are too small to warrant the expense of testing for mycotoxin contamination, the feeds should be disposed of in such a way that is not harmful to fish or other animals. Fish feeds should be kept free of infiltration and eventual damage by insects and rodents. In large numbers, these pests not only render feed unpalatable to fish, but also rob the feed of important nutrients and create conditions that are suitable for the development of mold growth on feed.

Testing for Mycotoxins in Aquaculture Feeds

Testing of fish feeds suspected of being contaminated with mycotoxins can be accomplished by submitting samples of the feeds to a commercial testing laboratory, or using kits that are available for such evaluation. Most of these kits are simple to use and rely on enzyme-linked immunosorbent assay (ELISA) technology. Many of these test kits provide only qualitative (yes/no) results as to the presence of a specific mycotoxin. Other ELISA kits also provide quantitative information about the concentration of the mycotoxin in the feed. The drawback to these types of mycotoxin testing kits is that, in order to obtain accurate mycotoxin concentration results, an electronic test reader needs to be used to interpret the concentration.

More accurate procedures for the determination of the concentration of mycotoxins in fish feeds can be obtained using a commercially prepared clean-up column, called a monoclonal immunoaffinity column. The column can isolate a specific mycotoxin with a chemical solvent (usually chemically derivatized methanol), and determine the concentration with a fluorometer instrument. Alternatively, an HPLC (high-performance liquid chromatography) procedure can be used to quantify mycotoxin after its elution from the immunoaffinity column. The last two methods are usually used only in research and some commercial testing laboratories.

Mitigation of Mycotoxin Contamination in Aquaculture Feeds

Methods to reduce the toxicity of some feed-borne mycotoxins have been devised. These may involve

the use of special, adsorbent clays know as hydrated sodium calcium aluminosilicates, or HSCAS. These clays seem to be very effective in animals that may consume aflatoxin-contaminated feeds by binding aflatoxins and blocking intestinal adsorption of this group of mycotoxins (Taylor 1999). The effectiveness of HSCAS in binding other mycotoxins has not been established, but it has been suggested that these reactive clay products do not bind very well to mycotoxins other than aflatoxin. The reason that HSCAS bind aflatoxins lies in the distinctive molecular structure and chemical composition of these compounds. The aflatoxin molecule has a rigid co-planar structure and possesses a 1,3 diketone moiety; both characteristics facilitate the binding properties of HSCAS to aflatoxin.

Claims that HSCAS and other mycotoxin binders will bind mycotoxins other than aflatoxin are usually based on *in vitro* evaluation in the laboratory rather than *in vivo* testing in the field. Ledoux and Rottinghaus (1999) found that *in vitro* evaluation of the mycotoxin binding efficacy does not always predict the *in vivo* binding capacity of mycotoxins in poultry feeds. Their research determined that various adsorbents tested with poultry feeds were not effective in binding feed-borne FB_1, moniliformin, OA, or DON in poultry feeds. Commercial HSCAS preparations are considered to be effective in binding feed-borne aflatoxin and therefore prevent aflatoxicosis in poultry, swine, and other farm animals (CAST 2003).

Mycotoxin binders such as activated carbon compounds (charcoal) have been used in animal feeds with varied success (Diaz and Smith 2005), but have not been evaluated in aquaculture feeds. Polymeric glucomannan preparations derived from the cell walls of the yeast organism *Saccharomyces cerevisiae* are commercially available as feed additives for the purpose of sequestering feed-borne mycotoxins and preventing intestinal absorption of the mycotoxins. Sequestering by the yeast-derived polymer, and therefore reduction of intestinal absorption of the feed-borne mycotoxin, varies with the mycotoxin that contaminates the feed. In some cases, sequestering appears to be complete because the added glucomannan prevents all of the symptoms of mycotoxicosis; in other cases however, addition of the polymeric yeast preparations to mycotoxin contaminated feeds appears to eliminate some but not all symptoms (Swamy et al. 2003).

The cooker-extrusion process that is used to manufacture commercial floating channel catfish feeds in the US appeared to be very effective in eliminating or destroying a substantial amount of the aflatoxin that was contained in diets prepared from naturally contaminated field corn (Manning et al. 2005a). A review of the literature indicates that aflatoxin is very sensitive to the application of external heat (Coomes et al. 1966). In their study, peanut meal containing 7000 ppb aflatoxin was autoclaved at 120°C for varying lengths of time. Meal that received the heat treatment for 30 minutes had an aflatoxin concentration of 5000 ppb, while meal that received the heat treatment for 4 hours had an aflatoxin concentration of 340 ppb (a 95% reduction). These researchers also performed a biological evaluation of the heat treatment procedure using ducks, an animal that is sensitive to dietary aflatoxin. Mortality of ducks fed a diet prepared with 10% unheated peanut meal was 100% (4/4), compared to no mortality (0/4) for ducks fed a diet using 10% of peanut meal that contained 1000 ppb aflatoxin as a result of being heat treated as described previously.

In a separate study, Coomes et al. (1966) determined that the UV absorption spectrum of aflatoxin that normally appears at 265 and 363 nm was replaced by a single band at 258 nm. It was determined that the breakdown product that absorbed at 258 nm could indicate the presence of *o*-coumaric acid, which could be formed as a result of the hydrolytic opening of the lactone ring of AFB_1. It was concluded that autoclaving aflatoxin at 120°C in the presence of moisture results in the formation of non-toxic breakdown products (Coomes et al. 1966). These conditions are very similar to those used in the cooker-extrusion process for the production of catfish feeds in the US (Manning et al. 2005a).

It is important to note that some extrusion methods are not exactly the same as the cooker-extrusion method referred to here. For example, some extrusion methods do not incorporate the cooking phase into the method, but instead expose the feed mixture to high temperatures (from steam and compression in the extrusion barrel) for a fairly brief period of time (of the order 20–30 seconds). In the cooker-extrusion method, the feed mixture is mixed with hot water and steam in a cooking compartment that is separate from the extruder for up to 3–4 minutes at 120°C before being mechanically transferred to the extrusion barrel, where the moist mixture is exposed to more steam from injection ports located along the barrel.

Additionally, the dough-like feed mixture is subjected to the effects of compression, kneading, and shearing as it is moved through the extrusion barrel by the turning action of the internal screw (augar). Transit time for the feed mixture in the barrel is approximately 30 seconds (Rokey 1993). Subsequently, extruded catfish feeds must be dried, which requires the application of heated, forced air at 120°C for approximately 20–25 minutes, as previously described.

Because virtually all commercial catfish feeds are manufactured by the cooker-extrusion method due to the preference for floating feed pellets by US catfish farmers, a substantial reduction in aflatoxin content of these feeds could be realized by utilizing this feed manufacturing method. By following the example outlined in Manning et al. (2005a), corn that contained 100 ppb aflatoxin could be used in producing catfish feeds or feeds for other warmwater fish that meet the FDA action limits of 20 ppb. The feeds were produced by combining the diluting effect of the other dietary ingredients with the reduction in aflatoxin content as a result of the cooker-extrusion and pellet drying processes. Typically, catfish diets contain about 35% ground corn (Robinson et al. 2001); for corn that contained 100 ppb aflatoxin in this example, the final diet mixture before extrusion possessing would contain 35 ppb aflatoxin. At the conclusion of the cooker-extrusion and drying possesses, the diet would contain 14 ppb aflatoxin after a 60% reduction in aflatoxin content, which is well below the FDA 20 ppb action limit. Since many aquaculture feeds are manufactured using the cooker-extrusion process, it might be possible to reduce the aflatoxin levels of these feeds to acceptable concentrations while making use of aflatoxin-contaminated grains.

Other mycotoxins are not as sensitive to heat treatment as aflatoxin; these include OA, fumonisin, moniliformin, DON, and other trichothecene mycotoxins. Little or no benefit could be expected as a result of using this process to mitigate the toxicity of these mycotoxins.

Conclusion

Understanding the problems that can be encountered by feeding aquaculture feeds contaminated with mycotoxins is important. Fish comprise substantial portions of the diets of people in many parts of the world. Eventually, fish grown using aquaculture technologies will comprise a larger part of the diet of people throughout the world because of the decline of wild fisheries. Even though problems with mycotoxins are not always present, over extended periods of time the threat of mycotoxin contamination of aquaculture feeds occurs periodically. These situations occur when environmental conditions are not optimum to allow for normal development of grain crops.

Adverse environmental conditions, such as drought and insect-caused damage, can increase plant stress and result in the invasion of ubiquitous mold organisms and the subsequent production of mycotoxins in grain crops. When mature grain crops cannot be harvested in a timely manner due to weather delays, opportunistic fungal organisms, especially *Fusarium* spp., can infect plants and contaminate grains with mycotoxins. Molds can develop on feed ingredients and manufactured fish feeds that are not stored properly. These mold organisms may not only produce mycotoxins but, from the fish nutritionist's point of view, may also reduce the nutritional value of the feed as they use nutrients for their own development. As more fish are grown on aquaculture farms, the use of plant-source ingredients in fish feeds will become more common and, as such, increase the opportunities for exposure to mycotoxin contaminated feeds. It is therefore essential to understand the effects that consumption of mycotoxin-contaminated feeds can have on fish productivity and health.

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Chapter 12
Nucleotides

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Introduction

Nucleotides serve numerous essential physiological and biochemical functions including encoding and deciphering genetic information, mediating energy metabolism, and signaling information to cells; they are also components of coenzymes, allosteric effectors, and cellular agonists (Carver and Walker 1995). Nucleotides can be *de novo* synthesized from amino acids such as glutamine, glycine, and aspartate by all creatures, including aquatic animals. Nucleotides have therefore traditionally been considered to be non-essential nutrients because neither overriding biochemical malfunctions nor classical signs of deficiency are developed in human or animal models. However, numerous research groups have presented evidence that dietary nucleotide deficiency may impair liver, heart, intestine, and immune functions (reviewed by Grimble and Westwood 2000a) and that exogenous nucleotides influence lymphocyte maturation, activation and proliferation, macrophage phagocytosis, immunoglobulin responses, and genetic expression of certain cytokines in humans and various animal models (reviewed by Grimble and Westwood 2000a; Gil 2002). Potential application of dietary nucleotides in aquaculture has received heightened attention since Burrells et al. (2001a,b) published studies showing beneficial effects of dietary inclusion of commercial nucleotide supplement Optimûn on various salmonid species. Commercial nucleotide products including Optimûn (Chemoforma Co., Basel, Switzerland) and Ascogen P (Canadian Biosystems Inc., Calgary, Alberta, Canada) were evaluated with various aquatic species, and presumed beneficial influences of nucleotide supplementation were rather consistent across those studies. Li and Gatlin (2006) reviewed research related to commercial nucleotide products with aquatic animals and concluded that nucleotides should be considered as a semi-essential nutrient for aquatic animals. However, Li and Gatlin (2006) also emphasized the restrictions on expansion of knowledge related to nutrition of nucleotides and application of nucleotides in aquaculture feeds in that review. In the subsequent years, improvements in experimental design, especially sources of nucleotides for experiments, have been made to better define roles of exogenous nucleotides and influences on lives of aquatic animals (Li et al. 2007a,b; Lin et al. 2009; Cao et al. 2011; Welker et al. 2011; Xu et al. 2011). The aforementioned researchers abandoned commercial nucleotide products in their studies and used mixtures of pure nucleotides, or pure individual nucleotides in

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the cases of Lin et al. (2009) and Song et al. (2012). These improvements precluded possible interfering effects of non-nucleotide compounds that may be present in various commercial products and clearly defined nucleotide nutrition with aquatic animals. At the same time, research with commercial nucleotide products progressed and provided additional insights into physiology, immunology, and proteomic changes that may be related to nucleotide nutrition. The use of nucleotides in aquatic feeds was initially intended to provide a promising immunostimulant and potential alternative to chemotherapeutics in aquaculture, but increasing evidence shows the additional beneficial effects of exogenous nucleotides on growth and feed efficiency of aquatic animals. Research pertaining to nucleotide nutrition in fishes is still rather limited to date. More research is needed to provide further insights concerning effects of diet composition, physiological stages, and environmental conditions on nucleotide metabolism, as well as demands for exogenous nucleotides. In addition, future research must provide practical solutions to enhance overall efficiency in the aquaculture industry.

Nucleotide Biochemistry

Nucleotides consist of various nitrogenous bases, sugars, and at least one phosphate group (Fig. 12.1). The nitrogenous bases can be either a purine or a pyrimidine. Pyrimidine bases are composed of six-membered rings and comprise uridine, cytosine, and thymine. Purine bases are composed of five-membered rings and comprise adenine, guanine, and hypoxanthine (Fig. 12.1). The phosphate group can be in mono-, di-, and tri- phosphate form (Table 12.1). It is commonly

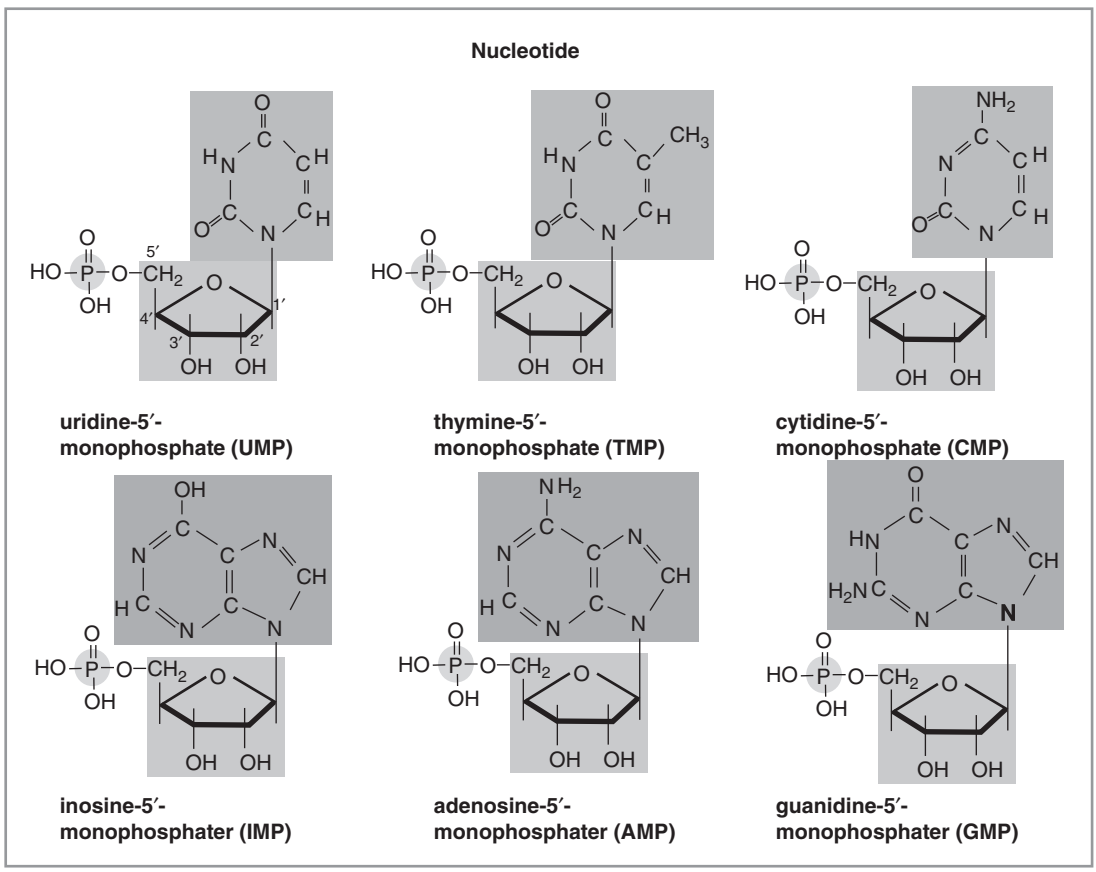

Figure 12.1 Structure of common nucleotides

Table 12.1 Nucleotide nomenclature.

Base-product	Nucleoside	Ribo-nucleotide[a]	Deoxyribo-nucleotide[b]	Diphosphate nucleotide[c]	Triphosphate nucleotide[d]
Purines					
Adenine	Adenosine	AMP	dAMP	ADP/dADP	ATP/dATP
Guanine	Guanosine	GMP	dGMP	GDP/dGDP	GTP/dGTP
Hypoxanthine	Inosine	IMP	–	–	–
Pyrimidines					
Cytosine	Cytidine	CMP	dCMP	CDP/dCDP	CTP/dCTP
Uracil	Uridine	UMP	dUMP	UDP/dUDP	UTP
Thymine	Thymidine		dTMP	dTDP	dTTP

[a]AMP: adenosine 5'-monophosphate; GMP: guanosine 5'-monophosphate; IMP: inosine 5'-monophosphate; CMP: cytidine 5'-monophosphate; UMP: uridine 5'-monophosphate.
[b]dAMP: deoxyadenosine 5'-monophosphate; dGMP: deoxyguanosine 5'-monophosphate; dCMP: deoxyeytidine 5'-monophosphate; dUMP: deoxyaridine 5'-monophosphate; dTMP: deoxythymidine 5'-monophosphate.
[c]ADP: adenosine 5'-diphosphate; dADP: deoxyadenosine 5'-diphosphate; GDP: guanosine 5'-diphosphate; dGDP: deoxyguanosine 5'-diphosphate; CDP: cytidine 5'-diphosphate; dCDP: deoxycytidine 5'-diphosphate; UDP: uridine 5'-diphosphate; dUDP: deoxyuridine 5'-diphosphate; dTDP: deocythymidine 5'-diphosphate.
[d]ATP: adenosine 5'-triphosphate; dATP: deoxyadenosine 5'-triphosphate; GTP: guanosine 5'-triphosphate; dGTP: deoxyguanosine 5'-triphosphate; CTP: cytidine 5'-triphosphate; dCTP: deoxyeytidine 5'-triphosphate; UTP: uridine 5'-triphosphate; dTTP: deoxythymidine 5'-triphosphate.

esterified to the C5'- hydroxyl group of the pentose sugar (Mateo and Stein 2004). Several common nucleotides containing ribose and deoxyribose sugars are depicted in Figure 12.1.

The purines and pyrimidines associated with nucleotides are either synthesized from *de novo* pathways or obtained from salvage pathways, as shown in Figure 12.2 (reviewed by Rudolph 1994; Carver and Walker 1995; Grimble and Westwood 2000a). Purine rings are synthesized in the cytosol of mammalian cells from glycine, aspartate, glutamine, tetrahydrofolate derivatives, and CO_2 with considerable energy input, while pyrimidines are synthesized from aspartate, glutamine, and CO_2 in the cytosol and mitochondria of mammalian cells. Research on nucleotide synthetic pathways with aquatic animals is extremely limited. Presumably, these mammalian pathways are also operative in fish. However, Dabrowski and Kaushik (1982) speculated that stomach-less fish larvae barely possess carbamyl phosphate synthase activity, and suggested that provision of dietary pyrimidine is essential for protein synthesis.

There is also a salvage pathway that conserves energy and maintains nucleotide homeostasis. Based on mammalian research, those salvage and *de novo* pathways vary markedly among various tissues and may be significantly influenced by metabolic needs or physiological functions. Based on mammalian models, nucleotide turnover in erythrocytes, lymphocytes, heart, and brain primarily depends upon supply from the salvage pathway. It has also been noted that dietary nucleotides modulate nucleotide metabolism of the liver (López-Navarro et al. 1995), which is the most important organ for nucleotide storage and inter-organ transport to meet physiological needs.

Nucleotides such as ATP, ADP, and AMP are modulators of calcium uptake across fish brush border membranes through P2-purinoreceptor (Klaren et al. 1997). In addition, neutrophil extracellular traps (NETs), made of deoxyribonucleic acid (DNA) and certain proteins, were discovered as an important defensive mechanism against bacterial infection (Brinkmann et al. 2004). The production of NETs by teleost neutrophils has been recently described in both zebrafish (*Danio rerio*) and fathead minnows (*Pimephales promelas*) (Palic et al. 2007a,b). However, much remains to be learned about the contribution of different neutrophilic granule classes to the composition of teleost NETs, as well as the ability of these NETs to prevent pathogen spread, contain potential tissue-damaging factors, and kill invading microorganisms (Rieger and Barreda 2011). To the best of our knowledge, the influences of exogenous nucleotides on calcium uptake modulation and NETs

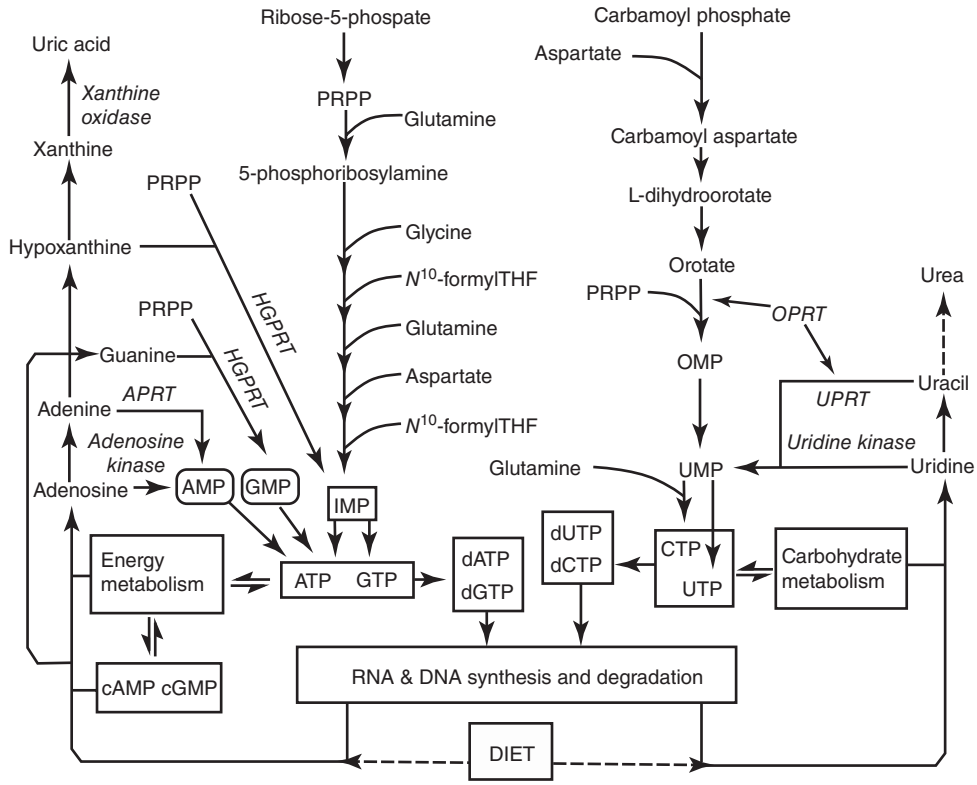

Figure 12.2 Overview of purine and pyrimidine salvage and *de novo* pathways based on mammalian research. PRPP: phosphoribosylpyrophosphate; N^{10} –formylTHF: N^{10} -formyl-tetrahydrofolic acid; APRT: adenine phosphoribosyltransferase; OPRT: orotidine phosphoribosyltransferase; HGPRT: hypoxanthine-guanine phosphoribosyltransferase (Adapted from Grimble, G. K. and O. M. R. Westwood. 2000a. Nucleotides. Page 135–144 in M. E. Gershwin, J. B. German, and C. L. Keen, editors Nutrition and Immunology: Principles and Practice. Humana Press Inc., Totowa, NJ, USA. Copyright © 2000, Springer Science + Business Media.).

formation have not been explored, even in mammalian models.

Nucleotide Content in Feedstuffs and Commercial Nucleotide Supplements

Nucleotides are naturally present in all feedstuffs of animal and vegetable origin as free nucleotides and nucleic acids. Analytical procedures for nucleotides are relatively complex and quite diverse across various laboratories. The data generated by different research groups varies considerably. Clifford and Story (1976) reported the contents of purines and RNA in some feedstuffs including organ meats, seafood, and dried legumes. Devresse (2000) also reported the total contents (after complete hydrolysis) of purine and pyrimidine bases in common aquafeed ingredients such as fishmeal (1.4%), press cake fishmeal (0.4%), fish solubles (2.8%), yeast (0.9%), yeast extract (2.3%), and single-cell proteins (2.1%). Rumsey et al. (1992) reported that 12–20% of the total nitrogen in brewer's yeast (*Saccharomyces cerevisiae*) can be composed of RNA nitrogen, mainly in the purine and pyrimidine bases of the nucleoproteins. Nucleotide content in various feedstuffs is summarized in Table 12.2, and Table 12.3 based on Mateo and Stein (2004) and Suresh et al. (2011).

Several different nucleotide supplements are now commercially available, as presented in Table 12.4. These products consist of various mononucleotides and/or oligonucleotides primarily derived from

Table 12.2 Nucleotide concentrations in some commonly used feed ingredients (as-is basis). Data from Mateo and Stein (2004).

			Nucleotide (mg kg^{-1})		
Ingredient	CMP	AMP	GMP	UMP	IMP
Barley	2	1	1	0	1
Casein	1	0	0	0	0
Corn	3	2	3	0	1
Fishmeal	26	11	2	1	35
Naked oats	3	3	3	1	1
Plasma protein, spray dried	2	2	2	0	1
Red blood cells, spray dried	0	44	3	2	6
Soy protein concentrate	0	1	2	0	1
Soybean meal, 44%	16	8	3	9	2
Whey, dried	270	19	0	1	4

single-cell protein feedstuffs such as yeast. Most of the research to date on dietary supplementation of nucleotides to fish has employed the products from Chemoforma Co. (Basel, Switzerland) such as Optimûn and Ascogen S; Ascogen P and NuPro have also been tested in some studies. Based on the limited description of those commercial products, total nucleotide content does not exceed 20%. In addition, specific concentrations of various constituents in those products are not disclosed. Concerns about non-nucleotide yeast constituents (over 80% of the product), such as β-glucans, have complicated the scientific basis of nucleotide nutrition research on aquacultured species.

Digestion and Absorption of Nucleotides and Related Metabolites

The presence of ribonucleases and deoxyribonucleases has been confirmed in hepatopancreases and/or pyloric caeca of African lungfish (*Protopterus amphibius*); rainbow trout (*Oncorhynchus mykiss*); and common carp (*Cyprinus carpio*) (reviewed by Dabrowski and Kaushik 1982). This discovery proves that both stomach-less fishes and fishes possessing a stomach can digest RNA and DNA. However, the efficiency of the digestion has not been determined. It has been suggested by Borda et al. (2003) that nucleotides in either their non-free form or in the form of nucleic acids tend to be tremendously stable and difficult to digest. Those authors therefore recommended a well-balanced cocktail of free nucleotides. To the best of our knowledge, the digestibility and bioavailability of nucleic acids in natural feed ingredients, such as marine protein sources or brewer's yeast, for fishes is currently unknown. However, it appears that fish such as rainbow trout can utilize yeast nucleic acid extracts for growth, nitrogen retention, and possibly non-essential amino acid synthesis (Rumsey et al. 1992). In addition, it has been hypothesized that the capacity for RNA and DNA digestion may be influenced by developmental stages. The nucleotide concentration in feed ingredients and bioavailability to fishes, as well as information about the nucleotide pools in fishes, need to be determined in order to obtain a greater understanding of nucleotide nutrition of fishes.

Table 12.3 Nucleotide concentrations in common animal proteins (as-is basis). Data from Suresh et al. (2012)

Nucleotide (ppb)	Fish meal	Fish hydrolysate	Krill	Squid liver meal	Poultry byproduct meal, pet-food grade	Poultry by-product meal, feed grade	Feather meal	Blood meal
Uridine	32	51	101	51	196	71	22	<10
Cytidine	14	<10	26	12	81	33	23	<10
Inosine	16	516	312	1440	589	205	31	<10
Guanosine	27	55	49	140	130	62	20	<10
Adenosine	22	21	40	35	259	74	19	<10
UMP	153	84	919	24	123	45	<10	<10
CMP	128	29	991	32	108	60	<10	<10
IMP	36	122	988	2230	182	88	22	<10
GMP	274	58	798	67	71	41	<10	<10
AMP	130	292	2270	443	461	127	27	<10

Table 12.4 Commercial "nucleotide" products.

Product name	Source	Nucleotide concentration	Manufacturer	Reference
Optimûn ®		15% (CMP, UMP, AMP, IMP, GMP and RNA)	Chemoforma, Augst, Switzerland	Burrells et al. (2001a,b); Leonardi et al. (2003); Low et al. (2003); Li et al. (2005, 2007a); Glencross and Rutherford (2010); Tahmasebi-Kohyani et al. (2011, 2012); Abtahi et al. (2013); Kenari et al. (2013)
Ascogen P®	Brewer's yeast		Canadian Biosystems Inc., Calgary, Alberta, Canada	Ramadan and Atef (1991); Ramadan et al. (1994); Adamek et al. (1996); Li et al. (2004); Cheng et al. (2011)
Aquagen™			NOVARTIS-Aqua Health Ltd., Charlottetown, Canada	Russo et al. (2007)
Nupro®	Yeast extract	Nupro® (5–7% nucleotide)	Alltech, Nicholasville, KY, USA	Panagiotidou et al. (2009); Salze et al. (2010); Peterson et al. (2012)
		Nupro®15 (15% nucleotide)		
LALTIDE	Yeast	Combination of purified nucleotides	Biotal Co., UK	
NUCLEO 5 PRIME™	Yeast	40% free 5' nucleotides and 6% free 5' nucleotides	Prosol S.p.a, Italy	
Nucleoforce Aqua	Yeast	With a minimum concentration of 24%	Bioiberica, S.A, Spain	
ENHANCE			The ROAN Group, Inc., Acampo, CA, US	
CJTIDE		IMP, GMP and I&G (50: 50 mixture of IMP and GMP	CJ Indonesia, Indonesia	
Xintun-1	Yeast	8.6% nucleotide	Guangzhou Xintun Aqatic technology Incorporation, China	Wei et al. (2007); Xiang et al. (2011)
Yeast nucleotide (Yikangbao)	Yeast		Guangzhou Leader Bio-technology Co. Ltd. Guangzhou PR. China.	
Yinyou100		Combination of amino acids and nucleotides	Guangzhou Fishtech Aquatic Sciences Co., Ltd.	
Nucleotide products		Purity: ≥99%	Nanjing Biotogether Co.,Ltd.	Cao et al. (2011)

Biological Effects of Nucleotides

Growth and Feed Utilization

One of the most remarkable findings with regard to nucleotide nutrition of aquatic animals in the past 10 years is the growth-enhancing effects of mixed, free nucleotides (AMP, GMP, UMP, CMP, and IMAP) with various fishes and shrimp. Those studies are summarized in Table 12.5. Li et al. (2007a) demonstrated that diets supplemented with 0.03% and 0.1% of a purified nucleotide mixture (equal amounts of AMP sodium, IMP sodium, CMP sodium, GMP

Table 12.5 Research on dietary supplementation of commercial "nucleotide" products with fishes.

Species	Initial age or weight (g)	Nucleotide product	Dose of commercial product tested	Length of administration	Dietary protein and protein source (% in diet)	Effect	Reference
Hybrid tilapia	21 d old	Ascogen S	2, 5 g kg^{-1} diet	16 weeks		Growth↑; survival ↑	Ramadan and Atef (1991)
Hybrid tilapia	30 d old	Ascogen	5 g kg^{-1} diet	120 days		Antibody titer after vaccination↑; mitogenic response of lymphocyte ↑	Ramadan et al. (1994)
Rainbow trout	23	Optimûn	0.5, 1, 1.5, 2 g kg^{-1} diet	8 weeks	CP: 48.25 FM: 43 SBM: 26 Wheat:19	Growth and feed efficiency ↑; complement, lysozyme and IgM ↑; survival after challenge with S*treptococus iniae* ↑	Tahmasebi-Kohyani et al. (2011)
Rainbow trout	23	Optimûn	0.5, 1, 1.5, 2 g kg^{-1} diet	8 weeks	CP:48.25 FM: 43 SBM: 26 Wheat:19	Serum alkaline phosphatase↓; lactate dehydrogenase↓; aspartate transaminase↓; alanine transaminase↓; white blood cell↑; lymphocyte↑; neutrophil↑; red blood cell↑; albumin↑; globulin↑; in acute stress challenge (0.2% NT): plasma cortisol ↓ plasma glucose↓	Tahmasebi-Kohyani et al. (2012)
Rainbow trout	23	Optimûn	0.5, 1, 1.5, 2 g kg^{-1} diet	8 weeks	CP:48.25 FM: 43 SBM: 26 Wheat:19	Glyceraldehyde-3-phosphate dehydrogenase-1↑; fast myotomal muscle troponin-t-1↑; creatine kinase↑; nucleoside diphosphate kinase a↑; adenylate kinase↓	Keyvanshokooh and Tahmasebi-Kohyani (2012)
Rainbow trout	53–55	Optimûn	2 g kg^{-1} diet, 1% bw d^{-1}	2 weeks		Survival after challenge with infectious salmon anaemia virus ↑	Burrells et al. (2001a)
All-female rainbow trout	80–100	Optimûn		120 days		B lymphocytes↑; resistance to ipn virus ↑; plasma cortisol ↓	Leonardi et al. (2003)

(*continued*)

Table 12.5 (*Continued*)

Species	Initial age or weight (g)	Nucleotide product	Dose of commercial product tested	Length of administration	Dietary protein and protein source (% in diet)	Effect	Reference
Rainbow trout	163.4–169.7 fish^{-1}	ASCOGEN	0.62, 2.5, 5 g kg^{-1} diet at 1% bw d^{-1}	37 days		Growth↑	Ademek (1996)
Rainbow trout	217±62	Optimûn	2 g kg^{-1} diet, 2% bw d^{-1}	3 weeks		Survival after challenge with *V. anguillarum* ↑	Burrells et al. (2001a)
Brown trout	12.3	Optimûn	1.5, 2.5, 3.5, 5 g kg^{-1} diet, feed to apparent satiation	8 weeks	CP 50% FM 69.4%	Growth ↑; feed efficiency ↑; serum alkaline phosphatase (0.25%)↓; lactate dehydrogenase (0.35%)↓; aspartate transaminase (0.35%)↓; alanine transaminase (0.25 and 0.35%) ↓; lysozyme (0.15, 0.25 and 0.35%) ↑; survival after salinity stress (0.25 and 0.35%) ↑	Kenari et al. (2013)
Atlantic salmon	34.7±9.6	Optimûn	2 g kg^{-1} diet, containing 0.03% NT at 1.5% bw d^{-1}	3 weeks before vaccination and 5 weeks post-vaccination		Antibody titer ↑; mortality ↓	Burrells et al. (2001b)
Atlantic salmon	43±3.0	Optimûn	2 g kg^{-1} diet, containing 0.03% NT at 1.5% bw d^{-1}	8 weeks		Plasma chloride ↓; growth ↑	Burrells et al. (2001b)
Atlantic salmon	205	Optimûn	2 g kg^{-1} diet, containing 0.03% NT	10 weeks		Intestinal fold ↑	Burrells et al. (2001b)
Common carp	100	Ribo-nuclease-digested yeast RNA	15 mg fish^{-1}	3 days		Phagocytosis ↑; respiratory burst ↑; complement ↑; lysozyme ↑	Sakai et al. (2001)
Common carp	1.1	Xintun-1	0.258, 0.516, 0.774, 1.032, 1.29% NT	50 days	CP 36% FM 30% SBM 40%	Growth (0.516%) ↑; feed efficiency (0.516, 0.774, 1.032, 1.29%) ↑; protein	Xiang et al. (2011)

					Wheat bran 15% Corn 10%	efficiency ratio (0.516, 0.774, 1.032, 1.29%) ↑; serum lysozyme ↑	
Turbot *Scophthalmus maximus*	121	Optimûn	2 g kg^{-1} diet, containing 0.03% NT to hand satiation daily	15 weeks		Altered immunogene expression in various tissues	Low et al. (2003)
Hybrid striped bass	9.1	Ascogen P	5 g kg^{-1} diet, fixed ration approaching satiation daily	7 weeks	CP 40% FM 57.7%	Neutrophil oxidative radical production ↑; survival after challenge with *Streptoccus iniae* ↑	Li et al. (2004)
Barramundi	17.9	Optimûn	2 g kg^{-1} diet, to hand satiation once daily	4 weeks	CP 52% FM 70% Wheat flour 14.4%	Growth ↑ at 30°C, but not at 37°C; feed efficiency ↑ at both 30°C and 37°C; feed intake ↓ at 37°C, but not at 30°C; protein retention ↑	Glencross and Rutherford (2010)
Red drum	7.1	Ascogen P	0.5 and 1%	6 weeks	CP 40% FM 57.8%	Intestinal fold↑; enterocyte height ↑; microvilli height ↑; head kidney macrophage O^{-}↑	Cheng et al. (2011)
Red-tail black shark	1.4	Aquagen	0.2%	24 days	CP 48% Krill meal 20% FM 40% Fish CS 5%	Survival after *Streptococcus iniae* challenge ↑	Russo et al. (2007)
Begula sturgeon	12.6	Optimûn	0.15, 0.25, 0.35, 0.5% and feed ration close to satiation	62 days	CP 45.5% FM 58% Wheat flour 21%	Growth (0.35%) ↑; feed efficiency ↑; whole-body protein (0.25 and 0.35%) ↑; total tissue saturate fatty acid (0.35%) ↓; total tissue unsaturated fatty acid (0.35%)↑	Abtahi et al. (2013)
Gastropod spotted babylon	0.3	NuPro	1 and 2%	4 months	CP 40.3% FM 40% SBM 17% Shrimp meal 3% Wheat gluten 7%	Growth↑; feed efficiency ↑; protein efficiency ratio ↑; survival after *Vibrio alginolyticus* challenge (30% vs 70% (1%) and 60% (2%)) ↑	Chaitanawisuti et al. (2011)

sodium, and UMP sodium coated with carboxymethyl cellulose, casein, and gelatin) significantly enhanced weight gain and feed efficiency of juvenile red drum (*Sciaenops ocellatus*) compared to fish fed an isonitrogenous basal diet. The same nucleotide mixture was tested with Pacific white shrimp (*Litopenaeus vannamei*), and the growth-enhancing effect of nucleotide mixture was also confirmed (Li et al. 2007b). In both studies, the basal diet formulations contained significant amounts of fish meal, which has high levels of nucleotides. The growth-enhancing effects of nucleotide supplementation with both species were achieved, indicating that their requirements for a balanced mixture of free nucleotides are high. Lin et al. (2009) supplemented the same nucleotide mixture to casein-gelatin based diets and found 0.15% mixed nucleotides (AMP: UMP: IMP: CMP: GMP 1:1:1:1:1) significantly increased weight gain and feed efficiency of juvenile grouper (*Epinephelus malabaricus*); however, other doses of 0.05%, 0.1%, and 0.2% did not confer significant growth-promoting effects. In their further studies, all individual nucleotide supplements (AMP, UMP, IMP, CMP, and GMP) at 0.15% enhanced growth and feed utilization of grouper. Mixed nucleotides and AMP conferred the optimal effects to grouper (Lin et al. 2009). Cao et al. (2011) also confirmed that the mixed nucleotides could enhance growth of Pacific white shrimp. Those studies present strong evidence that marine species such as red drum, grouper, and Pacific white shrimp require exogenous nucleotides for optimal growth and feed utilization. Intake of free nucleotides presumably reduces energy expenditure and amino acid degradation for nucleotide synthesis. Feed efficiency of the organism was therefore improved. Nucleotide intake can also alter the gastrointestinal tract (GIT) structure by increasing the height of fold and microvilli of fishes such as Atlantic salmon (*Salmo salar*) (Burrells et al. 2001b) and red drum (Cheng et al. 2011). This influence favors the absorption of nutrients from digesta and results in improved feed utilization.

