

Fish health challenge after stress. Indicators of immunocompetence

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Abstract

Changes in the nervous and endocrine systems of fish after stress episodes have consequences on their immune system and thereby affect the ability to maintain immunocompetence. Fish mainly depend upon innate immune responses, which include a rich and powerful array of mechanisms, that appear to be more potent than in higher vertebrates. Thus, fish provide a unique model to understand the evolution of immune defence system. When the organism is challenged by an antigen or by stressors, a number of responses of reactive nature are engaged in an attempt to counteract the threat and recover homeostasis. However, if the challenge is maintained, changes in the immune system become chronic, and suppression can be observed in several key immune mechanisms, leading to maladaptation. Therefore, the time factor is of key importance in immune assessment. Taking into account this dynamic pattern of infection and stress, specific indicators should be identified in order to detect functional changes in the immune system. Furthermore, there is a need for specific markers that reflect either activation in the initial stages or suppression in response to a chronic challenge.

Keywords: Immune suppression, fish, stress, fish health, immune indicators.

Resum

Els canvis en els sistemes nerviós i endocrí després d'episodis d'estrès generen conseqüències en el sistema immunitari que influeixen en la capacitat de mantenir la immunocompetència. Els peixos depenen especialment de la resposta immunitària innata, un ampli conjunt de mecanismes que sembla que actuen amb més potència que en vertebrats superiors. Així, els peixos es troben en una posició evolutiva única per a comprendre els models de mecanismes de defensa en els vertebrats. Quan l'organisme és afectat per un antigen o per situacions que provoquen estrès, s'inicien una sèrie de respostes de naturalesa reactiva per a contrarestar-ne els efectes i recobrar l'homeòstasi. Altrament, els canvis en el sistema immunitari esdevenen crònics i es pot observar una depressió en alguns mecanismes immunitaris que acaben amb una mala adaptació. Per tant, el factor temps és clau en la determinació de la resposta immunitària. Tenint en compte aquest patró dinàmic en la infecció i l'estrès, és important determinar indicadors específics que detectin canvis funcionals del sistema immunitari dels peixos. Els marcadors més apropiats són els que indiquen tant una activació dels mecanismes immunitaris en els estadis inicials com una depressió en situacions cròniques.

Fishes are ectothermic vertebrates that live in an aquatic environment. On a generalised functional and ecological basis, the fish immune response must contend with more wide-ranging parameters or threats compared to those which mammals experience. Two major factors that contribute to such differences are: (i) The physical nature and

ecology of the aquatic environment, which likely increases contact with possible pathogenic organisms. It was recently estimated that about 10^{29} prokaryotic cells exist in oceans [1], with viruses accounting for 10^{10} cells/l in aquatic habitats [2]. The aquatic environment also provides a medium in which not only most microorganisms may propagate efficiently, but which also favors their growth. Thus, a number of trophic chains may be less productive due to the ubiquity of heterotrophic bacteria, which take up 70% of marine biogenic carbon [3]. Therefore, in terms of their immune capacity, fish have to be able to cope with a diversity of microor-

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ganisms (bacteria, virus, parasites) that may be more or less active depending upon the physiochemical composition of the water environment, such as temperature, oxygen or solute composition. Furthermore, pathogens may act at specific periods of the year coinciding with periods of physiological changes related to seasonal conditions. (ii) Fish are generally poikilothermic; therefore, the components of their immune system need to function under a broader range of environmental conditions of which temperature is a major factor, although both oxygen levels and salinity also play an important role [4]. Despite this higher tolerance, significant environmental changes may result in immunosuppression, even for reactive responses such as acute-phase proteins and complement. When this combines with seasonally driven physiological changes (migrations, spawning), the effects can be severely aggravated due to the increased levels of cortisol and sexual steroids [5].

In order to cope with these differences, fish have several important immune features which are different from those of warm-blooded mammals. Importantly, the innate immune system rather than the adaptive immune system appears to play a more central role in response to infection, whereas the

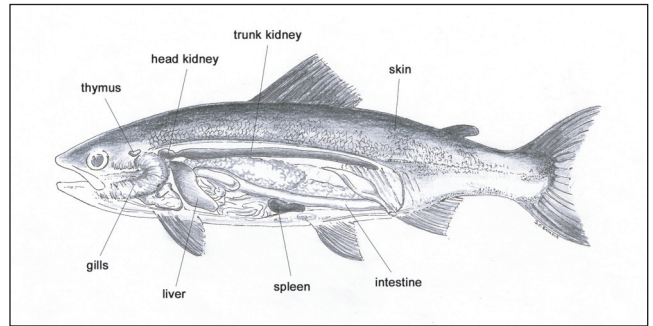


Figure 1. Organs with immune function in fish.

humoral antibody response—in fish Immunoglobulin M (IgM) is the major antibody isotype—appears to be weaker or slower [6]. Secondly, external barriers, including gills and skin, have a significant role in controlling potential portals of entry in a highly antigenic environment. For example, increases in both lysozyme and mucus production enhance the protective function of the skin (Fig. 1).

