

In the early days of aquaculture, nutritional pathology was limited to understanding of gross deficiency symptoms. Treatment of infectious diseases was limited to chemotherapy. As we gain insight on the physiological mechanisms relating nutrition and health, solutions to maintain the health of farmed fish are increasingly sought in the form of nutrients and additives that play a role in the immune system of animals. The preferred choice is to prevent diseases and promote sustainable farming rather than using chemotherapy to combat outbreaks. This article provides a fundamental understanding of the immune system and the role of nutrition in the immune modulation of aquatic animals.

Nutrition and immune modulation in aquatic animals

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In a recent column of the “Fish Vet's diary” (Fish Farming International, October 2004) Prof. Ronald J Roberts wrote “the significant vulnerability (of fish) to deficiency of vitamin C is somewhat surprising to the human pathologist, who is taught that only the great ape and the guinea pig are vulnerable to it.” He was referring to catfish in ponds that were “spinally twisted like hair curling implements”. This quote demonstrates how the science of nutrition carries the same philosophy across the different phyla: adequate nutrition is a pre-requisite for proper health.

The prime objective in any aquaculture operation is to optimize growth of the farmed organism and to have a quality product for the market. This objective can be materialized only if the organisms stay healthy throughout their life in the farm. Modern farming has brought in new challenges that threaten the health of farmed species: increased prevalence and spread of infectious diseases. Since chemotherapy is not a viable and sustainable option on the long-term, nutritional tools to maintain and manage health are increasingly sought after. There is wide recognition that control of diseases in aquaculture will be better achieved by prevention than cure. It is expected that feed should provide for better immunocompetence and disease resistance, besides satisfying the dietary nutrient requirements for maximum growth.

Since most farmed aquatic animals are dependent on formulated feeds, it is natural that nutritional aspects have a direct influence on their immunocompetence. Though malnutrition could logically be linked to poor immune status, in farming systems one must be concerned not with severe under-nourishment, but marginal or sub-optimal deficiencies that are often unnoticed. In general, the level of a nutrient required for better health could be over and above that reported for normal growth.

Immune system of aquatic animals

The immune system of aquatic animals can be divided into two categories:

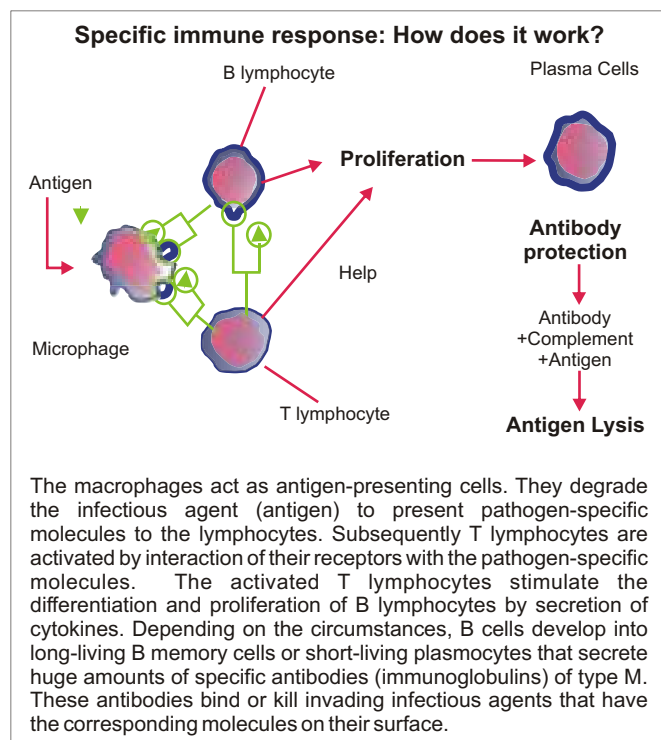
1. The **innate or non-specific immune response** prevents the entry of micro-organisms into tissues or, if they have gained entry, eliminates them prior to the occurrence of disease. It is characterized by a rapid deployment of humoral¹ and cellular defense mechanisms without prior recognition of the infectious agent. This type of response is observed in both invertebrate (molluscs and crustaceans) and vertebrate aquatic animals (fish).