Welker et al. (2011) used channel catfish (*Ictalurus punctatus*) as an experimental model to explore the potential benefits of nucleotide supplementation for freshwater fishes, but found supplementation of nucleotides does not enhance growth and feed efficiency of channel catfish (Table 12.6). The feed formulations were practical and water source did not contain phytoplankton, zooplankton, or other nucleotide sources. This study may indicate distinctions in nucleotide demand and metabolism across species, especially among herbivores, omnivores, and carnivorous species.

Larval Development

Person-Le Ruyet et al. (1983) reported that turbot (*Scophthalmus maximus*) larvae (approximately 100 mg/fish) fed an inosine-supplemented diet (1.3% of diet for 6 days, 0.13% for 45 days) had significantly enhanced growth and survival after a 51-day period. Their subsequent study showed that 10 or 20 days of feeding a diet supplemented with 0.77% inosine also significantly increased weight gain of turbot larvae (approximate initial weight of 230 mg/fish). Those researchers hypothesized that the growth-enhancing effect of inosine resulted from improved feed intake. Live foods were discontinued, which promoted more rapid feed intake, reduced nutrient leaching into the water, and possibly played other roles in metabolism (Métailler et al. 1983). Borda et al. (2003) reviewed research concerning dietary nucleotide application to sea bream (*Sparus aurata*) larvae and hypothesized that an exogenous supply of nucleotides may promote growth of fish in early stages to meet their high rate of cell replication.

Lane et al. (2012) compared the effects of enrichment of two commercial nucleotide products originating from brewer's yeast (*Saccharomyces cerevisiae*) on the nucleotide profile of rotifer and *Artemia*. Based on their study, the enrichment with the two commercial nucleotide products significantly increased the RNA concentration in rotifer by 10% (5.8% vs 6.2% on a dry-weight basis), but not the nucleotide profile. The influences of nucleotide-enriched rotifer on growth of Atlantic cod (*Gadus morhua*) larvae were insignificant. By contrast, enrichment of the two nucleotide products for *Artemia* significantly enhanced the total nucleotide concentration. Atlantic cod fed nucleotide-enriched *Artemia* gained significantly greater weight (34% on average) than cod fed normal *Artemia* at 38 days post-hatch. The researchers also tested the expression of various genes and concluded that hyperplasia was likely the main reason for rapid growth of Atlantic cod larvae.

Table 12.6 Research on dietary supplementation of pure nucleotide/nucleotide mixture with fishes and shrimp. Symbols represent an increase (↑) or decrease (↓) in the specified response.

Species	Ave initial weight (g)	Nucleotide composition	Dose tested (% of diet)	Length of administration (weeks)	Dietary protein and protein source (% in diet)	Effect	Reference
Red drum	10	Disodium salts of AMP, GMP, IMP, UMP, CMP (Sigma , St. Louis, MO, US) mixed equally and coated with gelatin and carboxymethyl cellulose	0.03, 0.1, 0.3	4	Crude protein: 40 Menanden fish meal 54.5 of diet	Growth and feed efficiency↑	Li et al. (2007a)
Grouper, *Epinephelus malabaricus*	5.9	Disodium salts of AMP, GMP, IMP, UMP, CMP (Sigma, St Louis, MO, US) mixed equally	0.05, 0.1, 0.15, 0.2	8	Crude protein: 46.75; Casein 51, mixed amino acid as attractants 0.5	Growth and feed efficiency (0.15% NT) ↑; head kidney superoxide anion (0.1, 0.15% NT) ↑; Ig (0.15%, 0.2%NT) ↑	Lin et al. (2009)
	10.3	Disodium salts of AMP, GMP, IMP, UMP, CMP and their mixture (Sigma, St Louis, MO, US)	0.15	8	Crude protein: 46.75; Casein 51, mixed amino acid as attractants 0.5	Growth and feed efficiency (AMP, IMP, GMP, UMP, CMP and equally mixed NT) ↑; head kidney superoxide anion (AMP, UMP, CMP and equally mixed NT) ↑; Ig (AMP and GMP) ↑	Lin et al. (2009)
Channel catfish, *Ictalurus punctatus*	14.4	Disodium salts of AMP, GMP, IMP, UMP, CMP (US Biochemical, Cleveland, OH, US) mixed equally	0.1, 0.3, 0.9, 2.7	8	Crude protein: 32 fishmeal 8 soybean meal 45, corn meal 25	Growth and feed efficiency: survival after *Edwardsiella ictaluri* challenge (2.7% NT) ↓; antibody titer (0.1, 0.3, 0.9%) ↑ Plasma cortisol (0.9, 2.7%) ↓; spontaneous hemolytic complement activity (0.3, 0.9%) ↑; bactericidal activity (0.1, 0.3, 0.9%) ↑	Welker et al. (2011)
Turbot *Scophthalmus maximus*	9.18	Disodium salts of AMP, GMP, IMP, UMP, CMP (Sigma, St Louis, MO, US) mixed equally	0.03, 0.1	7.5	CP: 50 FM: 42, 36 and 30 SBM: 26.4, 35.2, 43.7 wheat gluten: 3	Growth and feed efficiency: head kidney respiratory burst ↑; serum lysozyme ↑; intestinal fold height ↑; enterocyte height ↑; microvillus height ↑	Peng et al. (2013)

(*continued*)

Table 12.6 (*Continued*)

Species	Ave initial weight (g)	Nucleotide composition	Dose tested (% of diet)	Length of administration (weeks)	Dietary protein and protein source (% in diet)	Effect	Reference
Pacific white shrimp	0.84	Disodium salts of AMP, GMP, IMP, UMP, CMP (Sigma, St Louis, MO, US) mixed equally and coated with gelatin and carboxy methyl cellulose	0.04	6	CP: 25 and. 35 FM: 13.6 squid meal: 13.6 krill meal: 9.1 soy protein 11(in 35% CP only)	Growth ↑; whole-body protein ↑	Li et al. (2007b)
Pacific white shrimp	0.43	Disodium salts of AMP, GMP, IMP, UMP, CMP (Tongkaizhaoye Biochem. Nanjing, China) mixed equally	0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.12	5	CP: 43.4 FM 5 soy protein concentrate 26.4 casein 20 flour 23.6	Growth ↑; feed intake (0.04% NT) ↑; whole-body ash ↑; RNA in hepatopancreas ↑; RNA in intestine ↑; phenoxidase in gill ↑; lysozyme in hepatopancreas ↑; lysozyme in hemolymph ↑	Cao et al. (2011)
Pacific white shrimp	0.43	Disodium salts of AMP, GMP, IMP, UMP, CMP (Tongkaizhaoye Biochem. Nanjing, China) mixed equally	0.01, 0.02, 0.04, 0.06, 0.08, 0.1 and 0.12%	5	CP: 43.4 FM 5% soy protein concentrate 26.4 Casein 20 Flour 23.6	Total hemocyte count↑; SOD in hemolymph & hepatopancres ↑; malondialdehyde in hepatopancres ↑	Xu et al. (2011)

Attractability, Palatability and Feed Intake

The earliest studies of nucleotides in fish nutrition concerned their potential influences as palatability enhancers. Mackie (1973) first analyzed the low-molecular weight fraction of squid and hypothesized nucleotide (AMP) and nucleoside (inosine) components as the main chemo-attractants. Kiyohara et al. (1975) also reported the presence of chemoreceptors on the lips of puffer fish (*Fugu pardalis*) that responded to nucleotides (AMP, IMP, UMP, and ADP) based on electrophysiological responses. These early experiments substantiated the chemo-attractive effect of dietary nucleotides on fish. Subsequently, Mackie and Adron (1978) tested 47 nucleosides and nucleotides, and identified inosine and IMP as the most potent gustatory feeding stimulants for turbot based on feeding behavior. Ishida and Hidaka (1987) tested gustatory sensitivity of various marine teleosts and found UMP was the most effective for most species, although ADP and IMP were also effective. Rumsey et al. (1992) subsequently observed that dietary supplementation with 2.5% and 4.1% yeast RNA extract, 1.85% guanine, or 2.17% xanthine significantly increased cumulative feed intake of rainbow trout over a 12-week period. However, the behavioral or gustatory responses of fishes to exogenous nucleotides have not always been consistent. For example, it was reported that aigo rabbitfish (*Sigmanus fuscescens*) did not respond to any nucleotides in the same manner as most other marine teleosts (Ishida and Hidaka, 1987).

It has also been noted that the stimulatory effects of inosine or IMP on various fishes have not been consistently observed (Person-Le Ruyet et al. 1983; Métailler et al. 1983). Ikeda et al. (1991) found that IMP, GMP, UMP, UDP, and UTP were effective feeding stimulants for jack mackerel (*Trachurus japonicus*), while nucleosides (including inosine, adenosine, guanosine, and uridine) and other nucleotides (AMP, ADP, ATP, IDP, ITP, GDP, GTP, xanthosine 5'-monophosphate, 3'-IMP, 3'-UMP, 2-deoxy-IMP, and allyltio-IMP) were not. Only IMP, but not inosine, has therefore been reported to have stimulatory effects on feeding of fish species including jack mackerel (Ikeda et al. 1991) and largemouth bass (*Micropterus salmoides*) (Kubitza et al. 1997). However, a recent study with olive flounder (*Paralichthys olivaceus*) failed to show any positive influence of graded IMP supplementation (0.1, 0.2, 0.4, and 1%) on feed intake in a 14-week feeding trial (Song et al. 2012), although the commercial feed formulation was used and other feed attractants may have been included. IMP may serve as a primary candidate for feed attractant research to further explore complete replacement of fishmeal in aquafeeds for various species.

Effects on Gastrointestinal Tract

Studies with human and terrestrial animals have shown that dietary supplementation of nucleotides can alter gastrointestinal tract (GIT) morphology and potentially enhance digestive capacity (Carver and Walker 1995). Research into the influences of nucleotide supplementation on GIT morphology of aquatic animals has been limited. Burrells et al. (2001b) first demonstrated that supplementation of a commercial nucleotide product (Optimûn) can significantly increase fold height in proximal, mid, and distal intestinal of sub-adult Atlantic salmon by 18.7%, 18.0%, and 21.4%, respectively. Cheng et al. (2011) examined GIT morphology of red drum after a 6-week feeding trial with another commercial nucleotide product (Ascogen P), and confirmed that supplemental nucleotide supply significantly increased fold height in the proximal intestine. Cheng et al. (2011) also found that nucleotide supplementation increased enterocyte height in the pyloric caeca, proximal, and distal enteric sections, and increased microvilli height in all evaluated enteric sections. The increase in fold height, enterocyte height, microvilli height, and overall absorptive area might be another mechanism for improved feed utilization, but requires further research. Peng et al. (2013) were the first to use purified nucleotide mixture (equal amounts of AMP sodium, IMP sodium, CMP sodium, GMP sodium, and UMP sodium) to investigate the influences of nucleotides on gastrointestinal tract morphology and observed very similar results as Cheng et al. (2011). The purified nucleotide mixture significantly increased fold height, enterocyte height, and microvillus height at three soybean meal levels. However, improved growth or feed efficiency of turbot was not observed; even gastrointestinal morphology of turbot fed nucleotides was altered. To the best of our knowledge, the influences of nucleotide supplementation

on digestibility and nutrient availability of various feedstuffs have not been explored.

Health of the GIT is extremely important for livestock and aquatic animals because it is a major organ for nutrient absorption and immune responses through the mucosal associated lymphoid tissue (MALT). Burrells et al. (2001) and Cheng et al. (2011) observed that fish fed nucleotide-supplemented diets had up-regulated immune responses and altered GIT morphology. Potential involvement of MALT in immune systems and stimulation mechanisms due to nucleotide supplementation remain unclear.

Immune Responses to Nucleotides

Innate Immunity

It is well established that dietary nucleotides can influence macrophage activity, such as phagocytosis (Grimble and Westwood 2001b; Gil 2002) and activity of natural killer cells (Carver et al. 1990). Research with fish has also shown that exogenous nucleotides can influence both humoral and cellular components of the innate immune system (Table 12.5). Sakai et al. (2001) reported that exogenous nucleotides could increase serum complement (alternative pathway), lysozyme activity, phagocytosis, and superoxide anion production of head kidney phagocytes of common carp. In addition, Li et al. (2004) and Cheng et al. (2011) reported that hybrid striped bass (*Morone chrysops* x *M. saxatilis*) and red drum fed an oligonucleotide-supplemented (Ascogen P) diet had higher blood neutrophil oxidative radical production and head kidney macrophage superoxide anion production than fish fed the basal diet. Olive flounder fed diets containing 0.2% and 0.4% IMP showed significantly higher myeloperoxidase and lysozyme activity than fish fed the basal diet.

However, the influences of dietary supplementation of nucleotides on innate immune responses of aquatic animals have not been consistently observed. For example, the effect of dietary nucleotides on respiratory burst of head kidney cells of salmonids was not demonstrated (Burrells et al. 2001a). Variation among individual samples and limitations of some experimental designs, such as number of replicates, can mask potential dietary effects. Other factors such as dose, administration length, type of nucleotide products, and temperature are discussed further in this chapter.

Adaptive Immunity

Nucleotides have also been shown to influence lymphocyte activity and immunoglobulin production. Jyonouchi et al. (1993, 1994) and Navarro et al. (1996) suggested nucleotides exert their greatest impact on the immune system by modulating immunoglobulin production. Ramadan et al. (1994) first reported that dietary supplementation of nucleotides (Ascogen) had a marked immuno-potentiating effect on both humoral and cell-mediated immune responses of tilapia (*Oreochromis* sp.) after intramuscular injection or direct immersion with formalin-killed *Aeromonas hydrophila*. Antibody titers after vaccination, as well as mitogenic responses of lymphocytes from fish fed the nucleotide-supplemented diet, were much higher and significantly different from those of fish fed the basal diet. Similar phenomena also have been reported for other species such as rainbow trout (Burrells et al. 2001b; Leonardi et al. 2003) and hybrid striped bass (Li et al. 2004). For example, Burrells et al. (2001b) observed that Atlantic salmon fed a nucleotide-supplemented diet for 8 weeks had significantly enhanced specific antibody production compared to fish fed the basal diet. In addition, Leonardi et al. (2003) reported a significant enhancement of lymphocyte stimulation in rainbow trout fed a nucleotide-supplemented diet.

The antibody titer of hybrid striped bass fed an oligonucleotide-supplemented diet after vaccination with formalin-killed *Streptococcus iniae* was three-fold higher than that of fish fed a basal diet (Li et al. 2004). Low et al. (2003) reported that dietary nucleotides enhanced expression of immunoglobulin M and recombinase-activating gene in gill and spleen of turbot, but reduced their expression in kidney. Keyvanshokooh and Tahmasebi-Kohyani (2012) confirmed that dietary supplementation of Optimûn can alter the expression of proteins produced by the immune system of rainbow trout. Of all the studies that investigated influences of nucleotide on adaptive immune systems, Welker et al. (2011) provided the most convincing evidence that adequate inclusion level of mixed free nucleotides (0.1%, 0.3%, and 0.9%) can enhance antibody titers against *Edwardsiella ictaluri.*

Although the mechanisms of these various actions are practically unknown, nucleotides may be used as an "oral adjuvant" and thereby enhance vaccination efficacy. Burrells et al. (2001b) explored this strategy in vaccination and reduced mortality of vaccinated Atlantic salmon from 6% to 2%. In summary, research on modulation of adaptive immunity by exogenous nucleotides has shown consistent results in various fish species; further research on this subject is therefore warranted.

Disease Resistance

Although various tests have been developed to assess immunity of aquatic animals, it is unreliable to predict resistance against certain pathogens based on test results from immune responses. The infection routes of different type of pathogens can be different, and effective defensive mechanisms against various pathogens can also be different. Survival after challenge with certain pathogens is the only way to test if supplementation strategies can enhance resistance of tested animals against a specific pathogen. It has been reported that dietary nucleotides can enhance resistance of fishes against various pathogens including viral, bacterial, and parasitic pathogens, indicating a promising use of these biochemicals for health management in aquaculture.

Burrells et al. (2001) observed that Atlantic salmon fed a nucleotide-supplemented diet for 2 weeks had cumulative total mortality of 35.7%, compared to 48% for fish fed the basal diet, 53 days after initial contact with fish previously injected with infectious salmon anemia (ISA) virus. The difference in mortality between the two treatments after 39 and 45 days of cohabitation was statistically significant. Leonardi et al. (2003) also reported that all rainbow trout fed a nucleotide-supplemented diet for 60 days survived after injection with infectious pancreatic necrosis (IPN) virus, whereas all virus-injected fish fed the basal diet died.

Enhanced resistance to various pathogenic bacteria has also been reported for several fish species including salmonids (Burrells et al. 2001a), common carp (Sakai et al. 2001), hybrid striped bass (Li et al. 2004a), red-tail black sharks (*Epalzeorhynchos bicolor*) (Russo et al. 2007), and olive flounder (Song et al. 2012). In recent years, there have been growing concerns about the adverse effects of the bacterium *S. iniae* on the aquaculture of many economically important marine and freshwater fish species. *S. iniae* has therefore been widely used as pathogenic bacteria to test disease resistance of fishes (Li et al. 2004a; Russo et al. 2007; Tahmasebi-Kohyani et al. 2011; Song et al. 2012). Based on these published studies, nucleotide products such as Ascogen P, Aquagen ™, Optimûn, or free IMP significantly enhanced survival of hybrid striped bass, red-tail black shark, rainbow trout, and olive flounder after challenge with *S. iniae.*

Burrells et al. (2001a) reported that after bath challenge with *Vibrio anguillarum* rainbow trout fed a nucleotide-supplemented diet had cumulative mortality of 31%, while mortality of fish fed the basal diet and β-glucan-supplemented diet was 49% and 43%, respectively. Chaitanawisuti et al. (2011) challenged the gastropod spotted babylon (*Babylonia areolata*) with a *Vibrio* strain after feeding a nucleotide-supplemented (NuPro) diet, and found the nucleotide-supplemented diet increased their survival. Welker et al. (2011) challenged channel catfish with *Edwardsiella ictaluri* after feeding graded levels of mixed pure nucleotides (0.1%, 0.3%, 0.9%, and 2.7%) and did not find any protective effects of the mixed nucleotides on survival. However, they found that fish fed diets with nucleotides at 0.1%, 0.3%, and 0.9% had significantly elevated antibody titer after the challenge, as well as enhanced bactericidal activity and complement concentration after being stressed. Burrells et al. (2001a) also reported that cohabitation of coho salmon (*Oncorhynchus kisutch*) with fish infected with *Piscirickettsia salmonis*, a rickettsia-like intracellular γ-proteobacteria, resulted in 76.8% mortality in fish fed the basal diet, whereas only 46.9% mortality was observed in fish fed the nucleotide-supplemented diet. Sakai et al. (2001) intubated either nucleotide-suspended saline or an equal amount of dextrin (control group) to common carp and injected the fish intraperitoneally with a 0.1 mL suspension of *Aeromonas hydrophila* at 3×10^7 CFU mL^{-1}, and determined the bacterial numbers in blood, liver, and kidney at 2, 4, 8, and 12 hours after injection. In the blood, kidney, and liver of fish treated with nucleotides, no *A. hydrophila* colonies were detected 12 hours after challenge; however, the number of bacteria in the blood and liver of control fish reached 1×10^3 CFU mL^{-1}.

In addition to reducing viral and bacterial pathogens, Burrells et al. (2001b) found that dietary nucleotides significantly reduced the number of sea lice infecting Atlantic salmon. They also reported that dietary supplementation of nucleotides in conjunction with cypermethrin affected the development potential of early chalimus stages of sea lice, thereby reducing the numbers of mobile pre-adult lice available to cross-infect other fish. Rather consistent results from various experiments have therefore indicated that dietary nucleotides enhance resistance in fishes against a variety of different pathogens. This phenomenon may have important applications for disease control in aquaculture.

Stress Tolerance

One of the more readily accepted hypotheses associated with the observed beneficial effects of dietary supplementation of nucleotides in fishes is that typical stressors encountered by fish in aquaculture, such as poor water quality, excessive crowding, and frequent handling, place additional demands on available nucleotides beyond those provided in typical aquafeeds (Burrells et al. 2001b; Low et al. 2003). Burrells et al. (2001b) first raised the hypothesis that dietary nucleotides could enhance stress tolerance, and provided some evidence of this by comparing osmoregulatory capacity and growth performance of Atlantic salmon fed a nucleotide-supplemented diet and control diet after acute stress by seawater transfer. Leonardi et al. (2003) observed that dietary nucleotides reduced serum cortisol levels of healthy rainbow trout and fish infected with IPN virus after 90–120 days of feeding. This stress reduction associated with dietary nucleotides also resulted in enhanced disease resistance of challenged fish in that study. Welker et al. (2011) also observed that supplementation of mixed pure nucleotides at 0.1%, 0.3%, and 0.9% significantly reduced serum cortisol, but not lactate or glucose of channel catfish stressed by reduced water level (crowding test). However, Welker et al. (2011) observed no relationship between serum cortisol level and survival after challenge with *Edwardsiella ictaluri*. Tahmasebi-Kohyani et al. (2012) also conducted an out-of-water challenge for 30 seconds immediately followed by a crowding test ($100\,g\,L^{-1}$), and found that rainbow trout fed a diet supplemented with 0.2% Optimûn had significantly lower blood cortisol and glucose levels and significantly higher ion concentrations of sodium, chloride, calcium, and potassium. Currently, it remains unknown if exogenous nucleotides are involved in signaling pathways associated with stress responses or if various stressors have specific effects on nucleotide metabolism of fishes.

Body Composition

Several studies reported that dietary supplementation of nucleotide products can change the body composition indices and fatty acid profile of fish. For example, supplementation of 0.25% and 0.35% Optimûn was reported to increase whole-body protein and decrease whole-body saturated fatty acids of Beluga sturgeon (*Huso huso*) (Abtahi et al. 2013), although the results were not consistent. Juvenile red drum fed diets supplemented with Optimûn had significantly higher whole-body lipid content, but not intraperitoneal lipid deposition (Li et al. 2005). However, this phenomenon was not noticeable in hybrid striped bass (Li et al. 2004). Tacon and Cooke (1980) reported that dietary inclusion of a high level of nucleic acids (10% of the diet) in the form of yeast extract increased the ash content of rainbow trout carcass, although the possible influences of exogenous nucleotides on mineral absorption and metabolism is still poorly studied at this time. Although it is known that dietary nucleotides can influence levels of various lipids and/or fatty acids in certain tissues such as erythrocytes, plasma, liver, and brain of mammals (Carver and Walker 1995), such information on fish and their potential physiological consequences is limited and deserving of further investigation.

Factors Influencing Efficacy of Nucleotides and Research Interpretation

Limitations on Experimental Design

There has been considerable variation reported across studies that have investigated nucleotide supplementation. Based on published reports, weight gain, feed efficacy, and immune responses of various aquatic

animals have responded differently to nucleotide supplements (reviewed by Li and Gatlin 2006). Triplicate experimental units per dietary treatment are typically used in nutrient requirement studies with fish. Because influences of dietary supplements on growth and feed efficacy may be less noticeable than those of macronutrient variations in the diet, more replicates may be needed to demonstrate significant differences in dietary supplements within a limited experimental period. In addition, considerable variation in immune responses and hematological parameters among replicates has been observed, which may mask possible significant differences. However, there is no standard for experimental designs to be used in studies of nucleotide supplementation, or any other feed supplements, with aquatic species. It would be of great benefit for researchers to increase the number of replicates used to evaluate the effects of dietary nucleotide supplementation.

Interfering Nucleotide Content in Feedstuffs and Other Dietary Composition

To the best of our knowledge, nucleotides are present in most feedstuffs. Yeast, bacterial proteins, fish meal, and poultry meal contain large quantity of nucleotides, possibly in various forms. Inclusion of those ingredients in trial feed formulation could alter the results of experiments. In Tables 12.5 and 12.6, the major protein sources for each study are presented to better demonstrate the "nucleotide source."

Protein content is another factor for consideration because amino acids are precursors of endogenenous nucleotides, and additional amino acid supply may enhance *de novo* synthesis of nucleotides and mask effects of exogenous nucleotides. However, research pertaining to this issue is very limited. Li et al. (2007b) used two dietary protein levels (25% and 35%) to explore the influence of additional nucleotides on shrimp fed both protein diets. Nucleotide effects on growth and whole-body protein were shown in shrimp fed both high- and low-protein diets. The mixed free nucleotides spared some protein in shrimp feeds but the effect of dietary protein level on nucleotides was negligible in that study.

Source of Nucleotide Products

The selection of suitable sources of nucleotides should be a primary consideration so that appropriate interpretation of experimental data can be achieved. Although various studies have used mixtures of pure nucleotides to replace commercial products in an effort to better elucidate the effects of nucleotides, the composition of individual nucleotides may be very difficult to manipulate in commercial products. Several articles reported the source and concentration of individual nucleotides in Optimûn; this type of information is generally unavailable for other commercial products. In addition, nucleotides only account for less than 20% of the total weight of Optimûn and other commercial nucleotide products; the non-nucleotide yeast components could therefore potentially impart interfering effects and should be taken into consideration. In some instances, researchers have mistakenly reported the concentration of commercial products as the concentration of nucleotides. Those mistakes should be carefully corrected in the future. In addition, it is very difficult to quantitatively estimate or compare the effects of various nucleotide products on different species of fish. Researchers should not rely strictly on commercial nucleotide products, but should instead develop nucleotide supplements or combinations using pure nucleotides. More careful product and/or research design is needed to determine nucleotide effects if the nucleotides are present in the form of polynucleotides (oligonucleotides).

Dose

Li and Gatlin (2006) reviewed circumstantial evidence that dose of administration is important to the efficacy of nucleotides. Welker et al. (2011) found dietary supplementation of mixed free nucleotides at 2.7% significantly reduced survival of channel catfish challenged with *Edwardsiella ictaluri*. Additionally, the increase in antibody titers of fish fed nucleotides at lower inclusion rates was not observed in fish fed a diet with mixed nucleotides at 2.7%. Tacon and Cooke (1980) found that 10% inclusion of nucleic acid extracted from bacteria adversely affected weight gain and feed efficiency ratio of rainbow trout. Lin et al. (2009) compared four doses (0.05%, 0.1%, 0.15%, and 0.2%) of mixed free nucleotides and found 0.15% conferred the best growth and feed

utilization. Collectively, the dose of nucleotide should be a primary consideration in administration of nucleotide products. Overdose of nucleotides poses risk of decreased growth, feed utilization, disease resistance, and health status. The dose of nucleotide supplementation for individual species needs to be optimized, and should also take into consideration nucleotides present in feedstuffs such as fish meal and poultry meal.

Administration Duration and Frequency

The duration and frequency of administration to achieve optimum responses to immunostimulants such as nucleotides in aquaculture species are other areas requiring further research. In some instances, prolonged administration of immunostimulants such as peptidoglycan and levamisole may cause undesirable side effects on growth and disease resistance of cultured fish (Sakai 1999). Currently, there is no definitive evidence indicating that the efficacy of dietary nucleotides is strictly associated with administration duration. However, Leonardi et al. (2003) observed that mitogenic response of B-lymphocytes from rainbow trout was influenced by dietary nucleotides after 60 days of feeding, but not after 120 days. It has also been observed that hybrid striped bass fed an Ascogen P®-supplemented diet for 16 weeks failed to show any enhancement of innate immune responses, including blood neutrophil oxidative radical production, serum lysozyme, and extracellular and intracellular superoxide anion production of head kidney cells; this observation was inconsistent with the results after 8 weeks of feeding the same diet (Li et al. 2004). Although this evidence is rather circumstantial, such observations may suggest that administration duration should be taken into consideration in the use of dietary nucleotides. Use of free nucleotides for metabolism and growth enhancement might not be a major concern. However, oligonucleotides may be recognized by immune systems of aquatic animals, in which case overstimulation or prolonged stimulation should be taken into account.

Environmental Factors

Effects of environmental factors such as water temperature on efficacy of dietary nucleotide supplementation have only been studied to a limited extent. Glencross and Rutherford (2010) found that feeding barramundi (*Lates calcarifer*) an Optimûn supplementation for 4 weeks significantly enhanced growth rate, feed efficiency, protein retention, and energy retention of fish cultured at 30°C, but not of fish cultured at 37°C. This appeared to be due to the high temperature depressing growth, and the higher feed intake of fish fed the diet containing Optimûn.

Nucleotide Supply from the Water Environment

In extensive, semi-intensive, and some intensive aquaculture systems, bacteria contribute significantly to the food web. They may be directly eaten by the cultured species (e.g., tilapia) or by other organisms that the culture species consume (e.g., penaeid shrimp). The high concentration of nucleic acids in microbes may at least partially meet the physiological requirement of these aquacultured animals for nucleotides. The application of nucleotide products for large-scale aquaculture in ponds or cages needs further evaluation and justification. In addition, the nucleotide supplied by phytoplankton from various cultured waters should be quantified.

Other Factors

It has been hypothesized that younger fish may have higher demands for nucleotides, and dietary nucleotide fortification for early development stages may be more desirable than for sub-adult stages. In addition, most of the studies with carnivorous fishes have shown positive results of dietary nucleotide supplementation. However, some studies with omnivorous or herbivorous fishes have only shown marginal effects (Welker et al. 2011). The differences in nucleotide synthesis and metabolism of various fish species might contribute to these variable results. Research to further evaluate these various hypotheses is needed.

Conclusions

Based on the studies reviewed in this chapter, nucleotides undoubtedly have nutritional value, especially when pure nucleotides have been used in place of commercial products. However, current data on nucleotide composition of common feedstuffs is

incomplete and sometimes controversial. Research to develop a database of nucleotide composition of commonly used feedstuffs in aquaculture should be a prioritized task. As such information becomes available, the dietary levels of nucleotides provided by ingredients and supplements could be more readily quantified in future feed formulations.

Disclaimer

The authors of this chapter do not endorse any of the products specifically mentioned in this chapter.

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Chapter 13
Prebiotics

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Introduction

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health-promoting bacteria in the intestinal tract, thus improving the host's intestinal balance (Gibson and Roberfroid 1995). The definition of prebiotics was updated by Gibson et al. (2004) to "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health." These diet additives therefore indirectly alter the microbial composition of the host gastrointestinal (GI) tract by selectively modifying its microbiota. This is in contrast to the nature of probiotics, which were originally defined by Parker (1974) as "organisms and substances which contribute to intestinal microbial balance"; this definition was subsequently revised by Fuller (1989) to "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." As such, probiotics have a direct effect on the host's microbial composition. Moriarty (1998) proposed that the definition of probiotics also be extended to microbial "water additives."

Both prebiotics and probiotics exert a variety of effects that are mediated through the GI tract of host organisms. Many of the effects associated with prebiotics will be specifically addressed in this chapter, while the following chapter (Chapter 14) is devoted to probiotics.

Interest in the use of prebiotics in aquaculture increased considerably over the past several years due to the beneficial effects reported for these diet additives in humans and terrestrial animals, including enhanced production efficiency, increased nutrient utilization, and improved disease resistance (Flickinger et al. 2003; Patterson and Burkholder 2003). The potential application of prebiotics in aquaculture is appealing because infectious diseases cause considerable negative impacts, especially in intensive aquaculture, and cost millions of dollars in lost revenues due to mortality and reduced production efficiency. Prebiotics are therefore one group of feed additives that represents a potential alternative to traditional means of combating diseases. However, gaps in knowledge currently exist regarding our understanding of the effects of prebiotics on various aquacultured organisms and their pathogens (Ringø et al. 2010a). Nevertheless, based on the information available to date, further research is warranted for prebiotics to become well established as functional feed additives in aquaculture. The concept of functional feeds (Gatlin 2002; Nakagawa et al. 2007) and immunonutrition (Kiron 2012) represents an emerging paradigm to develop diets that extend beyond satisfying basic nutritional requirements of

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the cultured organisms to improving their health and resistance to stress and disease-causing organisms. Various functional feed additives, as well as nutrients, continue to receive considerable attention as alternative disease prevention and treatment strategies compared to more traditional uses of vaccines and drugs which are expensive, subject to regulatory constraints, and/or associated with inconvenient administration options.

Biochemical Characteristics of Prebiotics

To meet the definition of a prebiotic, a diet additive must satisfy the following three criteria: (1) be resistant to digestive processes in the upper part of the GI tract; (2) be fermentable by intestinal microbiota; and (3) selectively stimulate the growth and/or activity of a limited number of the health-promoting bacteria in the GI microbiota (Gibson et al. 2004). A number of different dietary ingredients have been introduced as potential prebiotics for humans and various animals. However, sufficient scientific evidence to fulfill the above-mentioned criteria exists for only a limited number of compounds, namely inulin, oligofructose, lactulose, and galactooligosaccharides, as specifically related to humans (Gibson et al. 2004). These prebiotic compounds are therefore characterized as being carbohydrates, primarily short-chain oligosaccharides consisting of 3–10 carbohydrate units. The most prominent prebiotic compounds in terrestrial animals and aquatic animals, which have been evaluated to a lesser extent, are described in the following.

