Immunostimulants: Towards Temporary Prevention of Diseases in Marine Fish

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Abstract

Due over the last years, marine aquaculture has grown into a very significant industry in many parts of the world, and in most developed countries marine fish are farmed intensively under conditions of high population density, infectious diseases pose a constant and highly cost threat to successful animal husbandry. Antibiotics provide a useful means of helping to control many bacterial diseases but there are many problems associated with the development of antibiotic resistance and recurrent outbreaks necessitating further, costly, treatments. Immunostimulants especially when administered through the diet have been perceived as potentially playing an important role in aquaculture. Efficiencies and strategic use of immunostimulation methods are presented and dose-effects between species analyzed. In addition, updated reviews of effects reported by several authors with nutritional and non-nutritional factors tested as immunostimulants in marine fish are presented; and those recent findings on the wide range of humoral and cellular innate immune responses affected by immunostimulation summarized. We conclude that actual knowledge of potential immunostimulants is still obscure in several aspects, especially in those related to pathways and mechanisms in which such substances can reach their specific cells targets. Nevertheless, immunostimulants as diet supplement, especially those of non-nutritional origin, should be good choice to induce a brief disease resistance enhancement in marine fish; although, methods and selected assess immune responses should be standardized between researchers in the aim of understanding better the complexity of disease resistance and immune function to make easier the development of these therapies until the production scale and not just as mare laboratory trials.

Key words: Immune system, immune tissue, marine fish, immunostimulants, immunostimulation methods.

Running title: Immunostimulants for marine fish

Introduction

Marine aquaculture represents one of the fastest growing food producing sectors and in the aim to increase productivity per unit space fishes are usually cultured in narrow spaces such as ponds or net cages under high densities, thus overcrowding trends to adversely affect the health of cultured fish making them a feasible target to infectious diseases, as a consequence, several studies have looked into the modulation of the fish immune system in order to prevent the outbreak of diseases as reviewed recently by Sakai (1999). The state of being immune is defined as inherited ability to resist infection, then, immunity is the result of the recognition of non-self or a foreign agent, with the subsequent response and memory in vertebrate animals. The response includes expansion of cells for the immune response, expression of the cells and molecules, and, finally, the coordination of the response by regulatory substances. Disease resistance is the innate defense mechanisms of an animal against foreign invaders.

Figure 1, is a schematic representation of the result of the response to a pathogen by fish. The study of fish immunity and disease resistance is relatively young in comparison to the study of mammalian immunity. Most early research on the fish immunology focused on the comparative aspect of the immune system with fish and other species. Nevertheless, recently research has focused on understanding how the fish immune system responds to foreign agents or how innate resistance can be selected by breeding to produce stock of fish with superior disease resistance.

Many chemical entities, either naturally occurring or synthetic are known to stimulate the vertebrate immune system. Between substances reported to be effective as immunostimulants in fish are included chemical agents, bacterial components, polysaccharides, animal or plant extracts, nutritional factors and cytokines (Raa 1996; Sakai 1999; Sealey and Gatlin III 2001). An important point to have in mind is that immunostimulants increase resistance to infectious disease, not by enhancing the acquired immune response, but by enhancing innate humoral and cellular defense mechanisms. It is well known that fish depends more heavily on nonspecific defense mechanism than do mammals (Anderson 1992). Among the nonspecific defense mechanisms important in fish are the “barriers in place”, such as the skin and scales, and lytic enzymes of the mucus and sera; cellular aspects include monocytes, macrophages, neutrophils, and cytotoxic cells (Secombes 1990).

In order to research the humoral and cellular component activations it is necessary to study a number of biologically relevant assays such as complement activity, lysozyme production, phagocytosis, chemotaxis or the generation of microbicidal products like reactive oxygen species (ROS), due those are good choice for monitoring whether the innate immune system is activated; since clearly they contribute directly to any increased killing activity and the way to analyze such responses are relatively simple.

(Shoemaker 2001).

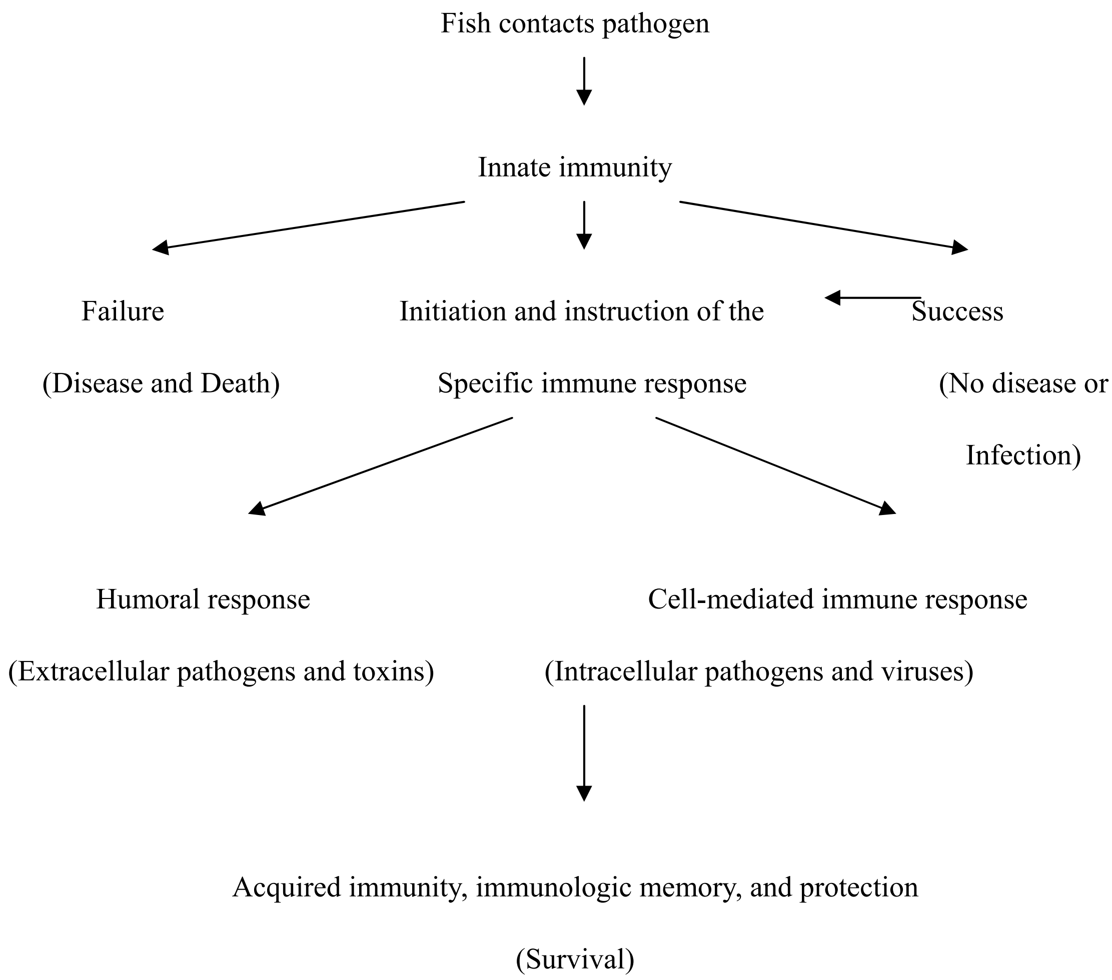

Fig. 1. Schematic representation of the response of a fish following an encounter with a pathogen