Stress is a well studied concept in physiology but is difficult to define. Taking a definition used by Brett [7] and others, stress is a real or symbolic (perception of a potential

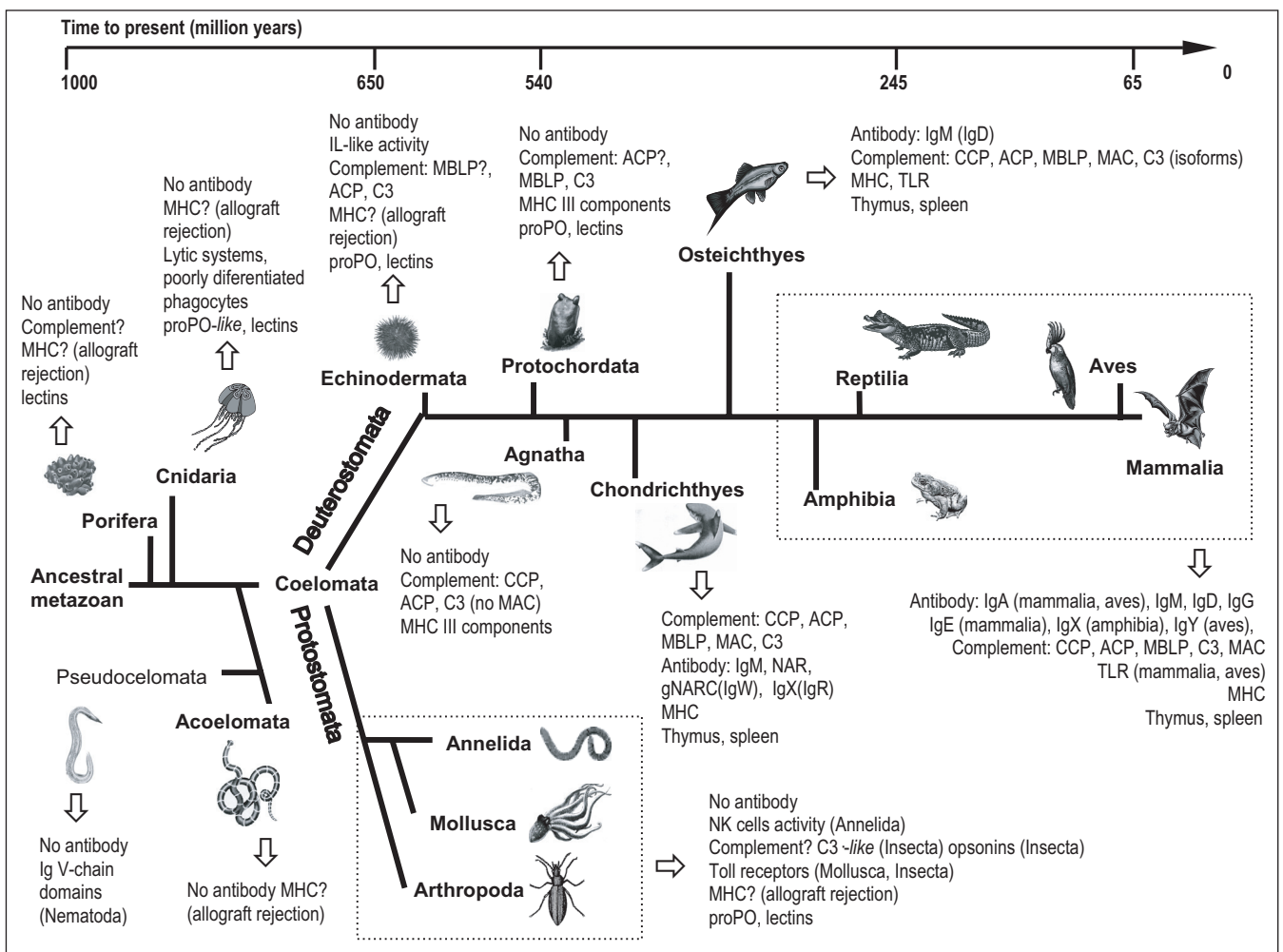


Figure 2. Innate immune responses in evolution. The figure shows the different types of immune mechanisms present in each group related to evolutionary time. The appearance or disappearance of such responses in evolution can be seen. ACP, alternative complement pathway; CCP, classical complement pathway; Ig, immunoglobulin; IL, interleukin; MAC, membrane attack complex; MBLP, mannose-binding lectin pathway; MHC, major histocompatibility complex; NAR, natural antibody repertoires; NK cells, natural killer cells; proPO, pro phenol oxidase; TLR, toll-like receptor

threat) state produced by an environmental or other factor that extends the adaptive responses beyond its normal physiological range. As in other vertebrates, fish experiencing stress may suffer a suppression of immune function due to dysfunction of other physiological regulatory systems, including both the neural and endocrine systems. Impairment of immune function may be due to increases or decreases in key regulatory molecules derived from the neuro-endocrine systems, such as steroids, which may act to reduce or block part of the immune response mechanisms. As a consequence, immunosuppression can occur and pathological conditions may develop more easily.

The immune system in lower vertebrates and fish: A crossroads between specific and innate responses

Fish occupy a strategic crossing point between specific-adaptive responses and innate ones. Figure 2 depicts the spectrum of immune responses found in the main groups of the animal kingdom. Fish are the first group to present an antibody response, although in a simple and initial form, and at the same time display an important range of innate responses, with significant diversity between molecular and cellular components (Fig. 3).