Inulin-type fructans are polymers of fructose that typically have a terminal glucose with prebiotic properties (Teitelbaum and Walker 2002). Various oligofructose compounds of different chain lengths can be produced from the partial enzymatic hydrolysis of inulin polymers or the enzymatic synthesis of fructooligosaccharides (FOS; Figure 13.1). Other oligosaccharides possessing prebiotic characteristics include mannanoligosaccharides (MOS; White et al. 2002), which are glucomannoprotein complexes derived from the cell walls of yeast (*Saccharomyces cerevisiae*), galacto-gluco-mannans (Zhou et al. 2010), and lactose (Szilagyi, 2002). Enzymatic transglycosylation of lactose produces a mixture of

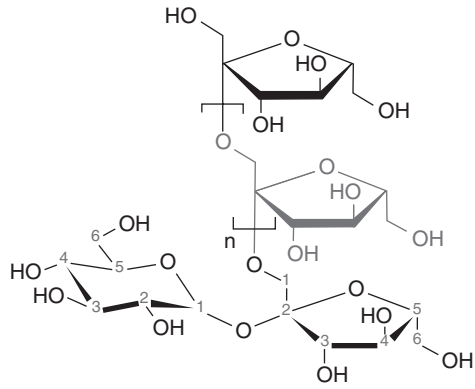

Figure 13.1 Structure of a fructooligosaccharide derived from inulin showing the fructose units joined by a β(2→1) glycosidic bond and a terminal glucose (modified from Hayashi et al. 1989).

oligosaccharides known as transgalactooligosaccharides (TOS). Another feed additive shown to possess prebiotic properties in several different aquatic species is GroBiotic®-A, a mixture of partially autolyzed brewer's yeast, dairy ingredient components, and dried fermentation products (Li and Gatlin 2005; Burr et al. 2008a).

Compared to probiotics, there is generally less concern about incorporating prebiotics into diets because they are not living microorganisms; viability of the bacteria during storage and processing is considered to be critical for probiotics to confer their beneficial effects to the host organisms. However, the application of dead cells, lyophilized cells, or cell-free supernatants or spores has shown some degree of success with various aquatic species (Merrifield et al. 2010).

Gastrointestinal Tract Microbiota

In recent years, it has become increasingly apparent that the microbiota of the GI tract of fish may influence a wide variety of metabolic processes (Dubert-Ferrandon et al. 2008). These influences are mediated by the microbiota, which stimulate epithelial proliferation and expression of numerous genes. Prominent among these are various physiological, biochemical, and immunological responses that must be maintained or enhanced to improve health status,

stress response, and disease resistance. In addition, various other responses may synergistically enhance weight gain and feed utilization of the cultured organism. The important functions of the GI tract microbiota and how they may be modulated by prebiotics will be highlighted in the following sections. Much of the basic information in these areas has already been obtained with humans and animal models, although similar types of responses are believed to occur in fish.

Before describing some of the detailed effects of prebiotics, it should be noted that the intestinal microbial populations are composed of two primary groups: those that are permanent colonizers (autochthonous bacteria); and transients (allochthonous bacteria). The autochthonous bacteria are resident populations that colonize the epithelial surface of the host organism's GI tract, including the microvilli. These health-promoting bacteria, such as lactobacilli, may provide a defensive barrier and protect against the invasion of bacterial pathogens via the GI tract. It is this group of bacteria that is generally targeted for manipulation by prebiotics and probiotics (Ringø et al. 2010b). Alteration of the GI tract microbiota of various fish species has been induced by prebiotics such as MOS (e.g., Dimitroglou et al. 2009) and GroBiotic®-A (e.g., Burr et al. 2008a; 2009). Establishment of bacterial pathogens in the GI tract may also be impeded by the mucus layer of the GI tract, which provides physical as well as various types of biochemical protection. Increased mucus production has been observed in European sea bass (*Dicentrarchus labrax*) fed diets supplemented with MOS (Torrecillas et al. 2011).

Pathogen Entrance

The GI tract is among the most common sites of pathogen entrance in fish because they are exposed to water that contains various types of potentially pathogenic bacteria. However, a healthy gut microbiota has the ability to prevent pathogenic bacteria from colonizing the intestine, thus preventing infection (Birkbeck and Ringø 2005; Stecher and Hardt 2008). The autochthonous bacteria of the GI tract that are present under normal conditions act to competitively exclude pathogens simply by their presence. By taking up space and resources along the mucosal lining of the GI tract, pathogenic bacteria are forced to continue in a transient state and the likelihood of damaging intestinal cells or causing infection is reduced. Autochthonous bacteria also have the capacity to produce antimicrobial substances, which enhances their ability to inhibit pathogens from colonizing the GI tract. However, when the natural equilibrium state of the microbiota is altered, conditions become more favorable for pathogenic organisms to flourish.

In order to help maintain the delicate balance between microbiota of the GI tract, prebiotics or probiotics may be included in the diet to reinforce the population of beneficial bacteria and decrease the number of potentially pathogenic bacteria. Probiotics help to directly accomplish this by providing an increased number of desirable bacteria when ingested. A regular supply of beneficial bacteria added to the GI tract will help to control or reduce the number of detrimental bacteria via the means described above. Prebiotics accomplish their goal more indirectly by acting as a food source, preferentially, to the beneficial bacteria. It has also been shown that some pathogenic bacteria may become bound to certain prebiotics, as opposed to attaching to the mucosal lining of the GI tract, and thus may be passed from the GI tract (Spring et al. 2000).

The Immune System

The first line of defense within the GI tract is the mucosa that separates the gut microbiota from direct contact with the epithelial cells of the GI tract (Dubert-Ferrandon et al. 2008; Pérez et al. 2010). It is because of this direct contact with the mucus that the immune system of the GI tract, often referred to as gut-associated lymphoid tissue or GALT, has developed mechanisms to distinguish between potentially pathogenic bacteria and the normal, commensal autochthonous bacteria. Consequently, the GALT can determine whether to mount an attack or tolerate the presence of specific bacteria (Pérez et al. 2010). In the event that potentially pathogenic bacteria are detected, the cellular and humoral mechanisms of the GALT activate the innate immune system, and subsequently the adaptive immune system, to prevent bacteria from causing and/or spreading infection (Gómez and Balcázar 2008). Components of the innate or non-specific immune response that can be activated to kill invading pathogens include factors

such as blood neutrophil oxidative radical production, serum lysozyme, and superoxide anion production in activated macrophages (Nayak 2010). These various responses are intended to kill a wide variety of foreign or invading microorganisms, and their enhancement may lead to significant reductions in mortality of the host when exposed to various pathogenic organisms. It has been difficult to consistently observe enhancement of these various non-specific immune responses with prebiotic supplementation (Kiron 2012). Reasons for these inconsistencies may be related to differences in the types of responses measured and the timing of such measurements after prebiotic supplementation, as well as other variables.

Adaptive immunity is a more complex component of the immune system that is activated and, to some extent, directed by the innate immune system. Components of the adaptive or specific immune system include lymphpocytes such as B-cells and T-cells, which allow the host to recognize and combat specific disease-causing organisms. The adaptive immune system allows vertebrates, including fish, to recognize and remember specific pathogens and generate immunity against future exposure to such pathogens. The response to prebiotic supplementation has not been extensively studied in this complex part of the immune system, but some of its components appear to be enhanced. Additional research in this area is warranted to more fully characterize the effects of prebiotics on adaptive immunity of the host organism.

Effects of Prebiotics on Integrated Animal Responses

Although research to evaluate the various effects of prebiotics on aquatic species is still rather limited compared to humans and terrestrial food animals, efforts have intensified and publications have proliferated in recent years. Table 13.1 summarizes the results of many studies conducted with various prebiotics and aquatic species, including the most prominent effects observed. Also provided is the level of prebiotic supplementation and duration of feeding in each study. The following sections highlight several of the integrated animal responses that have shown enhancement to prebiotic supplementation.

Disease Resistance

The ability of the cultured organism to resist disease from an infectious agent is one of the most important responses to the aquaculture producer, as it directly affects the production efficiency and profitability of the enterprise. Disease resistance is an integrated response or outcome that may be influenced by the organism's genetic makeup and various components of the innate and adaptive immune systems, as described earlier. The inclusion of pathogen challenges in studies of prebiotic efficacy is highly desirable (Ringø et al. 2010a).

Numerous studies have demonstrated that supplementation of prebiotics may enhance the ability of various aquatic species to resist disease from numerous bacterial, viral, and protozoan pathogens. For example, MOS has been shown to increase lysozyme activity, antibody titers, and survival of rainbow trout (*Oncorhynchus mykiss*; Staykov et al. 2007). In another study with rainbow trout, MOS supplementation enhanced hemolytic and phagocytic activity, as well as survival against *Vibrio anguillarum* (Rodrigues-Estrada et al. 2008). Supplementation of MOS and FOS individually in diets increased survival and non-specific immunity of hybrid tilapia (He et al. 2003). Survival of larval cobia (*Rachycentron canadum*) was also improved with MOS supplementation (Salze et al. 2008).

The evaluation of the prebiotic GroBiotic®-A (a mixture of partially autolyzed brewer's yeast, dairy ingredient components, and dried fermented products) has also been extended to a variety of fish species and potential pathogens. Enhanced survival of fish fed diets supplemented with GroBiotic®-A has been observed after the following controlled disease exposures: striped bass exposed to *Strepococcus iniae* and *Mycobaterium marinum* (Li and Gatlin 2004, 2005); Nile tilapia (*Oreochomis niloticus*) exposed to *Aeromonas hydrophila* (Zheng et al. 2011) and *Strepococcus iniae* (Peredo 2011); and golden shiner (*Notemigonus chrysoleucus*) exposed to *Flavobacterium columnare* (Sink et al. 2007; Sink and Lochmann 2008; Lochmann et al. 2010). Protection from other pathogens has also been reported. For example, rainbow trout fed a diet supplemented with GroBiotic®-A at 2% by weight had significantly greater survival after exposure to infectious hematopoietic necrosis virus (Sealey et al. 2007).

Table 13.1 Summary of prebiotics evaluated in aquaculture (adapted from Ringø et al. 2010a; Gatlin and Peredo 2012).

Prebiotic[a]	Dose (g kg^{-1}); duration of trial	Species	Initial weight (g)	Response[b]	Reference
Inulin	150; 4 weeks	Arctic charr (*Salvelinus alpines*)	218	Intestinal cell damage	Olsen et al. (2001)
	75; 3 weeks	Atlantic salmon (*Salmo salar*)	218	→ Intestinal cell damage; ↑ intestinal growth and relative mass of the gastrointestinal tract	Refstie et al. (2006)
	5 and 10; 1 week	Gilthead sea bream (*Sparus aurata*)	175	Significant inhibition of phagocytosis and respiratory burst in leukocytes	Cerezuela et al. (2008)
	20; 1 month	Turbot larvae (*Psetta maxima)*	n/a	↑ Growth rate; effects on gut microbiota (*Bacillus* and *Vibrio*)	Mahious et al. (2006)
MOS	10; 4 months	Atlantic salmon	200	↓ Oxygen consumption; ↓ protein and ↑ energy concentration in the whole body	Grisdale-Helland et al. (2008)
	2; 4 weeks	Channel catfish (*Ictalurus punctatus*)	16.0	→ Growth performance, hematology, or immune function	Welker et al. (2007)
	20 and 40; 67 days	European sea bass (*Dicentrarchus labrax*)	33.7	↑ Growth; → feed conversion; ↓ lipid vacuolization; ↓ presence of *Vibrio alginolyticus* on head kidney	Torrecillas et al. (2007)
	2; 90 days	Rainbow trout (*Oncorhynchus mykiss*)	30.0	↑ Growth and survival; ↑ antibody titer and lysozyme activity	Staykov et al. (2007)
	0.2; 43 days	Sea bream larvae	n/a	↑ Microvilli length	Dimitroglou et al. (2010)
	0 and 4; 12 weeks	Rainbow trout	13.2	↑ Growth; ↑ hemolytic and phagocytic activity; ↑ mucus weight; ↑ survival against *Vibrio anguillarum*	Rodrigues-Estrada et al. (2008)
	0, 2, and 6; 58 days	Hybrid tilapia (*Oreochromis niloticus* × *O. aureus*)	8.1	→ Growth rate; ↑ survival; ↑ non-specific immunity	He et al. (2003)
	10; 4 weeks	Red drum (*Sciaenops ocellatus*)	10.9	↑ Feed efficiency; ↑ survival following parasitic challenge; ↑ non-specific immunity	Buentello et al. (2010)

(*continued*)

Table 13.1 (*Continued*)

Prebiotic[a]	Dose (g kg^{-1}); duration of trial	Species	Initial weight (g)	Response[b]	Reference
FOS	10; 4 months	Atlantic salmon	200	→ Feed intake, growth or digestibility	Grisdale-Helland et al. (2008)
	10; 4 weeks	Red drum	10.9	↑ Non-specific immunity	Buentello et al. (2010)
	0, 2, and 6; 58 days	Hybrid tilapia	57.0	→ Growth rate; ↑ survival; ↑ non-specific immunity	He et al. (2003)
	20; 1 month	Turbot larvae	n/a	↑ Growth rate; effects on gut microbiota (*Bacillus* and *Vibrio*)	Mahious et al. (2006)
	20; 7 weeks	Beluga (*Huso huso*)	19.2	↑ Survival; elevated lactic acid bacteria	Hoseinfar et al. (2011)
	0.8 and 1.2; 8 weeks	Hybrid tilapia	5.6	↑ Growth rate, feed intake, feed conversion; → survival	Hui-Yuan et al. (2007)
scFOS	0.1 and 0.8; 6 weeks	White shrimp (*Litopenaeus vannamei*)	75.4	→ Weight gain; → survival; → feed efficiency; altered microbial community	Li et al. (2007)
GBA	10 and 20; 4 (Trial 1) and 7 (Trial 2) weeks	Hybrid striped bass (*Morone chyrsops* × *M. saxatilis*)	91.4 (Trial 1) and 19.7 (Trial 2)	↑ Feed efficiency; ↑ respiratory bursts; ↑ resistance against *Streptococcus iniae*	Li and Gatlin (2004)
	20; 16 weeks	Hybrid striped bass	64.5	↑ Growth performance; ↑ resistance against *Mycobacterium marinum*	Li and Gatlin (2005)
	10; 6 weeks	Red drum	2.4	→ WG or FE; → intestinal microbiota	Burr et al. (2009)
	10; 4 weeks	Red drum	10.9	↑ Feed efficiency; enhanced WG; ↑ survival following parasitic challenge; ↑ non-specific immunity	Buentello et al. (2010)
	20; 16 weeks	Golden shiner (*Notemigonus crysoleucas*)	1.06	↑ Resistance against *Flavobacterium columnare*	Sink et al. (2007)
	20; 10 weeks	Golden shiner	0.46	→ Survival; ↑ resistance against *Flavobacterium columnare*	Sink and Lochmann (2008)
	10; 3 weeks	Red drum	500	↑ Protein, lipid and organic ADC values	Burr et al. (2008a)
	10 and 20; 8 weeks	Hybrid striped bass	34.4	→ WG or FE	Burr et al. (2010)
	20; 9	Rainbow trout	14.3	→ WG or FE; → antibody levels	Sealey et al. (2007)
XOS	0, 0.15, 2.1, and 3.2; 45 days	Crucian carp (*Carassius auratus gibelio*)	17.0	↑ Growth; → survival; ↑ enzymatic activity	Xu et al. (2009)

[a]Abbreviations: MOS: mannanoligosaccharides; FOS: fructooligosaccharides; scFOS: short-chain fructooligosaccharides; GBA: GroBiotic®-A; XOS: xylooligosaccharides.
[b]Arrows indicate an increase (↑), decrease (↓), or no change (→) in the response.

Similar improvements in survival were observed for red drum (*Sciaenops ocellatus*) fed GroBiotic®-A at 1% by weight before being exposed to the parasitic dinoflagellate *Amyloodinium ocellatum* (Buentello et al. 2010).

Nutrient Utilization

Supplementation of various prebiotics has been associated with improved digestion of dietary nutrients and energy in some fish species. For example, red drum fed diets containing equal amounts of protein provided by fishmeal and soybean meal had higher digestibility coefficients for protein, energy, and organic matter when the diets contained GroBiotic®-A, MOS, or galactooligosaccharide (GOS), but not FOS in the form of inulin; each prebiotic was individually added to the diet at 1% by weight (Burr et al. 2008b). The specific mechanism for increased nutrient digestibility was not determined in that study. However, increased nutrient digestibility associated with prebiotic supplementation may be due to the favored microbial community producing enzymes that are either lacking or occurring only at low levels in the host (reviewed by Burr et al. 2005), and/or increased absorptive area in the GI tract as described in the previous section.

Weight Gain and Feed Efficiency

Improved nutrient utilization of the cultured organism, as well as enhanced metabolism associated with prebiotic supplements, may also result in increased weight gain and feed efficiency, as reported for various fish species fed several different prebiotics. For example, supplementation of FOS enhanced weight gain of turbot (*Psetta maxima*) larvae (Mahious et al. 2006), hybrid tilapia (Hui-Yuan et al. 2007), and red drum (Zhou et al. 2010). Red drum fed diets supplemented with GOS and galacto-gluco-mannans also had significant weight gain compared to fish fed a basal diet without prebiotic supplementation (Zhou et al. 2010). Positive effects of MOS supplementation on weight gain were observed in studies with rainbow trout (Staykov et al. 2007; Rodrigues-Estrada et al. 2008) and European sea bass (Torrecillas et al. 2007), but not with channel catfish (*Ictalurus punctatus*; Welker et al. 2007) or hybrid tilapia (He et al. 2003). Enhanced feed efficiency has been reported for hybrid striped bass fed GroBiotic®-A (Li and Gatlin 2004, 2005). In some of the preceding studies, it was observed that improvements in weight gain and feed efficiency in response to prebiotic supplementation were most apparent when organisms were cultured under less than optimal environmental conditions or in the presence of pathogenic organisms.

Gastrointestinal Tract Development

One of the first prebiotic studies that included inulin (15% of diet) found that it caused intestinal damage of Artic charr (*Salvelinus alpinus*; Olsen et al. 2001). Subsequent studies with Atlantic salmon showed that a lower inclusion of inulin (7.5% of diet) did not damage the distal intestine or alter nutrient hydrolytic or absorptive capacity, but it did stimulate intestinal growth (Refstie et al. 2006). A similar dietary inulin inclusion level reduced the diversity of gut microbiota in Atlantic salmon, which was also noted in Arctic charr fed 15% inulin by Ringø et al. (2006). Various studies have shown that MOS supplementation to the diet may enhance gut development of cobia larvae (Salze et al. 2008) and increase the absorptive area of the GI tract of juvenile rainbow trout (Yilmaz et al. 2007; Dimitroglou et al. 2009) and white sea bream (*Diplodous sargus*; Dimitroglou et al. 2010). Other recent studies with red drum and hybrid striped bass have reported quantitative changes in histological measurements, such as intestinal fold height, enterocyte height, and microvillus height, in response to dietary supplementation of galacto-gluco-mannans, MOS, FOS, GOS (Zhou et al. 2010), and GroBiotic®-A (Anguiano et al. 2013). Such changes also could contribute to increased nutrient absorption as previously described.

Other Physiological Responses

As research techniques to assess endocrine and molecular effects of prebiotic supplementation are more readily applied, additional insights on how these compounds may influence the host's metabolism are likely to emerge. For example, European sea bass larvae fed a *Lactobacillus* sp. probiotic via rotifers and *Artemia* had increased weight gain, which was associated with increased insulin-like growth factor (IGF)-I expression based on mRNA transcription and a decrease in myostatin mRNA transcription (Carnevali et al. 2006). It was also noted in the

study that whole-body cortisol of larval sea bass was reduced after 70 days of exposure to the probiotic compared to the control group, indicating that the probiotic reduced stress on the fish. Such a response is of considerable interest given the immunosuppressive effect of cortisol on fish. Golden shiner intentionally exposed to stress prior to disease exposure had significantly increased whole-body cortisol levels and higher mortality when fed a basal diet compared to fish fed a diet supplemented with GroBiotic®-A (Lochmann et al. 2010). The potential application of advanced molecular techniques should not only be considered for the host species, but also its gut microbiota to more fully characterize and understand changes brought about by prebiotic supplementation.

Practical Applications of Prebiotics

As previously mentioned, maintaining the viability of probiotics during storage and processing is important for them to exert their beneficial effects to the host organisms. Some logistical constraints may therefore be encountered with the cultivation of live microorganisms in conjunction with manufacturing feeds (Ringø et al. 2010). To ensure probiotic viability, its application to the feed must occur post-extrusion so the probiotic organisms are not subjected to excessive heat and pressure. Administration of probiotics in the form of lyophilized cells or spores may be less demanding. Feed manufacturing constraints are generally of less concern when dealing with prebiotics, because they are not living organisms. Although the efficacy of several prebiotics has been shown when incorporated into extrusion-processed feeds, the potential chemical alteration of prebiotic compounds during feed manufacturing has not been studied to any appreciable extent and deserves further consideration.

Administration regimes for specific prebiotics have not been widely studied to date. Although these compounds may have immunostimulating effects, it does not appear that long-term administration causes immunosuppression, as noted with other potent immunostimulants (Kiron 2012). These diet additives may therefore be administered for extended periods. However, developing more refined administration protocols for individual prebiotics or probiotics should be investigated to optimize their effectiveness. For example, administering probiotics or prebiotics at prescribed times, such as before the culture organisms are exposed to a stressful event or at particular times of the year when pathogenic organisms are most prevalent, may be the most efficient way to derive the benefits of these compounds under particular culture regimes.

Synbiotics

A synbiotic is a combination of prebiotics and probiotics administered at the same time. The administration of such a synbiotic is intended to improve the survival and implantation of the live microbial supplement in the GI tract. To date, there has been limited research on the use of synbiotics in aquaculture. Due to the numerous positive effects observed in various aquatic species fed prebiotics or probiotics, further research and development of application protocols for the administration of these compounds may be warranted. However, the incorporation of synbiotics in extruded feeds will require special care in order to maintain viability of the probiotic bacteria.

Conclusions

As highlighted in this chapter, prebiotics have been reported to have numerous beneficial effects on aquatic species including increased disease resistance and improved nutrient utilization, weight gain, and feed efficiency. As such, prebiotics have significant potential to increase the efficiency and sustainability of aquaculture production. However, additional research is needed to refine prebiotic administration protocols and fully characterize the responses of the intestinal microbiota to prebiotic supplementation.

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Chapter 14

Gastrointestinal Microorganisms of Fish and Probiotics

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Introduction

Well-being of farmed animals is the guiding factor for the implementation of preventive healthcare measures. Any impediment that jeopardises the physical and physiological conditions of the animal can affect the farming operation and, in turn, the supply of products from them. Aquaculture is a continually evolving and fast-growing food production sector that generates a sizable portion of products for human consumption. The economic impact of diseases in aquaculture, particularly those caused by emerging pathogens, is substantial. Akin to attempts in other arenas, the use of chemotherapeutants in farms should be reduced to minimize their adverse effects on the environment, ecosystem, and humans. Vaccines, which are part of health management strategies in aquaculture, have only been developed against the most prevalent pathogens of some commercially important farmed fish, particularly high-value species. Alternative means of disease prevention and control, such as nutraceuticals and biotherapeutics, are therefore being implemented in aquaculture production. These types of intervention strategies are being evaluated to gather more evidence on their efficacy to tackle diseases of farmed aquatic animals.

Over the past decade, research on identification and characterization of beneficial host-derived microorganisms, and efforts on their commercial use in aquaculture, have gained momentum. Such salubrious microorganisms are deemed to "support the life" of the respective farm animal by improving its health or by preventing the onslaught of pathogenic organisms. The need for developing sustainable practices in aquaculture has evoked widespread interest in research on these valuable microorganisms as summarized in different reviews (Verschuere et al. 2000; Balcázar et al. 2006; Kesarcodi-Watson et al. 2008; Wang et al. 2008; Denev et al. 2009; Qi et al. 2009; Merrifield et al. 2010a; Nayak 2010a; Ringø et al. 2010; Kiron 2012; Sugita et al. 2012).

In farming of crustaceans (primarily shrimp), beneficial microorganisms are applied directly into the production systems for the bio-control of pathogens. Further, their application also supports decomposition of organic matter, reduction of nitrogen loading, and control of ammonia and nitrate (Leonel Ochoa-Solano and Olmos-Soto 2006; Sahu et al. 2008). In fish farming, innocuous microorganisms are commonly administered via feed to derive a direct benefit on the target animal. This chapter will focus on this nutritional approach, which has been adopted to improve animal health. Because knowledge of the effects of such microorganisms on fish is rather limited, the description relies heavily on what is known from research on humans.

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Microorganisms of Fish

Bony fish possess mucosal surfaces that serve as primary barriers between the animals and their habitats. Microorganisms can attach themselves to or colonize these mucus layers, even during the egg stage (Cahill 1990; Hansen and Olafsen 1999). As an animal develops, microbes populate the mucosal organs: skin, gills, and alimentary canal. The symbiotic consortium of microbes in the gut of fish starts to develop with the first feeding during the larval phase, and possibly reaches a stable composition at the juvenile stage, following autogenic and allogenic succession (Hansen and Olafsen 1999; Raz 2009). Variations in the microbial profile of the epithelial surfaces can be expected depending on the species, anatomic site, and the developmental stage of fish. Individual-specific microbiomes are shaped by several factors including the availability of nutrients for the organisms, interactions between microbes, influence of the external environment, and elements associated with the host (Bevins and Salzman 2011). Based on their interrelationship with the host, microbes are categorized as either indigenous, those capable of colonizing and establishing in the host, or transient, those that are unable to persist on the mucosal surfaces (Brock et al. 1994). These microorganisms may be pathogenic or beneficial to the host animal. Facultative pathogens can also be found along with indigenous or normal microbiota in naïve fish (Denev et al. 2009; Ringø et al. 2010). A great deal of information exists on pathogenic microorganisms in fish, and has been collated in review articles or books (Gatesoupe 2007; Noga 2010; Roberts 2012; Sudheesh et al. 2012). Resident microorganisms may have co-evolved with the host fish to form part of a complex ecosystem, as described for mammals (Tlaskalová-Hogenová et al. 2004). The symbiotic relationship that prevails within the microbial ecosystem is crucial for the host physiology and homeostasis within the gut environment (Bevins and Salzman 2011).

Gut-Associated Microbiota of Fish

Our knowledge of the gut microbiota of fish is limited. As in the case of higher vertebrates, the digestive tracts of the aquatic animals can be considered as ideal ecological niches for a variety of microorganisms (Cahill 1990; Sugita et al. 2005; Austin 2006; McIntosh et al. 2008). The microbes that form communities in the gut of fish compete for space and nutrients while managing to thrive in the intricate environment. They are capable of trapping organic and inorganic components that become available within the gut micro-milieu (Munn 2004). Bacteria and yeasts are usually found in the intestine of fish. Table 14.1 lists the genera of bacteria and yeasts reported from fish. Although the information is imprecise, it is clear that some genera of bacteria and yeasts occur more frequently.

Bacteria

Bacteria form the predominant group of microorganisms in the fish intestine; their numbers depend on the density of the microbes present in the ambient water (Cahill 1990). The digestive tract of fish can harbour up to 10^8 heterotrophs g^{-1} and up to 10^5 anaerobes g^{-1}, and these amounts are much higher than that found in the surrounding environment (Austin 2006). These microorganisms form biofilms on intestinal surfaces and exist in a matrix comprising their own extracellular products, making it easier for them to adhere to the gut (Munn 2004). The profile of bacterial communities and their counts are dependent on the region of the digestive tract, fish size, feeding habits, nutritional status, and season. For instance, (1) detritivorous fish harbour a far greater number of bacteria compared to filter-feeders (Balasubramanian et al. 1992); (2) captive rearing and artificial feeding led to a reduction in the intestinal bacterial diversity in wild-caught Atlantic cod (*Gadus morhua*; Dhanasiri et al. 2011); and (3) feed types (inulin vs dextrin) affected the bacterial numbers in Arctic charr (*Salvelinus alpinus*; Ringø et al. 2006a).

Proteobacteria are the main phylogenetic type of bacteria (Cahill 1990; Huber et al. 2004; Romero and Navarrete 2006; Dhanasiri et al. 2011) in fish intestine; Actinobacteria and Firmicutes are also present (Sanchez et al. 2012). Bacteria belonging to genera such as *Vibrio*, *Aeromonas*, *Flavobacterium*, *Plesiomonas*, *Pseudomonas*, *Photobacterium*, *Micrococcus*, *Acinetobacter*, *Clostridium*, *Bacteroides*, *Fusobacterium*, *Eubacterium*, and members of the family *Enterobacteriaceae* commonly colonize the gastrointestinal (GI) tract of freshwater and marine

Table 14.1 Microbes known to be associated with the gastrointestinal tract of fish.

Fish	Habitat	Bacterial/yeast genera (species name is mentioned if reported)
Bacteria[a]		
Atlantic salmon (*Salmo salar*)	Sea farm	*Gelidibacter salicanalis*, *Pseudoalteromonas elyakovii*, *Psychrobacter aquimaris*, *Ps. cibarius*, *Ps. fozii*, *Ps. maritimus*, *Ps. okhotskensis*, *Ps. psychrophilus*, *Pseudomonas*, *Acinetobacter*, *Photobacterium*, *Vibrio Arthrobacter bergeri*, *A. psychrolactophilus*, *A. rhombi*, *Bacillus pumilus*, *B. subtilis*, *Exiguobacterium*, *Microbacterium oxydans*, *Planococcus maritimus*, *Sporosarcina ginsengisoli*, *Carnobacterium inhibens*, *Streptococcus*, *Lactobacillus*, *Brevibacterium*, *Microbacterium*, *Micrococcus*, *Lactococcus*
Atlantic salmon (*S. salar*)	Freshwater	*Shewanella*, *Pseudomonas*
Rainbow trout (*Oncorhynchus mykiss*)	Freshwater	*Carnobacterium*, *C. piscicola*, *Streptococcus*
Arctic charr (*Salvelinus alpinus*)	Lake	Dominant: *Aeromonas*, *Micrococcus*, *Lactobacillus*, members of Enterobacteriaceae Occasional: *Acinetobacter*, *Cytophaga*, *Flavobacterium*, *Moraxella*, *Pseudomonas*, *Vibrio*, *Coryneforms*, *Streptococcus*
Arctic charr (*S. alpinus*)	Freshwater	*Staphylococcus*, *Pseudomonas*, *Micrococcus*, *Psychrobacter*, *Streptococcus*, *Carnobacterium*, *Bacillus*
Atlantic cod (*Gadus morhua*)	Sea farm	*Brochothrix*, *Carnobacterium*, *Streptococcus Chryseobacterium*, *Ps. glacincola*
Atlantic cod (*G. morhua*)	Coastal	*Holdemania*, *Clostridia, Photobacterium phosphoreum*, *Ph. kishitanii, Mollicute*
Rohu (*Labeo rohita*)	Freshwater pond	*Bacillus licheniformis*
Common carp (*Cyprinus carpio*)	River	*Lactococcus garvieae*, *Pediococcus acidilactici*, *Enterococcus faecium*
Mossambique tilapia (*Oreochromis mossambicus*)	Freshwater farm	*Bacillus circulans*
Grass carp (*Ctenopharyngodon idella*)	Freshwater farm	*Bacillus megaterium*
Yeasts[b]		
European plaice (*Pleuronectes platessa*)	Coastal	*Rhodotorula*
Topsmelt (*Atherinops affinis littoralis*)	Coastal	*Metschnikowia zobelii*, *Kloeckera apiculata*
Bluefish (*Pomatomus saltatrix*)	Coastal	*Rhodotorula*
Pacific jack mackerel (*Tachurus symmetricus*)	Coastal	*Metschnikowia zobelii*, *Debaryomyces*
Turbot (*Scophthalmus maximus*)	Sea farm	*Candida zeylanoides*
Rainbow trout (*Oncorhynchus mykiss*)	Freshwater farm	*Saccharomyces cerevisiae*, *Debaryomyces hansenii*, *Cryptococcus*, *Leucosporidium*, *Trichosporon*, *Rhodotorula rubra*, *R. glutinis*
Large yellow croaker (*Pseudosciaena crocea*)	Coastal	*Lodderomyces elongisporus*
Mullet (*Mugil* spp.)	Coastal	Dominant: *Candida*, *Metschnikowia*, *Sporidiobolus*, *Clavispora* and *Sporobolomyces* Occasional: *Zygosaccharomyces*, *Endomycopsella*, *Debaryomyces*, *Pichia*, *Tremella*, *Rhodotorula*, *Phaffia*, *Dekkera*, *Hanseniaspora*, *Cryptococcus*, *Williopsis*

[a] Information on bacteria collated from: Ringø and Strøm (1994); Cai et al. (1999); Ringø et al. (2000, 2006a, b, 2008); Saha et al. (2006); Hovda et al. (2007); Navarrete et al. (2009); Roy et al. (2009); Dhanasiri et al. (2011).
[b] Information on yeasts collated from: Uden and Branco (1963); Andlid et al. (1995); Gatesoupe (2007); Laconi and Pompei (2007); Wang et al. (2007).

fish (Cahill 1990; Nayak 2010b; Ringø et al. 2010). Other bacterial genera that are present in the GI tract of different fish species are *Mycoplasma*, *Arthrobacter*, *Ochrobactrum*, *Psychrobacter*, *Sejongia*, *Micromonospora*, and *Rhodococcus* (Nayak 2010b; Sanchez et al. 2012). Lactic acid bacteria (LAB) that are commonly found among the normal intestinal microbial population of fish are *Carnobacterium* spp. and *Lactobacillus* spp. (Ringø et al. 2005, 2010).

Yeasts

Yeasts, the ubiquitously distributed polyphyletic group of basidiomycetous and ascomycetous fungi (Kutty and Philip 2008), are also found in the gut of both wild and farmed fish (Gatesoupe 2007). Although yeast cells are outnumbered by bacteria in the fish gut, accounting for only around 1% of the total isolates, they are over hundred-fold larger than bacteria and could be physiologically relevant for the host animal (Gatesoupe 2007). Observations from several studies revealed that the counts of yeasts in fish intestine are highly variable and could range from hardly any to 10^7 CFU g^{-1} (Gatesoupe 2007). Table 14.1 lists naturally occurring yeasts associated with marine and freshwater fish. *Debaryomyces hansenii*, *Metschnikowia zobelli*, and *Rhodotorula* sp. are among the commonly observed yeasts in the fish gut, and their occurrence is influenced by the feeding habits of the fish (Kutty and Philip 2008).

It is plausible that several of the organisms listed in Table 14.1 may be autochthonous, and could have a positive effect on the health of the host. Abundance and diversity of the intestinal microbiota in fish would signify their physiological relevance: through their role in digesting complex molecules, by producing vitamins and polymers, or via their ability to compete with pathogenic organisms (Austin 2006). From nutritional and health perspectives, the gut microbiota is of immense relevance for the well-being of the host.

Understanding Probiotics

Traditionally, bacteria and yeasts have been used to ferment raw human food ingredients in order to degrade carbohydrates and proteins, making the food more digestible when consumed. On the other hand, it is well accepted that gut bacteria in humans breakdown food constituents, including indigestible components, to facilitate efficient nutrient utilization by the host. Bacteria in the gut of humans are also helpful in producing energy for cells and the synthesis of DNA and fatty acids (Ackerman 2012). Broadly speaking, microorganisms that are vital for the well-being of the host can potentially be considered as probiotics. The current interest on probiotics in human nutrition stems from the perceived health benefits of these live microorganisms. When consumed adequately through diets, they traverse and survive the harsh conditions in the digestive tract to contribute both nutritionally and physiologically to the well-being of the host (FAO/WHO, 2002). Probiotics that have normal nutritional benefits are also considered as functional foods due to their mechanistically linked roles in preventing digestive tract-related diseases and improving immune functions (Roberfroid 2000; Yan and Polk 2006). The salubrious nature of these organisms has been widely commercially exploited, and probiotics for human consumption are marketed worldwide (Sanders and Huis in't Veld 1999; Sanders 2008).