2. The **acquired or specific immune response** is characterized by recognition of the infectious agent and the deployment of humoral and cellular mechanisms that are specifically directed against it. This type of response is found in fish although less developed than in mammals.

When an infectious agent penetrates the organism, non-specific defense mechanisms are stimulated. Their sole activation might be sufficient to stop the infection. If not, the infection would develop, leading to the induction of specific defense mechanisms (in the case of fish). The mechanisms (see box) then lead to the cure of the disease and the set-up of an immunological memory which would block the development of a new infection caused by the same pathogen. Vaccination takes advantage of the specific defense mechanism by intentionally introducing the pathogen in an inactive form to avoid infection. The introduction initiates a specific immune response to protect the organism against future infections for a certain period of time.

¹ Stricto sensus, in mammals, humoral immunity refers to antibody response. In fish it has somewhat been extended to all the soluble factors as well as humoral non-specific responses. Cytotoxic-T cells are not fully characterized in fish although some work has been done showing that this function exists in fish. Natural killer cells are linked with non-specific immune response. Fish lymphocytes can be divided into two categories as in mammals: B and T lymphocytes.

Fish versus shrimp: The major immunological difference between fish and shrimp is the apparent lack of specific defense mechanism in the latter. While both fish and shrimp share some non-specific defense mechanisms such as phagocytosis, there are differences as well.²



The non-specific immune response in fish is characterized by a first line of physical and biochemical defenses represented by the skin and the mucus. The mucus helps in preventing micro organisms from gaining access to the body through the integument, gills and gastrointestinal mucosa. It stops the adherence of bacteria to epithelial cells and contains several molecular components of the non-specific immune response.

In case the pathogen enters the body, macrophages and granulocytes and a number of soluble factors (Table 1) monitor and try to eliminate the pathogen. Some specialized cells called phagocytes ingest micro organisms and foreign particles in what is known as the phagocytic process and destroy them with the help of their enzymes. Macrophages and granulocytes are summoned to the sites of inflammation by their attraction to pro-inflammatory molecules such as interleukin 1. This phenomenon is called chemotaxis. The killing of bacteria is characterized by oxygen-dependent and -independent mechanisms. Following microbial invasion, phagocytes consume a high amount of oxygen to produce free radicals that are powerful microbicidal agents. Fish granulocytes kill extracellularly through the discharge of their hydrolytic and oxidizing enzymes rather than intracellularly via phagosome-lysosome fusion as in mammals.

Some components of the non-specific immune response in shrimp are significantly different from that of fish. Shrimp blood or hemolymph contains hemocyanin (O₂ carrier) and

² Understanding of the shrimp immune system has to advance vastly. There are several unanswered questions on the immune response of shrimp to various pathogens. For example, why the shrimps respond differently to bacterial and viral infections and how they develop a degree of immunity to viral infections. Discussion on the evolving concepts in shrimp immunity is out of the scope of this article. Interested readers are directed to a recent review on the topic by Loker et al. (2004).

soluble factors such as lectins and glycoproteins that can bind to surface molecules of pathogens. Lectins recognize the infectious agent, agglutinate it and make it ineffective. Hemocytes of shrimp then process the lectin-pathogen complex through phagocytosis. Other types of hemocytes encapsulate the foreign bodies. Lysozyme is used to digest the pathogens. Other key components of the crustacean immune system include the prophenoloxidase and the beta-1,3 glucan binding protein. The latter is mainly used in the recognition of antigens. Prophenoloxidase is present in the granules of hemocytes and is released by degranulation induced by beta-glucans, peptidoglycans and lipopolysaccharides. The protein is then cleaved in the plasma by a serine protease to form phenoloxidase. In its active form the phenol oxidase catalyses the oxidation of phenols to semi-quinones and quinones which due to their high reactivity, kill the microbes.