<i>Proteins</i>	<i>Superoxides peroxides</i>
CYTOKINES: Messengers CHEMOKINES: Messengers INTERFERON: Inhibit virus replication COMPLEMENT: Bacteriolytic, Opsonisation, Macrophage activation.	Produced by phagocytes. Bactericidal
LYSOZYME: Bacteriolytic	<i>Nitric oxide</i>
ACUTE PHASE: Agglutination, Precipitation LECTINS-AGGLUTININS: Agglutination IMMUNOGLOBULINS: Precipitation, agglutination, opsonisation, complement	Peroxidation, nitrating, nitrosating

Figure 3. Range of innate serum immune responses in fish fluids (serum, skin, mucus, eggs) and their main effect.

Higher vertebrates have developed an efficient adaptive response consisting of an increased diversity and specificity of antibody isoforms that yield a more potent secondary response to antigen. By contrast, the innate response of lower vertebrates is less efficient [8]. Therefore, it appears that the evolution of more sophisticated and regulated organisms, i.e. those having a higher complexity of homeostasis, has included the acquisition of a more potent adaptive response, whereas lower vertebrates, confronted with more variable conditions in both their internal and external environments, rely on more generalized innate responses. Fish thus represent a point of divergence in the evolutionary development of immune systems in vertebrates. Interestingly, fish have all the necessary cell types to generate an adaptive response whereas the absence of antibody isotypes, especially the IgG group, suggests that the B-lymphocyte memory re-

sponse may not be well developed. In addition, it remains to be proven whether the potency of fish cytotoxic T-lymphocytes is of significance [9]. Recent evidence supporting a physiological role for T cells describes allograft rejection in the rainbow trout (*Oncorhynchus mykiss*), and the involvement of CD8+ T lymphocytes clearly shows that adaptive cell-mediated cytotoxicity mechanisms are present in fish as in higher vertebrates [10].

The innate response in fish

Innate responses in fish comprise a wide repertoire of biological actions, including phagocytosis, opsonization, and lytic and cytotoxic cellular activity. As a whole, these actions are seemingly driven by pattern-recognition receptors capable of recognizing pathogen-associated molecular structures highly conserved during the course of evolution [11]. They are also characterised by non-specificity, being antigen-independent, rapidity and activity over a wide range of temperatures. Although many of these responses are present in mammals, it has been suggested that other, more specialized components also make up the innate response in fish, suggesting that some lower vertebrates, including fish, may have fine-tuned some aspects of their innate defense mechanisms. For example, the complement system is one of the main immune responses, resulting in activation of phagocytic cells, direct lysis of target cells and opsonization. The fish complement system contains multiple active isoforms of the key activation molecule C3. Mammals have one isoform of the C3 whereas there are three isoforms in trout and medaka [12,13], five isoforms in sea bream and carp [14,15] and three loci coding for three isoforms in zebrafish [16]. The differential functioning of trout and carp C3 isoforms and derived peptides has been analyzed [17,18]. The results suggest that in fish several isoforms of C3 proteins may have evolved; these are able to react with different surfaces thus increasing efficiency in which immunogens are eliminated. Furthermore, the bacteriolytic and hemolytic activity of complement is higher than in mammals [12]. However, recent studies have shown that the alternative complement pathway in fish, proposed as being potentially more potent than that of humans, does not display such higher activity [19]; therefore several major questions remain to be answered in this field.

Other relevant innate responses in fish are synthesis and secretion of interferon, lysozyme, and acute-phase proteins; cytotoxic cells; chemotaxis and the phagocytic process [20]. Interferons are cytokines produced by different cell types in both adults and young fish; they constitute a rapid (within 2 days in salmonids) and powerful response to viral infection [6]. Interferons probably act by stimulating the synthesis of proteins that inhibit the translation of viral mRNA [21]. Lysozyme activity has been found in most tissues, in serum and on external surfaces (skin, mucus or gills) and its mode of action is to disrupt bacterial walls by splitting glycosidic linkages in peptidoglycan layers [22]. Acute-phase

proteins (CRP, ceruloplasmin, fibrinogen, HsP) help to regulate phagocytosis and complement proteins and their levels in plasma or serum are greatly increased after tissue damage, infection or inflammation. The process of cell defense includes chemotaxis, killing activity by macrophages and neutrophils [6] and phagocytic activity in which bacteria attached to the phagocyte surface are trapped within the phagosome and subsequently killed by mechanisms based on reactive oxygen and nitric oxide responses.

The antibody response in fish

Fish lack recombination by antibody class switching, although somatic hypermutation has been demonstrated [23]; thus, immunoglobulin diversity in fish is rather restricted with respect to antibody structure. For this reason, it has been assumed that the antibody response is poor in fish compared with higher vertebrates; in fact, antibody production in salmonids takes 4-6 weeks. Therefore, protection afforded by antibodies would be important only in fish previously immunised, with faster responses driven by innate mechanisms. However, recent work on the structural characteristics of fish antibodies indicates that they may play a more significant role than expected. Fish serum antibodies are composed of cross-linked tetramers and may generate as many as six structural forms of immunoglobulin due to random polymerization thereby compensating for the poor isotopy [24]. It remains to be assessed whether such a mechanism of functional diversity produces a higher degree of efficiency and protection in fish. Affinity maturation in fish is also controversial: following immunization, there is an increase in the avidity of IgM for antigen [25], but it could not be demonstrated that this was due to the expansion of pre-existing cells producing high-affinity antibodies.