Although it has been approximately two decades since the concept of probiotics was introduced to aquaculture, the bulk of the research was conducted during the 2000s. The benefits of probiotics for humans and land animals are exclusively related to their health effects. However, the multiple advantages derived from these organisms in aquaculture have led to the widely accepted, all-encompassing definition provided by Verschuere and co-workers: "A probiotic is defined as a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" (Verschuere et al. 2000). This definition, excluding the effect on the quality of the ambient environment, is directly linked to the health and nutritional attributes of probiotics discussed in this chapter. The knowledge currently available on fish mainly covers the health benefits of probiotics, and is based on microbiological and immunological information rather than nutritional benefits.

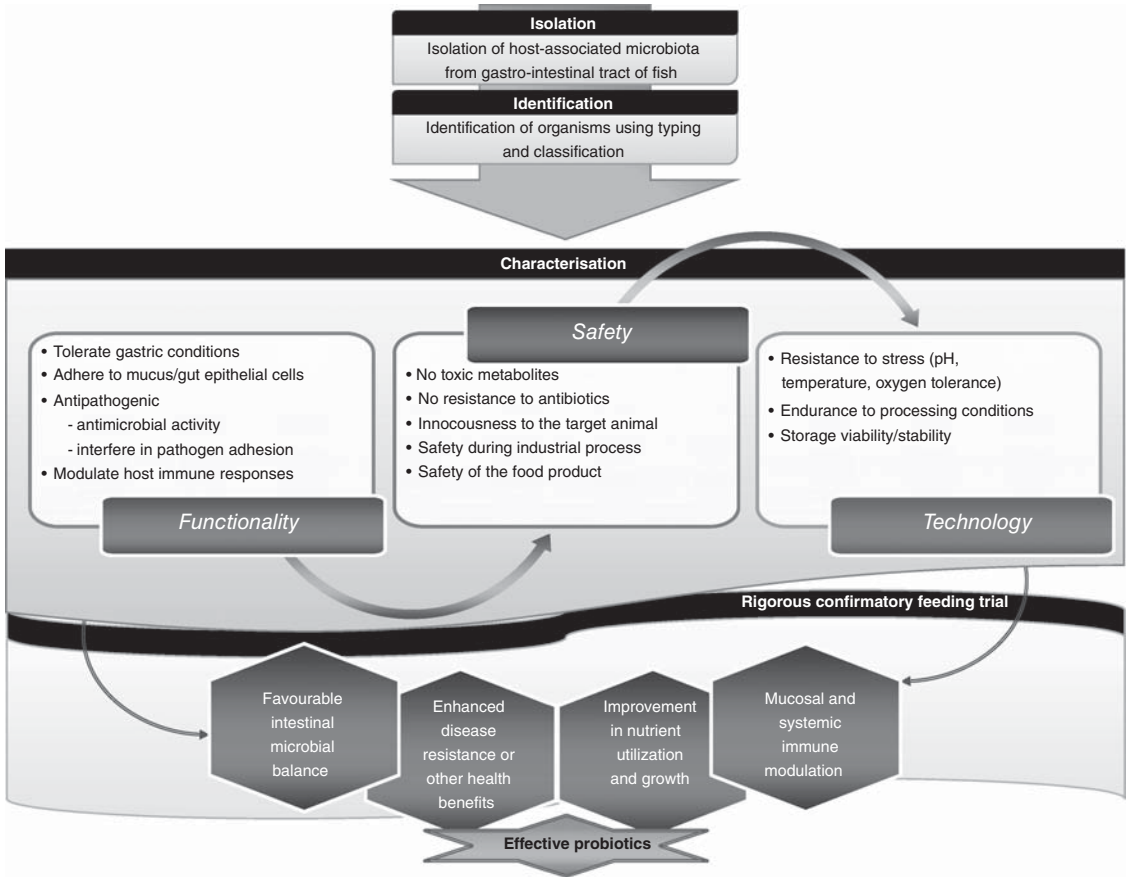

Figure 14.1 Pathway leading to the development of probiotics for farmed aquatic animals and the expected benefits in the target host upon their application as feed-delivered microbials. Microorganisms, ideally isolated from among the commensal microbiota of a target aquatic animal, are identified and characterized based on their functionality, safety, and technological suitability. A candidate probiotic has to be rigorously examined through controlled feeding studies to confirm the reproducibility of its observed beneficial effects. Confirmatory farm trials employing multiple batches of bulk-produced microorganisms should precede the commercialization of the probiont.

Beneficial Effects of Probiotics

Probiotics delivered through diets offer multiple advantages to the host (Collado 2009). This could include either one or more of the functions indicated in Figure 14.1, though the mechanisms underlying such benefits in fish are still not clearly known. In humans, it has been established that probiotics modulate health, primarily in three ways: (1) by excluding pathogenic bacteria; (2) by regulating signaling pathways; and (3) by modulating the intestinal immune system of the host. Research on farmed fish has now started describing some of these mechanisms.

The gut ecosystem can benefit from the supplementation of valuable probiotics such as lactic acid bacteria (LAB). They adhere to the protective mucus secreted by the epithelial cells, establish a biofilm, and colonize intestinal surfaces. The probiotic bacteria can either directly inhibit/compete with the pathogenic bacteria or indirectly influence them via autochthonous bacteria. Further, these beneficial organisms can help in the production of mucus and antimicrobial peptides, prevention of apoptosis, or improvement in tight junction

function, thereby enhancing epithelial barrier function. The inhibitory molecules of the probiotics can deter the pathogen from establishing in the gut. The probiotic will therefore be able to dominate the pathogens, if present, and prevent any breach of the mucosal defence barriers, which can otherwise lead to infection. Other functional properties of a probiotic are its ability to improve gut structure and nutrient utilization, which translates into better growth (Domeneghini et al. 2006; Ringø et al. 2010).

Most of the responses to probiotic intervention described in fish are limited to general immune responses (see "Current Knowledge on the Application of Probiotics on Selected Fish Species") and disease resistance. To appreciate the usefulness of probiotics, it is necessary to understand how the effector molecules of these organisms activate the immune cells of the host and, in turn, how the host responds by favorably equipping its immune system. In addition, it is essential to know which physiological mechanisms of the host are benefitted when the probiotic organisms support nutrition.

Intestinal Architecture and Immune Response to Luminal Microbes

The intestinal tract of fish is a multifunctional organ that is involved in digestion of food, absorption of nutrients, and immune defense. The fish intestine has three segments: anterior (1st) segment, 60–75% of gut length; mid (2nd) segment, 15–30% of gut length; and hind (3rd) segment, 5–15% of gut length (Rombout et al. 2011). These segments consist of an upper mucosal epithelium that segregates the intestinal lumen from the lower lamina propria, which is a loose vascular sterile connective tissue containing nerves and leucocytes (Wilson and Castro 2011). The epithelial layer is made up of columnar cells, most of which are absorptive enterocytes. In the anterior intestinal segment, the cells are engaged in nutrient uptake; in the mid-segment, they possess large supranuclear vacuoles that enable the uptake of macromolecules. In the hind-segment, they have a greater role in osmoregulation rather than in nutrient absorption. In mammals, enterocytes respond to the dense antigenic load in the lumen through the use of their array of receptors, antimicrobial peptides, and regulatory cytokines (Miron and Cristea 2012). The enterocytes of the mid-segment and hind-segment of fish are known to be involved in antigen uptake, and the triggering of local and systemic immune responses (Inami et al. 2009); however, this may be species-dependent. It could be presumed that probiotics exert their influence primarily on the last two segments.

The apical surface of the intestinal epithelium is constantly exposed to a wide array of luminal microbes, while the basolateral surface interconnects and coordinates the responses of innate and adaptive arms of the immune system with the underlying lamina propria cells (Xavier and Podolsky 2000). Goblet-type mucous cells are interspersed in the epithelium of fish, and the mucus they produce serves as a protective layer that limits direct contact with luminal microorganisms (Wilson and Castro 2011). The epithelial layer can hinder the entry of extraneous luminal materials such as bacteria and substances that induce inflammatory responses in the host (O'Hara et al. 2006). Innate immune components, such as the antimicrobial peptides, also help to reduce the bacterial load at the interface between the luminal elements and epithelium (Bron et al. 2012). Adjacent intestinal cells form tight junctions to permit the epithelium to perform its selective impermeable barrier function (Balda and Matter 2008). The luminal antigens that are allowed access by the intestinal epithelium are taken up via a liquid or a solid phase uptake route; they will then be processed by phagocytosing cells found under the intestinal epithelium. These antigen-processing cells can stimulate lymphocytes to develop an immune response.

As a key organ of the mucosal immune system, the intestine is the response-generating site. It responds upon identification of both resident and transient microbes, including probiotic organisms that are offered to evoke a potential health benefit. Very little information exists on the mechanisms in fish that facilitate tolerance towards the commensal and probiotic microorganisms. A variety of molecules from commensal or probiotic organisms, termed microbial or commensal-associated molecular patterns (abbreviated as MAMPs or CAMPs), interact with the pattern

recognition receptors such as Toll-like receptors (TLRs) of the host. TLRs expressed by the intestinal epithelial cells activate the immune cells in the lamina propria (Cario et al. 2002). The apically positioned TLRs dynamically survey the luminal microbial array and communicate with the immune cell populations. The interaction between epithelium, macrophages, and immune cells leads to lymphocyte differentiation and subsequently, immune homeostasis (Sansonetti and Medzhitov 2009). In this way, tolerance towards commensal and probiotic microbiota is established through the molecular crosstalk between bacteria and host cells, which nurtures the innate and adaptive immune systems (O'Hara et al. 2006; Chung and Kasper 2010; Lee and Mazmanian 2010). However, over-representation of certain bacterial groups can disrupt the homeostasis in the intestine through skewed exposure to microbial factors and alterations in collective microbial metabolism (Gaboriau-Routhiau et al. 2009; Bron et al. 2012).

The immune functions of the intestine are orchestrated by the gut-associated lymphoid system (GALT). GALT in fish is far less complex than in mammals and is diffusely organized with many lymphoid cells, macrophages, and eosinophilic and neutrophilic granulocytes (Rombout et al. 2011). Immunomodulatory effects of probiotics are assessed based on the changes they bring about in the GALT. Evidence suggests that probiotic feeding can modulate both humoral and cellular innate immune components in fish. The therapeutic potential of probiotics for use in aquaculture is currently described by assessing the intestinal immune responses (Abid et al. 2013). However, very little is known about the local immune responses and systemic immune responses of fish that are triggered by probiotics.

Other Benefits of Luminal Microbes

In the gut, a mutually beneficial relationship exists between commensal organisms and the host. A classic example of mutualism between the intestinal microbes and host is seen in human infants. Milk-derived oligosaccharides nourish genetically compatible bifidobacteria, which are able to establish the developing gut microbiota of the infant (Sela and Mills 2010; Bevins and Salzman 2011). The composition of the microbiota is influenced by the nutrients made accessible in the gut microenvironment; they obtain their nourishment from the degradation of host food-derived exogenous nutrients and endogenous products from the host, including bacterial secretions, sloughed epithelial cells, and mucins (Wallace et al. 2011). Bacteria employ their enzymes to degrade these materials to short-chain fatty acids, amines, phenols, and other neutral, acidic, and basic end products (Cummings and Macfarlane 1991; Wallace et al. 2011). It has been revealed that bifidobacteria have the coding capacity for the biosynthesis of nucleotides (pyrimidine and purine) and B vitamins (folic acid, thiamine, and niacin), and the ability to divert mono- and di-saccharides into the fructose-6-phosphate pathway (Ventura et al. 2009). Intestinal microbiota are also known to generate minerals and digestive enzymes for the host, as well as aiding in the absorption of minerals such as calcium, magnesium, and phosphorus (Chaia and Oliver 2008; Ventura et al. 2009).

The host-microbe symbiosis can have a significant role in the metabolism of endogenous and exogenous compounds. Probiotics have been found to temper several metabolic processes, including those related to amino acids, lipoproteins, short-chain fatty acids, and carbohydrates (Martin et al. 2008). For instance, probiotic supplementation in mice facilitated calorie recovery through greater catabolism of branched-chain amino acids, which produces acetyl-CoA and glucose via gluconeogenesis (Martin et al. 2008). The contribution of microbes to hepatic protein synthesis and amino acid homeostasis is also recognized (Metges 2000; Metges et al. 2006).

It is well known that the microbiota in fish gut assist in the digestive processes (Ray et al. 2012). Studies have demonstrated that GI tract bacteria have different enzyme activities that could be specific to the region of the gut (Bairagi et al. 2002; Mondal et al. 2008; Lazado et al. 2012). Few studies have indicated commensal microorganism-induced improvement of gut structure and digestion in fish. It has been reported that pathogen-induced alterations in epithelial microvilli, protrusion of epithelial cells, and damage of tight junctions were not prevalent in the intestine of Atlantic salmon treated with a probiont, *C. divergence* (Ringø et al. 2010).

Organisms Considered as Probiotics

Bacteria

At present, probiotics that are used in humans and land animals are Gram-positive obligate or facultative anaerobes, mainly lactic acid bacteria. *Lactobacillus* and *Bifidobacterium* are probiotics that are commonly used for human applications. The latter has been found to be effective in protecting from pathogens. However, organisms ideal for human/veterinary applications may not be applicable for aquatic animals. While selecting a probiotic for an aquatic animal, their feeding habits (aquacultured fish species are euryphagous carnivores/omnivores/herbivores) and their compatibility with the existing host intestinal microbiota, which typically contain Gram-negative facultative anaerobes, should be considered. Further, the chosen organism should be able to tolerate the pH conditions in the stomach and intestine of fish. It should be noted that pH of the region posterior to pylorus ranges from 7 – 8.5 (Payne 1978; Rust 2002; Deguara et al. 2003). The candidate microbes should produce additional useful substances for the host such as bacteriocin, hydrogen peroxide, and biosurfactants that inhibit the growth of different types of pathogens including harmful yeasts as indicated for humans (Barrons and Tassone 2008).

Lactic Acid Bacteria

Lactic acid bacteria comprise non-spore-forming rods, or cocci, belonging to several genera of Gram-positive bacteria. They can be from homo- or hetero-fermentative groups capable of producing lactic acid, ethanol, and/or other metabolites upon carbohydrate fermentation (Françoise 2010). The native microbiota in the gut of the studied freshwater fishes are partly constituted by LAB, and their numbers are higher than that in marine fishes (Ringø et al. 2010; Héléne and Ringø 2011). The prominent genera of this bacterial group associated with fish are *Lactobacillus*, *Leuconostoc*, and *Pediococcus*, and the peripheral *Carnobacterium*, *Oenococcus*, *Sporolactobacillus*, *Tetragenococcus*, *Weissella*, and *Bifidobacterium*. Diversity of LAB associated with fish, including a variety of rarely recorded species, has been revealed by adopting advanced molecular techniques (Michel et al. 2007). Several of the aforementioned species are generally regarded safe. However, LAB such as *Streptococcus iniae* and *Lactococcus garvieae* are fish pathogens (Gatesoupe 2008). *Lactobacillus* is the largest genus of the Lactobacillaceae family with many species and subspecies, and they are known to have therapeutic and prophylactic effects in humans (Kaur et al. 2009). These chemoorganotrophic and microaerophilic bacteria can replicate in an acidic environment and exhibit optimum growth in the pH range 5.5–6.2 (Zhu 2000). *Lactobacillus* sp. is found in both freshwater and marine fishes, and their probiotic potential is increasingly being studied.

Bacillus

Bacillus species are also used as probiotics. These aerobic, heterotrophic Gram-positive rods and cocci bacteria are heat stable and capable of surviving in extreme conditions, including the low pH of the GI tract (Cutting 2011). Psychrophilic, mesophilic, thermophilic, alkalophilic, neutrophilic, and acidophilic species are all included in this group (Priest 1977). *Bacillus megaterium*, *B. lincheniforms*, *Paenibacillus polymyxa*, and several strains of *B. subtilis* are currently used in aquaculture (Cutting 2011); the latter is the best studied to date. As *B. subtilis* are capable of forming endospores, they can preserve their genome safely during unfavourable environmental conditions (McKenney et al. 2013). It should be noted that certain Bacillus species, such as *Bacillus mycoides*, are considered pathogenic to some fish species.

Yeasts

Yeasts can also be considered as probiotic candidates. These organisms can withstand high pH variations. The most frequently occurring yeast species in the human gut is *Candida albicans*, yet *Saccharomyces boulardii* has been used as a probiotic against bowel diseases because they inhibit certain bacterial toxins, have antipathogenic activity, and exert an immunostimulatory effect on the intestinal mucosa (Czerucka et al. 2007). In ruminants, *S. cerevisiae* has been found to benefit the rumen bacteria, partly through their respiratory activity (Newbold et al. 1996). Yeasts, applied as probiotics for fish, can tolerate the conditions in the GI tract and resist antibacterial antibiotics (Czerucka et al. 2007). The optimum pH and temperature ranges at which yeasts thrive are

4.5–6.5 and 20–30°C, respectively (Czerucka et al. 2007). The non-pathogenic, genetically tractable *S. cerevisiae* is the most-studied yeast in aquaculture (Barnett 2007; Gatesoupe 2007).

Current Knowledge of the Application of Probiotics on Selected Fish Species

In recent years, testing the efficacies of newly discovered and well-known probiotic products is being pursued with immense interest to benefit aquaculture. The effect of a variety of probiotic organisms on different fish has been documented. This section will focus on three prominent farmed species as representatives of warmwater, temperate, and coldwater fishes: Nile tilapia, *Oreochromis niloticus*; common carp, *Cyprinus carpio*; and rainbow trout, *Oncorhynchus mykiss* (Table 14.2). References to recent studies on other fish are also considered, wherever possible.

Probiotic application has been shown to enhance immune responses, induce changes in gut microbial community, influence gut morphology, support production of digestive enzymes, and produce better survival of tilapia, carp, and trout (Table 14.2). The wide range of probiotic organisms examined includes *Lactobacillus acidophilus*, *L. plantarum*, *L. rhamnosus*, *L. casei*, *B. subtilis*, *B. amyloliquefaciens*, *Pediococcus acidilactici*, and *S. cerevisiae*. The rate of application of bacteria in the feed ranged from 10^6 to 10^{10} CFU g^{-1}, while the rate of application of yeast varied widely. The beneficial effects of these organisms were visible after administration for a period of 2–14 weeks; in most cases, the responses were documented by about 4 weeks.

A competent immune system supports the host in its defense against pathogens. Different studies suggest that probiotic organisms equip the target fish with the necessary elements to improve their health and survival. Fish that were fed probiotics and then challenged with pathogens had improved survival (Table 14.2). Resistance to disease in farmed fish is perhaps the ideal measure of probiotic efficacy, but it is imperative that studies are performed in a rigorous manner. Priming of the host immune defense prior to a pathogen encounter would increase the chances of survival. Feed-delivered microbes exert their influence through the intestinal immune system. However, the extent of activation is dependent on the type and form of bacteria (Pérez et al. 2010), which is clearly evident from various studies. For instance, viable *L. rhamnosus* produced higher immune response in rainbow trout than non-viable heat-killed bacteria (Panigrahi et al. 2005).

The microbial-associated molecular patterns (MAMPs) interact with the TLRs of the epithelial cells to signal the immune cells in the lamina propria in order to evoke a response. It has been suggested in mammals that stimulatory and regulatory DNA motifs control the induction of immune responses in the GI tract (Bouladoux et al. 2012). The signaling of immune cells might have resulted in the increase in gene expression of soluble mediators, such as interleukin 1β (*il1b*) and tumor necrosis factor α (*tnfa*), which are inflammatory cytokines, and interleukin 10 (*il10*), an anti-inflammatory cytokine, as observed in rainbow trout and Nile tilapia (Pérez-Sánchez et al. 2011; Pirarat et al. 2011; Ridha and Azad 2012). In addition, enhancement of phagocytic function has been frequently reported for the three target fish species (Table 14.2). *L. acidophilus*, *L. rhamnosus*, and *B. subtilis* increased phagocytic-killing ability of the fish. An increase in respiratory burst in rainbow trout has been associated with this cellular response. Complementing this effect, an upsurge in superoxide dismutase activity of leucocytes has been reported for both Nile tilapia and rainbow trout. The two aforementioned responses suggest that the respective probiotic organisms may also be influencing the oxidative balance in fish. It has been pointed out in humans that multifunctional probiotics influence oxidative stress and anti-oxidative defense (Kullisaar et al. 2012).

In Nile tilapia and rainbow trout, total immunoglobulin increased upon probiotic feeding (Andani et al. 2012; Ridha and Azad 2012). Association of non-specific secretory mucosal immunoglobulin (IgA) and enhancement of probiotic adhesion, in connection with colonization of the mammalian gut, has been revealed by Mantis et al. (2011). In rainbow trout, IgT, a mucosal-epithelial immunoglobulin that binds to resident bacteria in the gut (Flajnik 2010), was significantly expressed in the gut of fish that were offered L. *plantarum* subsp. *plantarum* and challenged with *Lactococcus garvieae* (Pérez-Sánchez et al. 2011).

Table 14.2 Summary of representative studies that have examined the effect of feed-delivered microorganisms (probiotic) in three major farmed fish: Nile tilapia (*Oreochromis niloticus*); common carp (*Cyprinus carpio*); and rainbow trout (*Oncorhynchus mykiss*).

Fish studied	Probiotic organism	Inclusion rate (CFU g^{-1}), feeding duration (weeks)	Reported responses upon probiotic feeding
Nile tilapia[a]	*Lactobacillus acidophilus* (a reference strain)	1×10^7 8	Immune responses: increased phagocyte function, neutrophil adherence, lysozyme activity Challenge test: intraperitoneal (IP) injection with *Streptococcus iniae*- improved survival
Nile tilapia[b]	*Lactobacillus plantarum, L. brevis* (from tilapia intestine)	1×10^8 2	Immune responses following IP injection with *Enterococcus durans*: increased number of total leukocytes- lymphocytes, neutrophils and monocytes
Nile tilapia[c]	*Lactobacillus rhamnosus* (ATCC 53103)	1×10^{10} ≈ 4	Gut morphological changes: improved proximal and mid-intestine villous height, higher number of intraepithelial lymphocytes and acidophilic granulocytes in proximal and distal intestine Immune responses: higher serum complement activity, phagocytosis, killing ability of head kidney leukocytes, expression of *tnfa*, *il1*
Nile tilapia[d]	*Lactobacillus* sp. (from dairy yogurt)	1×10^8 ≈ 14	Immune responses: increased serum lysozyme activity, head-kidney leucocyte superoxide dismutase activity, total immunoglobulin level
Nile tilapia[e]	*Lactobacillus acidophilus* (from dairy yogurt)	1×10^6 ≈ 2	Challenge test: IP injection with *Aeromonas hydrophila*- higher survival at 20 days Immune responses: increased expression of cytokine gene *il1b* and antibacterial gene *tf* in spleen and kidney
Common carp[f]	*Lactobacillus acidophilus* (a laboratory strain)	2 g/100 g feed ≈ 6	Microbiological changes: high total heterotrophic microbial count
Rainbow trout[g]	*Lactobacillus casei* (from common carp intestine)	5×10^7 ≈ 4	Immune responses: increased lysozyme activity, alternative complement activity and total immunoglobulin level Challenge test: IP injection with *Yersinia ruckeri*- higher survival
Rainbow trout[g]	*Lactobacillus plantarum* (from common carp intestine)	5×10^7 ≈ 4	Challenge test: IP injection with *Yersinia ruckeri*- higher survival
Rainbow trout[h]	*Lactobacillus plantarum* (from rainbow trout)	1×10^6 ≈ 5	Immune responses: increased expression of cytokine genes *il1b*, *il10*, *tnfa* in head kidney Challenge test: cohabitation with *Lactococcus garvieae* infected fish: higher percentage survival of probiotic fed fish; higher expression of cytokine genes *il10*, *il8* and immunoglobulin gene *igt* Microbial changes: no detectable levels of the probiotics or the pathogen in the distal intestinal mucosa
Nile tilapia[a]	*Bacillus subtilis* (ATCC 6633)	1×10^7 8	Immune responses: increased phagocyte function, neutrophil adherence, lysozyme activity
Nile tilapia[d]	*Bacillus amyloliquefaciens* (from yellow fin bream gut)	1×10^8 ≈ 14	Immune responses: increased serum lysozyme activity, head-kidney superoxide dismutase activity, total immunoglobulin level, and serum bacterial agglutination titres Microbiological changes: gut microbiota dominated by *B. amyloliquefaciens* (even up to 61 days following termination of probiotic feeding)
Common carp[i]	*Bacillus sp.* (from common carp ponds)	1 g/kg feed ≈ 9	Nutritional aid: high protease and amylase activities
Rainbow trout[j]	*Bacillus* sp. JB-1 (from digestive tract of rainbow trout)	5×10^7 2	Immune responses: induced transferrin protein in serum

Table 14.2 *(Continued)*

Fish studied	Probiotic organism	Inclusion rate (CFU g^{-1}), feeding duration (weeks)	Reported responses upon probiotic feeding
Rainbow trout[k]	*Bacillus subtilis*	1×10^7 ≈ 6	Hematological changes following IP injection of *Streptococcus iniae*: increased leucocyte count-higher percentage of lymphocytes, but lower percentage of neutrophils
Rainbow trout[l]	*Bacillus subtilis AB1* (from intestine of rainbow trout)	1×10^7 2	Immune responses: stimulated respiratory burst, serum lysozyme and peroxidase activity, phagocytic killing, total and α1-antiprotease activity Hematological changes: increased leukocyte populations
Rainbow trout[m]	*Pediococcus acidilactici MA185 M*	1.5×10^6 ≈ 21	Morphological change: low frequency of malformed fish fry
Nile tilapia[n]	*Saccharomyces cerevisiae* (autolyzed)	2 g/100 g feed 10	Immune responses following injection of *Aeromonas hydrophila* into swim bladder: greater accumulation of total cells due to inflammation in swim bladder, greater number of thrombocytes, but lesser number of neutrophils, macrophages and lymphocytes at 6 and 24 hours. Leucocytosis occurred at 24 hours, indicating increased non-specific acute inflammatory response.
Common carp[f]	*Saccharomyces cerevisiae* (a laboratory strain)	3 g/100 g feed ≈ 6	Microbiological changes: high total heterotrophic microbial count
Rainbow trout[o]	*Saccharomyces cerevisiae* (hydrolyzed)	5 g/kg feed ≈ 7	Nutritional aid: increase in trypsin and amylase activities Morphological changes: higher density of the goblet cells per villus in proximal intestine
Rainbow trout[p]	*Saccharomyces cerevisiae* (hydrolyzed)	5 g/kg ≈ 7	Immune responses: increased lysozyme, protease, alkaline phosphatase, and esterase activities Greater hemagglutination and antibacterial activity of fish mucus against *Yersinia ruckeri*
Rainbow trout[q]	*Saccharomyces cerevisiae* PTCC5052 (allochthonous strain)	1×10^8 yeast g/kg feed 7	Health: improved survival of larval fish

[a] Aly et al. (2008)
[b] Jatobá et al. (2008)
[c] Pirarat et al. (2011)
[d] Ridha and Azad (2012)
[e] Villamil et al. (2014)
[f] Ramakrishnan et al. (2008)
[g] Andani et al. (2012)
[h] Pérez-Sánchez et al. (2011)
[i] Yanbo and Zirong (2006)
[j] Brunt et al. (2008)
[k] Kamgar and Ghane (2012)
[l] Newaj-Fyzul et al. (2007)
[m] Aubin et al. (2005)
[n] Reque et al. (2010)
[o] Heidarieh et al. (2012)
[p] Sheikhzadeh et al. (2012)
[q] Zargham et al. (2011).

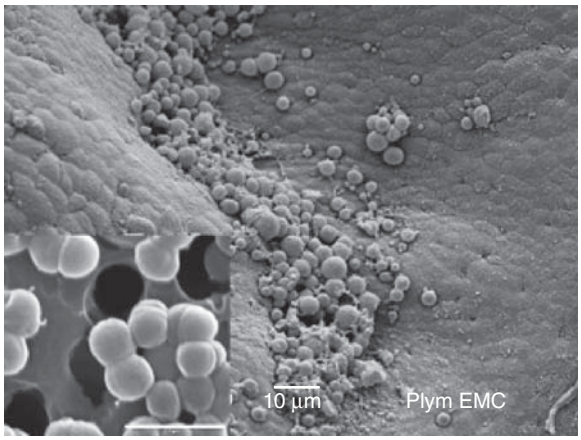

Figure 14.2 Colonization of the intestine of rainbow trout by feed-delivered microbials. Scanning electron micrograph reveals the colonization of the distal region of rainbow trout intestinal mucosa by *Pediococcus acidilactici*-like cells. Scale bar: 10 µm. Inset: *P. acidilactici* on 1 µm nucleopore filter; scale bar: 2 µm (Merrifield et al. 2010, Aquaculture Research, 41:1268–1272. Copyright © 2010, John Wiley & Sons, Inc.).

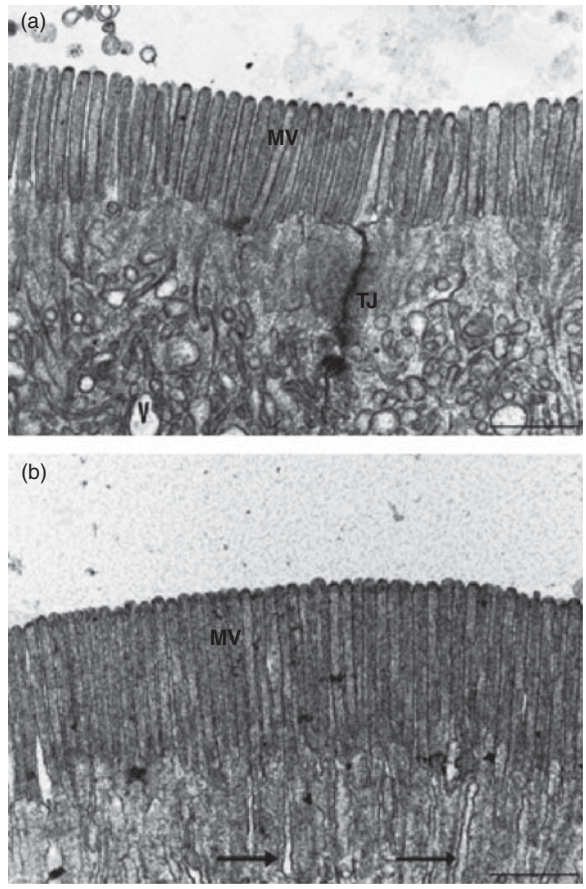

Figure 14.3 Beneficial effect of feed-delivered microbial *Pediococcus acidilactici* on intestinal morphology of rainbow trout. Transmission electron micrographs of the proximal intestine of (a) control fish that did not receive microbial additive and (b) *P. acidilactici*-fed fish. Significantly longer microvilli (MV) were seen in the *P. acidilactici* group. Higher endocytic activity was also observed in the probiotic-fed fish (b), depicted by arrows. TJ: tight junction; V: vacuole. Scale bar: 1 µm (Merrifield et al. 2010, Aquaculture Research, 41:1268–1272. Copyright © 2010, John Wiley & Sons, Inc.).

The influence of feed-delivered microbes on the morphology of fish intestine has been shown in different studies. Colonization patterns of the probionts and their impact on microvilli were examined in rainbow trout fed *Enterococcus faecium*, *Bacillus* spp., and *P. acidilactici* for 5 weeks by Merrifield et al. (2010b). *P. acidilactici* alone was found attached to proximal and distal intestinal epithelium (Fig. 14.2). Endocytic activity in the distal intestine was high when the probiont was present (Fig. 14.3). In another study, feeding yeast to rainbow trout also influenced the morphology of the proximal intestine; a high density of goblet cells per villus was recorded (Heidarieh et al. 2012). On the other hand, significant intestinal damage was reported in gilthead sea bream (*Sparus aurata*) that were offered a feed containing *B. subtilis* (10^7 CFU g^{-1}) for 4 weeks (Cerezuela et al. 2012). A thick mucus layer over the apical part of enterocytes, enterocyte vacuolization, intercellular spaces, shorter and disrupted microvilli, and a decrease in bacterial diversity were among the observed damages. The aforementioned contradictory observations resulting from probiotic feeding indicate the importance of examining intestinal structural changes in fish while evaluating the probiotic efficacy of fed microbial preparations.

Probiotic-feeding can affect the digestive enzyme activities in the host. *Bacillus* sp. offered via feed was found to increase the protease and amylase activities in the gut of common carp (Yanbo and Zirong 2006). Candidate probiotics *Pseudomonas* sp. and *Psychrobacter* sp., isolated from the GI tract of

Atlantic cod (*Gadus morhua*) and delivered through feeds (10^6 CFU g^{-1}), influenced the amylase and cellulase activities in the anterior intestine of fish by day 40 (Lazado et al. 2012). On the other hand, feeds containing the yeast *S. cerevisiae* increased trypsin and amylase activities in rainbow trout (Heidarieh et al. 2012). In rainbow trout fry fed *S. cerevisiae* var. *boulardii* at the rate of 10^6 CFU g^{-1} of feed, the activity of the brush border enzymes, namely alkaline phosphatase, γ-glutamyl-transpeptidase, and leucine-amino-peptidase, was high (Waché et al. 2006). The potential of probiotics to serve as nutritional supplements for fish has not been adequately documented. This has to be considered as a fertile area of investigation, particularly for advancing the technology of culturing fish larvae, a life stage in which additional nutritional support will be advantageous.

Developing Probiotics for Fish

A systematic pathway that has to be followed while developing probiotics for farmed aquatic animals is outlined in Figure 14.1. The key steps are (1) isolation of potentially beneficial organisms; (2) identification and characterization (including their safety status) of these organisms; and (3) establishing their benefits, initially through controlled small-scale trials and later via larger on-farm trials. In the first stage, culturable microorganisms are isolated from different niches, including those from commensal microbiota of the eventual target animals. While organisms that do not originate from the target host have been proven to be beneficial (Aubin et al. 2005; Sanders 2006; Panigrahi et al. 2007, 2011), those isolated from the host itself may not necessarily produce the intended composite effects (Lamari et al. 2013). Nevertheless, the straightforward approach would be to isolate potential probiotic organisms from target animals because this can be considered as safe for the host and sustainable for the target industry. The risk to the consumer and the environment also must be adequately evaluated. Sourcing of probionts from the host itself has the added advantage that multiple species could be simultaneously developed. As to their efficacy for the target animal, it has been demonstrated that multispecies (or strains) probiotics could in some cases function synergistically and provide greater benefits compared to monospecies (Timmerman et al. 2004; Ramos et al. 2013). Depending on the type of the intended effect, and particularly in the case of "competition exclusion", application of a probiotic cocktail, which is a combination of indigenous microbial strains of the designated host, would be advantageous. Such strains could occupy different niches in the intestine and complement the properties of each other to prevent the development of opportunistic bacteria within the community. As pointed out earlier however, autochthonous bacteria may not be the best choice (Merrifield et al. 2010b; Lamari et al. 2013); every probiotic candidate should be assessed on a case-by-case basis.

After isolation, proper and efficient identification and characterization of these potentially beneficial microorganisms should be undertaken. Identification is currently performed using advanced molecular techniques that provide a far greater degree of accuracy than previously attained through conventional microbiology. Documentation based on the phenotype, growth, fermentation profile, and serology is complemented by molecular fingerprinting techniques (e.g., polymerase chain reaction-denaturing/temperature gradient gel electrophoresis, Ribosomal intergenic spacer analysis), which use different genetic markers to differentiate subspecies or even strains.

Characterization of the microbes intended for probiotic applications is an elaborate process that should address their functionality, safety, and technological suitability (Fig. 14.1). In aquaculture, benefits of the chosen organisms to the host should be confirmed through dependable *in vivo* feeding studies.