Table 1: Non-specific soluble factors in fish

Factor	Molecule	Mode of action
Lectins	Glycoproteins Natural agglutinins	Recognition, precipitation, agglutination. Neutralising bacterial components released by pathogens
Acute-phase proteins	C-reactive protein Ceruloplasmin	React with molecules at the cell surface of micro-organisms and acts as an opsonisation factor to facilitate phagocytosis or activate the complement system
Lytic enzymes	Lysozyme	Splits mucopolysaccharides from bacterial cell walls and lead to the destruction of pathogens
Iron-binding proteins	Transferrin Lactoferrin	Protective role in fish, as an iron-binding protein that limits the amount of free iron in the bloodstream, making it unavailable for bacteria during infection
Complement	Cascade of enzymes	Alternative pathway of activation related to non-specific immunity
Interferons	Proteins	Produced during viral infections and increase the resistance of cells to viral invasion

Factors influencing immune response: The development of immune responses in aquatic animals can be influenced by several factors (Table 2). Nutrients can exert influence either directly on the immune system or indirectly through diet induced alterations in metabolism that could be hormonal, neural etc. The environmental conditions, particularly the water temperature and the light regimes have a significant role in the physiological responses of the aquatic animals. Studies on nutritional immunology should account for these factors in either experimental design or interpretation of results.

Orally deliverable immune modulators

Both macronutrients and micronutrients influence the resistance of aquatic animals to viruses, bacteria, fungi and parasites. Additionally, there are immune modulating compounds that are not essentially nutrients, but are conveniently delivered orally to the aquatic animals.

Table 2: Factors that influence the immune responses in aquatic animals

Factor	Influence
Environment (Temperature, season)	Slows down metabolic process at low temperatures. Decreases cell membrane fluidity and permeability, receptor and enzyme activity, cellular cooperation
Stressors (Handling, crowding)	Induces immunodepression as secondary stress reaction, cell depletion
Pollution (Water quality, heavy metals)	Effects depend on nature of the substance. Can induce a stress response. Drugs can also be immunosuppressive
Nutrition (Feed quality, nutrient availability, antinutritional factors)	A well-balanced diet and a planned feeding regime are essential for adequate host defense mechanisms
Pathogen	The type, the virulence and the level of infection influence the capacity of the animal to counter the pathogen
Animal	Individual variation, age, species or strains
Genetics	Differences in capacity to develop non-specific or specific immune response

Lipids: The beneficial effect of polyunsaturated fatty acids (PUFA) particularly of the n-3 series have been well documented in fish and other animals. However, PUFA are extremely prone to oxidation. At the cellular level, several nutrients act in concert to prevent the oxidative damage. Particularly important are antioxidant vitamins and carotenoids.

Carbohydrates: Dietary carbohydrates play a role in the immune responses through their interactions with the intestinal bacterial flora and the gut-associated lymphoid tissue in fish. The insoluble dietary fibers can trap pathogenic bacteria and prevent their access to gut mucosa. On the other hand, recent attempts in fish include the use of fructooligosaccharides as a prebiotic, in combination with probiotics to achieve long-term health benefits via the gastro intestinal immune system.

Antioxidant vitamins and carotenoids: Ascorbic acid plays a major role by protecting the cells from oxidative damage. Indeed, high concentrations of ascorbic acid were measured in phagocytes and lymphocytes. A positive correlation between the concentration and cellular activities has also been established. Vitamin E concentration in the immune cells is also related to its dietary intake as well as on lipid level in the diet. Dietary vitamin E is partly used to protect the cellular lipids (including those of the immune cells) from peroxidation. It is present in the membranes and is important for their fluidity and the receptor-based functioning of the immune response. Carotenoid pigments are potent antioxidants. Antioxidant vitamins at elevated doses are generally regarded as short-term boosters of the immune response. They also play an important role in the intercellular communication, which is vital for the coordination of all biological activities in tissues.