The key regulators of the immune response: cytokines

Cytokines as modulators of the immune response have been studied not only in higher vertebrates but also in fish and a significant number of these compounds are functionally active in teleosts [26]. The most intensively studied cytokine, interleukin-1 β (IL1- β), mainly produced by macrophages, has been characterized in various vertebrates including, birds, amphibians, bony fish and cartilaginous fish (for review see: [27]). IL1- β is an important mediator of inflammation in response to infection. In the trout, IL1- β has been reported to directly affect hypothalamic-pituitary-inter-renal (HPI) axis function, stimulating cortisol secretion [28]. Thus, clear evidence exists for the multi-functional role of this cytokine, and that fish cytokines clearly have an important role to play in the regulation of neuro-immunoendocrine responses. While such networks of intercommunication are likely conserved in lower vertebrates [29], it has been suggested that the absence of several IL-1 family members in fishes in-

dicates the development of highly specific adaptive immunity in higher vertebrates only [30].

Another potentially important cytokine, tumor necrosis factor α (TNF- α), has also been cloned in various fish species [31-34], and TNF-like activity has been shown to induce apoptosis as well as enhance neutrophil migration and macrophage respiratory burst activity [35]. Due to the diversity of functions ascribed to TNF- α in mammalian vertebrates, it is likely that the cytokine will also prove to be a key player in neuro-immunoendocrine responses in fish.

Other cytokines and regulatory molecules reported in fish include: transforming growth factor β (TGF- β) [36,37], an interferon regulatory factor (IRF)-1, [38] and a recently identified interferon-like peptide in zebrafish [39]. Undoubtedly, future studies will identify increasing numbers of fish cytokines, especially interleukins. Identifying and mapping of the interactions between specific regulatory molecules from each system will lead to a more complete picture of the humoral component of the fish immune system.

Stress and the activation of system responses

Stressors are primarily perceived by sensors of the nervous system and the initial response is induced in integrating brain centers, specifically in the hypothalamus where the two major regulatory axes are stimulated: the sympathetic-chromaffin (SC) axis, via nervous fibers innervating chromaffin cells, and the HPI axis, via an endocrine cascade (Fig. 4). In fish, both axes act upon the same organ, the head kidney. The first hormone in the HPI axis is corticotropin-releasing hormone (CRH), which is released by hypothalamic neurons of the preoptic region. CRH is found in fish [40], along with another hypothalamic peptide, thyroid-releasing hormone (TRH), which also has activation properties [41]. TRH and CRH stimulate the release of adrenocorticotrophic hormone (ACTH) from the pituitary, which in turn induces production and release of the major stress steroid, cortisol, by interrenal cells located within the head kidney. Nevertheless, little data are available concerning specific interactions with other peptides of endocrine or immune action.

Responses to stress are of different durations depending on the stressor and the indicator measured and therefore,

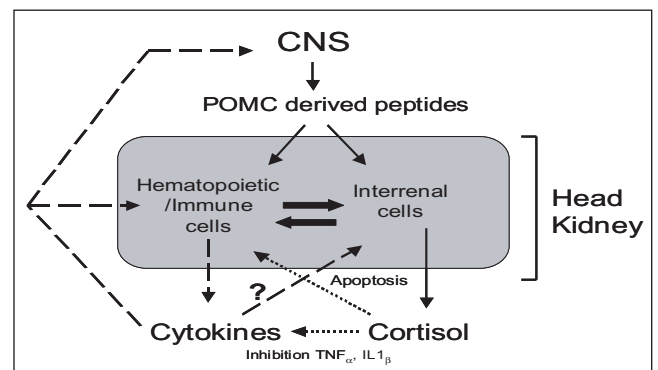


Figure 4. Interactions of cytokines and cortisol in the head of the kidney in fish.

time is an important factor for recovery. The activation of neural systems and the release of the first mediators from SC and HPI cells takes place within seconds, cortisol release within minutes, and other metabolic responses over minutes to hours. While these are general responses to all stress situations, the duration of the stressor significantly influences the overall physiological status. Thus, acute stressors involve rapid and high levels of secretions, followed by recovery a few hours after induction, such that the overall costs of the stress episode are reduced. However, chronic or repeated stressors are characterized by lower, more constant levels of steroid secretion, often involving longer recovery periods and much higher energetic and performance costs due to both persistence of the stressor and the derived effects of the stress-response effectors. While recovery and adaptation may occur in the first case, both a lack of recovery and maladaptation occur during and after periods of chronic stress exposure. This maladaptation involves alterations at several physiological levels, from impaired growth and reduced reproductive capacity to immune suppression.