Functionality

The probiotic organisms' mechanisms of action and the health benefits they bestow on a host during and after the application of the "microbial supplements" need to be examined in-depth. As the GI tract is the main route of infection in fish, the primary role of the feed-delivered probiotic organism is to safeguard the gut mucosal barrier and prevent pathogen dissemination within the host. The probiotic organisms should tolerate the gastric conditions prior to their arrival at the intestine, where they may adhere and possibly colonize. This can eventually result in a healthy intestinal ecosystem supported by the beneficial organisms. Once the probiotic organisms manage to establish in

the intestine, they should exert antimicrobial activity against pathogens and interfere with the adhesion of disease-causing microbes. However, after an infection or a disturbance in the community, alteration to a population favoring the applied probiotic alone should not be equated to maintenance of microbial stability, but instead should confirm the reestablishment of the original composition of the intestine (Sanders 2008). The probiotic organisms should also beneficially modulate the host immune system, or function as a nutritional aid (Panigrahi et al. 2007; Lazado et al. 2012; Standen et al. 2013).

Safety

Probiotic organisms for aquaculture need to be first assessed for their innocuousness. Their safety, not only for the host animal but also for the users and even the consumers of the fish product, needs to be properly documented. Several countries have now developed regulations for the application of microbial feed additives (Anadón et al. 2006). The safety issues with respect to the target animal are to be considered during the development phase of a probiotic organism. Concerns pertaining to the users/consumers have to be taken into account when a selected probiotic is being evaluated for commercial use. Existing evidence points out that the regular use of feeds containing probiotic candidate with weak safety background could result in previously unknown disease problems in aquatic animals (Wang et al. 2000; Cerezuela et al. 2012). Investigations on the target animal should therefore include tolerance testing (up to 10 times the suggested level) to determine the safety margin of the microbial additive. These studies should examine zootechnical parameters (weight gain, feed intake and conversion, flesh quality) and clinical signs (including morbidity and mortality), the latter being assessed through blood chemistry, hematology, and histopathology. From a consumer safety perspective, oral-toxicity studies in model organisms as well as *in vitro* and *in vivo* genotoxicity studies are suggested to test for mutagenicity (Anadón et al. 2006). As certain bacteria produce toxins (e.g., *B. cereus*), genetic and biochemical tests are needed to understand their toxins and virulence factors. Bacteria are also known to possess transferable resistances, and if the candidate probiotic strain demonstrates resistance to antibiotics, it is considered unsuitable for application in feeds. Further, as consumption of raw fish is gaining popularity, harmful residual probiotics, if any, could be a food safety issue. Hence, regulatory practices have been implemented for commercial probiotics; they should be generally regarded as safe (GRAS) in the United States, or follow the qualified presumption of safety (QPS) in Europe (EFSA 2005). Most of the commercially available probiotic preparations, currently registered in Europe or USA, usually fulfill the GRAS and QPS requirement. At the industry level, the bacteria will have to be checked for irritancy and inhalation toxicity, considering the safety of the worker. This is applicable both at the site of production of the microbe and the feed manufacturing plant. Finally, in rare cases, the environmental risk of the microbial additive also has to be considered while gauging the safety of the product. This is particularly true for microbial preparations intended for direct application into the environment of the host.

Technological Aspects

Once the desirable properties of a probiotic organism are characterized, the technological issues related to its incorporation into feeds are to be considered. Feed manufacturing and storage conditions affect the viability of the organisms. The organisms should be able to endure pH, temperature, pressure, and other physical stressors encountered during feed processing to preserve their viability in order to produce the desired effect in the host when delivered through feeds. Probiotic bacteria that can form spores, for example *Bacillus* sp., are therefore often considered as more appropriate for in-feed application, as they can withstand a harsh environment and heat (McKenney et al. 2013). Most of the probiotics employed for fish are non-spore formers. However, the aquafeed industry generally prefers not to include even spore-forming bacteria or protected bacterial forms (microencapsulated bacteria) in the mill before processing. In such circumstances, top coating of the feed with the chosen probiotic is preferred but these feeds would need specific cold storage conditions and may have a limited shelf life. Currently, only one commercial probiotic fulfilling these prerequisites is approved for use in aquafeed within the European Union; it is applied after pelleting in several commercial feeds

for salmonids, marine fish, and shrimp (Castex and Aarestrup 2011).

Efficiently delivering the adequate and intended number of viable organisms to the site of action through feeds, following a prescribed dosing regimen, is necessary to produce the desired health effects on the host organism. Even though inactivated forms of probiotic organisms may possess immunostimulatory effects, induced by their intact microbial cell wall components including lipopolysaccharides and peptidoglycans, the benefits may not match those derived from the live microbes (Panigrahi et al. 2005).

Commercial Aspects of Aquaprobiont

If the identified and characterized commensal organisms are accepted as a commercial probiotic for farmed aquatic species, extensive *in vitro* and *in vivo* evidence on the different aspects mentioned in the previous sections needs to be gathered. Moving forward, this section briefly covers some of the technological aspects related to the production of probiotic strains and discusses some of the currently available products for farmed fish.

Production technology

The intended effects of the feed-delivered probiotics on the target animal would depend on several factors, including those related to the viability of the probiotic organism. Most of the strains currently employed for aquaculture are those that are widely accepted for human/animal applications. Bulk production of host-derived microbes may demand additional steps to maintain the viability and performance of the organism, as it has been noted that the therapeutic potential of commercial probiotics fluctuates significantly depending on their production process (Schillinger 1999; Masco et al. 2005). A specific set of industrial production conditions (from fermentation to downstream processing) should therefore be designed to ensure optimal viability/stability/effectiveness of the resulting product.

The microbes suitable for commercialization are stored as seed stock at −80 °C in a cell master bank. As required, they serve as inoculum for pre-culture that will later be taken into batch fermentation and suspension culture under sterile conditions to produce the organism in commercial volumes. By optimizing the fermentation conditions, the viability, stability and functionality of the microorganism can be improved, thereby enhancing the probiotic properties and performance. In the continuous fermentation process, the microorganisms are highly susceptible to contamination and the cells lose their characteristics over time, making this technique unacceptable under industrial conditions (Soccol et al. 2010). Through technological advances however, cells with different physiologies are produced under well-controlled conditions (Lacroix and Yildirim 2007). Unlike stirred tank reactors, membrane bioreactors enable the production of a large number of bacteria of stable quality. Continuous feeding of fresh medium into these bioreactors, which are equipped with ultrafiltration or microfiltration membranes, helps to retain pure cells (Soccol et al. 2010) as inhibitory metabolic products permeate through the membrane. The concentrated cell fraction that is harvested either batch-wise or continuously does not normally need additional downstream treatment before freezing or freeze-drying. To protect the probiotics physically and chemically, different processing or formulation technologies such as an addition of protectants or microencapsulation using alginate, chitosan, and carboxymethylcellulose have been adopted (Rokka and Rantamäki 2010).

Commercial Products

Certain companies have taken some of the probiotic organisms through the necessary developmental phases to introduce them into the market. In Europe, Lallemand is the first company to have registered a probiotic (*P. acidilactici* MA18/5M) for use in Aquaculture; regulation (EC) N°911/2009 (salmonids and shrimp) and N°95/2013 (all other fish). *P. acidilactici* (Bactocell®), marketed for premixes and pelleted feeds, has been found to effectively reduce vertebral compression syndrome and improve intestinal health in salmonids and to prevent deformities in marine fish (Aubin et al. 2005; Abid et al. 2013). Outside Europe, several of the commercial preparations employ *Bacillus* isolates. *B. cereus* var. *toyoi* isolates

(Toyocerin®) have been developed as feed additives. *B. amyloliquefaciens* (Ecobiol®) are available in different grades, either for pelleted feeds/premixes or for coating. *B. cereus* (Esporafeed®) are also used for pelleted feeds/premixes. Although Gram-positive bacteria (e.g., strains of *Lactobacillus* and *Bacillus*) dominate among the probiotics applied to fish, Gram-negative probiotic bacteria (e.g., strains of *Aeromonas* and *Pseudomonas*) are not uncommon in countries having less-stringent regulations (South Asia, South East Asia, Central America). At present, there are only a few examples of microbial feed additives that have distinct beneficial effects. The above-mentioned products are marketed as liquids or powders and in different concentrated forms, to ensure their application at different stages of feed production.

Conclusions

The concept of health feeds has gained importance in the aquafeed industry, and microbial feed additives are generally expected to impart improved health and reduced mortality of farmed fish. Several beneficial effects of various probiotic candidates in fish have been reported. In most cases however, the limited focus of the research groups, the reproducibility constraints, and the lack of interest by industry to document the effects/mechanisms of promising probiotic candidates make it difficult to vouch for their net health-improving effects. These issues and the availability of unproven and undocumented products in the market constitute the present-day problems in the development of probiotics for aquaculture. There is certainly a need for standardizing the protocols that examine and rank the biological effects of a probiotic organism and promote its use as an eco-friendly additive. This could be done after conducting further elaborate trials using established probiotic organisms on concerned fish species. Until guiding information is made available, through comprehensive characterization of the effects of a given probiotic candidate, an unconfidently labeled and newly developed microbial additive would fail to win the confidence of consumers, and could even undermine the concept that probiotics are sustainable options to antibiotics in aquaculture.

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Chapter 15

Organic Acids and Their Salts

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Introduction

Because of its potential for high production and economic return, intensive fish farming is expanding very rapidly and becoming an important enterprise worldwide. Under this production practice, outbreaks of infectious diseases have been identified as a major economic loss to producers. Short-term feeding of antibiotic-medicated feeds is a common practice to treat bacterial infections. Long-term feeding of low doses or sub-therapeutic levels of antibiotics as growth promoters and for disease prevention in aquaculture has also been practiced, although not as extensively as in livestock and poultry production. In recent years, the growing concern over the possible development and proliferation of antibiotic-resistant pathogens, and presence of antibiotic residues in animal products and the environment, have made this practice less acceptable (Ravindran and Kornegay 1993; Canibe et al. 2001; Ricke 2003). On 1 January 2006 the European Union banned the use of antibiotics and related drugs as growth promoters in feeds of food-producing animals (Castanon 2007). This ban has implications in the international trade of animal products because the European Union only imports foods obtained from animals that were not fed with antibiotic-containing diets. Furthermore, the increased public awareness and objection to the use of antibiotics as growth promoters (AGP) in animal feeds has led some major restaurant chains to mandate their suppliers to stop using antibiotics that are important in human medicine as growth promoters in food animals after 2004 (Hayes and Jensen 2003). Consumer advocate groups support these actions and are calling for more widespread bans. It is therefore expected that worldwide use of antimicrobials in animal production practices will decrease in the future. This has compelled researchers to evaluate a variety of products ranging from plant extracts, immunostimulants, enzymes, prebiotics, probiotics, and organic acids and/or their salts as alternatives to in-feed antibiotic growth promoters.

Among these feed additives, the use of organic acids, their salts or their combination in livestock feeds has received much attention during the past few years. Although the growth performance benefits of these substances have been known for almost half a century (Cole et al. 1968), the ban on AGP resulted in an increased scientific focus on organic acids. Improvements in growth performance and feed efficiency have been documented in numerous studies in pigs and poultry (Ravindran and Kornegay 1993; Partanen and Mroz 1999; Dibner and Buttin 2002; Mroz 2005; Lückstädt 2007a; Lückstädt and Mellor

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2011; Jacela et al. 2009; 2010; Adil et al. 2010; Samanta et al. 2010; Suryanarayana et al. 2012). In poultry, evidence of the performance enhancement by dietary acidification is not as convincing as in pigs (Desai et al. 2007). However, it has been shown that the response of pigs to acidifiers is affected by age, with newly weaned pigs showing the greatest growth improvement (Dibner and Buttin 2002). Interest in the use of these substances as growth promoters in aquaculture diets is more recent, and results obtained for various fish species are often inconsistent (Lückstädt 2006, 2007b, 2008a, b; Lim et al. 2010a; Ng and Koh 2011).

This chapter provides a brief summary of the chemical and physical properties of organic acids and salts commonly used as feed additives. The role of organic acids, their salts or their combinations in feed preservation and their possible mode of action in digestive tract (antimicrobial effects) and on nutrient utilization are presented. Since available information on these research areas for aquatic species is limited, information related to livestock will also be considered. An overview of the influence of dietary organic acids, their salts or their combinations on growth performance, feed utilization efficiency, and disease resistance in various fish species is also provided.

Chemical and Physical Characteristics

Organic acids are organic carboxylic compounds of general structural formula R–COOH whose acidity is associated with their carboxyl group (–COOH). They are weak acids because they partially dissociate in water to form a hydrogen ion (H^+) and a carboxylate ion ($-COO^-$). At any one time, most of the organic acids will be present in solution as unionized molecules. The amount of ionization/dissociation is determined by the strength of acid, referred to as acid dissociation constant or acid ionization constant (pK_a). Values of pK_a can be determined by adding one equivalent of alkali to two equivalents of acid and measuring the resulting pH. The pK_a value is therefore the pH value of a 50% neutralized solution. The lower the value of pK_a, the stronger the acid or the larger the extent of dissociation. The chemical formulae, physical form, molecular weight, density, dissociation constant, and odor of acids and salts commonly used as feed additives are listed in Table 15.1. Acids with two carboxyl groups, di-carboxylic acids (fumaric and malic) and three carboxyl groups, tri-carboxylic acid (citric acid) have two and three pK_a values, respectively. The solubility of organic acids in water varies

Table 15.1 Formulae and chemical and physical characteristics of some organic acids and salts commonly used in animal diets. Modified from Dibner and Buttin (2002) and Mroz (2005).

Name	Formula	Physical form	Molecular weight	Density	Dissociation constant (pK_a)	Odor
Formic	$HCOOH$	Liquid	46.03	1.220	3.73	Pungent
Acetic	CH_2COOH	Liquid	60.05	1.049	4.76	Pungent
Propionic	CH_3CH_2COOH	Liquid	74.08	0.993	4.88	Pungent
Butyric	$CH_3CH_2CH_2COOH$	Liquid	88.12	0.958	4.82	Rancid
Lactic	$CH_3CH(OH)COOH$	Liquid	90.08	1.206	4.76	Sour milk
Sorbic	$CH_3CH=CHCH=CHCOOH$	Solid	112.14	1.204	4.76	Mildly acrid
Fumaric	$COOHCH=CHCOOH$	Solid	116.07	1.635	3.03/4.44	Odorless
Malic	$COOHCH_2CH(OH)COOH$	Solid/liquid	134.09	1.609	3.46/5.10	Apple
Citric	$COOHCH_2C(OH)(COOH)CH_2COOH$	Solid	192.14	1.665	3.1/4.8/6.4	Odorless
Ca-formate	$Ca(HCOO)_2$	Solid	130.11	2.02		Neutral
Ca-lactate	$C_6H_{10}CaO_6$	Solid	218.22	1.49		Neutral
Ca-propionate	$C_6H_{10}CaO_4$	Solid	186.21	1.55		Neutral
K-diformate	$KH(COOH)_2$	Solid	130.14	–		Neutral
Ca-butyrate	$C_8H_{14}CaO_4$	Solid	214.27	–		Rancid
Mg-citrate	$C_6H_6MgO_7$	Solid	214.41	1.74		Neutral
Na-lactate	$C_3H_5NaO_3$	Solid	112.06	1.33		Neutral

with the number of carbon atoms. Acids with up to four carbon atoms are relatively soluble in water. The solubility rapidly decreases with increasing numbers of carbon atoms. Organic acids with more than six carbon atoms are minimally soluble to insoluble in water.

Role in Feed Preservation

Manufactured aquaculture feeds currently used for modern fish farming comprise a mixture of animal and plant feedstuffs and vitamin and mineral premixes at proportions that provide adequate levels of essential nutrients and digestible energy required for optimum growth and health of the cultured species. The proportion of animal to plant ingredients in the formulations varies depending on fish species. For instance animal ingredients, particularly fish meal and oil, are major protein and oil sources in feeds for carnivorous species, while plant ingredients are the major components in feeds of omnivores and herbivores. However, due to the increased demand, high cost, and limited supply of fish meal and oil, emphasis is placed on increased use of renewable plant ingredients as alternatives to animal feedstuffs. The increased reliance on plant ingredients could create the risk of potential mycotoxin contamination in compounded diets because plant feedstuffs such as grains, grain by-products, and oil-seed meals are often contaminated with mycotoxins, toxic metabolites produced by a diverse species of fungi or molds (NRC 1993, 2011; Manning 2001). Mold toxins vary in their toxicity among different aquaculture species and their life stages. Most mycotoxins are produced primarily by three genera of molds: *Aspergillus*, *Penicillium*, and *Fusarium*. Mold growth in plant feedstuffs could occur in the field during growing season (pre-harvest) and/or post-harvest during processing and storage (CAST 2003; NRC 2011). Feed manufacturers should avoid using ingredients suspected to be contaminated with mycotoxins because steam pelleting or extrusion processing does not destroy most of the mold toxins and spores as they are resistant to heat (CAST 2003; Hughes et al. 2006; Jones 2008). However, even in the absence of contaminated ingredients, feeds may come into contact with moldy old feed residues in various locations during the feed manufacturing process, storage, and transport. This old feed may seed the new feeds with mold, thus increasing the chance of mold growth and mycotoxin contamination (Jones 2012). The growth of mold can be accelerated if the finished feed has a moisture content of more than 12% and is stored under warm and humid conditions for a prolonged period (NRC 1993; Higgins and Brinkhaus 1999; Manning 2001).

Organic acids have long been used in fish feeds as one of several strategies to supplement good management practices to reduce the potential threat of microbial contamination, including pathogenic bacteria and molds or fungi that may grow during feed storage. Dibner and Buttin (2002) indicated that the first effect of organic acids in animal agriculture is related to feed preservation, particularly to control the growth of molds. The most commonly used mold inhibitors are: (1) individual or combinations of organic acids such as propionic, sorbic, benzoic, and acetic acids; and (2) salts of organic acids such as calcium propionate, potassium sorbate, and sodium benzoate. Solid or liquid forms work equally well if the inhibitors are evenly dispersed though the feed. Acid forms are more effective mold inhibitors than their corresponding salts. However, salts have advantage over free acids because of their solid and less volatile form, and they are generally odorless and easy to handle in the feed-manufacturing process. They are also less corrosive and more soluble in water than free acids (Higgins and Brinkhaus 1999; Partanen and Mroz 1999; Schnürer and Magnusson 2005; Eissen et al. 2010; Jones 2012). Combinations of different anti-mold agents that work synergistically are more effective mold inhibitors because individual compounds have different efficacies against different molds (Auerbach 2011). Commonly used mold inhibitors contain various levels and combinations of organic acids, with propionic acid being the principle ingredient (Eissen et al. 2010).

The effectiveness of mold inhibitors is also affected by several other factors. The smaller the particle sizes of the mold inhibitors or their carriers, the greater the effectiveness. Dietary nutrients such as protein and minerals tend to reduce the effectiveness of free acids by neutralizing and converting them to their corresponding salts, which are less active as inhibitors. Dietary fat tends to enhance the activity of organic acids, probably by increasing their penetration into feed particles (Jones 2012). Surfactants may also

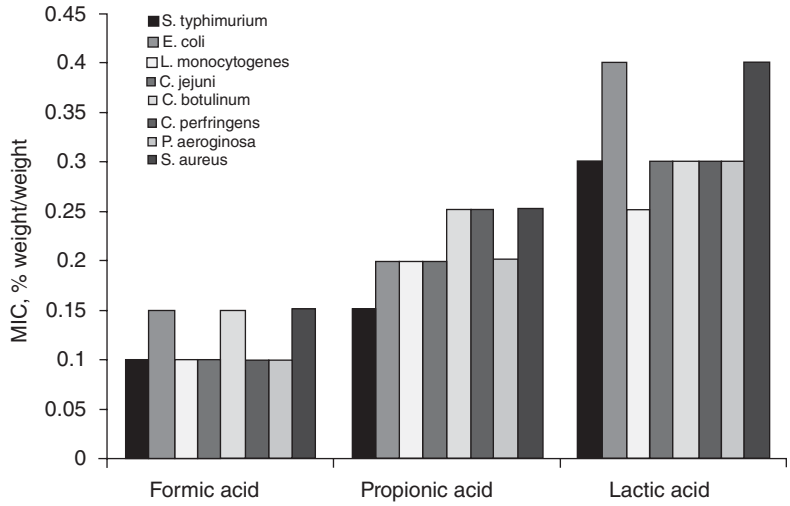

Figure 15.1 The antimicrobial effects of formic, propionic and lactic acids against different bacteria (Modified from Dibner and Buttin 2002 and Mroz 2005).

be added to liquid organic acid blends to enhance their effectiveness by ensuring that mold inhibitors and water are evenly distributed throughout the feed particles. Surfactant also improves the water-binding capacity of feed ingredients, thus lowering water activity in feeds (Eissen et al. 2010).

Antimicrobial effects of different organic acids against the various molds vary from one acid to another. Higgins and Brinkhaus (1999) showed that valeric acid had the highest inhibitory activity against mold (*Aspergillus* spp., *Geotrichum* spp., *Mucor* spp., *Fusarium* spp., *Penicillium* spp. and *Scopulariopsis* spp.), followed by propionic acid and butyric acid which have similar efficacy. For these three acids, a complete growth inhibition was obtained at concentrations of less than 0.35%. For other acids, such as acetic acid, lactic acid, and benzoic acid, a concentration of 0.5% or more was required for effective mold inhibition. These authors also suggested that, due to its relative good palatability and high efficacy at low inclusion levels (0.1–0.2%), as well as its relatively low cost, propionic acid may be considered as one of the most economical organic acids for use as a mold inhibitor.

Furthermore, organic acids are well known for their antibacterial action. They appear to be both bacteriostatic and bactericidal, which is especially important for protein-rich ingredients commonly used in fish feed such as fish meal, poultry by-product meal, and soybean meal. Okoli et al. (2006) reported that up to 90% of locally produced fish meal in Nigeria is contaminated with *Salmonella*; it is therefore also of great concern to counteract the bacterial load of raw materials important for fish feed with organic acids. Studies have quantified the effects of a number of organic acids against different bacteria *in vitro* (Strauss and Hayler, 2001; Fig. 15.1). The minimum inhibitory concentration (MIC) is commonly used as an index of the efficacy of antimicrobial substances and the minimum concentration of these needed to inhibit bacterial growth.

Despite their differences in antimicrobial activity, all organic acids appear to share a common mode of action. Freitag (2007) described how, in animal nutrition, acidifiers and their salts exert their performance-promoting effects via three different ways: feed, intestinal tract, and metabolism (Table 15.2). The efficacy of acids as antimicrobial agents increases at lower pH values. In solution, the concentration of undissociated acid increases as the pH decreases (Brul and Coote 1999; Lambert and Stratford 1999; Dibner and Buttin 2002; Ricke 2003). The inhibitory action of organic acids on the growth of molds and bacteria has been attributed to the undissociated forms of acids which are lipophilic and can freely pass through the cell membrane of microorganisms into the cytoplasm. Inside the cell, due to higher (near neutral) pH in the cytoplasm, the

Table 15.2 Effects of organic acids and salts in animal nutrition (adapted from Freitag 2007).

	Effective form	Effects
Feed	H^+	- pH reduction
	H+ and Anion	- Reduction of acid-binding capacity
		- Reduction of microbial growth
		- Antibacterial effects
Intestinal tract	H^+	- pH reduction in stomach and duodenum
	Anion	
	H^+ and Anion	
		- Improved pepsin activity
		- Complexing agents for cations (Ca^{++}, Mg^{++}, Fe^{++}, Cu^{++}, Zn^{++})
		- Antibacterial effects
		- Modulation of microbial population
Metabolism		- Energy supply

organic acid molecules dissociate into protons and anions which cannot cross the cell membrane. The accumulation of protons within the cell reduces the intracellular pH, causing disruption of cell membrane permeability and inhibition of enzymatic reactions and nutrient transport systems. Moreover, to maintain intracellular homeostasis, the cell is required to use cellular energy (adenosine triphosphate) to expel excess protons leading to depletion of cellular energy (Brul and Coote 1999; Lambert and Stratford 1999; Davidson 2001; Dibner and Buttin 2002; Ricke 2003). Accumulation of anions is responsible for the toxic effect of organic acids for microorganisms that are more resistant to organic acids because they are capable of growing at lower intracellular pH (Russell 1992). The action of organic acid against Gram-negative bacteria is depicted in Figure 15.2.

Samanta et al. (2010) reported that a significant decrease in total coliforms in starter and finisher diets of broiler chickens treated with 1 or 2 g of organic acid blend (comprising, per kilogram of powdered-acid blend, orthophosphoric acid 400 g; formic acid 150 g and 30 g each for propionic acid and calcium propionate). *Escherichia coli* and *Clostridium* in both diets decreased linearly with the dosage of organic acid blend. Tests for *Salmonella* were negative for the control and the organic-acid-blend-treated dietary groups.

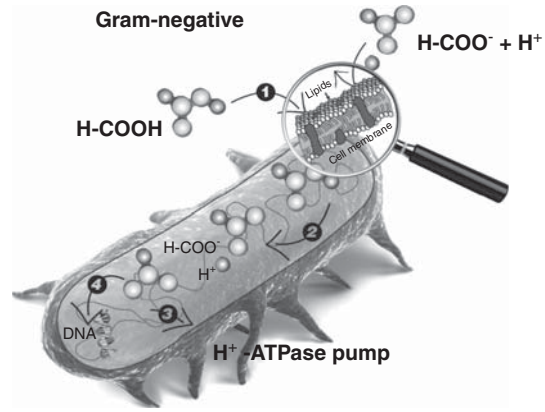

Figure 15.2 Action of organic acids against Gram-negative bacteria ©ADDCON 2012. For color details, please see color plate section.

Effect in Gastro-Intestinal Tract

The mode of action of organic acids in the digestive tract involves two different mechanisms: (1) the reduction of pH level in the stomach and particularly in the small intestine through delivery of H^+ ions; and (2) the inhibition of growth of Gram-negative bacteria through the dissociation of acids and the production of anions inside bacterial cells.

During periods of high feed intake, such as when the animals are young or when the feeds are high in protein, hydrochloric acid concentrations in the stomach are reduced. This reduction negatively impacts pepsin activation and pancreatic enzyme secretion and impairs digestion. Providing acidifiers in the feed addresses this problem and aids feed digestion (Eidelsburger 1997). Positive effects of organic acids on protein hydrolysis have been demonstrated (Mroz et al. 2000). Similarly, feed supplementation with organic acids has been shown to lead to lower duodenal pH, improved nitrogen retention and increased nutrient digestibility (Øverland et al. 2000; Kluge et al. 2004). Metzler and Mosenthin (2007) reviewed available data on the effects of various organic acids on nutrient digestibility indices in pigs (Table 15.3).

Few studies have evaluated the effects of dietary supplementation of organic acids and/or their salts on gastro-intestinal pH and nutrient digestibility of fish species. In the Indian major carp, *Labeo rohita*, Baruah et al. (2005) showed that dietary inclusion of citric acid decreased the pH of gut digesta leading

Table 15.3 Influence of organic acids on the apparent total tract digestibility of crude protein and energy as well as nitrogen (N) retention in pigs (adapted from Metzler and Mosenthin 2007). D: digestibility of non-acidified control diet; ΔD: percentage unit change in the digestibility relative to the non-acidified control diet; R: N-retention of non-acidified control diet as % of intake; ΔR: percentage unit change in the N-retention relative to the non-acidified control diet.

	Level	Crude protein		Gross energy		N retention	
Organic acid	(%)	D (%)	ΔD	D (%)	ΔD	R (%)	ΔR
Formic acid	1.4	80.6	+1.4	82.2	+0.7	48.3	+4.9*
Butyric acid	2.7	80.6	+1.4	82.2	+1.6*	48.3	+4.0*
Fumaric acid	1.8	80.6	−1.0	82.2	+0.7	48.3	+2.9*
Propionic acid	2.0	80.2	+2.3	77.9	+1.4	–	–

*Significantly different from the control diet ($P<0.05$).

to significant increase in bone ash, P and Mn concentrations. It was suggested that the decrease in pH of diet and intestinal digesta is a contributing factor leading to significant improvement in growth and mineral utilization of this carp species (Baruah et al. 2007a, b). Dietary supplementation of citric acid has also been reported to decrease the pH in the diets and feces with subsequent improvements of nutrient retention, particularly some minerals, in rainbow trout, *Oncorhynchus mykiss* (Vielma et al. 1999; Pandey and Satoh 2008). Dietary formic acid has also been shown to significantly decrease intestinal pH and increased mineral absorption in rainbow trout (Vielma and Lall 1997). With the same species, Sugiura et al. (2006) observed that dietary acidifications with mineral acids (hydrochloric or sulfuric acid) or organic acid (acetic acid) did not reduce gastric luminal pH sufficiently to substantially improve P digestibility. These authors suggested that exogenous acids may have an inhibitory effect on endogenous acid production. Likewise, Gao et al. (2011) noted that supplementation of diets with a blend of Na-formate and Na-butyrate had no significant effect on the pH levels in stomach, pyloric caeca, and middle and distal intestines of rainbow trout. Lückstädt (2008b) reported that dietary addition of potassium diformate (KDF) had no effect on intestinal pH of Atlantic salmon, *Salmo salar*, but significantly improved the digestibility of protein, dry matter, and gross energy. In hybrid tilapia, however, Ng et al. (2009a) reported that dietary KDF decreased the diet pH and the stomach and gut digesta.

The growth rates of many Gram-negative bacteria such as *E. coli* or *Salmonella* in the digestive tract are reduced at a pH level below 5. Low pH also forms a natural barrier against ascending microbes from the ileum and large intestine. Moreover, low-molecular-weight acids are lipophilic and can diffuse across the cell membranes of Gram-negative bacteria. In the more alkaline cytoplasm, they dissociate and reduce pH. This reduction alters cell metabolism and enzyme activity, thus inhibiting growth of intraluminal microbes, especially pathogens. Several studies have demonstrated a reduction in bacterial counts in the stomach (Kluge et al. 2004) and the duodenum (Kirchgessner and Roth 1991; Hebeler et al. 2000; Hellweg et al. 2006), while acid-tolerant, beneficial *Lactobacilli* seem to be unaffected or may even be enhanced in number (Hellweg et al. 2006).

A study with red hybrid tilapia (*Oreochromis* sp.) by Ng et al. (2009a) demonstrated that the total fecal and adherent gut bacterial count, particularly *Aeromonas hydrophila*, significantly decreased in fish fed organic acid blend or KDF-containing diets and 0.3% organic acid blend was as effective as 0.2% KDF. In another study with hybrid tilapia, Zhou et al. (2009) showed that dietary KDF affected the gut bacterial population by stimulating the growth of some and inhibiting the colonization of others.

Most organic acids have high gross energy contents. Short-chain organic acids are generally absorbed through the intestinal epithelia by passive diffusion and can be used in various metabolic pathways for energy generation, for instance, for ATP generation in the citric acid cycle. As the energy content of organic acids is high and is completely used in metabolism, their values should be included in the energy content of feed rations. For example, propionic acid contains one to five times more energy than wheat (Diebold and Eidelsburger 2006).

Effects on Growth Performance, Nutrient Utilization, and Disease Resistance

Salmonids

The earliest study on the use organic acids (succinic and citric acid) in fish diets was performed by Fauconneau (1988) using rainbow trout, *Oncorhynchus mykiss*. Fish were fed for 12 weeks with fish meal diets supplemented with 12% of a purified protein (casein or corn gluten), an amino acid (alanine, aspartic acid, or glutamic acid) or an organic acid (succinic or citric acid). Fish fed amino-acid- or organic-acid-supplemented diets consumed significantly less feed than those fed the control commercial diets or protein supplemented diets, but minimal variation in protein and energy utilization efficiency. However, later studies with salmonids using organic acids, their salts, or their mixtures have shown more promising results. Ringø (1991) fed Artic charr, *Salvelinus alpinus* a commercial diet with and without supplementation of 1% Na-lactate or Na-propionate for 12 weeks; it was reported that fish fed the Na-lactate-supplemented diet had significantly higher weight gain compared to those fed the control diet. Dietary inclusion of 1% Na-propionate resulted in significantly depressed weight gain, however. Analyses of gut contents showed that fish fed the diet supplemented with Na-lactate had significantly less water and gross energy. Highest water and gross energy contents were observed in gut contents of fish fed the Na-propionate diet. Contrary to the results obtained with Artic charr (Ringø 1991), a study with Atlantic salmon, *Salmo salar* showed no growth-stimulating effect in fish fed the diet supplemented with 1.5% Na-lactate (Gislason et al. 1994). To ascertain the differences of the effects of Na-lactate on growth performance between these two species, Artic charr and Atlantic salmon were fed a commercial diet with or without 1.5% Na-lactate (Gislason et al. 1996). Dietary inclusion of Na-lactate significantly improved the growth of Arctic charr, but had no effect on Atlantic salmon. The authors suggested that the growth differences observed between these two species were probably related to the feed retention time in the digestive tract. Results of the performance trials conducted using sodium salts of organic acids in Arctic charr and Atlantic salmon are summarized in Table 15.4. In charr, approximately 82% of the initial radioactivity (^{14}C-lactate) in the stomach was registered after 6 hours as compared to only about 41% for Atlantic salmon. The enhancement effect of dietary sodium lactate (1% in a commercial diet) on weight gain of Arctic charr has also been demonstrated in another study by Ringø et al. (1994). They suggested that this growth improvement may be due to increased feed intake and increased lipid deposition. However, feeding the diet containing Na-lactate did not alter fatty acid composition of fillets. Lückstädt (2008b) evaluated the effect of KDF added at 1.35% at various stages of feed production (added to the raw fish before fish meal production, during feed preparation, and without KDF control) on growth and digestibility in Atlantic salmon. Weight gain and specific growth rate significantly increased in fish fed the diet in which KDF was added before fish meal preparation. Significant increase in dry matter, protein, and energy digestibility was obtained in both diets containing KDF. Another study with Atlantic salmon showed that inclusion of fish meal enriched with 0.8 or 1.4% KDF tended to improve growth. However, feed efficiency (FE) significantly increased for diets containing KDF enriched fish meal (Christiansen and Lückstädt 2008).

Table 15.4 Effect of the sodium salts of different organic acids on the performance of Arctic charr and Atlantic salmon (adapted from Lückstädt 2008a).

Fish species	Acid/acid salt	Dose (%)	SGR (% day^{-1})[b]	FCR[c]
Arctic charr	Control	0	0.61	–
	Na-lactate	1	0.83[a]	
	Na-propionate	1	0.49[a]	
Arctic charr	Control	0	0.51	1.20
	Na-formate	1	0.58	1.08
	Na-acetate	1	0.70[a]	0.96
Arctic charr	Control	0	0.79	1.30
	Na-lactate	1	1.12	0.91
Atlantic salmon	Control	0	0.97	–
	Na-lactate	1.5	0.97	
Arctic charr	Control	0	0.28	–
	Na-lactate	1.5	0.51[a]	
Atlantic salmon	Control	0	0.76	–
	Na-lactate	1.5	0.79	

[a] Significantly different from the control diet ($P<0.05$)
[b] Specific growth rate = [(ln final body mass − ln initial body mass)/culture period (days)] × 100
[c] Feed conversion ratio = feed intake/live weight gain

Recent environmental concern regarding nutrient discharge from aquaculture operations has stimulated scientists to develop strategies to improve dietary nutrient availability. Vielma and Lall (1997) evaluated the effect of dietary formic acid (4 and 10 mL kg^{-1} diet) on the availability of phosphorus (P) from a fish-meal-based diet by rainbow trout, *Oncorhynchus mykiss*. The apparent digestibility coefficient (ADC) of P significantly increased from 69.5% for the control diet to 73.6 and 75.0% diets supplemented with 4 and 10 mg formic acid kg^{-1} diet, respectively. The ADC of Ca and Mg also significantly increased with the addition of formic acid. Another short-term study (Sugiura et al. 1998) evaluating the effects of dietary acidification on the availability of minerals in fish meal in rainbow trout diets showed that citric acid at 50 g kg^{-1} diet increased the ADC of Ca, P, Mg, Fe, Mn, and Sr. Inclusion of 50 g Na-citrate also increased Mn availability in fish meal diet. Morken et al. (2011) reported that supplementation of sodium diformate (1.06 g kg^{-1} diet) and increasing extrusion temperature (from 110 to 141°C) significantly improved digestibility of all major nutrients and individual amino acids of barley protein concentrate-based diets for rainbow trout. Vielma et al. (1999) fed juvenile rainbow trout for 6 weeks with a low-P diet supplemented with finely or coarsely ground fish bone meal. A coarse bone-meal diet was supplemented with 0, 4, 8, or 16 g citric acid kg^{-1}. Weight gain and whole-body moisture, protein, and lipid were unaffected by bone meal supplementation. Addition of both coarse and fine bone meal to the basal diet significantly increased body ash contents. Phosphorus in the fine bone-meal diet had higher availability than P in the coarse bone-meal diets. Dietary citric acid significantly increased whole-body ash but had no significant effect on whole-body P content. Whole-body iron concentrations, however, linearly increased with increasing dietary levels of citric acid. Pandey and Satoh (2008) observed that dietary supplementation of 1% citric acid or liquid trace minerals to the low fish-meal, low-P diets improved the growth of rainbow trout to a level comparable to the P-added diet. Poor growth was observed in fish fed a low-P diet with or without inclusion of 1% lactic acid or methionine hydroxy analog. The authors suggested that it may not be necessary to add P in the low fish-meal-based diet if certain organic acids such as citric acid are used.