Authors like Landolt 1989, Blazer 1992 and Waagbø 1994 reviewed the importance of diet in fish immune response and such authors concluded that potential of dietary enhancement of disease resistance in fish culture certainly exists. Mechanisms involved remain as yet rather obscure, although some information exists.

The aim of this paper was to present the latest findings reported after immunostimulant administration to several marine fish; as well as describe briefly most of the innate cellular and humoral responses affected by such treatments; results are compared and conclusions presented.

How to Describe Immunostimulants

By definition, an immunostimulant is a chemical, drug, stressor, or action that enhances the innate or non-specific immune response by interacting directly with cells of the system activating them. In practice, immunostimulants are promising dietary supplements to potentially aid in disease control of several organisms including marine fish and increase disease resistance by causing up regulation of host defense mechanisms against opportunistic pathogen microorganisms in the environment.

Immunostimulatory compounds are often grouped by either function or origin and consist of a heterogeneous group (Anderson 1992). Non-nutritive compounds that have been examined most frequently for their ability to increase the nonspecific immune response of fish include several substances such as β-glucan, peptidoglycan or LPS. Nevertheless, animal-derived products like chitin (Sakai et *al*. 1992; Siwick, et *al.*1994), abalone extract (Sakai et *al*. 1991), bacterial-derived products such as muramyl dipeptide (MDP), alginates (Fujiki et *al*. 1997) or spirulina (Duncan and Klesius 1996b) also have been examined (Table 1). Much of the research on immunostimulants to date has focused on routes of administration other than through the diet, but information is presented here to indicate the potential application of these products as dietary supplements and the need for further research in the area of their oral administration.

Table 1 Immunostimulants tested for marine and freshwater fishes

Groups	Substances
Biological substances	Animal and plant extracts: EF-203 (Chicken), Ete (Tunicate), Hde (Abalone), Firefly squid, *Quillaja saponica* (Soap tree), Glycyrrhizin (Licorice), Laminaran (Seaweed) Bacterial derivatives: Peptidoglycan, B-glucan (MacroGard, VitaStim, SSG, Eco-Activa, Betafectin), FCA,EF-203, Lypopolysaccaride (LPS), *Clostridium butyricum* cells, *Achromobacter stenohalis* cells, *Vibrio anguillarum* cells Hormones, cytokines and others: Growth hormone, Interferon, Interleuckin-2, Lactoferrin, Nucleotides, Prolactine, TNF Nutritional factors: Vitamin C, E, A, Nucleotides, Trace elements (Zinc, Iron, Copper, Selenium), Protein, Carbohydrate Polysaccarides: Chitin, Chitosan, Lentinian, Oligosaccaride, Sclerotium, Schizophyllan
Synthetic chemicals	Avridine, Bestatin, DW-2929, FK-156, FK-565, Fluoro-quindone, Freund's adjuvant, Isoprinosine, Levamisole, Muramyl dipeptide (MDP),

In fish, after immunostimulation research, from among the responses that are routinely reported are macrophage activation, increased phagocytosis by neutrophils and monocytes, increased lymphocyte numbers, increased serum immunoglobulins, and increased lysozyme (Secombes 1990; Raa 1996; Sakai 1999; Sealey and Gatlin III 2001). Immunostimulants which are effective in fish diets in a laboratory setting act within the nonspecific immune system at several levels. The first immune system defenses are substances found in mucus secreted by endothelial cells, macrophages attacking pathogens directly, and 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 non-virulent microorganisms or their by-products. The compounds are recognized by the cellular components of the nonspecific immune system and initiate the same humoral and cellular response as pathogenic organisms. 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 diet is based on the evolutionary history of immune system development in aquatic organisms (Manning et *al*. 1982). Survival in the aquatic environment requires an immune system that can combat the constant challenge of waterborne pathogens. Immunomodulators present in the diet stimulate the nonspecific immune system, while antigenic substances such as bactrins or vaccines initiate the more prolonged process of antibody production and acquired immunity. Aquatic organisms evolved immediate, generalized responses to compensate for the continual exposure and delayed, specific response that require time for acquired immunity to develop. Fish immunologists have concentrated their investigations of immunostimulation on laboratory research designed to explain the actions of individual immune response components to immunomodulation. Responses have generally been associated with innate immune system, although antigen-antibody based enhancement has been reported (Raa 1996; Sakai 1999).

Use of immunostimulants is a unique approach for fish culturists as they undertake methods of controlling disease losses in their facilities. The interest in using this approach is heightened by the problems of viral, bacterial, parasitic, and fungal diseases that are limiting factors in culture at many fish farms, hatcheries, and aquaculture stations.

More over, a serious problem is that few approved chemotherapeutics agents are available for use in food fish because of growing concerns for consumer liability and for accumulation of substances in the environment. Use of antibiotics (Terramycine, Sulfadimethoxine or Ormetoprim) in fisheries is extensive, and there is concern about increases in antibiotic-resistant strains of bacteria in the aquatic environment surrounding locations where the drugs are used. Indeed, while these antibiotics are often effective in the treatment or control of some diseases agents, additional methods are needed to control these and other fish diseases. Problems with present antibiotic, drug, and chemical treatments to prevent diseases in fish, set the stage for this newly concept in disease prevention.

Efficiencies and strategic use of immunostimulation methods

Different farm circumstances have given rise to a number of different methods of administering immunostimulants. The basic methodologies adopted are injection, immersion and oral. Injection and immersion methods are suitable only for intensive aquaculture and both require the fish to be handled or at least confined in a small space during the procedures. Many authors reported that injection of immunostimulants enhances the function of leucocytes and protection against pathogens (see table 3 and 4). However, this method is labor intensive, relatively time-consuming and becomes impractical when fish weigh less than 15g. By immersion, efficacies had been demonstrated by several authors (Baba et al. 1993; Anderson *et al*. 1996; Jeney and

Anderson 1993a), although, since dilution, exposure time and levels of efficacy are not well defined, caution must be taken in account by applying this method. Oral administration is the only method economically suited to extensive aquaculture, is non-stressful and allows mass administration regardless of fish size, but of course, can be administered only in artificial diet.