Connections between the nervous, endocrine and immune systems

The head kidney, or pronephros, is also the major hematopoietic organ in fish, in which immune cell types are also produced. In the zebrafish (*Danio rerio*), erythroid, lymphoid and myeloid elements have been localized in the pronephros both at the ultrastructural and molecular level. Furthermore, the transcription of regulatory genes involved in distinct hematopoietic lineages, such as *rag1* (lymphoid), *spi1/pu.1*, *c/ebp1*, *mpx/mpo* (myeloid) and *Bik1f* (erythroid) has been demonstrated [42-46]. Although similar data are scarce for other Teleostei, it may be assumed that the head kidney is the major location for hematopoiesis in bony fish. As mentioned before, this organ also plays a major role in the secretion of cortisol, the major glucocorticoid and mineralocorticoid in fish, from interrenal cells and contains chromaffin cells, which are components of the sympathetic nervous system (see [47] for an extensive review).

Therefore, fish represent an interesting comparative model in which to study the effects of regulatory stimuli (nervous and endocrine system) on the immune system, due to the structure and composition of the head kidney. The concept of a neuro-immunoendocrine connection relating specific tissues with specific systems, e.g. lymphoid tissue/immune system, gland/hormones or neurones/nervous system, is no longer valid in that these tissues or cells may also play a role in another system [48]. Clearly, in this case the head kidney in fish represents a tissue in which all three regulatory systems are integrally connected, forming a centralized network to coordinate both endocrine, neural, and the immune (humoral and cellular) responses.

Communication between these three physiological systems has been poorly studied in fish compared to mammals, possibly due to the lack of experimental tools. However, re-

cent studies suggest that such functional connections are indeed present in fish. For example, intra-peritoneal injection of recombinant trout IL-1 β increases the plasma cortisol concentration in rainbow trout [28]. Cortisol, in turn, is able to inhibit TNF- α mRNA expression [49] (MacKenzie et al, manuscript in preparation). Therefore functional immune-endocrine and endocrine-immune interactions clearly exist in fish [50].

Such networks of connected responses may well be an early evolutionary mechanism based on several reasons: (a) a small family of molecules appears to have similar functions in all vertebrates; (b) invertebrates have neuro-immunological connections [51]; (c) other general responses to stressors, such as heat-shock proteins and metallothioneins, are found in most animals and they play different roles related to cell integrity, antioxidant properties and components of regulatory systems [52,53].

Recently, an increasing number of studies has pointed to connected pathways and molecules, as well as the concerted action of the three regulatory systems [50]. For example, proopiomelanocortin (POMC)-derived peptides have been shown to be involved in the regulation of the immune system. Interestingly they can also be synthesized and released by immunocytes; and a leukocyte factor induces the release of cortisol in vitro [54]. Growth hormone (a peptide with structural similarities to some interleukins, can directly act on hematopoietic cells of sea bream (*Sparus aurata*) [55] and stimulates immune system activity [56]. Sympathetic neurotransmitters can modulate the respiratory burst activity of trout phagocytes [54,57]. Melanotropins, melanocyte-stimulating hormone (MSH), and melanocyte concentrating hormone (MCH) have also been found to exert relevant stimulatory effects on the immune system [58,59]. Furthermore, mammalian POMC is produced not only by neuro-endocrine tissues, but also by lymphoid cells, which reinforces the neuro-immunoendocrine connection, although this has not yet been studied in fish [60].

As these interactions will be dependent on specific receptors, it will be of great interest to analyze differential receptor expression, including both immune and endocrine regulators in tissue and cells. The results will no doubt provide important physiological data allowing neuro-immunoendocrine regulatory sites to be elucidated.

Activation and suppression

Time course of the immune responses

An overall view of the immune response after contact with an infectious agent is shown in Fig. 5. Initially, the innate immune responses are directly activated by antigen recognition in parallel with neuro-endocrine activation. Later stages, requiring minutes to hours, include the subsequent activation of effector pathways involving complement and lysozyme, and increased phagocytic activity. Nevertheless, there is little research in fish on the activation of specific cell types. However, there are extensive data on the endocrine response, centering specifically on the gluco/mineral corticoid hormone cortisol.

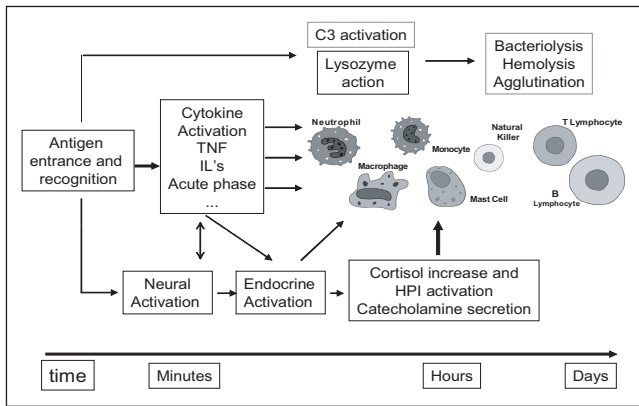


Figure 5. Time course of immune responses after an antigen challenge and the interconnections between endocrine and immune events.

Stress and immune activation

The consequences of a stress situation are currently considered to be mostly suppressive or deleterious in that the stress response diverts energy from normal metabolic processes including growth, to physiological systems activated to adapt to the stressor. When this situation becomes chronic, the energy reserves are depleted.