Beta-1,3/1,6 glucans: These are polysaccharides extracted from yeast cell walls. Their immunostimulating function is linked to the presence of specific receptors at the surface of macrophages and other phagocytic cells of fish. In shrimp, it is linked to the presence of beta-1,3 glucan binding proteins,

which specifically recognize this molecule. The oral delivery of beta-1, 3/1,6 glucan leads to significant increase in resistance to diseases in both fish and shrimp.

Nucleotides: These are low molecular weight compounds that are important in almost all biological processes. They are particularly important in production of new cells since they supply the building blocks of nucleic acids. Though nucleotides are synthesized or salvaged by organisms, the rate of nucleotide generation by the body may be insufficient to meet the requirement at certain lifestages and during times of stress and disease. At such times, dietary delivery of nucleotides is probably essential. In fish, clear benefits have been demonstrated on disease resistance and selected immune parameters. Early results in shrimp show a positive effect on haemocyte counts and prophenoloxidase activity. It is also noteworthy that some changes in feed formulation such as fishmeal replacement reduce the amount of nucleotides available from the diet, requiring the addition of nucleotides from another source.

Lactoferrin: This is an iron-binding protein that has been linked to host defense against primary infection at mucosal surfaces through its antibacterial properties. It is also associated with inflammation and immunomodulation processes, including regulation of phagocyte activity and lymphocyte proliferation. It may be digested into small cationic domains that exhibit enhanced anti-microbial activities. It may also be an important prophylactic tool because of its direct bacteriostatic effect at the intestine level. In mammals, lactoferrin is found mainly in neutrophils. In fish, a protein with similar characteristics has been identified. Dietary supplementation of the protein has been shown to enhance disease resistance in both fish and shrimp. However, further work has to be done in order to define the effective dose.

Probiotics: The use of antagonistic bacteria to control populations of potential pathogenic strains through competitive exclusion or enhancement of immunity of the host is of growing interest. An increasing number of probiotic bacteria are being discovered for farmed aquatic animals. The bacteria could be delivered orally either through live feed for the larval stages or along with formulated feeds for the larger sizes. Probiotics have the advantage of a long-term effect as opposed to other immune modulators.

Antimicrobial peptides: Gene-encoded, cationic antimicrobial peptides have been identified in virtually all species of life. Many of these peptides have been shown to play roles in host defense, providing local non-specific protection against infectious microbes. They are characterized by a broad spectrum of activity against bacteria, fungi, and viruses. Examples of cationic antimicrobial peptides are the cecropins, melittins, magainin, and defensins. A new family of antimicrobial peptides has been identified in shrimp: penaeidins. Research is being carried out on their potential in controlling disease by analyzing the expression of the encoding genes in response to infections. Antimicrobial peptides experimentally injected to coho salmon lead to better survival against *Vibrio anguillarum*. Although a lot of research has to be done, these antimicrobial peptides constitute an interesting alternative to the use of antibiotics in the control of diseases.

Others: Herbs, plant extracts, polyphenols, flavonoids, fucoidans are substances that are being promoted as nutraceuticals for humans and are of interest for development as aqua feed additives.

Evaluating the efficacy of immune modulators

Immune response involves a broad range of molecules, cells and other modulating factors. When evaluating an immune modulator, it is important to measure various aspects of the response. It is noteworthy that the immune modulators described above have various modes of action, but response parameters that are measured are often the same. Table 3 shows various parameters and their sensitivity to immune stimulation.

In fish, alternative complement activity is a fairly reliable parameter of humoral defense, though its measurement involves a tedious process in arriving at the lytic values. Phagocytic functions (phagocytosis and oxidative burst) are of great importance since phagocytes and hemocytes are major players in the response of fish and shrimp, respectively. The phagocytic activity is usually determined by the ingestion of inert or antigenic particles. This could be measured either by microscopy or flow cytometry. While the former is a simpler technique, the latter is preferred for its sensitivity. The phagocyte killing mechanisms can be assayed among others by measuring the oxygen radical production either spectrophotometrically or by flow cytometry.