Table 2 Administration methods of immunostimulants to fish

Route	Immunostimulant dose	Exposure time
Injection	variable	1 or 2 doses
Immersion	2 – 10 mg/l	10 min to hours
Oral	0.01 – 4 %	Some days or longer

After analyzing some research trials results in which several substances were tested as immunostimulants, suggested dose and exposure times as well as relative advantages and limitations to achieve the best immunostimulation are summarized in tables 2 and 3.

Table 3 Summary of advantages and limitations of immunostimulation methods

Method	Advantages	Limitations
Injection	Most potent immunization route. Allows use of adjuvants. Most cost effective method for large fish.	Useful only in intensive aquaculture. Labor hard. Stressfully (anesthesia; handling). Fish must be > 10 ~ 15g.
Immersion	Allows mass immunostimulation of small (< 5 g) fish. Most cost effective method for small fish. Bath: not stressful.	Only for intensive aquaculture. Dip rise handling stress. Potency not as high as injection route.
Oral	Only non-stressful method for aquaculture. Allows mass immunostimulation of fish any size. No	Poor potency. Requires large amounts of immunostimulants to achieve protection. Suitable only for

extra labor costs.	fish fed artificial diet.

Evaluation methods for immunostimulants

There are two main procedures for evaluating the efficacy of an immunostimulant: (a) *in vivo*, such as protection test against fish pathogens; (b) *in vitro*, such as the measurement of the efficiency of cellular and humoral immune mechanisms. Protection tests against fish pathogens are currently used with many successful results, as shown in Tables 4 and 5. These experiments have shown a relevant increase of immune response and/or resistance to several pathogens in marine fish, suggesting a relevant role in aquaculture. Knowledge of the immune system is, however, very limited for most fish species, and information on the mode of action of most immunostimulatory substances is even more restricted. The evaluation of an immunostimulant by the *in vitro* methods which test the effects of that substance on the immune system is to be preferred in preliminary studies. Nevertheless, if possible *in vitro* tests should be performed together with *in vivo* experiments in order to elucidate the basic mechanisms responsible for the protection. *In vitro* evaluation should be based at least on the following parameters: serum lysozyme, complement, total leucocytes and erythrocytes count, respiratory burst phagocytosis, chemotaxis, chemokinesis, and lymphocyte proliferation. Some other recommended parameters to be measured are: C-reactive protein, natural cytotoxic activity, and MAF. Techniques involved goes from relatively simple and inexpensive methods to the use of immunoassays, flow cytometry or bio-molecular approaches.

Table 4 Updated review of nutritional factors tested as immunostimulants in marine fish

Spp.	Immunostimulant	Dose	Immune response	Disease resistance	Reference
Atlantic salmon *(Salmo salar)*					
	PUFA's	300 - 5200	No change	Yes	Thompson *et al.* 1996
	Protein hydrolisate	1 - 25 In vitro	ROS		Gildberg *et al.* 1996
	Bovine lactoferrin + Vitamin C	140	No change	No	Lygren *et al.* 1999
	Vitamin C	50 - 2000	No change		Lall 1988
		5000	No effect on antibody production	No	Sandnes *et al.* 1990
		2980	Antibody increase		Erdal *et al.* 1991
	Ascorbil 2-sulfate	4770		No	Erdal *et al.* 1991
		2750	Complement		Hardie *et al.* 1991
		Megadose		No	Lall and Olivier 1993
		82 - 3170	Antibody increase		Thompson *et al* . 1993
		4000	Lysozyme	Yes	Waagbo *et al.* 1993
		1000	ROS, Lymphocyte		Verlhac and Gabaudan 1994
	Ascorbate 2-monophospate	20 -1000	No change		Waagbo *et al.* 1996
	Vitamin E	> requirement		No	Lall 1988
		800	No change		Hardie *et al.* 1990
	Piridoxine	5	No change	No	Lall and Weerakoon 1990
Chinook salmon *(Oncorhynchus tshawytscha)*	Vitamin C	2500		No	Leith and Kaatari 1989
	Vitamin E	> requirement		No	Leith and Kaatari 1989
		300		No	Thorarinsson et al. 1994
	Pyridoxine	> requirement		Yes	Hardy *et al.* 1979
		> requirement	No change	No	Leith and Kaatari 1989
	Riboflavin	> requirement	No change	No	Leith and Kaatari 1989
	Panthothenic acid	> requirement	No change	No	Leith and Kaatari 1989
	Folic acid	> requirement	No change	No	Leith and Kaatari 1989
Coho salmon *(Oncorhynchus kisutch)*	Vitamin C	400 - 1000	Improved wound healing		Halver 1972
Gilthead sea bream *(Sparus aurata)*	Vitamin C + Vitamin E	2900 1200	Lysozyme and NCCS		Cuesta *et al.* 2002
	Vitamin C + Vitamin E	Many concentrations In vitro	Migration, Phagocytic ROS (Mix)		Mulero *et al.* 1998
	Vitamin A (retinol)	50 - 300	ROS		Cuesta *et al.* 2002
	α -tocopherol	600 - 1800	Complement		Ortuno *et al.* 2000
	Vitamin C	3000	Phagocytic, ROS, Complement		Ortuno *et al.* 1999
Japanese flounder *(Paralychtys olivaceus)*	Axtahantin	100	Chemotaxis, NBT	Yes	Galindo-Villegas *et al.* 2002
	Vitamin C	6100	NBT	Yes	Galindo-Villegas *et al.* 2002
	Vitamin E	600	Lysozime, Phagocytic	Yes	Galindo-Villegas *et al* . 2002
	Arginine	150	NBT, Lysozime	No	Galindo-Villegas *et al.* 2002
Red sea bream *(Pagrus major)*	Vitamin C	10000	Phagocytic		Yano *et al.* 1990
Sockey salmon *(Oncorhynchus nerka)*	Vitamin C	> requirement	No change		Bell *et al.* 1984
Turbot *(Scopthalmus maximus)*	Vitamin C	300 - 2000	Phagocytic, Lysozyme		Roberts *et al.* 1995
	Vitamin E	500	Phagocytic		Pulsford 1995
Yellow tail *(Seriola quinqueradiata)*	α -tocopherol	120 - 880		Yes	Ito *et al.* 1999
	α -tocopherol acetate	119 - 5950	Lysozyme, phagocytic	Yes	Hosokawa 2000
	Vitamin C	122 - 6100	Lysozyme, phagocytic	Yes	Hosokawa 2000
		2%		Yes	Ito *et al.* 2000