A study by de Wet (2005), comparing the growth of trout fed diets supplemented with 0, 0.5, 1.0, and 1.5% organic acid blend (sorbic acid and formic acid and its salt) or 40 mg kg^{-1} of flavomycin, showed that weight gain (WG) significantly increased in fish fed diets with 1.0 or 1.5% acid blend. The growth of fish fed the antibiotic diet was similar to that of fish fed the 1.5% acid blend diet, but the latter tended to have better FE. Gao et al. (2011) found no significant improvement in growth rate or feed utilization of rainbow trout fed fishmeal or plant protein-based diets added with 10 g acid moiety kg^{-1} organic acid salts blend (OAB) at two points of feed production (before and after extrusion). Their 24-day digestibility trial showed that trout fed a plant-protein-based diet with the OAB added before extrusion had significantly lower dry matter, organic matter, crude lipid, and most amino acids. Supplementation of OAB after extrusion reduced the digestibility of crude fat both in fish-meal- and plant-protein-based diets. However, addition of OAB to either fish-meal- or plant-protein-based diets before extrusion significantly increased FE and middle intestine to body weight ratio.

Tilapia

In recent years, several studies have been conducted on the use of organic acids, their salts, or their blend in diets of tropical species, particularly tilapia. Xie et al. (2003) evaluated the effects of diets supplemented with different concentrations of organic acids (citric, propionic, acetic, lactic, and oxalic) on the stimulatory feeding behavior (feed-biting frequency) of Nile tilapia. They observed that citric acid at concentrations of 10^{-2}–10^{-6} M, propionic acid at 10^{-4}–10^{-6} M and lactic acid at 10^{-2}–10^{-5} M stimulated feeding response. Propionic acid at 10^{-3} M tended to repel feeding. Acetic acid at 10^{-5} M and oxalic acids at 10^{-6} M had no effect on fish feeding. A growth trial comparing the performance of diets supplemented with an organic acid/salt blend (calcium formate, propionate, lactate, and phosphate and citric acid) at 0, 0.5, 1.0, and 1.5% or 0.5% oxytetracycline showed non-significant improvement in WG and FE among treatments, although the group fed the 1.5% acid/salt blend diet gained 11% more than the negative control. WG and FE were similar in fish fed 1.5% acid blend and 0.5% oxytetracycline diets (Petkam et al. 2008). Ramli et al. (2005) fed tilapia diets containing 0,

0.2, 0.3, and 0.5% KDF 6 times daily for 12 weeks. The fish were orally challenged at day 10 with 10^5 CFU *Vibrio anguillarum* daily for 20 days. Significant improvement in feed intake, WG, FE, and protein efficiency ratio (PER) were obtained in all the treatments with added KDF. The improvement was greater for diets supplemented with 0.2 and 0.5% KDF. Survival 20 days post-challenge was significantly higher in fish fed diets containing KDF, but significantly highest in treatment fed the 0.5% KDF diet.

Ng et al. (2009a, b) conducted a feeding study with red hybrid tilapia, *Oreochromis* sp. to compare the effects of various dietary levels (0, 0.1, 0.2, or 0.3%) of a commercial organic acid blend and 0.2% KDF on growth performance, antimicrobial activity, nutrient digestibility, and survival 16 days after challenge with *Streptococcus agalactiae*. They reported that dietary KDF at 0.2% decreased the diet pH and reduced the pH of the digesta in the stomach and gut. However, WG, FE, PER, and net protein utilization (NPU) were not affected by dietary treatments, but there was a trend toward improved results in fish fed acid blend or KDF-containing diets. Total fecal and adherent gut bacterial count, particularly *Aeromonas hydrophila*, significantly decreased in fish fed organic acid blend or KDF-containing diets, and 0.3% organic acid blend was as effective as 0.2% KDF. Cumulative mortality 16 days post-challenge with *Streptococcus agalactiae* was significantly reduced in fish fed diets supplemented with organic acid blend or KDF. Another study investigating the effects of KDF (0, 0.3, 0.6, 0.9, or 1.2%) and antibiotics, flavomycin (8 mg kg^{-1}), quinocetone (100 mg kg^{-1}) or flavomycin + quinocetone (4 + 50 mg kg^{-1}) on growth performance and gut microflora in hybrid tilapia (*O. niloticus* x *O. aureus*) showed that WG, FE, and survival of various treatments did not differ from those of the control. Among the treated groups however, diets with 0.3 and 0.6% KDF provided significantly better growth and FE than the diet supplemented with the antibiotic mixture. It was also observed that both KDF and antibiotics affected the bacterial population but in different ways that are not as simple as earlier believed (Zhou et al. 2009).

A study conducted by Lim et al. (2010b) with mixed-sex Nile tilapia, *O. niloticus* in which fish were fed for 12 weeks diets supplemented with graded levels of KDF (0, 0.25, 0.50, 0.75, 1.00, 1.25, and 1.50%) showed a trend of increased WG in fish fed diets with increasing levels of KDF up to 1.0%. Fish fed 1% KDF diet had significantly higher WG and FE than those fed diets with 1.25 or 1.50% KDF, but did not differ from the groups fed lower levels of dietary KDF. Hematological parameters and innate immune responses were not affected by dietary treatments. Likewise, mortality 14 days post-challenge with *S. iniae* and antibody titer against the same bacterium was not affected by dietary treatments. The same test diets were used in a recent 10-week feeding trial with sex-reversed all male hybrid tilapia (*O niloticus* x *O. aureus*). No significant differences were observed among WG and FER of fish in all treatments. However, fish fed the 0.5% KDF diet had an 8.3% and 6.4% increase in WG and FER, respectively. No differences were found among hematological parameters, innate immune responses, and survival 15 days post-challenge with *S. iniae*. Cuvin-Aralar et al. (2011) reported that a 10-week study at the Aquaculture Department of the Southeast Asian Fisheries Development Center, Philippines yield significant improvement in growth and FE of juvenile Nile tilapia fed the diet with 0.3% KDF or 0.3% sodium diformate. However, a similar 6-week trial conducted in Germany showed a lack of significant increase in WG and FE in Nile tilapia fed the 0.3% KDF diet (Lückstädt and Mellor 2011).

Recently, Lückstädt (2012a) analyzed published and unpublished data from 18 trials involving the use of KDF in tilapia diets. The performance parameters evaluated include WG, FE, and survival following bacterial challenge. Data were subjected to statistical analysis and a probability level of 0.05 was used in all tests, expressed as a percentage of the negative control. The average level of KDF used in all studies was 0.41%. Only a numerical increase in feed intake (2.1%) was observed as compared to fish fed the control diet. However, the performance of tilapia, based on WG, was significantly increased by 5.6%. Feed efficiency of fish fed the KDF-containing diet was also significantly improved by 4.5%. Data on mortality were inconclusive due to variation in experimental conditions, type and dosage of bacteria, and method of challenge.

Other Species

Baruah et al. (2005) evaluated the effects of dietary citric acid (0 and 3%), microbial phytase (0 and 500 phytase units (U) kg^{-1}), protein (25 and 35%), and their interactions on intestinal digesta pH, bone ash, and bone mineralization of an Indian major carp, *Labeo rohita*. Results showed that the addition of 3% citric acid to either a low- or high-protein (25% and 35%) diet resulted in a significantly decreased intestinal digesta pH, whereas bone ash, P, and Mn content were significantly increased. They also observed that the effectiveness of phytase, particularly in the low-protein diet, increased as a result of addition of citric acid. A follow-up study evaluated the effects of citric acid, phytase, protein level, and their interaction on growth performance and nutrient digestibility of this agastric Indian carp (Baruah et al. 2007b). Inclusion of citric acid to both low- and high-protein diets significantly increased WG and FE and phosphorus digestibility in carp juveniles. The growth-promoting-effect of citric acid and phytase was higher in fish fed the low-protein diets. The decrease in dietary and intestinal digesta pH was suggested as a contributing factor to the significant improvement in growth and mineral utilization of this carp species (Baruah et al. 2007b). Owen et al. (2006) evaluated the effect of inclusion of 0.2% Na-butyrate in fish-meal- and soybean-meal-based diets of African catfish, *Clarias gariepinus*. Slightly better growth and FE were observed in catfish fed the fishmeal diet supplemented with Na-butyrate, compared to the unsupplemented control diet. No improvement in these parameters was observed in fish fed the soybean-meal-based diet with added 0.2% Na-butyrate. Sodium butyrate supplementation also appeared to increase the proportion of Gram-positive bacteria in the hindgut of *C. gariepinus*, although this increase was not statistically significant.

Sarker et al. (2005) investigated the effects of phosphorus, citric acid, and amino-acid-chelated trace mineral supplement in diets on growth and nutrient retention in red sea bream, *Pagrus major*. They reported that supplementation of 3% citric acid to a fish-meal-based diet without addition of inorganic P improved fish growth, FE, P absorption and retention as well as nitrogen retention. A later study with the same species evaluated the effects of citric acid, malic acid, and lactic acid (each at 1% level) and other supplements on growth and P utilization (Hossain et al. 2007). Similar to previous findings, results of this study also showed that supplementation of citric acid to the diet without inorganic P supplementation improved fish growth, FE, and P absorption and retention.

The effects of citric acid, formic acid (each at 0.5%), and P supplementation in diets containing increasing levels of plant proteins as replacements of fish meal on growth, and feed and P utilization in yellowtail, *Seriola quinqueradiata* were evaluated by Sarker et al. (2012a). The addition of citric acid and formic acid to low-P plant protein diets without inorganic P supplementation improved fish growth and FE and increased P retention. The addition of citric acid to plant protein diets exhibited better performance than formic-acid-supplemented diets. Another study with the same species investigated the supplemental effects of citric acid, formic acid, and/or lipid to plant-protein-based diets on growth and N and P utilization (Sarker et al. 2012b). Similar to earlier results (Sarker et al. 2012a), this study also showed that supplementation of citric acid or formic acid to plant-protein-based diets with no P supplementation improved growth, FE, and P retention. Citric acid at concentration of 2 and 3% in diets has also been shown to significantly increase Ca and P content in muscle and serum of beluga, *Huso huso* (Khajepour and Hosseini 2010). A more recent study (Khajepour and Hosseini 2012) indicated that the addition of citric acid at 3% to soybean-meal-containing diet of beluga improved fish growth, FE, and protein and P digestibility.

The effects of KDF in milkfish, *Chanos chanos* were tested by Lückstädt (2012). It was found that a 0.3% inclusion of the acid salt tended to improve daily gain and FE.

Recently, organic acids or their salts have also been investigated in diets of shrimp and abalone. Tung et al. (2006) reported that 0.5% sodium citrate with inactivated *Lactobacilli* boosted the growth of the Kuruma shrimp, *Masurpenaeus japonica*. He et al. (2006) reported on the effects of KDF in *Litopenaeus vannamei* larvae (initial weight 57 mg). The authors fed the larvae for 40 days with 0.8% KDF and noted significantly improved survival rates as well as FE. Kühlmann et al. (2011a) reported that supplementation of a diet with 0.2% KDF had no effect on white-leg

shrimp, *Litopenaeus vannamei* productivity index relative to those fed the control diet, but the value of this parameter significantly increased when the KDF inclusion level was increased to 0.5% of diet. Both dosages led to significantly improved weight gain, however. A subsequent study by Kühlmann et al. (2011b) with the similar dosages of KDF significantly reduced mortality in white-leg shrimp challenged with *Vibrio harveyi* for 10 days. da Silva et al. (2013) reviewed the use of various sodium salts of organic acids in marine shrimp nutrition and found that sodium propionate may have the biggest potential among tested additives as a diet supplement for *L. vannamei*.

Goosen (2007) determined the role of organic acids (1% acetic and 1% formic acid; 1% benzoic and 1% sorbic acid) and their salts (2% Na benzoate and 1% K sorbate) as growth promoters in the South African abalone, *Holiotis midae*, as well as their effect on gut microflora. It was observed that diets supplemented with organic acids or salts enhanced the growth rate of abalone relative to the control and the diet containing 30 ppm of avilamycin (antibiotic growth promoter). None of the treatments had a significant effect on FE and feed intake. The effects of treatment on gut microflora were inconclusive. Lastly, Chao (2011) tested varying dosages of KDF (0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2%) in Japanese sea cucumber, *Apostichopus japonicas* with an initial weight of 2.1 g over a period of 8 weeks. There was no documented effect on survival (100% in all groups), but a significant improved specific growth rate of the sea cucumber with the 1.0% and 1.2% KDF dosages compared to the negative control.

Conclusion

Organic acids, their salts, or their blends have been used for decades as preservatives in feed ingredients and animal feeds, including aquafeeds, to prevent the growth of molds/fungi and bacteria or improve the shelf life of these products. Their utilization has also been established as a potential alternative to prophylactic use of in-feed antibiotics to improve growth, feed utilization efficiency, and health of pigs and chickens. Their addition to the diets has been shown to lower the pH and buffering capacity of the diets, decrease gastro-intestinal pH, improve protein and mineral digestibility, inhibit the proliferation of detrimental bacteria, and improve nutrient utilization efficiency. Despite the reported beneficial effects of acidifiers in swine and poultry diets, limited research has been performed on the use of acidifiers in diets of aquaculture species. Moreover, published data on the beneficial effects of dietary inclusion of organic acids, their salts, or their combination on fish performance is inconsistent. Several factors such as fish species, fish size or age, nature and levels of organic acids, salts or their combination, composition and nutrient content of experimental diets, buffering capacity of dietary ingredients, culture environment and conditions, and feeding management may contributed to the variation among published results. However, despite the discrepancy among data of published studies, it appears that organic acids and/or their salts have good potential as dietary supplements to improve growth performance, feed utilization efficiency, and nutrient digestibility, alter gut microflora population, and increase disease resistance of aquaculture species. However, more research is needed to better understand their mechanism of actions affecting various performance parameters.

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Chapter 16

Plant Extracts

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Introduction

Immunostimulants used in fish culture are of interest as they offer an alternative to the drugs, chemicals, and antibiotics currently used to control fish diseases. Immunostimulants enhance the innate (or non-specific) immune response (Galeotti 1998; Sakai 1999), and can be applied via injection, bathing, or oral administration; the latter seems to be the most practicable (Jeney and Anderson 1993; Sakai 1999; Yin et al. 2006). The best-known immunostimulants are glucans (Engstad and Robertsen 1993), but synthetic compounds, polysaccharides, vitamins, and animal and plant extracts can also enhance the immune response and disease resistance of fish (Anderson 1992; Raa et al. 1992; Jeney and Anderson 1993; Sakai 1999; Raa 2000).

Medicinal plants (plant remedies) are deeply rooted as an integral component of the daily lives of many people for the maintenance of good health and are an important part of the cultural heritage in many countries (Chang 2000). About 70–80% of the world's population, particularly in developing countries, relies on traditional or non-conventional medicine in their primary healthcare as reported by the World Health Organisation (Akerele 1993). Other populations in more-developed countries have been beneficiaries of traditional medicine since the early 19th century. In the present era of rapid advances in biomedical science and technology, it is astonishing that the public in these developed countries spends a significant amount of money on herbal products and related non-conventional therapies. The growth in popularity of nutraceuticals and medicinal products from plants or other natural sources has taken a very large share of the healthcare market (Johnson 1997).

There are many herbs currently used in aquaculture for a variety of purposes, including growth promotion, antimicrobial agents, and supplying nutrients. Furthermore, there is growing interest in the use of herbs to prevent and control diseases in fish. For intensive aquaculture operations, the application of antibiotics, chemotherapeutants, and vaccines is quite expensive and leads to undesirable effects such as bioaccumulation, pollution, and antibiotic resistance. In this context, plant extracts or their by-products contain several active compounds that have been shown to be effective alternatives to traditional chemotherapies and vaccines, including phenols, polyphenols, alkaloids, quinones, terpenoids, lectines, and polypeptides. Several herbal immunostimulants administered at various concentrations via the oral route or injection have been shown to enhance the innate and adaptive immune response in different freshwater and marine fish and shellfish against various bacterial, viral, and parasitic diseases. Modulation of immune response

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in fish by using medicinal plant products as a possible therapeutic measure has become the subject of active scientific investigations (Jeney et al. 2009; Chakraborty and Hancz 2011; Harikrishnan et al. 2011b).

Sources, Chemical, and Physical Characteristics

Herbal and medicinal plant products have been available in various forms for hundreds of years to aid in the treatment of diseases in both Eastern and Western cultures. About one-quarter of marketed pharmaceutical medicines are either derived from plant sources or from derivatives of secondary plant metabolites. Various chemicals and biotechnological products are being screened by major multinational pharmaceutical industries in the hope of discovering new cures for diseases (Chan 2003). The rapid development of recombinant DNA technology and related procedures provide biomedical proteins and related biological products for use as therapeutic drugs, prophylactic vaccines, and diagnostic agents (Chan 1996).

The putative efficacy of herbs mainly relies on empirical data and tradition of use, which frequently cannot satisfy the requirements of application in livestock production. It is therefore necessary to establish the pharmacological basis for the actions of herbs. Currently, the approach is to extract the active ingredients from herbs. However, some studies showed that the pharmacological effect of extracts of herbs isolated in their pure state differs from the effect of the whole herbs (Chang 2000; Chan 2003; Liu et al. 2011). Moreover, several herbs are usually mixed to achieve a pharmacological effect, thereby making it extremely difficult to attribute the effect to a particular herb. Problems and difficulties arise in the quality assurance of herbal medicinal products because there are many unidentified chemical entities in the finished products, and the actual bioactive components are seldom known. Recent advances in analytical chemistry and related disciplines have helped to elucidate the complex chemical compositions of Chinese medicinal herbs (Tables 16.1 and 16.2). Sufficient evidence exists to suggest that extracts of medicinal herbs, once isolated in their pure state, can produce pharmacological effects that differ significantly from those of the whole herb (Chang 2000).

Table 16.1 Properties of medicinal plants and products (Chan 1995).

Properties	Remarks
Physico-chemical properties	
Active ingredients	Often unknown
Availability of pure compounds	Rare
Availability of raw material	Limited
Quality of raw material	Variable
Stability of preparation	Uncertain
Biomedical properties	
Mechanism of action	Often unknown
Toxicological tests	Usually not available
Empirical data	Very important
Specific adverse effects	Rare
Frequent tolerance of therapy	Usually good
Therapeutic window	Wide usually
Suitability for chronic use	Often well tested
Controlled clinical trial	Usually not available

Table 16.2 Some pharmacologically active components isolated from Chinese medicinal plants (Chan 2003).

Chemical type	Number of compounds isolated
Alkaloids	213
Terpenes	
Monoterpenes	36
Sesquiterpenes	39
Diterpenes	49
Triterpenes	65
Cardiac glucosides	25
Phenolic compounds	
Quinonis	34
Chromones	9
Flavonoids	49
Coumarins	34
Lignans	42
Phenyl propanoids	25
Others	75
Acids, amides and miscellaneous	66
Total	761

Medicinal plants used in aquaculture contain a number of bioactive compounds such as glycyrrhizin and its aglycon glycyrrhetic acid, liquiritin, liquiritin apioside, isoliquiritin and glabridin, polysaccharides, alkaloids and/or flavonoids, saponin, and azadirachtin (Jang et al. 1995; Ninomiya et al. 1995; Logambal and Michael 2000, 2001; Cinatl et al. 2003; Harikrishnan et al. 2009). Astragalus (*Astragalus membranaceus*)

has been used in Chinese traditional medicine as an immune booster for nearly 2000 years. Analysis shows that Astragalus root contains polysaccharides, monosaccharides, flavonoid, alkaloid, choline, betaine, folic acid, various amino acids, mucoitin, gum, cellulose, and 14 trace minerals, including selenium, zinc, and iron, which are essential micronutrients for humans and animals. Recently published research has found that some components such as polysaccharides, organic acids, alkaloids, glucosides, and volatile oil can enhance immune function (Wang et al. 1999; Liu 2002). Currently, most isolated Chinese herbal polysaccharides are not optimized: different extraction and separation methods might also result in differences in structure and activity of polysaccharides (He et al. 2012).

Chinese Toon (*Toona sinensis*), which consists of triterpenes and phenolic compounds such as methyl gallate, gallic acid, kaempferol, quercetin, quercitrin, rutin, kaempferol-D-glucoside, catechin, epicatechin, β-sitosterol, stigmasterol, β-sitosteryl-glucoside, stigmasterolglucoside, phytol, and toosendanin, has been reported to increase the immune activity in tilapia (*Oreochromis mossambicus*) (Wu et al. 2010). Furthermore, a study aimed at assessing the effects of the water-soluble and hexane-soluble fractions of the Indian herb *Solanum trilobatum* (containing sobatum, β-solamarine, solaine, solasodine, glycoalkaloid, diosogenin, and tomatidine as active phytochemicals) on the non-specific immune mechanisms of tilapia found that intraperitoneal injection of the water-soluble and hexane-soluble fractions significantly enhanced the native immune system (Divyagnaneswari et al. 2007). In addition, the water-soluble fraction of the leaves of the Indian medicinal plant *Tinospora cordifolia*, containing different phyto-constituents such as alkaloids, diterpenoid lactones, glycosides, steroids, and sesquiterpenoids, has been shown to act as an immunostimulating substance (Alexander et al. 2010).

The practices of most ethnic herbal medicine include the use of crude or raw herbs, that are collected from the wild or from cultivated fields, and their processed or ready-made (formulated mixture of herbal or other natural materials) products. One problem that arises is the variable quality of the raw materials. For example, Chan (2003) noted some of them contain toxic contaminants, which may come from:

- the environment and/or conditions in which the medicinal plants are grown or collected;
- the conditions under which they are dried and processed;
- the storage conditions and conditions during transport; and
- the manufacturing processes involved in producing the medicinal products.

Note that Chinese herbs from different regions and seasons have varying efficacy and contents of active substances. At present, it is difficult to make a correct assessment of efficacy and quality because of a lack of uniform quality standards and standard formulas (Liu et al. 2011). Further research is therefore required to standardize methods for their extraction and manufacture (Chang 2000; Chan 2003).

Effects on Growth Performance

Feed accounts for more than 50% of the total production costs in modern intensive aquaculture. Increasing feed efficiency, especially by improving the metabolic assimilation of dietary nutrients, is of high priority in contemporary aquaculture. The inclusion of synthetic substances such as antibiotics and steroid hormones, which have been conventionally used to increase the efficiency of feed assimilation by fish, is either prohibited or will soon be prohibited in several countries. In the European Union, for example, the use of antibiotics as growth promoters in animal feeds has been banned since 2006. A huge demand for inclusion of natural substances in aquatic feeds as feed efficiency enhancers and growth promoters is expected from the industry (Francis et al. 2005).

Plants commonly contain protein, carbohydrate, fat, vitamins, and minerals which are necessary nutrients for the growth of animals. Polysaccharides, organic acids, alkaloids, and essential oils present in herbs can improve the immune function of livestock. In general, Chinese herbs containing abundant proteins, carbohydrates, vitamins, lipids, and minerals can play an important part in enhancing growth performance and modifying physiological function of animals (Li 2001).

Natural plant products have been reported to reduce stress, promote growth, and stimulate appetite and antimicrobial properties in cultured finfish and shrimp due to the active components of alkaloids, flavanoids, pigments, phenolics, terpenoids, steroids, and essential oils (Sivaram et al. 2004; Xie et al. 2008; Jeney et al. 2009). For example, Immanuel et al. (2004) increased specific growth rate of shrimp (1.46–2.15%) by feeding them *Artemia* enriched with herbal extracts from *Ricinus communis*, *Phyllanthus niruri*, *Leucus aspera*, *Manihot esculenta*, and seaweed (*Ulva lactuca* and *Sargassum wightii*).

Supplementation of Jiaogulan (*Gynostemma pentaphyllum*), a traditional Chinese herbal medicine, in feed resulted in increased weight gain, feed conversion efficiency, and specific growth rate in grass carp (Wu et al. 1998). Common carp (*Cyprinius carpio*) fed a diet containing the extract of rubarb (*Rheum officinale*) had a significantly increased specific growth rate (Xie et al. 2008).

Olive flounder (*Paralichthys olivaceus*) fed an herbal-mixture diet containing ginseng (*Panax ginseng*), Chinese liquorice (*Glycyrrhiza uralensis*), and bark (*Acanthopanax koreanum*) had significantly increased growth from weeks 6 to 12 as compared to fish fed a diet without supplementation (Harikrishnan et al. 2010b). Improved growth performance has also been reported in parrotfish (*Oplegnathus fasciatus*) (Kim et al. 2003) and greasy grouper (*Epinephelus tauvina*) (Sivaram et al. 2004) that were fed diets which included a mixture of medicinal herbs.

Dietary saponins derived from different plants have been considered as the causative factor for depression of feed intake and reduced weight gain in salmonid (Bureau et al. 1998). On the other hand, common carp and Nile tilapia (*Oreochromis niloticus*) fed diets containing *Quillaja* saponins (QS) had significantly higher weight gain (Francis et al. 2001, 2002). The average final weights of carp and tilapia fed QS were about 18% and 20% higher, respectively, than those of the fish fed the control diet. Dietary QS significantly increased the activity of carp gut enzymes, amylase, trypsin and liver enzymes lactate dehydrogenase (LDH), and cytochrome *c*-oxidase (CO) (Serrano et al. 1998). This shows that QS could stimulate digestion of proteins and carbohydrates in the gut and promote both the respiratory chain and lactate fermentation.

Numerous studies have demonstrated the antioxidative and antimicrobial efficacy of Chinese herbs. Liu et al. (2004) used *Astragalus*, woad root (*Isatis tinctoria*), poria (*Poria cocos*), and houttuynia (*Houttuynia cordata*) in carp feed and observed that fish fed diets containing herbs had more intestinal bacteria, including *Bacillus* and *Corynebacterium*, compared to the control group. Moreover, these Chinese herbs changed the composition of predominant bacteria, increasing the proportion on *Bacillus* and reducing the populations of *Aeromonas*, *Plesiomonas*, *Pseudomonas*, *Vibrio*, and *Enterobacter*.

There is also some indication that Chinese herbs may improve the flavor of meat, although the mode of this action is still unclear. Further complications arise because herbal feed additives may vary widely with respect to botanical origin, processing, and composition. Regardless, Guo et al. (2005) claimed that clove flower bud (*Syzygium aromaticum*) and orange peel improved the flavor of fish feed, and that herbal feed additives could improve the meat quality of crucian carp (*Carassius carassius*). In their study, crucian carp fed with herbal additives containing baical scullcap root (*Scutellaria baicalensis*) as a main active substance showed higher body weight gain, body fat, and protein content in flesh of fish, and lower feed conversion.

Effects on Immune Responses

Medicinal plants can modulate the innate immune response (Jeney et al. 2009). Medicinal plants activate several components of the immune system. Plants or their extracts are commonly used by feeding or intraperitoneal injection to enhanced innate immune parameters (such as lysozyme, complement, antiprotease, meloperoxidase, reactive oxygen species, reactive nitrogen species, phagocytosis, respiratory burst activity, nitric oxide, total hemocytes, phenoloxidase, glutathione peroxidase, and phenoloxidase) and adaptive immune parameters (such as antibody titre, bactericidal activity, and hemagglutination against bacterial diseases) in different fish and shellfish. Plants may directly initiate activation of the innate defense mechanisms acting on receptors and triggering intracellular gene activation that may result in the production of antimicrobial molecules (Bricknell and Dalmo 2005). It leads to an increase in various

components of immunity such as phagocytic activity, complement activities, lysozyme activity, and disease resistance, as well as serum Ig levels. It has been reported that application of plant extracts orally significantly enhances the phagocytic activity in various fish species (Kim et al. 1998, 1999; Logambal et al. 2000; Logambal and Michael 2001; Jian and Wu 2003, 2004; Ardó et al. 2008; Cao et al. 2008a, b).

Astragalus polysaccharide (APS) is the major active component of Astragalus root. The role of APS on the specific and non-specific immune responses has been reviewed (Shan et al. 2000). APS modulates the functions of the immune cells including T-cells, B-cells, and macrophages (Kong et al. 2003). This Astragalus APS is a well-studied immunostimulant (Tan and Vanitha 2004). In murine macrophage-like cells it could enhance the expression of cytokine genes, for example IL-1, IL-6, or TNF-α (Song et al. 2000), the nitric oxide production of these cells, and the expression of the inducible nitric oxide synthase (iNOS) gene (Lee et al. 2005). Yuan et al. (2008) studied the effects of APS on the expression of immune response genes in the head kidney, gill, and spleen of the common carp. After injection of APS, the IL-1β mRNA level increased in a dose-dependent manner in the head kidney of carp, while no significant changes were found in the gill and spleen. Results of this study constitute a first step toward the understanding of APS effect on cytokines and immune-related gene expression in different organs of common carp. In experiments with common carp, it has been shown that Astragalus extract had a positive influence on the immune system by acting as a booster and protected the liver from damage (Yin et al. 2004; Cao et al. 2008a, b; Jia et al. 2012). Astragalus extract has also been shown to significantly enhance the phagocytic activity of leukocytes isolated from Nile tilapia as early as one week after the start of feeding. This elevated activity was maintained during the entire experiment (Yin et al. 2006; Ardó et al. 2008).

Baical Scullcap root is an important medicinal herb with antibacterial and antiviral effects (Kim et al. 1999) that has been used successfully for the treatment of various ailments including fever, ulcer, cancer, and inflammation in humans (Horvath et al. 2005). Our previous study found that feeding Nile tilapia (*Oreochromis niloticus*) diets containing higher doses (0.5 and 1.0%) of Baical Scullcap root extract depressed the function of phagocytic cells, while there was no effect on phagocytic activities when fish were fed a low dose of Baical Scullcap root (0.1%) (Yin et al. 2006). Lingzhi mushroom (*Ganoderma lucidium*) is an important medicinal herb containing polysaccharides. *G. lucidium* polysaccharides have been reported to be effective in modulating immune functions, inhibiting tumor growth (Lin and Zhang 2004), preventing oxidative damage (You and Lin 2002), protecting the liver from damage, and reducing serum glucose levels, while producing no toxic effects at the applied dose (Zhang et al. 2002). Lingzhi mushroom is a traditional Chinese medicine used for the prevention and treatment of various human diseases in China and other Asian countries (Lin 2001). It has been shown that an aqueous extract of *G. lucidum* promoted phagocytosis by macrophages in mice immunosuppressed by cyclophosphamide, stimulated proliferation of lymphocytes induced by concanavalin A or lipopolysaccharide, and influenced gene expression of cytokines (Wang et al. 1997). In carp fed with different doses of *G. lucidium* extract, enhanced respiratory burst activity of phagocytic cells, phagocytosis, and plasma lysozyme were observed (Yin et al. 2009). Japanese honeysuckle (*Lonicera japonica*) flower extract contains many different active components. One of them, chlorogenic acid, can activate macrophages through the calcineurin pathway in human cell line (Wu et al. 2004). *L. japonica* has been known as an anti-inflammatory agent and is used widely for upper respiratory tract infections, diabetes mellitus and rheumatoid arthritis (Lee et al. 2001). Nile tilapia treated with *Lonicera* extract demonstrated enhanced immune responses compared to the untreated fish (Ardó et al. 2008).

More often, herbal plants have been used in combination or as a mixture. Plant extracts of four Chinese herbs (*Rheum officinale*, *Andrographis paniculata*, *Isatis indigotica*, *Lonicera japonica*) increased phagocytosis of white blood cells of crucian carp (Chen et al. 2003). An earlier study had shown the immunostimulatory effects of a Chinese herb mix (C-UPIII) administered to Nile tilapia (Chansue et al. 2000). In common carp and large yellow croaker (*Pseudosciena crocea*), respiratory burst activity of phagocytic cells and plasma lysozyme activity were significantly increased after feeding with a ration containing a mixture of *A. membranaceus* and danggui

or "female ginseng" (*Angelica sinensis*) (Jian and Wu 2003, 2004). Common carp had been fed diets containing a mixture of *A. membranaceus* (root and stem), *Polygonum multiflorum*, woad (*Isatis tinctoria*), and European liquorice (*Glycyrrhiza glabra*). The results showed that the herbal mixture significantly increased phagocytosis, respiratory burst activity, and levels of total protein in serum (Yuan et al. 2007). The mixture of Astragalus and Japanese honeysuckle extracts was able to enhance the respiratory burst and phagocytic activity of blood phagocytes and plasma lysozyme activity of Nile tilapia (Ardó et al. 2008). The same results were noted when carp were fed with a combination of Astragalus and Lingzhi mushroom (Yin et al. 2009).

The use of immunostimulants in combination with fish vaccines shows great potential as a means for increasing the protective capabilities of fish while decreasing the size of the vaccine dose (i.e., the immunostimulant will boost the potency of the vaccine, thereby decreasing the dose necessary for the same effect; Jeney and Anderson 1993). Yin et al. (2009) fed Lingzhi mushroom and Astragalus in combination with a vaccine to common carp, and observed that these fish showed a significantly enhanced respiratory burst activity of phagocytic cells as well as enhanced phagocytosis and lysozyme activities in plasma. The specific immune response was also elevated, although there were no significant differences between the vaccinated group not fed herb extracts and the vaccinated fish fed herb extracts. Fish fed both herbs and the vaccine showed the best survival following infection with *Aeromonas hydrophila*. Misra et al. (2006) observed that the antibody titers against *A. hydrophila* in Indian carp (*Labeo rohita*) fed herbs were not significantly different from those of the control fish. It was found that the medicinal plant *Azadirachta indica* (neem) enhanced the primary and secondary antibody responses in an inverse dose-dependent mode in tilapia (Logambal et al. 2000). The administration of leaf extract of holy basil (*Ocimum sanctum*) before, along with, or after vaccine showed changes both in the magnitude of antibody response and the day of maximal response in tilapia. The results showed a significant enhancement in the antibody responses in fishes injected with the leaf of holy basil extract in all cases (Venkatalakshmi and Michael 2001). Other studies where higher doses of immunostimulants were used together with vaccine have been found to be even more suppressive (Logambal and Michael 2001). Conversely, there are several publications which report that the use of immunostimulants conferred no beneficial effects (Bricknell and Dalmo 2005). Indeed, some of these preparations have caused inhibition of the immune system (Yin et al. 2006). It is important to remember that there are more fish species in existence than mammalian species and the diversity between them is reflected in their immune system.

As in case of other immunostimulants, the effect of herbs is dose-dependent and species-dependent (Anderson 1992; Galeotti 1998; Bricknell and Dalmo 2005; Harikrishnan et al. 2011b). It is difficult to compare results as the quality of herbs and extracts from the same plant may differ (Jeney et al. 2009; Harikrishnan et al. 2011b) and it is difficult to make a correct assessment of efficacy and quality because of a lack of uniform quality standards and standard formulas (Liu et al. 2011). With further research quantifying the type of standardized herb extract, the appropriate doses, and timing, the use of these immunostimulants for protection of fish against diseases promises to become an important addition to fish culture.