A similar situation may take place in the immune system, where the capacity of response is limited, leading to its eventual suppression (see Fig. 6). There is an extensive literature on the effects of stress on the fish immune system, and many types of stressors, from natural environmental changes (reproductive status, water characteristics) to chemical products in water and husbandry procedures have been studied [41,61,62]. Those related to aquaculture include capture, crowding and dietary deficiencies [63, 64,65]. Table 1 shows that all these stressors, including transport, grading, confinement, handling and crowding, elicit a variety of responses, often species specific and stressor specific. While the overall conclusion suggests that stress induces immunosuppression, the extent of such perturbations is variable and likely depends on a number of key factors. The nature of the response is related to the stressor and to the organism. For the

stressor, the type, intensity and persistence of a stressor over time generates differential responses, as has been shown in relation to plasma cortisol concentration [66]. The organism's response is made up of multiple components and can be measured at several sites in the body (blood, organs or peripheral tissues); one or a sum of these components may be indicative of the health status of the fish.

During the primary phase of activation following an acute stress, immunocompetent cells in lymphoid tissues, such as head kidney, the spleen or thymus, may display a mitotic/proliferative response [67]. Acute stress, e.g. holding the fish out of the water, increases the number of receptors on total splenic and pronephric leukocytes and decreases the affinity of these receptors in splenic but not pronephric cells [68]. However, other stressors, such as transport or crowding, have been reported to cause a significant degree of mitotic suppression in lymphocytes from the head kidney [69], [70]. Chemical stressors, including pesticides, metals and organic compounds, usually suppress immune function, but a parallel increase in immune indicator activity has also been recorded, specifically phagocytic indices [71].

Such discrepancies can be explained by the circumstance in which the indicator is measured, more accurately, the time elapsed following initiation of the stressor effect and the temporal dynamics of the particular immune mechanism. Therefore, a number of indicators will increase when the immune system is in the primary phase of reaction against the challenge. Thus, an increase or decrease in the number of lymphocytes would be recorded depending on where the measurement is taken (blood or lymphoid tissue) and the time after the onset of the stressor. Phagocytic indices are also contradictory depending on the conditions of the experiments. Nevertheless, it is clear that, other than the first reaction to stressors, the medium/long-term effect of stress on the immune system is suppression and maladaptation, particularly when the stressor is chronic or repeated [52,72].

Is cortisol immunosuppressive?

A number of works have tried to ascertain whether cortisol is immunosuppressive, as has often been proposed. Some studies have shown significant effects of cortisol treatments: inhibition of chemiluminescence, plaque-forming response and nitric oxide production by macrophages after in vitro cortisol treatment [73-75]; an increased number of leukocytes in the thymus and head kidney but a decrease of spleen leukocytes of coho salmon fed with cortisol [76]. Other authors describe significant changes in the mitogenic response to bacterial lipopolysaccharide (LPS), in Ig-positive lymphocytes, or after incubating leukocytes with cortisol-enriched medium [77,78]. Some studies, however, have not shown significant effects [57], for example, a lack of depression of macrophage phagocytic activity in vitro after injection of 200 nM cortisol. Furthermore, inconsistent results were also reported in *Salmo salar* depending on the indicator analyzed. Similarly, after cortisol injection, no changes were observed in the level of blood granulocytes, lymphocytes or monocytes but there was a significant increase in the number of thrombocytes [79].

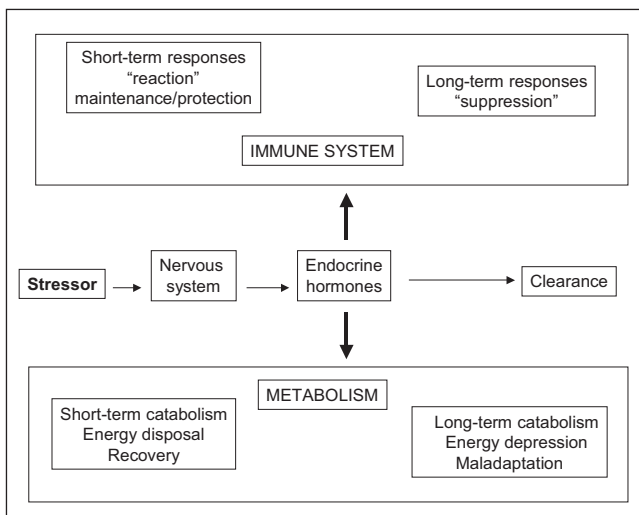


Figure 6. Effects of stress on the activation and suppression of metabolism and the immune system.

In carp, a series of recent experiments assessed the effects of cortisol on immune cells *in vivo* and *in vitro* [80]. Regarding the leukocyte response, there was a reduction of circulating B-cells but an increased number of neutrophils after corticosteroid treatment [81]. By contrast, cortisol did not seem to affect the *in vitro* respiratory burst activity of phagocytes. Experiments *in vitro* showed that cortisol inhibits lymphocyte proliferation probably by enhancing cell apoptosis [82], but only when lymphocytes have been previously stimulated with mitogen [82,83]. Regarding phagocytosis during *in vitro* experiments, again there were no effects on respiratory burst or phagocytic activity after cortisol treatment [57,75,81] unless nonphysiological doses were used [84]. According to these studies, corticosteroids appear to affect mainly B-cell function but not phagocytosis. The authors therefore concluded that cortisol actions are not always inhibitory but depend on the stressor, so that some non-specific responses are enhanced while specific responses are inhibited [80].