Table 5 Updated, non-nutritional factors tested as immunostimulants in marine fish

Spp.	Immunostimulant	Dose	Immune response	Disease resistance	Reference
Atlantic salmon					
(Salmo salar)		15 mg/kg; inj	ROS, Lysosomal		
	B-glucan	150 mg/kg; oral and anal	acid phosphatase		Dalmo *et al.* 1996
		1 ml/ fish; inj.	Lysozyme, complement		Engstad *et al.*. 1992
		1 ml/ fish; inj.	Antibody production		Aaker *et al.* 1994
		50 - 200 ug/kg; inj.		Yes	Dalmo *et al* . 1996
	B-glucan	1 - 250 ug/ml			
	+ LPS	10 ug/ml	Lysozyme		Paulsen *et al.* 2001
	B-glucan				
	+ FKC	0.5 mg/fish; inj.		Yes	Rorstad *et al.* 1993
	IFA	0.1 ml/fish; inj.		Yes	Olivier *et al.* 1985
			ROS, phagocytic and		
	Levamisole	2.5 mg/l; bath	lysozyme		Findlay *et al.* 2000
Coho salmon					
(Oncorhynchus kisutch)	B-glucan	5 and 15 mg/kg; inj.	No change		Nikl *et al.* 1991
	Levamisole	5 mg/kg; inj.	No change	No	Nikl *et al.* 1991
		0.1 ml/fish; inj.		Yes	Olivier *et al.* 1985
	DID	12.5 mg/kg; inj.	No change	No	Nikl *et al.* 1991
	MCFA	5 mg/kg; inj.	No change	No	Nikl *et al.* 1991
		5 mg/kg; inj.		Yes	Olivier *et al.* 1985
	WY-18, 251	10 mg/kg; inj.	No change	No	Nikl *et al.* 1991
	MDP	50 ug/kg; inj.		No	Nikl *et al.* 1991
Sockey salmon					
(Oncorhynchus nerka)	CFA	5 mg/ kg; inj	Antibody production		Cipriano and Pyle 1985
Gilthead sea bream					
(Sparus aurata)	B-glucan	500 ug/ml; *i.v*.		Yes	Mulero *et al.* 1998
			Phagocytosis, complement,		
	Levamisole	125 - 500 ug/ml; oral	lymphokine, ROS		Mulero *et al.* 1998
		0.5 - 500 ug/ml; *i.v.*	ROS		Castro *et al.* 1999
		75 - 300 mg/kg; oral	NCCT		Cuesta *et al.* 2002
	Chitin	0.1 ml/fish; inj.	No change		Esteban *et al.* 2000
		1.0 mg/fish; IP	Humoral and cellular		Esteban *et al.* 2000
		25 - 100 mg/kg	NCCT, ROS, Phagocytic		Esteban *et al.* 2001
	Fungi	10 g/kg; oral	No change		Rodriguez *et al.* 2002
	Yeast	1 - 10 g/kg	Cellular response		Ortuno *et al.* 2002
Japanese flounder					
(Paralychthis olivaceus)	B-glucan	3.0 g/kg; oral	NBT	No	Galindo-Villegas *et al.* 2002
	B-glucan + Mannose	1%; oral	NBT, Lysozyme		Honda *et al.* 2004
		122 mg/kg			
	B-glucan + FKC	34 mg/kg			
	+ Quillaja saponica	5 mg/kg	Agglutination titers	Yes	Ashida *et al.* 1999
	Levamisole	125 - 500 mg/ml; oral	Phagocytic, NBT, Lysozyme		Caceres *et al.* 2004
			Phagocytosis, complement,		
	Peptidoglycan	1.5 - 4.5 g/kg; oral	MAF, ROS	Yes	Galindo-Villegas *et al.* 2003
Pink snapper					
(Pagrus auratus)	B-glucan + Mannose	0.1 - 1.0% w/w; oral	ROS, macrophage activation		Cook *et al* . 2001
	B-glucan + Mannose				Cook *et al.* 2002
Sea bass					
(Dicentrarchus labrax)	B-glucan	2% wet body weight; oral	Humoral activation		Bagni *et al.* 2000
	Myxosporean	multiple ;i.v.	ROS		Munoz *et al* . 2000
Dab					
Limanda limanda	B-glucan	0.5ug/kg; i.v.	ROS		Tahir and Secombes 1996
Dentex					
(Dentex dentex)	B-glucan	0.5%; oral	No change	Yes	Nikl *et al.* 1991
	B-glucan	1 g/kg; oral		Yes	Efthimiou 1996
Flounder					
(Platichthys flesus)	Microsporidian	106 spores; inj.	Antibody production		Pomport-Castillon *et al.* 199
Turbot					
(Psetta maxima)	B-glucan	0.5 - 500 ug/ml; i.v.	ROS		Castro *et al.* 1999
Turbot					
(Scopthalmus maximus)	B-glucan	2 g/kg; oral	Increase leukocyte number	No	Ogier *et al* . 1996
		1 g/100 ml; oral		Yes	Ogier *et al.* 1996
Blue Gourami					
Trichogaster trichopterus	Laminaran	20 mg/kg; inj.	Chemiluminescence	Yes	Samuel *et al.* 1996
Red Sea Bream					
(Pagrus major)	LPS	1mg/fish; inj.	Phagocytic		Salati 1987
Yellow tail					
(Seriola quinqueradiata)	B-glucan	2 - 10 mg/kg; inj	Phagocytic	Yes	Matsuyama *et al.* 1992
	Peptidoglycan	0.2 mg/kg; oral	Phagocytic	Yes	Itami *et al.* 1996
		640 ug/kg; inj		No	Kawakami *et al.* 1998
	Chitin	4 mg/kg; inj		No	Kawakami *et al.* 1998
	Glycyrrhizin	0 - 50 mg/kg; oral	Complement	Yes	Edahiro *et al* . 1991
	Quillaja	0.5 -50 mg/kg	Chemotaxis		Ninomiya *et al.* 1995

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Lymphoid Organs and Immune Tissues Affected by Immunostimulants

The most important immunocompetent organs and tissues of fish affected by immunostimulant include the kidney (anterior and posterior), thymus, spleen, liver and the external mucous. The kidney is important in hematopoiesis and immunity in fish. Blood cell differentiation occurs here instead of in bone marrow, as in mammals. Early in development, the entire kidney is involved in production of immune cells and the early immune responses. As the fish matures, the anterior kidney becomes the most important site of blood cell formation and immune functions, whereas the posterior kidney is primarily involved in blood filtration and/or urinary functions. Blood flow through the kidney is slow, and exposure to antigens occurs. There appears to be a concentration of melanomacrophage aggregates or immune cells in the anterior kidney of most teleost fish. These melanomacrophage centers are aggregates of reticular cells, macrophages, lymphocytes, and plasma cells; they may be involved in antigen trapping and may play a role in immunologic memory (Secombes *et al.* 1982).