Effects on Disease Resistance

Disease outbreaks in commercial aquaculture may be prevented by the enhancement of innate and adaptive immunity through the application of plants extracts (Jeney et al. 2009), but this has not been accepted by the scientific community due to the lack of product standardization and quality control (Liu 2002; Liu et al. 2011). Herbs have been used in various countries to prevent diseases in shrimp and fish, and successful results have been reported in Mexico, India, Thailand, and Japan (Auro de Ocampo and Jimenez 1993; Direkbusarakom et al. 1996; Logambal and Michael 2000).

It is generally accepted that the feeding of immunostimulants to immunologically mature fish is beneficial, providing improved protection against bacterial and, to a lesser extent, viral diseases (Bricknell and Dalmo 2005). Plants such as *Stella aquatica*, *Impatiens biflora*, *Oenothera biennis*, *Artemisia vulgaris*, and *Lonicera japonica* have exhibited potentional antibacterial and antiviral properties in fish (Shangliang

et al. 1990). The use of natural products including plant extracts in the treatment of Epizootic Ulcerative Syndrome (EUS; Campbell et al. 1998) and lymphocystis disease virus (LDV; Harikrishnan et al. 2010d) has been reported. Micol et al. (2005) successfully controlled salmonid rhabdovirus, viral hemorrhagic septicemia virus (VHSV), by using the plant extract derived from olive tree leaf (*Olea europaea*) and its major compound oleuropein (Ole).

The use of plant extracts to treat some parasitic diseases in farmed fish has been reported (Dügenci et al. 2003; Harikrishnan et al. 2010b, c; Chakraborty and Hancz 2011). Ponpornpisit et al. (2001) fed C-UPIII to guppies (*Poecilia reticulata*) challenged with *Tetrahymena pyriformis* and observed an improved survival rate. Ekanem et al. (2004) immersed goldfish infected with the ciliate *Ichthyophthirius multifiliis* in baths for 72 hours with the crude methanolic extract of leaves of velvet bean or cowitch (*Mucuna pruriens*), containing bioactive substances such as free phenols, tannins, saponins, nicotine, physostigmine, bufotenine, serotonin, N, N-dimethyltryptamine, and 5-methoxy-DMT. Fish were then immersed for 96 hours in baths with the petroleum-ether extract (90%) of seeds of papaya (*Carica papaya*). Through this trial, researchers observed a reduction in the numbers of parasites and, consequently, a reduction in parasite-induced fish mortality. It was also reported that the crude extract of the green tea (Camellia sinensis) was effective in control of the flagellate fish parasite *Ichthyobodo necator* in chum salmon (*Oncorhynchus keta*) and masu salmon (*Oncorhynchus masou*) (Suzuki et al. 2006). Furthermore, intraperitoneal administration of three traditional Korean herbs, *Punica granatum*, *Chrysanthemum cinerariaefolium*, and *Zanthoxylum schinifolium* was reported to enhance disease resistance in olive flounder against *Uronema marinum* (Harikrishnan et al. 2010b), while dietary administration of this herbal mixture to the same fish species resulted in a reduction of mortality against *Philasterides dicentrarchi* infection (Harikrishnan et al. 2010c).

In many studies *Aeromonas hydrophyla* was used as a model bacteria for experimental challenges of fish treated with herbs or their extracts. Lingzhi mushroom and Astragalus extracts in combination with vaccine in common carp (Yin et al. 2009), *Solanum nigrum* leaves in spotted snakehead (*Channa punctatus*) (Rajendiran et al. 2008), garlic peel powder in African catfish (*Clarias gariepinus*) (Thanikachalam 2010), *Achyranthes aspera* root extract in Indian carp (*Labeo rohita*) (Rao et al. 2006), Astragalus and *Lonicera japonica* in Nile tilapia (Ardó et al. 2008), and a mixture of herbs in goldfish (*Carassius auratus*) (Harikrishnan et al. 2010a) decreased mortality rates against *A. hydrophila* infection.

Baical Scullcup root extract is effective against many types of bacteria, including *Streptococcus, Mycobacterium*, and *Pseudomonas* (Tan and Vanitha 2004). Nile tilapia fed diets supplemented with the herb *Andrographis paniculata* showed a dose-dependent reduction in mortality following a *Streptococcus agalactiae* infection challenge (Rattanachaikunsopon and Phumkhachorn 2009b). Similar results were observed with cinnamon (*Cinnamomum verum*) oil in tilapia infected with *Streptococcus iniae* (Rattanachaikunsopon and Phumkhachorn 2010b). Another Thai herb, *Cratoxylum formosum*, was reported to elevate the innate immune response and enhance disease resistance against *S. agalactiae* in tilapia (Rattanachaikunsopon and Phumkhachorn 2010a). Nile tilapia fed a diet supplemented with Chinese chive (*Allium tuberosum*) oil showed no mortalities following an infection challenge with *Flexibacter columnaris* (Rattanachaikunsopon and Phumkhachorn 2009a). Bath treatments containing different concentrations of Asiatic pennywort (*Centella asiatica*) extract resulted in a dose-dependent decrease in mortality in Nile tilapia infected with *Flexibacter columnare* (Rattanachaikunsopon and Phumkhachorn 2010c).

Kelp grouper (*Epinephelus* bruneus) fed a diet enriched with green tea showed higher relative percent survival than the control group after intraperitoneal administration of *Vibrio carchariae* (Harikrishnan et al. 2011a). Dietary administration of the combination of Astragalus root and Chinese Angelica root resulted in high survival rates in large yellow croaker infected with *V. alginolyticus* (Jian & Wu 2003). Won et al. (2008) observed that olive flounder fed a diet supplemented with Siberian ginseng (*Eleutherococcus senticosus*) residuum extract developed resistance to *Edwardsiella tarda* and *V. anguillarum* infections. Rock bream (*Oplegnathus fasciatus*) that were intraperitoneally inoculated with *E. tarda* showed high relative percent survival after being fed a diet enriched

with the herb Baical skullcap *Scutellaria baicalensis* (Harikrishnan et al. 2011c).

Conclusions

The theoretical benefit of medicinal plants is significant. They promote the growth and elevate the innate defense mechanisms of fish prior to exposure to a pathogen, and also improve survival following exposure to a specific pathogen. Multiple plants have been found to be effective against the following pathogens.

- Bacterial infections: improved resistance obtained with Ganoderma, Astragalus, *Scutellaria* Asiatic pennywort (*Centella asiatica*), Chinese chive (*Allium tuberosum*), Siberian ginseng *Eleutherococcus senticosus*, and *Cratoxylum formosum.*
- Viral infections: improved resistance obtained with *Stella aquatica*, *Impatiens biflora*, *Oenothera biennis*, *Artemisia vulgaris*, *Lonicera japonica*, and olive tree leaf (*Olea europaea*).
- Parasitic infections: improved resistance obtained with green tea, *Carica papaya*, and *Mucuna pruriens.*

A majority of the herbs and herb extracts can be given orally, which is the most convenient method of immunostimulation. However, the effect is dose-dependent and species-dependent (Anderson 1992; Galeotti 1998; Bricknell and Dalmo 2005; Harikrishnan et al. 2011b), and there is always a potential for overdosing (Yin et al. 2006). At present, it is difficult to make a correct assessment of efficacy and quality of herbs because of a lack of uniform quality standards and standard formulas (Liu et al. 2011). Most immunostimulation strategies involve pulse feeding the plant for a short period, usually 4–6 weeks, to up-regulate the immune response. The immunostimulant is then withdrawn for a similar period of time and the level of immunostimulation falls back towards the resting level before another dose of immunostimulant is given. This tends to cause the host immune system to oscillate from the resting level to an enhanced level then back to the initial level again. Such a strategy offers immense flexibility in fish farming, as the immunostimulant can be fed during periods of increased disease risk (Anderson 1992; Bricknell and Dalmo 2005).

Potentially, plants can make cost-effective dietary supplements due to the relatively low cost of their sources. Plant extracts have a potential application as an immunostimulant and growth promoter in fish culture, primarily because they can be easily obtained, are inexpensive, and act against a broad spectrum of pathogens when used at an optimum level. The use of plant products in fish culture systems may also be of environmental value because of their biodegradability. While the exact mechanism of the action of herbal extracts on fish and their immune systems is still unclear, it is envisioned that many issues will be resolved following the development of genomic and proteomic tools for several fish species.

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Chapter 17

Feeding Practices and Fish Health

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Introduction

The global aquaculture industry has expanded rapidly over the past few decades and is expected to continue to grow in the years to come due to the unpredictability and high cost of harvesting fish from the oceans, as well as the increased demand for fish as a result of rapid population growth, increased disposable income, and preferences for fish over other animal protein for personal, cultural, and health reasons. According to FAO (2012), world food fish production from aquaculture expanded at an annual average rate of 8.8% from 1980 to 2010, but has slowed to an annual growth rate of 6.3% from 2010 to 2012. Paralleling the growth of the industry has been a trend toward intensification of culture practices, where fish are stocked at high densities in an effort to obtain higher yield per unit area. In contrast to extensive and semi-intensive culture system, where fish derive all or most of their nutritional needs from natural pond-food organisms, fish reared under intensive production systems depend largely or solely on compounded feeds and may be subjected to more stressful conditions due to poorer water quality, which leads to growth reduction, immune suppression, and susceptibility to infectious diseases. It has generally been recognized that among other factors, adequate nutrition and good feeding practices are two of the most important requisites for sustainable, successful fish production in intensive culture operations. Without adequate intake of nutritionally balanced diets, fish are unable to optimally grow, reproduce, and maintain the ability to withstand stress and resist disease-causing agents (Landolt 1989; Blazer, 1992; Lovell et al. 1998b; Sealey and Gatlin 1999; Lall 2000; Lim and Webster 2001; Gatlin III 2002; Lim et al. 2008; Oliva-Tales 2012). However, considerable progress has been made over the last decade toward understanding dietary factors, including essential dietary nutrients (such as vitamins, minerals, amino acids, and fatty acids), additives (such as immunostimulants, prebiotics, probiotics, nucleotides, and organic acids and/or their salts), plant extracts, antinutritional factors, and toxins, and their effects on growth performance, stress, immune responses, and disease resistance in several fish species. The potential role and effects of these components on fish performance and health constitute major topics of some chapters of this book.

Dietary Nutrients, Additives and Fish Health, First Edition. Edited by Cheng-Sheng Lee, Chhorn Lim, Delbert Gatlin III, and Carl D. Webster.

Dupree (1984) indicated that good feeding practices are as important to the aquaculturist as the availability of good-quality and nutritionally sound diets. However, many problems are encountered in feeding fish as compared to feeding terrestrial animals. Fish are not fed on an *ad libitum* basis, as are domestic animals. Because fish are fed in water, feed that is not consumed within a reasonable time period represents not only an economic loss, but also causes environmental degradation that can bring about stress, poor growth, susceptibility to diseases, low survival, and poor harvest. Considerable research has been conducted to develop standard feeding practices (daily feed allowance, method of feeding, daily feeding frequency, and daily feeding schedule) for various fish species to provide adequate levels of nutritionally balanced diets to optimize growth, improve nutrient utilization, decrease production costs, maintain normal health, and minimize the impact of waste outputs on the environment. The feeding of fish is still an art rather than a science because the feeder, not the fish, determines how much, how often, when, how, and where to feed. Nevertheless, numerous publications addressing recommended feeding practices for various fish species are available (Stickney and Lovell 1977; Lim 1989, 1991; Cho 1990, 2004, 2007; Wilson 1991; NRC 1993, 2011; Robinson et al. 1994; Robinson and Li 1996; Lovell et al. 1998a, 2002; Robinson 1998; Gatlin III and Hardy 2002; Webster and Lim 2002; Riche and Garling 2003; Lim et al. 2006; Gatlin III 2010).

It is commonly known that feeding levels may influence the nutritional status of fish, which could ultimately affect their immune system function and resistance to infectious microorganisms (Gatlin III 2002; Alcorn et al. 2003). Underfeeding or overfeeding can directly or indirectly result in poor growth performance, and increase the susceptibility of fish to stress and disease-causing agents. However, sparse research has examined the effects of feeding allowances in relation to fish health. This chapter attempts to provide an overview of the effects of feeding practices on the immune responses and disease resistance in fish. The effects of winter feeding on disease resistance as well as the feeding of diseased fish are also discussed.

Feeding Level and Fish Health

Effects on Hematological Parameters

Hematological parameters have long been used as the most reliable tools for the diagnosis of diseases in human and domestic animals (Hesser 1960; Arnold 2009), but have not been widely used in the assessment of fish health (Clauss et al. 2008). However, hematological characteristics of fish are commonly determined as an integral part of studies evaluating their health status (Ighwela et al. 2012). Clauss et al. (2008) indicated that hematological data can be useful in monitoring the health status of fish provided that interpretation of results take into account intrinsic and extrinsic factors affecting blood cell morphology and the quantitative values obtained. Care must be taken when comparing data, as many factors including fish species, strain, sex, size, age, water quality, season, nutritional status, diseases, sampling technique, handling, and stress affect the hematological values and characteristics (Clauss et al. 2008; Arnold 2009).

Studies with fish have shown that, among other factors, starvation or insufficient feeding have significant effects on hematological values. Weinberg et al. (1973) examined the effects of food deprivation on erythropoiesis in red paradise fish, *Macropodus opercularis*. They observed that the values pertaining to red blood cell count (RBC) and hematocrit (Hct) were significantly lower in fish starved for 7 days when compared to normally fed fish. A significant increase in the mean corpuscular hemoglobin concentration (MCHC) was observed in the group of starved fish; however, there were no significant changes from the baseline values for white blood cell count (WBC) and hemoglobin (Hb) concentrations. A study with the kissing gourami, *Helostama temminki* by Weinberg et al. (1976) showed that after 9 days of starvation, there was a marked depression of erythropoiesis as indicated by a 45% reduction in the RBC, a decrease in Hct and Hb values, and a marked reticulocytopenia as compared to the values at day 0. There was a significant increase in mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCHC, and white blood cell count (WBC). Differential WBC counts showed no significant differences among the control and the fish starved for 9 days for the percentages of lymphocytes, thrombocytes, and monocytes,

although there appeared to be a trend of increasing percentage of neutrophils in starved fish.

Mahajan and Dheer (1983) evaluated the effects of 8 weeks of starvation followed by a 15-day recovery period on hematology and hematopoiesis of an air-breathing fish, *Channa punctatus*. The control group of fish received regular feeding at 2% body weight daily throughout the experiment. Weekly sampling data showed that, in starved fish, the RBC and related values (Hct, Hb, MCV, MCH, and MCHC) and the WBC progressively increased for 5 weeks, but all values significantly decreased at week 6. This trend continued until the end of the starvation period (week 8). Leukocytes and thrombocytes showed a change similar to RBC and WBC. Neutrophils were consistently increased throughout the 8-week period. The recovery was rapid after the normal feeding regimen was restored. Hematopoietic studies of spleen and head kidney imprints revealed that reticulocytes and mesomyelocytes were unable to keep pace with the changing peripheral blood picture. Blood glucose and liver and muscle glycogen progressively decreased during the 8-week starvation period. Lim and Klesius (2003) evaluated the effects of four feeding regimens (no feeding, feeding daily to apparent satiation, feeding every other day to apparent satiation, and no feeding for 3 weeks followed by feeding once daily to satiation during the 4th week) on hematological parameters of channel catfish, *Ictaluris punctatus*. The RBC of the fish that were fed every other day was significantly lower than that of the fish fed daily. The RBC value for the fish that were not fed was similar to that of the fish fed daily at week 4; both were significantly lower than the other two groups of fish. The WBC, Hct, and Hb of fish fed daily were significantly higher than those of the groups that were not fed or fed daily during the 4th week, but did not differ from those of fish fed every other day. The values of these parameters for the non-fed fish did not differ significantly from the group that was fed daily at week 4. Lim and Klesius (2003) concluded that juvenile channel catfish that were not fed for 4 weeks or not fed for 3 weeks followed by 1 week of satiation feeding developed anemia.

Based on these data, the duration of starvation required to induce anemia in fish varies among species. Other factors, such as nutritional status of fish, age and size, experimental conditions, and culture and feeding management may also contribute to the discrepancies between research results. Shoemaker et al. (2003) subjected juvenile channel catfish of the same strain used by Lim and Klesius (2003) to the following three feeding regimens for 4 weeks: no feeding; feeding once every other day to apparent satiation; and feeding once daily to apparent satiation. They measured hematology, blood glucose, and liver glycogen at weeks 2 and 4; values for RBC, WBC, and Hct at the end of weeks 2 and 4 and Hb at week 2 did not differ among various treatments. At week 4, Hb values were similar for fish fed once daily and once every other day, but these were significantly lower than those of the non-fed fish. Blood glucose and liver glycogen concentrations at weeks 2 and 4 did not differ for the fish that were fed daily and fed every other day. These values were significantly higher than those of the non-fed fish. The differences in hematological indices obtained in studies of Lim and Klesius (2003) and Shoemaker et al. (2003) could be due to larger size fish used (22.5 g vs 36.0 g) in the latter study, as experimental conditions, feed and feeding, and the strain of fish used were similar in both studies. Caruso et al. (2011) evaluated Hct, serum cortisol, and glucose of sub-adult European sea bass, *Dicentrarchus labrax*, and blackspot sea bream, *Pagellus bogaraveo*, that were starved or fed for 31 days. Blood samplings for measurement of these parameters were collected at days 0, 11, 20, and 31. Results of this study showed that Hct and concentrations of serum cortisol and glucose in both species were not significantly affected by starvation. The disparity among the values of these parameters could be attributed to the short (31 days) experimental duration.

Effects on Immune Responses and Disease Resistance

The influence of feeding regimens (no feeding, feeding once daily to satiation, feeding every other day to satiation, and no feeding for 3 weeks followed by feeding once daily to satiation during the 4th week; Table 17.1) on weight gain, macrophage chemotatic response, and resistance of juvenile channel catfish to *Edwardsiella ictaluri* infection were evaluated by Lim and Klesius (2003) in two separate feeding trials (Study I and II, respectively). After being subjected to various feeding

Table 17.1 Feeding regimens for channel catfish during the 4 weeks before and 2 weeks after challenge with *E. ictaluri* (Adapted after Lim, C. and P.H. Klesius. 2003. Influence of feed deprivation on hematology, macrophage chemotaxis, and resistance to Edwardsiella ictaluri challenge of channel catfish. Journal of Aquatic Animal Health 15: 13–20.) *1:

Before challenge	After challenge
Not fed	Not fed
	Fed daily
Fed daily	Fed daily
	Not fed
Fed every other day	Fed every other day
	Not fed
Fed daily at week 4*	Fed daily
	Not fed

*No feeding for the first 3 weeks

regimens for 4 weeks (Study I), five fish from each of the triplicate tanks were intraperitoneally (IP) injected with squalene and continued on the same feeding regimens for an additional 5–7 days prior to collection of macrophages. The researchers observed that the final weight gains differed significantly among treatments (Table 17.2). The unfed fish lost weight while those fed daily gained the most, and the fish fed daily starting at week 4 gained the least. Feed efficiency was highest for fish fed every other day and lowest for the group fed daily at week 4. Survival rate did not differ among treatments; however, feeding regimens had no effect on macrophage migration in the absence of *E. ictaluri* exoantigen (Table 17.3). In the presence of exoantigen, the mean macrophage migration significantly differed among all treatments with fish that were fed daily having the highest mean macrophage migration, followed in descending order by fish that were fed every other day, fed daily at week 4, and not fed. When expressed in terms of macrophage chemotaxis ratio, the value of the group that was not fed was similar to that of the fish that were fed daily at week 4, but both of these values (macrophage migration and macrophage chemotaxis ratio) were significantly lower than those of other treatments. Fish fed daily throughout the 4-week period had the highest chemotaxis ratio.

To determine if cessation or continuation of feeding during an enteric septicemia of catfish (ESC) epizootic (after *E. ictaluri* challenge) reduces fish mortality, fish from each of the four treatments subjected to the 4-week feeding regimens as described in Study I were divided into two groups of three replicates each. One group continued to receive the same feeding regimen, whereas the other groups were switched to fed daily or not fed for two weeks, depending on the feeding regimens used prior to challenge (Table 17.1). Weight gain and survival (Table 17.2) followed the same patterns as those observed in Study I. Feed efficiency, however, did not differ among treatments. The number of days to first mortality following *E. ictaluri* challenge was significantly less for the groups that were not fed throughout the study and not fed for 4 weeks and then fed daily; however, these values did not differ from those of fish fed daily during the 4th week and not fed thereafter (Table 17.4). The group

Table 17.2 Mean percentage weight gain, dry matter (DM) feed intake, feed efficiency ratio and survival of channel catfish receiving various feeding regimens for 4 weeks (study I and II). Means in the same column with different superscripts are significantly different ($P < 0.05$) (Adapted after Lim, C. and P.H. Klesius. 2003. Influence of feed deprivation on hematology, macrophage chemotaxis, and resistance to Edwardsiella ictaluri challenge of channel catfish. Journal of Aquatic Animal Health 15: 13–20.).

Feeding regimen	Weight gain (%)	Feed intake(g DM/fish)	Feed efficiencyratio	Survival (%)
Study I				
Not fed	–22.2^{d}	–	–	99.5^{a}
Fed daily	107.2^{c}	21.80^{c}	1.11^{c}	100.0^{a}
Fed every other day	73.7^{b}	12.89^{b}	1.29^{b}	100.0^{a}
Fed daily at week 4	16.2^{a}	4.31^{a}	0.84^{a}	98.8^{a}
Study II				
Not fed	–21.6^{d}	–	–	91.7^{a}
Fed daily	140.5^{c}	20.17^{c}	1.29^{a}	100.0^{a}
Fed every other day	72.5^{b}	10.59^{b}	1.27^{a}	98.6^{a}
Fed daily at week 4*	23.5^{a}	3.69^{a}	1.18^{a}	95.7^{a}

*No feeding for the first 3 weeks

Table 17.3 Mean macrophage migration and macrophage chemotaxis ratio of channel catfish receiving various feeding regimens for 4 weeks (study I). Means in the same column with different superscripts are significantly different ($P < 0.05$) (Adapted after Lim, C. and P.H. Klesius. 2003. Influence of feed deprivation on hematology, macrophage chemotaxis, and resistance to Edwardsiella ictaluri challenge of channel catfish. Journal of Aquatic Animal Health 15: 13–20.).

	Mean number of macrophage migration		
Feeding regimen	Without exoantigen	With 50 μL exoantigen	Macrophage chemotaxis ratio
No feeding	1.27[a]	2.87[d]	0.69[a]
Fed daily	1.51[a]	6.18[c]	0.81[c]
Fed every other day	1.44[a]	4.44[b]	0.76[b]
Fed daily at week 4*	1.58[a]	3.69[a]	0.70[a]

*No feeding for the first 3 weeks

Table 17.4 Mean number of days to first mortality and cumulative mortality after immersion challenge with *E. ictaluri* of channel catfish receiving various feeding regimens (study II). Means in the same column with different superscripts are significantly different ($P < 0.05$) (Adapted after Lim, C. and P.H. Klesius. 2003. Influence of feed deprivation on hematology, macrophage chemotaxis, and resistance to Edwardsiella ictaluri challenge of channel catfish. Journal of Aquatic Animal Health 15: 13–20.).

Feeding regimen	Days to first mortality	Cumulative mortality (%)
Not fed throughout study	3.7[a]	100.0[c]
Not fed for 4 weeks, then fed daily	3.3[a]	98.7[c]
Fed daily throughout study	7.0[c]	25.3[a]
Fed daily for 4 weeks, then not fed	4.3[bc]	53.3[ab]
Fed every other day throughout study	8.0[c]	32.0[ab]
Fed every other day for 4 weeks, then not fed	6.0[bc]	53.3[ab]
Fed daily at week 4*, then fed daily	6.7[c]	54.7[ab]
Fed daily at week 4*, then not fed	4.0[ab]	60.0[b]

*No feeding for the first 3 weeks

that was fed every other day throughout the study had the highest number of days to first mortality, but this was not significantly different from the groups fed daily throughout the study, fed daily for 4 weeks and then not fed, fed every other day for 4 weeks and then not fed, and fed daily at week 4 and after challenge. Cumulative mortality 2 weeks post-challenge for the groups that were fed daily or every other day, both before and after challenge, was significantly lower than the groups that were not fed before or after challenge, or not fed prior to challenge and fed afterwards. All fish that were not fed throughout the experiment died after 8 days post-challenge, and 98.8% of the fish that were not fed for the first 4 weeks and then fed daily after challenge died by the end of week 2 post-challenge. Results of these studies indicated that, in a controlled environment where natural food was absent, the starvation of juvenile channel catfish for 4 weeks resulted in weight loss, reduced macrophage chemotaxis, and early onset and high mortality following *E. ictaluri* challenge. Discontinuing feeding after the challenge increased the mortality due to ESC regardless of the feeding regimen prior to challenge. It is therefore suggested that juvenile channel catfish be fed at least once every other day to apparent satiation even during an ESC epizootic, to increase their resistance to *E. ictaluri* infection.

Klesius et al. (1999) conducted two studies to determine the effects of short-term feed deprivation on innate resistance and antibody production to *Flavobacterium columnare* in channel catfish. In Study I, fish were subjected to two feeding regimens: non-fed and fed twice daily to apparent satiation for 10 days before and after *F. columnare* challenge by intramuscular injection. Results showed that 100% of fish that were not fed died as compared to only 11.7% in the fed group. Deaths due to columnaris for both treatments began at day 3 and ended at day 7. In Study II, fish were subjected to 3-day and 7-day fed and non-fed. In the 3-day fed treatment, fish were not fed for the first 4 days, but were fed to apparent satiation twice daily 3 days before and 10 days after *F. columnare* challenge. In the 7-day fed treatment, fish were fed twice daily to satiation for 7 days prior to challenge and 10 days post-challenge. For the 3-day

Table 17.5 Mean cumulative mortality and antibody of channel catfish fed and non-fed for 3 or 7 days before and 10 days after challenge with *Flavobacterium columnare* injected with phosphate buffer saline (PBS). Means in the same column with different superscripts are significantly different ($P < 0.05$) (Adapted after Klesius, P.H., C. Lim and C.A. Shoemaker. 1999. Effect of feed deprivation on innate resistance and antibody response to Flavobacterium columnare in channel catfish, Ictalurus punctatus. Bulletin of the European Association of Fish Pathologists 19: 156–158.).

Feeding regimen	Mean cumulative mortality (%)	Mean agglutination titer
PBS (control):		
Non-fed, 3 days	0.0^{c}	18.5^{b}
Non-fed, 7 days	0.0^{c}	11.4^{b}
Fed, 3 days	0.0^{c}	8.3^{b}
Fed, 7 days	0.0^{c}	11.4^{b}
F. Columnare		
Non-fed, 3 days	11.7^{bc}	64.6^{a}
Non-fed, 7 days	70.0^{a}	54.5^{a}
Fed, 3 days	18.3^{b}	84.3^{a}
Fed, 7 days	11.7^{bc}	70.0^{a}

non-fed and 7-day non-fed treatments, fish were not fed for 3 days (days 5–7) and 7 days, respectively, before challenge and 10 days post-challenge. In this study, a group of fish from each treatment was injected with *F. columnare* culture, whereas the others groups were injected with phosphate buffer saline (PBS) and served as controls. Agglutination titers of fish in all treatments 10 days post-challenge with *F. columnare* were positive and were significantly higher than those of the control fish injected with PBS (Table 17.5). However, there were no significant differences among antibody titers of fish in all treatments, although the values of this variable tended to be lower in fish deprived of feed for either 3 or 7 days. Cumulative mortality did not differ in fish that were not fed for 3 days or fish that were fed for 3 and 7 days. However, significantly increased mortality was observed in fish that were not fed for 7 days.

Data from these two studies indicate that deprivation of feed for 7 days in an environment where natural food was absent appeared to be sufficient to decrease the innate resistance of juvenile channel catfish to *F. columnare*, with mortality reaching 70% and 100% in the 7- and 10-day starved fish, respectively. Shoemaker et al. (2003) evaluated the effect of longer periods of feed deprivation (no feeding, feed once every other day to satiation, and feeding once daily to satiation 4 weeks before and 2 weeks after challenge) on serum protein, specific antibody response, and susceptibility of channel catfish to *F. columnare* infection challenge. Two weeks after the start of the trial, serum protein was not affected by feeding regimens. At week 4 after feeding, however, serum protein was significantly lower in the non-fed group than the daily fed group, but neither value differed from that of the fish fed once every other day. The rate of cumulative mortality 2 weeks post-challenge of the fish that were not fed (78.3%) was significantly higher than those of the groups fed daily and fed every other day (0.0 and 1.7%, respectively). Antibody levels did not differ significantly among treatments; however, this value for the unfed group was 16% and 23% lower that the values for fish that were fed daily and fed every other day, respectively. This study suggested that, in the absence of natural food, juvenile channel catfish should be fed at least once every other day to apparent satiation to maintain proper immune function and improve resistance to *F. columnare*.

Studies on the effects of feeding regimens on health of other fish species are limited and only involve the evaluation of immune response parameters without infection challenge. Sakai (1983) assessed the health condition of salmonids by measuring serum non-specific hemolytic (SH_{50}) activity of juvenile coho salmon, *Oncorhynchus kisutch*, and matsu salmon, *O. matsu*, that were starved for 10 and 20 days and then IP injected with *Aeromonas salmonicida* and *Vibrio anguillarum*. A correlation between the decrease in the SH_{50} activity and an increase in severity of starvation and diseases was observed. The author also reported that a similar correlation was observed in naturally diseased salmonids. Mazur et al. (1993) investigated the effect of two feeding levels (67% and 100% of satiation) and three stocking densities (3000, 5000, and 7000 fish per 720 m^3 cage; 0.044, 0.074, and 0.104 kg m^{-3}, respectively) on the prevalence of *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD) in chinook salmon, *O. tshawytsha*, that were transferred from fresh- to seawater and reared for 272 days. A thirteenth cage (360 m^3) was stocked with 5000 fish (or 0.148 kg m^{-3}) and fed to satiation (control). Two cages at each density received 67% of satiation, while the other two received 100% of satiation. Thirty fish

from each of the two hatchery tanks and 30 fish from each treatment (combination of feeding level and density) were sampled and had their kidneys removed after 69, 171, and 263 days of rearing. Feeding levels had no significant effect on the prevalence of *R. salmoninarum*. However, a significant effect of density on *R. salmoninarum* infection was observed during the last sampling period (day 263). Fish at the highest rearing density had significantly greater prevalence of *R. salmoninarum* than fish reared at lower densities at both feeding levels. The control, which had the highest rearing density and was fed to satiation, had the highest prevalence rate of *R. salmoninarum.*

Alcorn et al. (2003) evaluated the effects of ration levels on immune functions of chinook salmon reared in tanks for a period of 54 weeks. Fish in each of the duplicate tanks were fed an experimental diet at 100, 64, and 40% satiation level, while another group of fish fed a commercial diet at 64% satiation served as the control. Fish were sampled at weeks 30 (fall), 39 (winter), and 54 (spring) for measurement of humoral and cellular immune responses, as well as the indices of disease resistance. These included Hct, leucocrit, differential leucocyte counts, plasma protein, serum lysozyme, serum complement (ACH50), superoxide anion production, phagocytosis of pronephros macrophages, and myeloperoxidase pronephros neutrophils. No differences were observed in the values of these indices between the control group (fed a commercial diet at 64% satiation) and the group fed an experimental diet at 64% satiation, except that the control fish had higher concentration of plasma protein. The authors (Alcorn et al. 2003) reported that among these nine immune parameters tested, consistent effects of the ration levels were obtained in plasma protein levels, leucocrit values, and phagocytic activity of pronephros macrophages. Plasma protein concentrations and leucocrit levels tended to increase among the experimental groups as the ration levels increased from 40% to 100% satiation. The percentage of macrophage phagocytic activity was inversely proportional to ration levels, and it was suggested that ration levels might not be as important as the composition of diets when it comes to the immune response of fish.

Caruso et al. (2011) assessed non-specific immune parameters (lysozyme, serum hemagglutinating titer, and respiratory burst) of sub-adult European sea bass, *Dicentrarchus labrax*, and blackspot sea bream, *Pagellus bogaraveo*, that were subjected to two feeding regimens: starved or fed for 31 days. Lysozyme activity in mucus, plasma, and kidney (not in blackspot sea bream), and serum hemagglutinating titer were measured at days 0, 11, 20, and 31, while whole-blood respiratory burst activity was measured following the treatment with zymosan and phorbol-12-miristate 13-acetate (PMA) at days 0 and 31. There were some significant differences among the values of the immune parameters tested at different time periods for each of the fish species receiving different feeding regimens. In starved sea bass, mucus lysozyme activity at day 31 was twice the level observed at day 0. Hemagglutinating titers of starved sea bass determined at day 31 were significantly lower than those of the fish that were fed. In blackspot sea bream, a slight non-significant reduction in the value of the hemagglutinating titer was observed after 11 days of starvation. Respiratory burst activity significantly decreased for both fish species that were starved. Caruso et al. (2011) reported that, unlike the hematological parameters (reported previously in this chapter), lysozyme activity, hemagglutinating titers, and respiratory burst activity were more sensitive indicators in the assessment of health status of staved sub-adult European sea bass and blackspot sea bream.

Henken et al. (1987) evaluated the relationship between feeding regimes (feed deprivation, feeding at maintenance level, and feeding above maintenance) on hemagglutination antibody titers against O-antigen of *Yersinia ruckeri*. Four replicate groups of fish received each of the three feeding regimens for 72 days. On day 73, two of the four replicate groups remained at the same feeding level, while each of the other two replicate groups were reassigned to the two remaining feeding levels. Two weeks later, fish in all groups were immunized by intramuscular injection with *Y. ruckeri* O-antigen. Blood samples were taken on the day of immunization and at weeks 1, 2, 3, and 5 following immunization. Fish fed continuously above maintenance level had consistently higher antibody titers than those continuously starved or fed at maintenance level. Significant differences in antibody titers were observed for the values at 1 and 5 weeks. Peak values of fish fed continuously above (6.19) and at maintenance (2.36) level were registered at weeks 1 and 2 after immunization, respectively.

The early- and high-peak values observed in the fish fed continuously above maintenance level could be important in those cases where antibodies play a major role in disease resistance. Antibody titers of starved fish were similar to those fed at maintenance level, but the starved fish showed more variability in response. This may be an indication of lower degree of immune regulation. Differences in antibody titers between groups switched to alternate feeding regimens from day 73, but with the same preceding feeding regimen, were not significant. Immune responsiveness of fish reassigned to different feeding regimens tended to be dependent on the preceding feed level, as antibody titers of fish continually received a certain feeding level and fish reassigned from that regimen to another were not significantly different. Results of this study indicate that antibody production of African catfish is affected by feeding levels, and long-term starvation or maintenance feeding induced a decrease in antibody production against *Y. ruckeri* O-antigen.

Seasonal Feeding and Health of Channel Catfish

Based on water temperature, the optimum growing season of channel catfish in ponds in the southern United States is approximately six months from mid-spring to mid-fall. Generally, catfish do not feed consistently when the water temperature drops below 21°C (Stickney and Lovell 1977), and generally stop eating when water temperature drops to 10°C or below (Li and Robinson 2006). Based on research results however, it has been recommended that catfish should be fed throughout the winter months to prevent weight loss (Lovell and Sirikul 1975), maintain health, and improve their resistance to infectious diseases (McMillan 1985). Even so, some fish producers choose not to feed at all during winter, whereas others continuously feed their fish based on the guidelines recommended by Stickney and Lovell (1977).

In the southern United States, infectious diseases such as *E. ictaluri*, the causative agent of enteric septicemia of catfish (ESC), is most virulent during spring (March–June) and fall (September–October) when water temperature is 22–28°C (McMillan 1985; Plumb and Brady 1990; Plumb 2001). In spring, this favorable environmental condition for an ESC epizootic coincides with the poor nutritional condition of commercially grown catfish that do not consume much feed during winter (Lovell at al. 2001). This section therefore briefly describes the results of two studies conducted at Auburn University on the effects of winter-feeding regimens on the response of juvenile (year 1) and food-size (year 2) catfish to *E. ictaluri* challenge.