Recently, we have found that application of exogenous cortisol to pronephros leukocyte cultures significantly reduces monocyte adhesion to *in vitro* culture dishes after 5 day of culture. Furthermore, this treatment induces apoptosis in the small leukocyte cell population, which may contain both lymphocytes and hematopoietic precursor cells, identified by flow cytometry (MacKenzie et al, manuscript in pre-

paration). The possibility that the increased cortisol secretion in the head kidney resulting from external stressors directly affects the hematopoietic potential of fish via paracrine signaling represents an exciting avenue for further endocrine-immune studies in this interesting comparative model. Furthermore, alterations in immune function have been described during the reproductive period of fish in response to steroids other than cortisol. Lower resistance to diseases was found in salmonids with high levels of testosterone [68]. Other authors showed a decrease in antibody-producing cells (APC) in peripheral leukocytes of migrating female salmon with corticosteroid levels of 200 ng/ml [85]. However, yet other studies reported different results; no changes were observed in males and APC levels remained unchanged after 3 weeks of acclimation to constant environmental conditions.

Therefore, at present, it is difficult to draw conclusions regarding a uniform or consistent role for cortisol. One hypothesis may be that cortisol acts as a regulator of the immune system rather than purely as a suppressor [80]. In fact, previous observations suggest that the early responses activated by cortisol may help to maintain key defense mechanisms, at least during the short term, whereas other actions of cortisol over longer periods of time result in suppression.

Table 1. Changes in fish immune indicators after different husbandry stressors. Values are calculated as the reduction in percentage of each indicator compared to respective controls, which were set at 100%. PI Phagocytic index

Stressor	Indicator	Change (%)	Species	Reference
Transport	PI	93.2	<i>Ictalurus punctatus</i>	Ainsworth et al., 1991
Transport	Blood PI	83.2	<i>Ictalurus punctatus</i>	Ainsworth et al., 1991
Transport	Head of the kidney PI	44	<i>Oncorhynchus mykiss</i>	Narnaware et al., 1994
Transport	Lysozyme activity	57.6	<i>Oncorhynchus mykiss</i>	Möck and Peters, 1990
Transport	Spleen PI	61	<i>Oncorhynchus mykiss</i>	Narnaware et al., 1994
Transport	Mitogenesis	45.4	<i>Oncorhynchus tshawytscha</i>	Maule et al., 1989
Grading	Antibody-producing cells	19	<i>Oncorhynchus tshawytscha</i>	Maule et al., 1989
Chronic	Head of the kidney PI	87	<i>Oncorhynchus mykiss</i>	Narnaware et al., 1994
Chronic	Spleen PI	76	<i>Oncorhynchus mykiss</i>	Narnaware et al., 1994
Confinement	Bactericidal activity	37.5	<i>Salmo salar</i>	Thompson et al., 1993
Confinement	Respiratory burst	55.6	<i>Salmo salar</i>	Thompson et al., 1993
Confinement	Thymic mitosis	222.5	<i>Sparus aurata</i>	Cubero and Molinero, 1997
Confinement	Lymphocytes	52.3	<i>Sparus aurata</i>	Cubero and Molinero, 1997
Handling	Antibody-producing cells	24	<i>Oncorhynchus tshawytscha</i>	Maule et al., 1989
Handling	Thymus leukocytes	173	<i>Oncorhynchus kisutch</i>	Maule and Schreck., 1990
Handling	Serum hemolysis	70.1	<i>Sparus aurata</i>	Sunyer et al., 1995
Handling	Agglutination	79.5	<i>Sparus aurata</i>	Sunyer et al., 1995
Handling	Lysozyme activity	143.3	<i>Oncorhynchus mykiss</i>	Möck and Peters, 1990
Handling	Respiratory burst	85.7	<i>Sparus aurata</i>	Ortuño et al., 2003
Deficient diet	Agglutination	64.7	<i>Sparus aurata</i>	Tort et al., 1996
Deficient diet	Serum hemolysis	56.1	<i>Sparus aurata</i>	Montero et al., 1998
Deficient diet	Serum hemolysis	60.5	<i>Sparus aurata</i>	Montero et al., 2001
Deficient diet	Hemagglutination	89.3	<i>Sparus aurata</i>	Montero et al., 2001
Feeding alteration	Phagocytic index	89.7	<i>Oncorhynchus tshawytscha</i>	Alcorn et al., 2003
Crowding	Serum hemolysis	68.3	<i>Pagrus pagrus</i>	Rotllant et al., 1997
Crowding	Agglutination	79.6	<i>Pagrus pagrus</i>	Rotllant et al., 1997
Crowding	pf _c /10 ⁶ lymphocytes	42.9	<i>Salmo salar</i>	Mazur and Iwama, 1993
Crowding	Serum hemolysis	88.8	<i>Sparus aurata</i>	Tort et al., 1996
Crowding+anesthetics	Complement	44.4	<i>Sparus aurata</i>	Ortuño et al., 2002
Crowding+anesthetics	Phagocytic activity	60.8	<i>Sparus aurata</i>	Ortuño et al., 2002
Chemical HgCl ₂	Apoptosis/necrosis	73.3	<i>Salvelinus namaycush</i>	Miller et al., 2002

Immune indicators in fish

Indicators of immunocompetence

The number of immune-system parameters that may be used to indicate altered immune status is considerable when we consider the diversity of possible responses. One of the major difficulties in identifying appropriate indicators of the immune response after stress is the lack of basic knowledge concerning immunological mechanisms in fish. Therefore, suitable immune indicators in fish could be defined as those that are representative of an immune response accomplishing two basic requirements: firstly, to respond to a wide range of antigens, and secondly, to achieve maximum efficiency in terms of rapidity and potency.