The thymus is a paired, bilateral organ situated beneath the pharyngeal epithelium dorso-laterally in the gill chambers. Evidence suggests that the thymus is responsible for the development of T-lymphocytes, as in other jawed vertebrates. It is regarded, as a primary lymphoid organ where the pool of virgin lymphocytes is produced and which then emigrates to join the peripheral pool of lymphocytes in the circulation and other lymphoid organs. The thymus appears to have no executive function. However, much of the data supporting this is indirect evidence, obtained either by immunizing with T-dependent antigens (Ellsaesser *et al.*, 1988) or by using monoclonal antibodies as cell surface markers (Passer *et al.* 1996) and functional *in vitro* assays.

The spleen is a secondary immune organ in fish which contains fewer haemopoietic and lymphoid cells than the kidney, being composed mainly of blood held in sinuses and it is believed to be involved in immune reactivity and blood cell formation (Manning 1994). Most fish spleens are not distinctly organized into red and white pulp, as in mammals, but white and red pulp are identifiable. Lymphocyte and macrophages are present in the spleen of fish, contained in specialized capillary walls, termed ellipsoids. Most macrophages are arranged in melanomacrophage centers, and it is believed that they are primarily responsible for the breakdown of erythrocytes.

The liver is included under this section because, in mammals, it is responsible for production of components of the complement cascade and acute-phase proteins, which are important in the natural resistance of the animal. Fletcher (1981) suggests that the liver of fish plays a similar role. However, research to support this claim is lacking.

External innate immunity is comprised by the mucous membranes of the gill, skin, digestive system, and genitor-urinary tract. These surfaces are physical barriers, but also contain and release anti-microbial agents. Mucous or goblet cells secrete mucus, which has at least three different types of defensive roles. First, mucus interrupts establishment of microbes by being continually sloughed off. Second, if establishment is accomplished, mucus acts as a barrier to be crossed. Finally, the mucus on skin, and presumably the other surfaces, contains a variety of humoral factors with anti-microbial properties. These include lysozyme, complement, lectins, and proteolytic enzymes (Alexander and Ingram 1992; Ellis 1981; Shephard 1994). Recently, several additional defenses have been discovered in fish mucous membranes (Bols *et al.* 2001). Examples of these are the production of nitric oxide by the gill (Campos-Perez *et al.* 2000) and of anti-microbial peptides and proteins by skin (Ebran *et al.* 1999).

Fish Immune System Description

Acquired System

Acquired or specific system plays an important role in the protection against recurrent infections by generating memory cells (cell-mediated immunity), and specific soluble-and membrane-bound receptors (humoral defense), such as T cell receptors and immunoglobulins (Ig), which allow for the fast and efficient elimination of the specific pathogens. The development of vaccines relies on the principle of acquired immunity. The presence of an acquired immune system, however, has not made innate immunity obsolete. On the contrary, by functioning as a first line in host defense, innate immune responses can award off many microbial attacks or keep them in check until an efficient acquired immune response has been developed. Since its complexity and due this component of the immune system is out of the scope of this brief review, will not be described here-in.

Innate System

Fish are in intimate contact with their environment, which can contain very high concentrations of bacteria and viruses. Many of these are saprophytic, some are pathogenic and both are very capable of digesting and degrading the fish tissues. However, under normal conditions the fish maintains a healthy state by defending itself against the potential invaders by a complex system of innate defense mechanisms.

These mechanisms are both constitutive and responsive and provide protection by preventing the attachment, invasion or multiplication of microbes on or in the tissues. Immunostimulants should act through the enhancement of the innate immune response.

Cellular mediated mechanisms

Varieties of leukocyte types are involved in innate cellular defense of fish, and include monocytes/macrophages, granulocytes and nonspecific cytotoxic cells (Table 6). Monocytes and or tissue macrophages are probably the single most important cell in the immune response of fish. Not only are important in the production of cytokines (Clem *et al.* 1985), but they also are the primary cells involved in phagocytosis and the killing of pathogens upon first recognition and subsequent infection (Shoemaker *et al.* 1997). Vallejo *et al.* (1992) also suggest the macrophage as begin the primary antigen - presenting cell in teleosts, thus linking the nonspecific and acquired immune responses.

In fish, Granulocytes (especially neutrophils) are the primary cells involved in the initial stages of inflammation, between 12 to 24 hours (Manning 1994). Granulocytes are highly mobile, phagocytic, and produce reactive oxygen species. These cells appear to possess both Fc and complement receptors, as evidence in opsonization studies (Secombes 1996). The role that neutrophils play in immunity probably varies with species of fish. Eosinophilic granular cells found in the stratum granulosum of the gut, gills, skin, meninges, and surrounding major blood vessels, are not considered to be eosinophils but rather mast cells (Vallejo and Ellis, 1989; Reite 1998).

Table 6 Fish defense mechanisms against bacteria, modified from Ellis, 1999

Humoral	CMI
Non-specific	Non-specific
(a) Inhibitors	(a) Neutrophils
(i) Transferrin (different genotypes)	(i) Respiratory burst → O_2^-, H_2O_2, $OH^·$
(ii) Antiproteases (α1 antiprotease; α2 macroglobulin)	(ii) Halide + H_2O_2 (MPO) → hypohalite ions
(iii) Antibacterial peptides	(iii) Lysozyme
(iv) Lectins	
(v) Interferon	
(b) Lysins	(b) Macrophages
(i) Proteases	(i) Hydrolytic enzymes
(ii) Lysozyme	(ii) Respiratory burst
(iii) CRP (reacts with phosphorylcholine; activates complement)	(iii) NO (+ O_2^- → peroxynitrite → $OH^·$)
(iv) Complement (lytic, proinflammatory, chemotactic, opsonic interacts with CMI	
	(c) Macrophage/Neutrophil cooperation
	(d) Natural cytotoxic cells
	(i) Lysis of tumor target cells
Specific	Specific
Antibody:	Activated macrophages:
(i) Anti-adhesins	Specific T lymphocytes and antigen
(ii) Anti-toxins	↓
(iii) Anti-invasins	Cytokines (IFNγ, TNF)
(iv) Activates classical complement pathway	↓
	Activate macrophages (enhanced RB, enhanced bactericidal activity)

CRP, C-reactive protein; MPO, myeloperoxidase; IFNγ, interferon gamma; TNF, tumour necrosis factor; CMI, cell mediated immunity; NO, nitric oxide; O^{2-}, superoxide anion; OH^-, hydroxyl free radical; RB, red blood.