In Study I (Kim and Lovell 1995), 22 g (year 1) and 420 g (year 2) channel catfish were stocked separately in 400 m^2 earthen ponds at 13,750 and 5000 fish ha^{-1} for year 1 and year 2, respectively. The 6-month feeding experiment began on 1 November 1992 and ended on 23 April 1993. Three ponds containing each age group of fish were randomly assigned to the following overwinter feeding regimens: continuous feeding, partial feeding, and no feeding. Fish on the continuous feeding regimen were fed throughout the study period, when the water temperature at a depth of 1 m was above 6°C. The year-2 fish were fed 2.0, 1.75. 1.5, 1.0, 0.5, and 0% of body weight when the water temperatures were 18, 15–18, 12–15, 9–12, 6–9, and below 6°C, respectively. Year-1 fish were fed 33% more feed than the year-2 fish at the prescribed water temperatures. Fish on the partial feeding regimen were fed in the same manner as fish on the continuous feeding regimen, except they were not fed during December, January, and February. The non-fed fish received no feed throughout the 6-month period. Crumbles and slow-sinking pellets containing approximately 32% protein and 3000 kcal kg^{-1} were fed to year-1 and year-2 fish, respectively.

At the end of the feeding trial, when the afternoon water temperature was approximately 23°C, fish from each pond were removed, weighed, and transferred to separate static 1 m^3 circular tanks (year 2) or 70 L aquaria (year 1). Water in holding tanks and aquaria were changed at a rate of about 10%, and salt was added to maintain a concentration of 200 mg NaCl L^{-1} to prevent nitrite toxicity. Fish in tanks and aquaria continued to receive the feeding regimens they were subjected to in the ponds. Water temperature in the raceways was 23–25°C during the daytime and 1–2°C cooler at night. Water temperature in the aquaria was maintained at 25 ± 2°C. Fish were maintained in raceways or aquaria for 5 days prior to challenge with *E. ictaluri* and IP injection with 0.1 mL of suspension at a predetermined LD50 dose. Mortality was recorded daily for 14 days. Serum agglutination antibody titers were determined for year-2 fish that had survived the

challenge. Antibody production of year-1 fish was not determined because all the fish on the non-fed regimen died 5 days post-challenge.

In Study II (Okwoche and Lovell 1997), which had similar objectives to Study I, 43 g (year 1) and 660 g (year 2) channel catfish were stocked separately in 400 m^2 earthen ponds at 13,750 and 3750 fish ha^{-1} for year-1 and year-2, respectively. The 6-month experiment began on 1 November 1993 and ended on 30 April 1994. Feeding regimens were the same as those described in Study I, except slow-sinking commercial pellets were used. Also, at the end of the feeding trial, fish from ponds were transferred to separate 1 m^3 circular raceways (year 2) or 70 L flow-through aquaria (year 1), all of which were supplied with a continuous flow of pond water. Water temperatures in holding raceways and aquaria were similar to those recorded in Study I. Bacterial (*E. ictaluri*) challenge and antibody production measurements were the same as described earlier in Study I. Post-challenge mortality was recorded daily for 16 days. However, because an insufficient amount of unfed year-1 fish survived the challenge, non-challenged fish from each treatment were IP injected with formalin-killed *E. ictalur*i for 14 days prior to the measurement of antibody production. Phagocytic activity of heat-killed *E. ictaluri* by head kidney phagocytic cells (pronephros phagocytes) of fish from both size groups was also determined.

Results of Study I showed that there were no significant differences in weight gain between the partially fed and continuously fed groups of year 1 and year 2 fish (Table 17.6). The unfed fish of each age group lost weight, but still appeared healthy and in satisfactory condition. Weight gain and the condition of fish at the end of the feeding trial followed a similar trend to that of fish in Study I; the unfed year-1 fish began to die 1 day post-challenge with *E. ictaluri* and all fish died at day 5 (Table 17.6). The cumulative mortality rate 4 days post-challenge was similar for the partially fed and continuously fed fish (50 and 48%, respectively), while that of the unfed fish significantly increased to 94%. In contrast, the unfed year-2 fish had significantly lower mortality than the partially and continuously fed fish, which had similar mortality (80 and 78%, respectively). There were no significant differences among antibody titer of year-2 at day 14 post-challenge.

Table 17.6 Weight change and post-challenge mortality of year-1 and year-2 channel catfish receiving three feeding regimens during winter. Means in the same column with different superscripts are significantly different ($P < 0.05$) (after Lovell et al. 2001).

Age group	Feeding regimen	Weight change (%)	Post-challenge mortality (%)*
Year 1	Non-fed	-9^a	94^a
	Partially fed	50^b	50^b
	Continuously fed	64^b	48^b
	Pooled SEM	14.1	4.4
Year 2	Non-fed	-10^a	23^a
	Partially fed	42^b	80^b
	Continuously fed	49^b	78^b
	Pooled SEM	3.8	3.9

*Cumulative mortality at day 4 for year-1 fish and day 12 for year-2 fish.

In study II, weight gain and the condition of fish at the end of the feeding trial followed a similar trend to that of fish in Study I (Table 17.7). Mortality of year-1 fish subsequent to *E. ictaluri* challenge was significantly higher for the unfed group (98.3%) compared to 55.8% and 52.5% for the partially fed and continuously fed fish, respectively (Table 17.7). In the year-2 fish, however, the unfed group had significantly lower mortality (9.3%) than partially fed (69.3%) and continuously fed (77.3%) fish. At both age groups, there were no significant differences in mortality rates of partially fed and continuously fed fish. Mortality in year-1 fish began 4 days post-challenge, peaked at days 5–7, and ceased at day 9. In year-2 fish, post challenge mortality in the partially fed and continuously fed groups began on day 6, peaked at days 8–11, and ceased at day 14. The unfed year-2 fish did not begin to die until day 10 days post-challenge, and stopped dying on day 12.

Antibody titers against *E. ictaluri* were not detected in fish of both age groups prior to challenge. Following challenge, antibody titers of the year-1 fish that were not fed were significantly lower than in the partially fed and continuously fed groups, which showed no differences (Table 17.7). For the year-2 fish, antibody titer of the unfed group was significantly higher than in the partially and continuous fed fish, which had similar values. Feeding levels for both age groups influenced the phagocytic indices of *E. ictaluri* by pronephros phagocytes. The unfed year-1 and year-2 fish had significantly lower phagocytic index than

Table 17.7 Weight change, post-challenge mortality, antibody titer and phagocytosis of year-1 and year-2 channel catfish receiving three feeding regimens during winter. Means in the same column with different superscripts are significantly different ($P < 0.05$) (after Lovell et al. 2001).

Age group	Feeding regimen	Weight change (%)	Post-challenge mortality (%)	Antibody titer to *E. ictaluri* (reciprocal titer)	Phagocitic index (bacteria/phagocyte)
Year 1	Non-fed	−12[a]	98.3[a]	128.8[a]	2.38[a]
	Partially fed	92[b]	55.8[b]	186.6[b]	5.06
	Continuously fed	106[b]	52.5[b]	288.9[c]	5.56
	Pooled SEM	7.6	2.2	7.9	0.09
Year 2	Non-fed	−7[a]	9.3[a]	52.8[a]	3.11[a]
	Partially fed	38[b]	69.3[b]	138.7[b]	5.69[b]
	Continuously fed	39[b]	77.3[b]	142.2[b]	6.32[b]
	Pooled SEM	5.6	5.4	4.3	0.21

the partially and continuous fed fish. However, there were no differences in phagocytic indices among the partially and continuous fed fish at both age groups.

Based on the similarity in weight gain data obtained from these two studies for the partially fed and continuously fed fish of both age groups, it is not beneficial to feed channel catfish during the months of December, January, and February if feeding is to be reintroduced in March and continued through April. Fish starved for 6 months (November 1 to April 30) were immunosuppressed and had decreased resistance to *E. ictaluri* infection challenge in small (year-1) fish, but enhanced resistance to *E. ictaluri* infection in large (year-2) fish. These findings may be of limited application to the current practice of channel catfish industry because current food fish production uses mixed size/age classes of fish. More research is therefore needed before appropriate feeding levels to improve the resistance of different sizes of channel catfish to *E. ictaluri* can be recommended.

Feeding Diseased Fish

Paralleling the rapid expansion and intensification of aquaculture production, there has been a trend of dramatic increase in the occurrence of fish disease cases (Plumb 2001). Disease outbreaks cause substantial economic loss to producers as a result of increased mortality, decreased growth rate, poor feed efficiency, treatment costs, and harvest delay. The use of medicated feed is the most effective method of treatment for bacterial infections in fish reared in large culture systems (Gatlin, III 2002, 2010; Kelly 2013). Li et al. (2006) indicated that systemic bacterial infections in cultured fish could be successfully treated with medicated feeds containing antibiotics if the diseased fish are treated at the early stage of disease outbreak. However, the use of medicated feeds should not be substituted for preventive measures, such as good water quality management, handling, sanitation, and adequate nutrition. Medicated feeds are costly and their efficacy varies depending on the state and nature of disease. Currently there are only three antibiotics that have been approved by the US Food and Drug Administration (FDA) that could be incorporated in feeds for the treatment of specific diseases of certain fish species intended for human consumption (http://www.fda.gov/AnimalVeterinary/DevelopmentApprovalProcess/Aquaculture/ucm132954.htm). These are florfenicol (Aquaflor®), oxytetracycline dihydrate (Terramycin® 200 for fish), and sulfadimethoxine/ormetoprim (Romet-30®).

Florfenicol, sold under the trade name of Aquaflor®, is a broad-spectrum antibiotic for use in the control of ESC in channel catfish caused by *E. ictaluri*, furunculosis associated with *Aeromonas salmonicida*, and coldwater disease associated with *Flavobacterium psychrophilum* in freshwater-reared salmonids. In addition, columnaris disease caused by *F. columnare* in freshwater-reared finfish and streptococcal septicemia caused by *Streptococcus iniae* in freshwater-raised warmwater finfish could also be treated with florfenicol. The antibiotic can be used only under the professional supervision of a licensed veterinarian through a veterinary feed directive order. Aquaflor®-medicated feed is fed for 10 days at a rate to deliver 10 mg active florfenicol per kilogram of

fish per day for ESC and furunculosis outbreaks, and 15 mg per kg of fish per day for columnaris disease and streptococcal septicemia. The withdrawal period for florfenicol for all these fish species is 15 days (Li et al. 2006; Kelly 2013).

Terramycin® 200, or oxytetracycline dehydrate, is also a broad-spectrum antibiotic that is approved by the FDA for the treatment of bacterial hemorrhagic septicemia, *A. liquefaciens*, and pseudomonas disease, *Pseudomonas* spp., in channel catfish. This drug is also approved for the treatment of ulcer disease, *Hemophiluspisium*, furunculosis, bacterial hemorrhagic septicemia, and pseudomonas disease in salmonids, as well as coldwater disease caused by *Flavobacterium psychrophilum* in freshwater-reared salmonids and columnaris disease in all freshwater-raised rainbow trout, *O. mykiss*. Terramycin medicated feed is fed for 10 days at a rate to deliver 2.5–3.75 g of active oxytetracycline per 45 kg of fish per day for bacterial hemorrhagic septicemia and pseudomonas disease in channel catfish, and for ulcer disease, furunculosis, and bacterial hemorrhagic septicemia and pseudomonas disease in salmonids. The dose required for the treatment of coldwater disease caused by *Flavobacterium psychrophilum* in freshwater-reared salmonids, and for columnaris disease in all freshwater-reared rainbow trout, is 3.75 g of active oxytetracycline per 45 kilogram of fish per day. For these fish species, a withdrawal period of 21 days is required before fish are slaughtered and processed for human consumption (Li et al. 2006; Kelly 2013).

Romet-30® is a combination of two drugs, sulfadimethoxine and ormetoprim, at a ratio of 5:1 (Li et al. 2006). In combination, these two drugs are more effective than either drug used alone. This product is approved by the FDA for the control of ESC in catfish and furunculosis in salmonids. Romet-medicated feed is fed at a rate to deliver 50 mg of active antibiotics per kilogram of fish per day for 5 days. The withdrawal period is only 3 days for channel catfish, but for salmonids the required withdrawal period is 42 days (Kelly 2013). Romet-medicated feeds are not as palatable to channel catfish as the regular non-medicated feeds due to the bitter taste of ormetoprim. However, this problem can be partially overcome by increasing the dietary fish meal level to 16%. Romet is heat stable, so can be conveniently incorporated in extruded pellets (Robinson 1998).

Although medicated feeds are effective for the treatment of certain bacterial infection in channel catfish, a common practice among some commercial catfish producers in the southern US is to withhold feeding fish during the manifestation of ESC. Such practices have been reported to reduce the severity and decrease mortality. However, the efficacy of this practice as a means of controlling ESC has not been well documented (Robinson and Li 1996). Wise and Johnson (1998) evaluated the effect of feeding regimens and Romet-medicated feed on survival and antibody response of naturally induced *E. ictaluri* infection in fingerling channel catfish reared in net-pens placed in a pond. Following detection of *E. ictaluri*, infected fish were subjected to the following feeding regimes: no feeding, fed medicated feed for five days, and fed medicated and non-medicated feed every day, every other day, and every third day for a period of 28 days. Survival was better among fish that were completely withheld from feed or those fed medicated feed every other day or every third day as compared to fish receiving other feeding regimens. Feeding non-medicated feed every other day or every third day was as effective in reducing *E. ictaluri*-associated mortality as feeding medicated feed on a daily basis. Agglutinating antibody titers of fish fed medicated feed for the duration of the study were lower than those of the other groups.

Conclusions

There has been little research examining the effects of feeding practices in relation to fish health. Moreover, available information on this subject is limited and often inconsistent. The differences between the results of various studies could be attributed to differences in fish species, strain, sex, size and age, water quality, nutritional status, diet quality, feeding level, experimental duration, environmental conditions, handling stress, and sampling technique. The type of bacteria, intensity and method of bacterial challenge, and the virulence of the bacterial strain may also account for the different results. More research is therefore needed to better understand the effect of feeding practices on disease prevention, management, and control. However, published data appear to indicate

that starvation or insufficient feeding adversely affect hematological parameters, immune response, and disease resistance. In a controlled environment where natural food was absent, starvation of juvenile channel catfish for 4 weeks resulted in decreased immune response and increased mortality following *E. ictaluri* challenge. Discontinuing feeding after infection also increased the mortality due to ESC, regardless of feeding regimens used prior to challenge. Under similar environmental conditions, deprivation of feed for a period as short as 7 days decreased the innate resistance of channel catfish to *F. columnare*. Feeding to apparent satiation at least once every other day prior to, and after, challenge is needed to maintain proper immune function and improve the resistance of fish against *E. ictaluri* or *F. columnare*. In ponds, small (year 1) catfish deprived of feed for 6 months during cold weather were immunosuppressive and more susceptible to *E. ictaluri* infection, but enhanced resistance to *E. ictaluri* infection was obtained in large (year 2) fish. At both age groups, there were no significant differences in mortality rates of partially fed and continuously fed fish. This information may be of limited application to the channel catfish industry since the current production practices use mixed-size fish.

The use of antibiotic-medicated feed has been reported as the most effective method of treatment for bacterial infections in fish reared in large culture systems. For the treatment to be successful, proper drugs should be initiated at the early stages of disease outbreak. However, a common practice among some commercial catfish producers in the US is to withhold feeding fish during the manifestation of ESC. This practice, although not well documented, has been reported to reduce the severity of the disease and decrease mortality. Withholding feed or feeding with Romet-medicated feed every other day or every third day has also been shown to increase the survival of naturally induced *E. ictaluri* infection in fingerling channel catfish, as compared to fish receiving medicated feed for 5 days or every day, and fish fed non-medicated feed every day, every other day, or every third day for a period of 28 days. Feeding non-medicated feed every other day or every third day was as effective in reducing *E. ictaluri*-associated mortality as feeding medicated feed on a daily basis.

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Index

Dietary Nutrients, Additives and Fish Health, First Edition. Edited by Cheng-Sheng Lee, Chhorn Lim, Delbert Gatlin III, and Carl D. Webster.

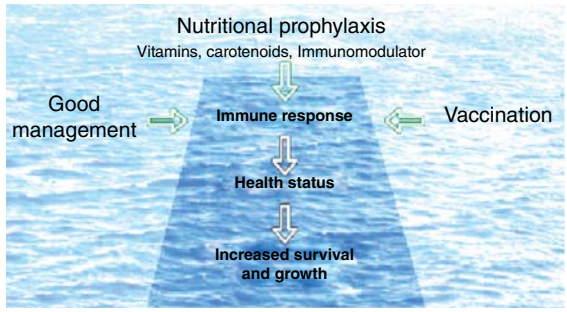

Figure 7.1 Benefits of nutritional prophylaxis in aquaculture.

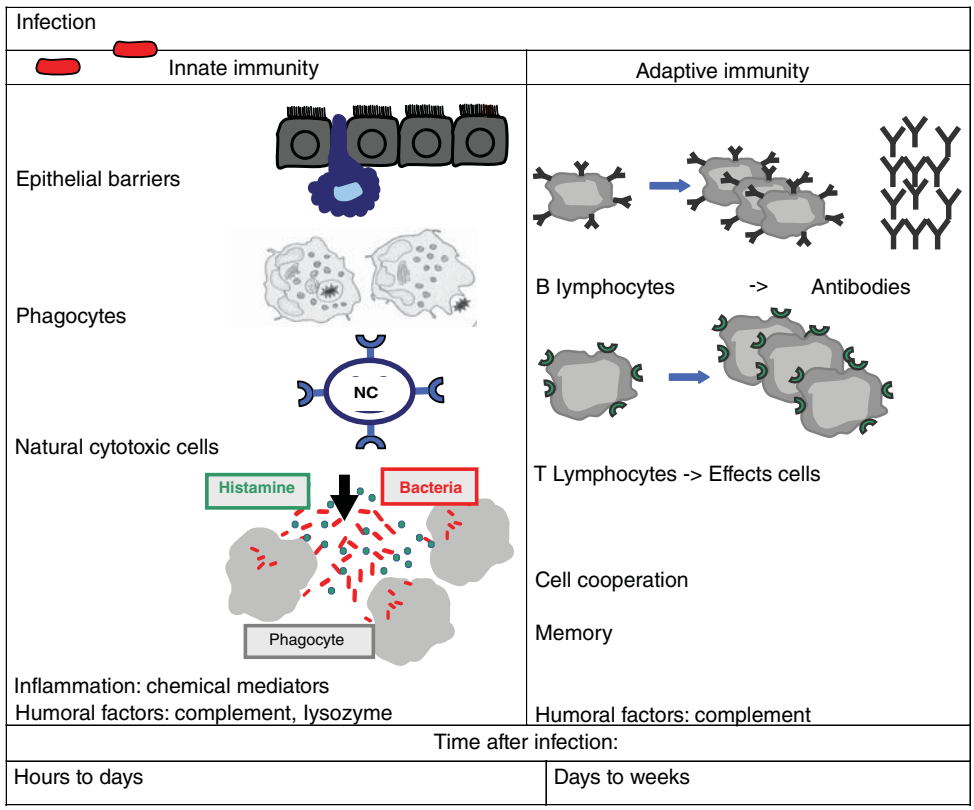

Figure 7.2 Distinction between innate and specific immunity. Source: V. Verlhac Trichet 2010. Nutrition and immunity: an update. Aquaculture Research 41(3): 356–372.

Dietary Nutrients, Additives and Fish Health, First Edition. Edited by Cheng-Sheng Lee, Chhorn Lim, Delbert Gatlin III, and Carl D. Webster.

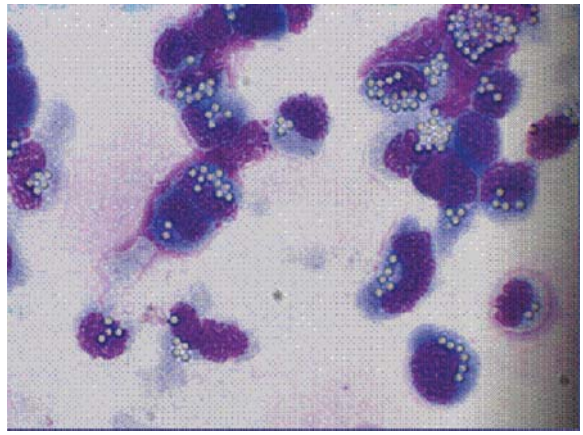

Figure 7.3 Phagocytosis of latex beads by trout macrophages.

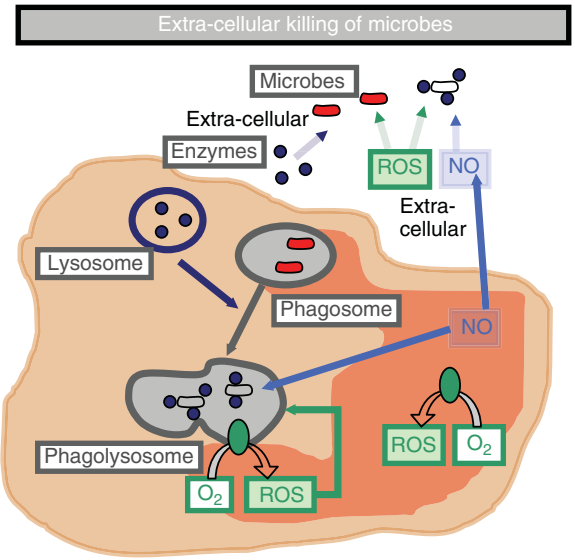

Figure 7.4 Microbicidal mechanisms of phagocytosis. ROS: reactive oxygen species. Source: V. Verlhac Trichet 2010. Nutrition and immunity: an update. Aquaculture Research 41(3): 356–372.

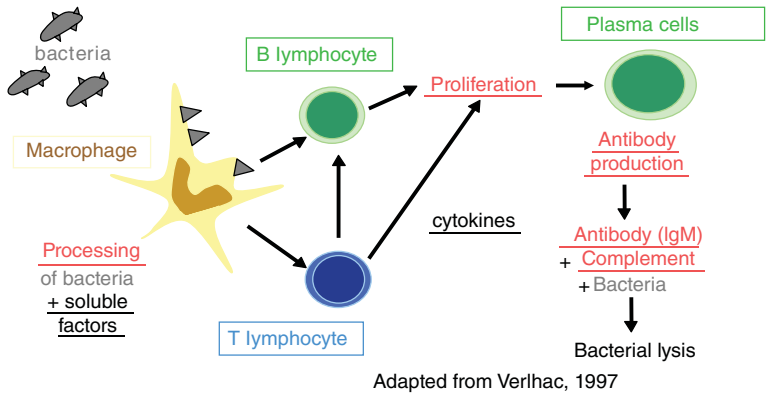

Figure 7.5 Specific immune response: cellular cooperation and antibody production.

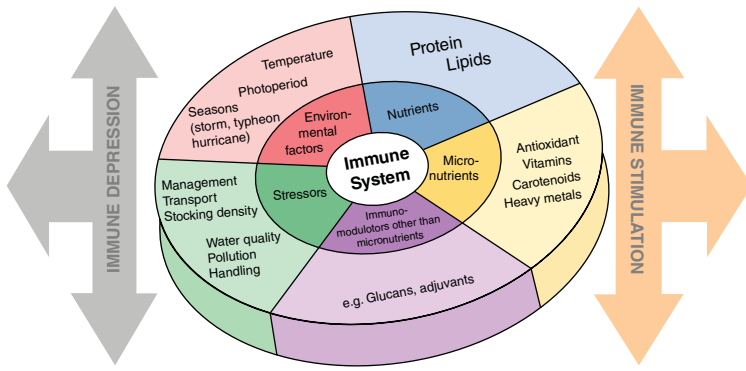

Figure 7.6 Factors influencing the immune response.

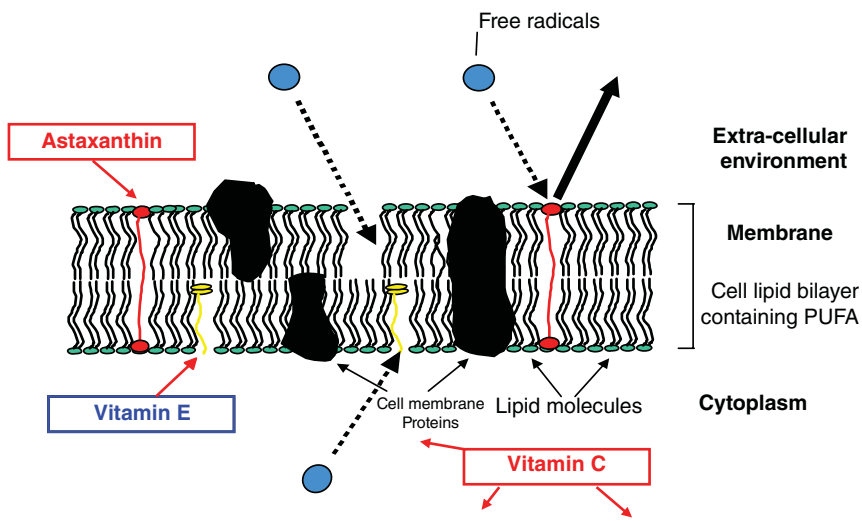

Figure 7.8 Antioxidant vitamins at cellular levels.

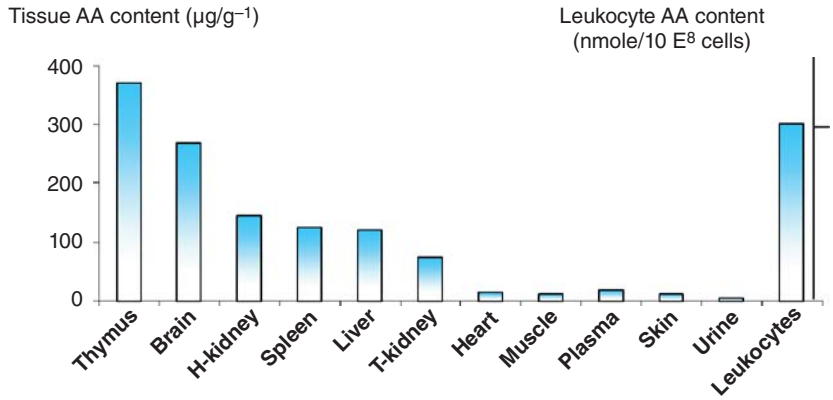

Figure 7.9 Ascorbic acid (AA) concentration in various tissues of rainbow trout fed a diet supplemented with 200 mg ascorbic acid (AA) per kilogram feed (Gabaudan and Verlhac, unpub. data, 1992).

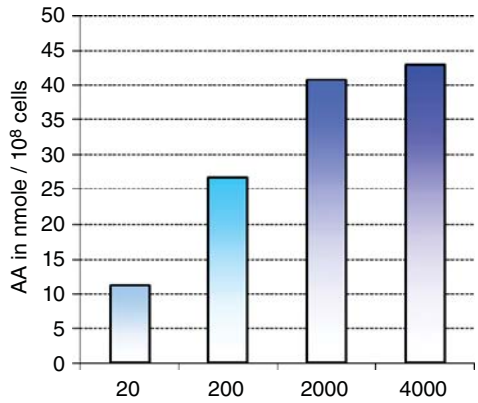

Figure 7.10 Influence of dietary intake of vitamin C on leucocyte ascorbic acid (AA) concentration in rainbow trout fed graded doses of vitamin C as ascorbate phosphate for four weeks (Verlhac, unpub. data).

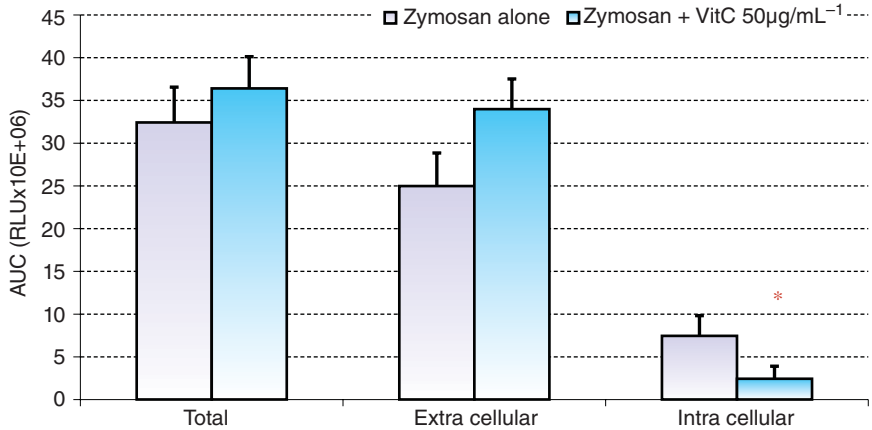

Figure 7.11 *In vitro* influence of ascorbic acid on total, extra- and intracellular oxidative burst of from rainbow trout phagocytes stimulated by zymosan particles (Verlhac et al., unpub. data).

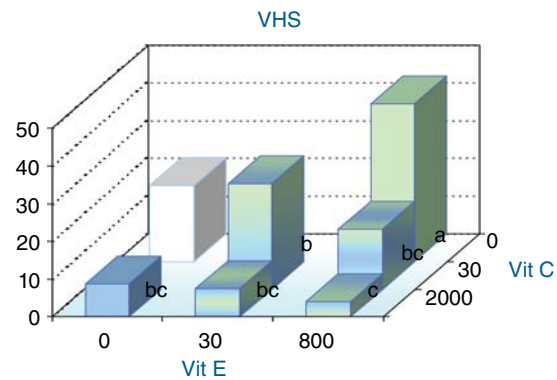

Figure 7.15 Influence of dietary combinations of vitamins C and E on resistance of rainbow trout to VHS virus infection. Source: T. Wahli, V. Verlhac, J. Gabaudan, W. Schüep and W. Meier 1998. Influence of combined vitamins C and E on non-specific immunity and disease resistance of rainbow trout (Oncorhynchus mykiss). Journal of Fish Diseases 21: 127–137.

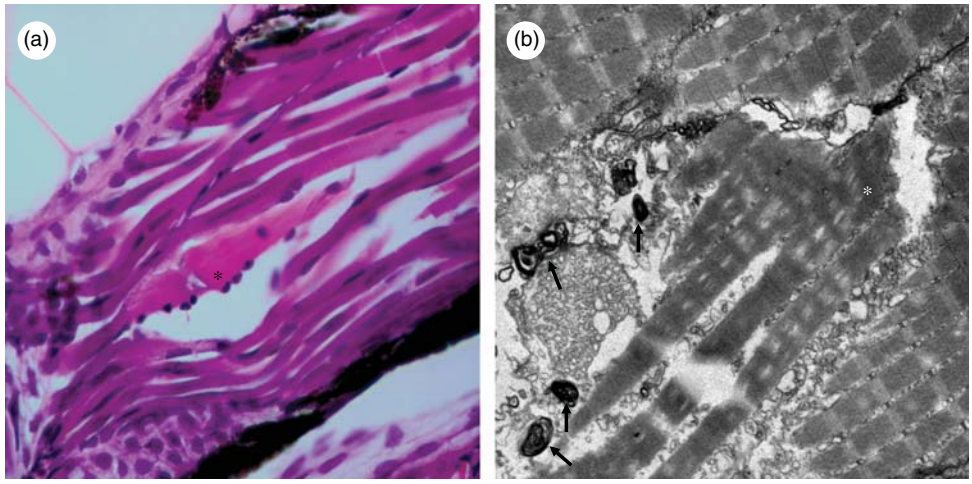

Figure 8.4 (a) Longitudinal optical microscopy and (b) transmission electron microscopy sections of sea bass larvae displaying mutirional muscular dystrophy. (a) Affected muscular swollen fiber (*) displacing the adjacent fibers and showing a marked eosinophilia. (b) Fragmentation of an affected muscle fiber (*) surrounded by myelin figures (arrow).

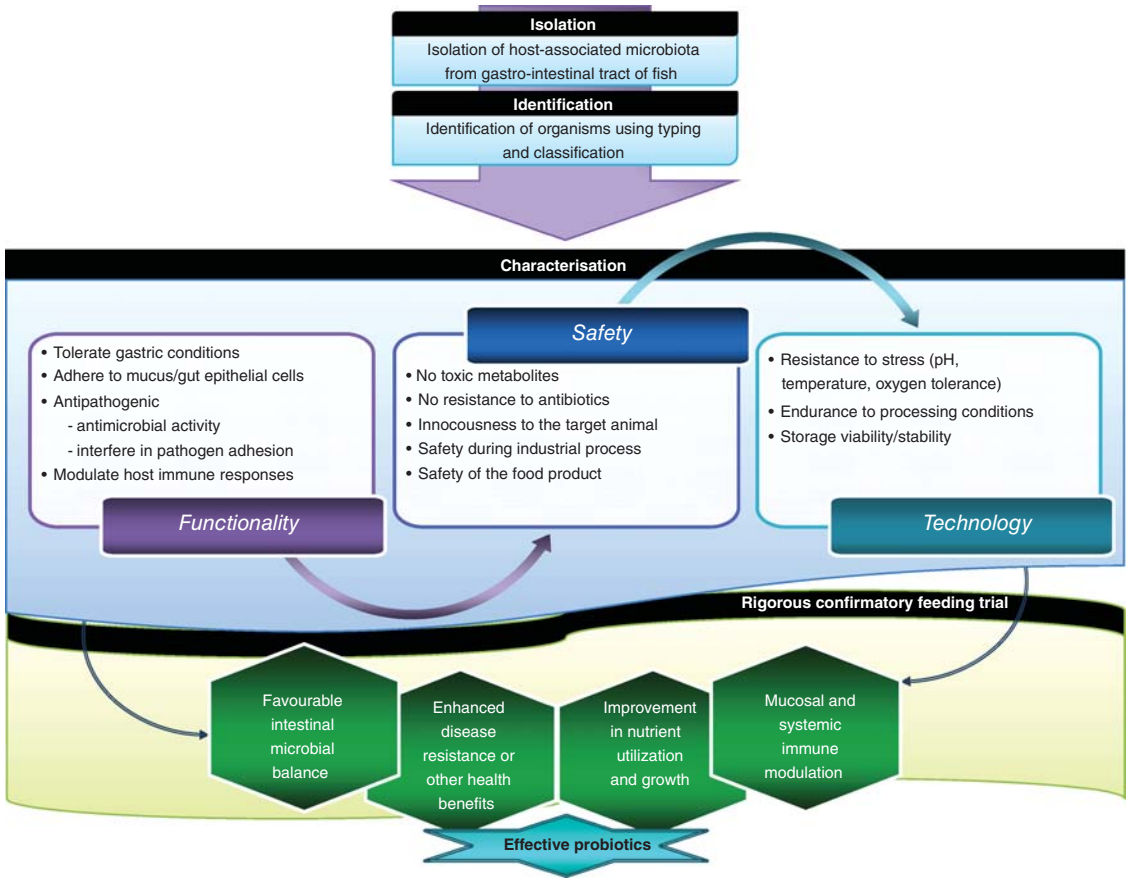

Figure 14.1 Pathway leading to the development of probiotics for farmed aquatic animals and the expected benefits in the target host upon their application as feed-delivered microbials. Microorganisms, ideally isolated from among the commensal microbiota of a target aquatic animal, are identified and characterized based on their functionality, safety, and technological suitability. A candidate probiotic has to be rigorously examined through controlled feeding studies to confirm the reproducibility of its observed beneficial effects. Confirmatory farm trials employing multiple batches of bulk-produced microorganisms should precede the commercialization of the probiont.

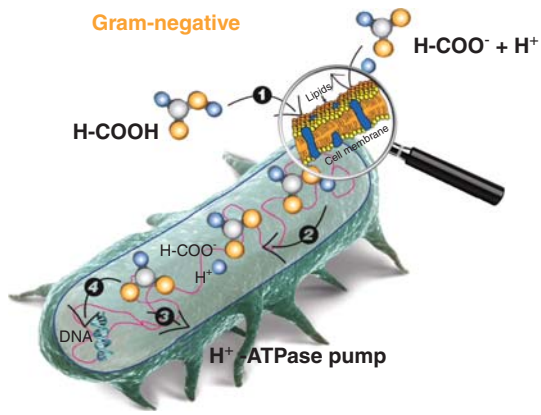

Figure 15.2 Action of organic acids against Gram-negative bacteria © ADDCON 2012.