As in other vertebrates, these responses rely on immune cells or plasma/serum molecules and may be either specific or non-specific. In consideration of the two requirements mentioned in the case of lower poikilothermic vertebrates, indicators representative of a central immune response should be of the non-specific type.

Thus, regarding cellular activity, it has been demonstrated that fish possess a powerful phagocytic response that can be detected using several indicators: the respiratory burst reaction, as measured by the levels of oxygen radicals produced; phagocytic indicators, such as the reduction of nitroblue tetrazolium (NBT) caused by the oxygen radicals; cell adherence; and the ability of macrophages to engulf particles. Also, with respect to cell responses, fish react to challenges by modifying the number and distribution of their white cells; thus, cell type, function and overall number should also be considered as a factor of importance. Many studies concentrate on the phagocytic capacity of macrophages although macrophages represent a small percentage (1-5%) of the total number of leukocytes. Few studies provide a complete picture of the status of white cells in fish during stress, i.e., determination of the number and type of cells (leukocytes or lymphocytes) both in blood and in immune organs (spleen, head kidney and thymus).

Regarding molecular mechanisms, it has been shown that immunoglobulin function in fish is less developed than in mammalian species [25,86]. Thus, the number of immunoglobulin forms in fish is greatly reduced (IgM and IgD) compared to higher vertebrates. This indicates a less potent response; additionally, the memory response is both less potent and slower, requiring 3-5 weeks to reach a maximum. Other innate responses, however, are enhanced. For example, induction of the lytic properties of the alternative complement system in fish is ten times greater than in mammals, and the system is active over a wide range of temperatures and pH [12]. Similarly, the fish immune system can use other non-specific components, such as lysozyme activity in blood, tissues or skin, and a range of other molecules in the serum, such as lectins or agglutinins, with higher efficiency than in homeothermic vertebrates [72,87]. Whilst many immune indicators may signal changes in specific parts of the immune response, challenge tests are always a very useful tool to determine the efficiency of the immune system under

the particular conditions assayed. Disease resistance tests with either specific pathogens or opportunistic ones indicate the overall ability of the immune system to overcome the challenge. Nevertheless, the timing of the responses and the nature of the stressor have to be taken into account. It may well be of interest to detect whether fish have been challenged and the state of the early reactive response activating the immune system. Alternatively, we may be interested in the chronic response, in which signs of suppression usually predominate (Fig. 7).

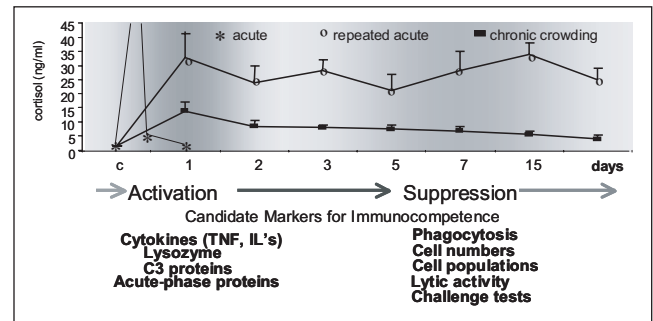


Figure 7. Activation and suppression phases after stress, and candidate markers

Practical applications of immunological indicators that identify stress

Dependent upon the goal of the study, a number of markers may be employed. Those which specifically analyze key molecules, such as acute-phase proteins, C3 proteins, lysozyme and cytokines, involved in the early response may provide invaluable information toward assessing activation status. Alternatively, when studying immune suppression phagocytic activity, lytic activity, cell numbers and trafficking may more adequately describe longer-term physiological changes within the immune system. Therefore, the ability to target and mount an immune response would be a strong indicator of adaptation, whereas alterations of these latter responses would imply the occurrence of a maladaptive process. In the absence of "gold standard" markers, a combination of these techniques should provide an excellent basis from which to study fish health.

Fish provide an interesting model to examine immune function as well as the interactions between regulatory systems (neuro-immune-endocrine). As a group, fish are in the baseline of vertebrate "radiation" [88], and their immune system anticipates the mammalian repertoire of combinatorial recognition (antibodies, lymphocytes, T-cell receptor and MHC complexes, RAG genes) while they conserve important features of non-specific defenses (complement diversity, antibacterial peptides). Fish are also the first group showing a neural crest and a significant increase of nervous system structure and complexity, including recognition, memory and targeted responses [89]. As these traits are also shared by the other physiological systems with regulatory functions (endocrine and immune) in fish, future research will most likely reveal further evidence of interactions between these three systems.

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