Cells mediating the lytic cycle to occur and destroy tumor target cells lines following receptor binding in fish have been denominated nonspecific cytotoxic cells (NCC). Evans and Jaso-Friedeman (1992) provide an excellent overview of these cells. NCC's, appear to be important in parasitic (Evans and Gratzek 1989) and viral (Hogan *et al.* 1996) immunity. Mulero *et al*. in 1994 described the ultra structural features of *Sparus aurata* L. and *Dicentrarchus labrax* NCC, founding that ultra structural change in the target cells are similar to those described as mediated by mammalian cytotoxic cells.

Inflammation

As stated by Ellis in 2001, the initiation of inflammation is highly complex and multifactorial. A number of blood enzyme systems, including the clotting system, the kinin system and the complement system play a major role and while little is known of the details in fish it is clear that they share many similarities to their mammalian counterparts (Secombes 1996). During the activation of the complement system by bacteria (directly by the alternative pathway or indirectly by lectins or CRP) the anaphylactic factors C3a and C5a are produced (Yano 1996). In mammals, these factors induce the release of vasoactive amines (histamine or 5-hydroxytryptamine; 5-HT) from platelets and mast cells. In fish, thrombocytes and eosinophilic granular cells (EGCs) probably play an equivalent role, though histamine does not appear to be present in fish and the observed degranulation of EGCs by bacterial products may result in the release of 5-HT (Reite 1998; Matsuyama 2001). The amines induce local vasodilatation and extravasation of neutrophils and monocytes into the infected site. The C5a component of the activated complement also has chemotactic activity for fish phagocytes (Yano 1996) and thus they accumulate at the site of infection. This influx of phagocytes is further stimulated by cytokines and eicosanoids. In mammals, bacteria and LPS stimulate macrophages to secrete interleukin-1 IL-1) which sequentially stimulates the release of eicosanoids, which have pro-inflammatory and chemotactic activity (Davidson *et al.* 1998). In fish, a similar process is apparent as LPS has shown to induce IL-1 production by fish leucocytes (Secombes *et al.* 1999) and the production of eicosanoids (with leukocyte chemotactic activity) by a variety of leucocytes has been reported (Rowley *et al.* 1995).

Phagocytosis

Phagocytosis occurs in fish and is the most primitive defense mechanism. The initial step in phagocytosis is the movement of the immune cell (mainly neutrophils and macrophages) in response to the foreign agent. The movement is by chemokinesis (nondirectional movement of the phagocyte) or chemotaxis (directional movement of the phagocyte). Weeks-Perkins and Ellis (1995), and Klesius and Sealy (1996) were among the first to demonstrate that fish macrophages possess the ability to move by chemokinesis or chemotaxis in response to bacterial antigen either *in vitro* or *in vivo*. After movement in response to the foreign agent, attachment occurs. Ainsworth (1994) demonstrated that attachment occurs via lectins and is enhanced by opsonization. The next step in phagocytosis is engulfment of the foreign agent. Engulfment is simply moving the foreign agent into the cell with subsequent phagosome formation. Killing and digestion of the foreign agent is the final step of phagocytosis.

In fish, destruction or killing can occur by oxygen-dependent or oxygen-independent mechanisms. Oxygen-independent mechanisms involve low pH, lysozyme, lactoferrin, and proteolytic and hydrolytic enzymes. Oxygen-dependent mechanisms are well defined in salmonids, and the pathways are becoming better defined in other species.

Humoral mediated mechanisms

The serum, mucus and eggs of fish contain a variety of substances that nonspecifically inhibit the growth of infectious microorganisms. These substances are predominantly proteins or glycoproteins and many of them are believed to have their counterparts or precursors in the blood and hemolymph of invertebrates. They are specific in that they react with just one chemical group or configuration, but they have been called “nonspecific” because of the substances with which they react are very common, and they do not influence the growth of only one microorganism (Yano 1996). Most nonspecific humoral molecules involved in the natural resistance of fish are presented in Table 6.

Inhibitors

These substances interfere with the metabolism of parasites either by depriving them of essential nutrients, or by interrupting metabolic pathways within the cell.
Transferrin is an iron-binding glycoprotein that plays a central role in the transport of iron between sites of absorption, storage, and utilization in all vertebrate organisms (Putnam 1975). The amount of transferring in host blood is therefore an important parameter in deducing the condition of a pathogen-susceptible host (Yano 1996).

Antiproteases are enzyme inhibitors within the serum. Their basic function is to maintain the homeostasis of the blood and other body fluids and to regulate the action of their mechanisms such as complement and coagulation. The ability of these enzymes to lyse formalin-killed *Vibrio anguillarum* has led to the suggestion that they may play a role in defense against bacteria but their action on live bacteria does not appear to have been studied yet (Ellis 2001).

Antibacterial peptides are substances that have been identified from mucus secretions of a number of fish species (Smith *et al.* 2000) but little is yet known about their ability to kill fish-pathogenic bacteria. These peptides may provide an important line of defense before development of the specific immune response in larval fish. Synthetic pleurocidin has recently been shown to protect coho salmon from infection by *V. anguillarum* (Ellis 2001).

Lectins are important for nonspecific binding to sugars located on the surface of bacteria and pathogens, resulting in precipitation and agglutination reactions. Lectins are Ca^{2+} dependent and can agglutinate a number of fish bacterial pathogen. Sharon and Lis (1993) suggest lectins are also involved in cell recognition and binding, thus playing an important role in cellular communication as well as defensive actions.

Interferons are proteins that inhibit virus replication. Three interferons have been described: Type I interferon which includes IFN-α and INF-β and type II interferon or IFN-γ (Alexander and Ingram 1992). The type I interferon system is a rapid and powerful antiviral defense mechanism in vertebrates. Interferons are pH-resistant cytokines which are produced by many cell types in response to a viral infection. Production occurs very rapidly after virus infection. In *Salmo salar* macrophages stimulated with poly I:C, peak interferon production occurred within 24 h and peak Mx protein production after 48 h. Thus IFN-mediated antiviral defense mechanism are able to respond during the early stages of a viral infection, which is mediated by the innate non-specific IFN responses while long-term protection is mediated by the acquired immune system (Ellis 2001).