The specific immune responses can be evaluated by measuring the antibody production either by the relatively simple technique of passive hemolytic plaque assay or more quantitatively by determining the antibody titres by ELISA technique. Though the latter method measures a response fairly

accurately, it has to be standardized thoroughly for the species and pathogen in question.

The best measure of a total response of the immune system of an animal is the disease-challenge (artificial infection) study. Though both nutrients and non-nutrients have been tested by this means, the results obtained are often questionable for their repeatability. The design and conduct of such experiments is a great challenge and should be performed by professionals under well-defined standard conditions.

The major issue one might encounter in studies on the immune system where several lots of animals have to be compared is the variability in the results within an identically treated group. Tests should be performed with a large number of individuals in a group to reduce the influence of individual variations. In a unique experimental design, Gullian et al. (2004) evaluated the efficacy of an immune stimulant in shrimp by calculating an individual immunity index based on five parameters. They also took into account the deviation of the individual and deviation of the test from historical data. This mode of evaluation could be adopted, taking into consideration factors such as survival and performance of the animals in deciding the efficacy of dietary immune modulators.

Systemic versus mucosal immune response: As a majority of immune stimulants are applied via feed, their first contact with macrophages would be at the gut mucosa. Consequently, the investigation of the immune response at the

Table 3: Measures of immune responses and their sensitivity

Parameters	Methodology	Determination	Sensitivity of the parameter to immune stimulation
Chemotaxis	Migration test in specific chambers and macroscopic observation	Migration index	High
Phagocytosis - ingestion of yeasts - ingestion of latex beads - ingestion of bacteria	- Staining and microscopic observation - Flow cytometry - Fluorescence	- Phagocytic activity/index - Phagocytic activity/index - No precise quantification	High Medium
Oxidative burst - intra cellular - extra cellular - Total	-Flow cytometry -Chemiluminescence with isoluminol -Chemiluminescence with luminol or colorimetric assay	Reactive oxygen species and free radical production	High
Lymphocyte proliferation induced by mitogen or antigen-specific	Uptake of tritiated thymidine Flow cytometry Tetrazolium salts	Stimulation index	High
Non specific cytotoxicity (NCC)	Chromium release assay Time resolved fluorescence Flow cytometry LDH assay	Percentage lysis of target cells	Fair
Lysozyme	Turbidimetry Fluorimetry	Units: decrease in optical density (plasma, tissue, mucus)	Medium
Complement - alternative pathway (non specific IR) - classical pathway (specific IR)	Spectrophotometry	CH50 units determined using a 50% hemolytic assay	High
Specific antibody	ELISA Passive hemolytic assay	Antibody levels or titers Plaque counts	High
Total immunoglobulin	Spectrophotometry	Molecular weight based precipitation	Low

Footnote: The sensitivity of the parameter to the immune stimulation depends on vaccination status and environmental factors. Our evaluation refers to fish grown under controlled conditions and not immunised. IR: immune response

gut level should be considered as very important. The efficacy of oral vaccination is also dependent on the knowledge on gut immunity and how it can be stimulated.

Perspectives

Disease prevention through dietary immune modulation is a strategy that would become more widely recognized as codes of conduct in aquaculture place further constraints on chemotherapy. Improved vaccine efficiency would also become more important and derive synergies from dietary immune modulation.

Efforts to further understand the mechanism of how nutrients impact immune modulation are needed. It would also be worth to investigate the synergistic effects of the more effective components in order to maintain disease resistant stocks of farmed animals.

A species-based approach, considering the life-stage (e.g. maturity) as well as environmental factors (e.g. temperature, salinity) will be necessary.

Many of the immunoassays currently being employed require standardization across laboratories if comparative values are aimed at. Since much of the knowledge on immune system is fragmentary, it would be ideal to have working disease model systems that could be effectively used in closely observing the nutrient effects. Molecular biological investigations are increasingly becoming important, along with the selection of aquatic animals for disease resistance. Some of the less-studied areas include mucosal immunology and skin protection mechanisms, and defence against parasites. Several of these issues linked to the immune system are influenced by nutrition. ■

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