Lysins

Lysins are substances that cause cell lysis. These are all enzymes and may be comprised of either a single substance such as the hydrolases, lysozymes, and chitinases, or a cascade of several enzymes such as that observed in the complement system.

Three hydrolases have been identified in the tissues of fish. They act on glycosides and may have defensive functions. These are: N-acetylmuramide glycanohydralase, Chitin glycanohydrolase, and Chitobiose acetylaminodeoxyglucohydrolase.

Lysozyme is found in a wide range of vertebrates (Osserman *et al.* 1974), and is one of the defensive factors against invasion by microorganisms. It splits the β-(1-4) linkages between N-acetylmuramic acid and N-acetylglucosamine in the cell walls (peptidoglycan layers) of Gram-positive bacteria, thus preventing them from invading (Salton and Ghuysen, 1959). In the case of Gram-negative bacteria, which are not directly damaged by lysozyme, the enzyme becomes effective after complement and other enzymes have disrupted the outer cell wall, thereby unmasking the inner peptidoglycan layer of the bacteria (Glynn, 1969; Neeman *et al.* 1974; Hjelmeland *et al.* 1983). In addition to a direct antibacterial effect, lysozyme promotes phagocytosis as an opsonin, or by directly activating polymorphonuclear leukocytes and macrophages (Klockars and Roberts, 1976; Jollės and Jollės, 1984).

In fish lysozyme play an important role in the host defense mechanisms against infectious diseases (Fänge *et al*. 1976; Murray and Fletcher, 1976; Lundblad *et al*. 1979; Lindsay, 1986; Lie *et al*. 1989). In plaice, lysozyme activity has been identified histochemically in monocytes and neutrophils (Murray and Fletcher, 1976). These cells probably contribute to the serum lysozyme activity since their number increases concomitantly with serum lysozyme levels (Fletcher and White, 1973).

Chitinase has been found in the lymphomyeloid tissues (Fänge *et al*. 1976; Lie et al. 1989) and blood (Fletcher and White 1973; Fänge *et al*. 1976) of fish; however, the function of chitinases in the serum and other fish tissues is uncertain (Yano 1996).

Chitobiase has also been found in a number of fish (Lindsay 1986; Yoshida and Sera 1970). There seems to be no correlation between the levels of chitinases and chitobiase activities in the gut of fish. It would appear that if chitinases and chitobiase have a defense function then it is to destroy organisms with chitin in their outer membranes (Alexander and Ingram 1992).

Acute-phase proteins (serum proteins involved in nonspecific defense) appear in the serum and brains of animals following tissue injury or infection. Three such proteins are

C-reactive protein, ceruloplasmin and pentraxins, those three of which have been identified in fish serum.

Pentraxins are capable of binding to a number of polysaccharide structures in the presence of Ca^{2+} ions. Their role in defense is not well understood but in mammals they are capable of activating complement and phagocytes have receptors for them. Ceruloplasmin is responsible for the binding of copper and was shown to be elevated in the presence of cadmium by Syed *et al.* (1979). The defense role of caeruloplasmin would appear to be more troughs its ferroxidase activity than as chelator of copper, because in converting ferrous to ferric iron it increases the removal of iron from the environment, and then decreases its availability to microorganisms.

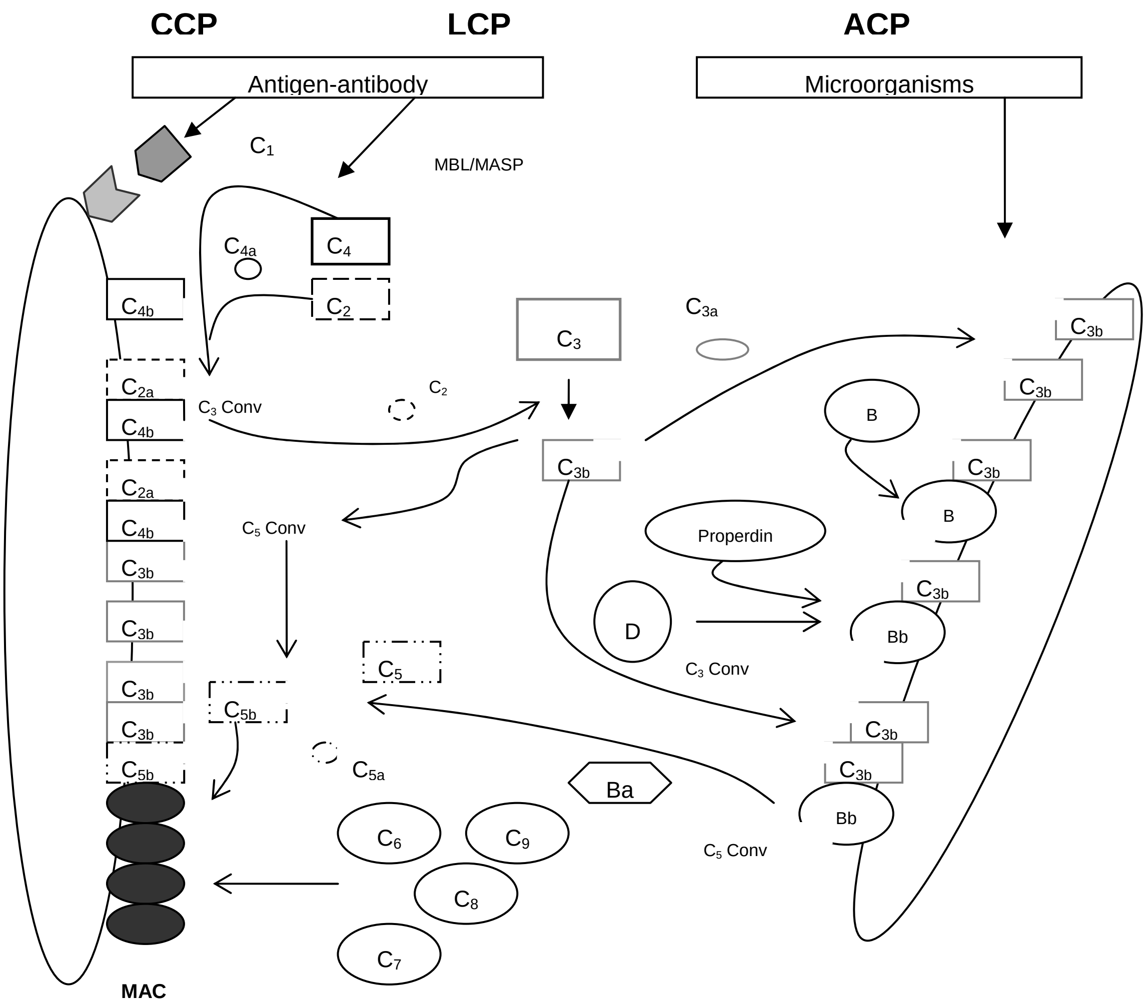
C-reactive protein was found to be elevated in response to elevated cortisol levels (Wingfield and Grimm 1977) and endotoxin stimulation (White *et al.* 1981). Kiron *et al.* (1995) also suggested that nutrition (protein level) influenced levels of C-reactive protein. Research suggests that these proteins are probably produced in response to stress and play a role in natural resistance to infection.

Complement is an essential part of the innate immune system, functions of complement are numerous but it is most well known for its capacity to kill pathogens by creating pores in their surface membranes. Complement-mediated killing occurs when complement is activated either directly by microorganisms or by antibody-antigen (Ag-Ig) complexes. This activation by Ag-Ig complexes makes complement an important effecter mechanism for the adaptive immune response. In addition, complement plays a role in immune complex clearance and participates in inflammatory reactions by attracting phagocytic cells to the site of injury. By opsonising pathogens, complement proteins can stimulate phagocytosis, a process that is mediated by complement receptors on the surface of phagocytic cells.

Complement also plays a role in modulating the adaptive immune response by binding to specific receptors on mammalian lymphocyte surfaces and follicular dendritic cells (Fearon and Locksley 1996; Carroll and Prodeus 1998; Sahu 2001). Complement thereby provides an important link between adaptive and innate immune response.

Complement activation can take place trough three pathways: the classical complement activation pathway (CCP), the alternative complement pathway (ACP) and the lectin complement pathway (LCP). All three activation pathways have been identified in fish, with the exception of the jawless fishes, which appear to lack the CCP and the lytic pathway (Fujii *et al.*, 1992; Nonaka 1994). Holland and Lambris (2002) wrote that CCP, the first pathway to be discovered, is triggered by the binding of antibody to cell surface. The ACP is activated directly by viruses, bacteria, fungi or even tumour cells and is independent of antibody. The LCP varies from the CCP in the way it is activated. Instead of being activated by Ag-Ig complexes, this pathway is initiated by binding of a protein complex consisting of mannose-binding lectin (MBL) and the serine proteases, mannose-binding lectin associated proteases 1 and 2 (MASP-1 and -2) to mannans on the bacterial cell surfaces; thus, its activation is independent of antibody (Fig. 2).

Fig. 2.- The three complement activation pathways. Details described in text.



Relation Between Nutrition and the Innate Immune System

The influence that dietary factors may have on disease outbreaks in cultured fish has been recognized for many years. Knowledge of fish immune system and nonspecific disease resistance factors has increased, and so has the methodology for examining mechanisms of diet-induced effects on infectious disease. Many recent investigations have examined the effects of various nutrients on specific immune functions, as well as on mortality and morbidity. Of course, knowledge of fish nutrition and nutritional requirements has also advanced. Now researchers are beginning to realize that feeds producing the fastest growth may not provide for the best disease resistance. In early fish nutrition studies, requirements were based strictly on growth, feed conversion, and lack of deficiency syndromes. Now attention is focused on the complex interactions of nutrients, physiological effects, disease susceptibility, and overall health.

There are several problems associated with studying nutritional effects on disease in fish, and hence in comparing results from various researchers.

First, there are no universally accepted test diets even for intensively cultured fish, such as salmonids. We do not have the luxury of defined synthetic diets that are well utilized by a variety of species. Nor is there complete and identical environmental control of test systems as are available for homeotherms nutritional studies. There are published test diets for the more commonly cultured fish (NRC, 1981; NRC, 1983). Many researchers do use these formulations; however, they are synthetic feeds with different digestibility and utilization than practical feeds. Other researchers prefer to use practical feeds, which may have more relevance to the fish culturist, but for which quality control is more difficult. There are pros and cons to both approaches. Since there are important interactions of various dietary components, any change in diet formulation may significantly affect the observed results.

Second, fish are not only aquatic organisms but poikilotherms. There are problems determining the actual amount of food eaten and the amount of certain nutrients, particularly B vitamins that may be lost to leaching into the water prior to consumption. The actual requirement for many essential nutrients changes with age and water temperature. Consequently, a finding in one species may not be universally relevant.

A third consideration is the interaction of stress, nutrition, and infectious diseases. Fish exhibit a classic stress response to environmental problems (toxicants, temperature and oxygen extremes, rapid environmental changes), husbandry factors (crowding, capture, hauling), and social interactions. Susceptibility to infectious disease is increased when fish are stressed. The effects of cortisol have been shown to affect a variety of disease resistance mechanisms (Barton and Iwama, 1991). The production of cortisol leads to ascorbic acid depletion and cortisol significantly affects a variety of metabolic process, and dietary factors (such as carbohydrate level fed prior to stress) may affect the stress response (Hemre, *et. al.* 1991). Although circulating cortisol levels are not routinely measured in nutrition and disease experiments, this may be a confounding factor.

Conclusions

Innate defense mechanisms activated by selected immunostimulants may have practical application for aquaculture. If relatively enhanced increase in resistance results, without accompanying undesirable side-effects, these substances may substitute the need for certain vaccines which are to expensive to produce commercially as well as the use of antibiotics to prevent diseases. Immunostimulants as diet supplements, especially those of non-nutritional origin, seems to be a good choice in the aim to induce some level of disease resistance enhancement against pathogens that commonly outbreak in marine fish culture facilities. It is strongly recommended to adapt the prophylactic and therapeutic administration of immunomodulators to each cultured species in anticipation of recognized pathogens, under known environmental conditions since high variability in results was observed. Actual knowledge of potential immunostimulants is still obscure in several aspects, especially in those related to pathways and mechanisms in which such substances can reach their specific cells targets. Understanding studies on the development of the cellular and humoral innate immune systems in marine fish have shown great improvement therefore every time must be encouraged the addition of new parameters in addition to those regularly analyzed. Methods and selected assess immune responses should be try to become standardized between researchers to open a possibility of better understanding the complexity of disease resistance and immune function and then make easier the development of these therapies until the production scale and not just as mare laboratory trials. Further larger number of immunostimulants and more research applying the oral method for their mass administration would be of extreme value.

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