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Fish immune system. A crossroads between innate and adaptive responses

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EL SISTEMA INMUNITARIO DE LOS PECES. UNA ENCRUCIJADA ENTRE LAS RESPUESTAS INMUNITARIAS INNATAS Y ADAPTATIVAS

RESUMEN

Considerados como la base evolutiva vertebrada tras su radiación adaptativa en el Devónico, los peces constituyen en la actualidad el grupo más exitoso y diversificado de vertebrados. Como grupo, este conjunto heterogéneo de organismos representa una aparente encrucijada entre la respuesta inmunitaria innata y la aparición de una respuesta inmunitaria adaptativa. La mayoría de órganos inmunitarios de los mamíferos tienen sus homólogos en los peces. Sin embargo, su eventual menor complejidad estructural podría potencialmente limitar la capacidad para generar una respuesta inmunitaria completamente funcional frente a la invasión de patógenos. Se discute aquí la capacidad de los peces para generar respuestas inmunitarias exitosas, teniendo en cuenta la robustez aparente de la respuesta innata de los peces, en comparación con la observada en vertebrados superiores.

PALABRAS CLAVE: Inmunidad innata / Inmunidad adaptativa / Vertebrados / Peces / Evolución.

ABSTRACT

Fish, as the first vertebrate group appearing in evolution after adaptive radiation during the Devonic period, still represent the most successful and diverse group of vertebrates. This heterogeneous group of organisms occupy an apparent crossroads between the innate immune response and the appearance of the adaptive immune response. Importantly, immune organs homologues to those of the mammalian immune system are present in fish. However, their structural complexity is less, potentially limiting the capability to generate fully functional adaptive immune responses against pathogen invasion. The ability of fish to mount successful immune responses with apparently more robust innate responses than that observed in higher vertebrates is discussed.

KEY WORDS: *Innate immunity / Adaptive immunity / Vertebrates / Fish / Evolution.*

INTRODUCTION

A recent estimation assumes that about 10^{29} prokaryotic cells exist in oceans[1], which are mainly responsible for the marine biomass. The aquatic media not only allows transport but also the growth of microorganisms. Thus, a number of trophic chains are less productive due to the ubiquity of heterotrophic bacteria which utilize 70% of the marine biogenic carbon[2] and viruses that may account for 10^{10} cells/L in aquatic habitats[3].

The adaptation of autocatalytic functions is a coevolutionary process, which in case of the immune system involves competition related to the number of microorganisms and their pathogenicity. Although most effector mechanisms of allorecognition in invertebrates are not known, recent studies in comparative immunology stress two general patterns: (a) conservation of the defensive signal pathways that are relevant to nonspecific immunity, and (b) restriction of adaptive immunity to vertebrates (Fig. 1).

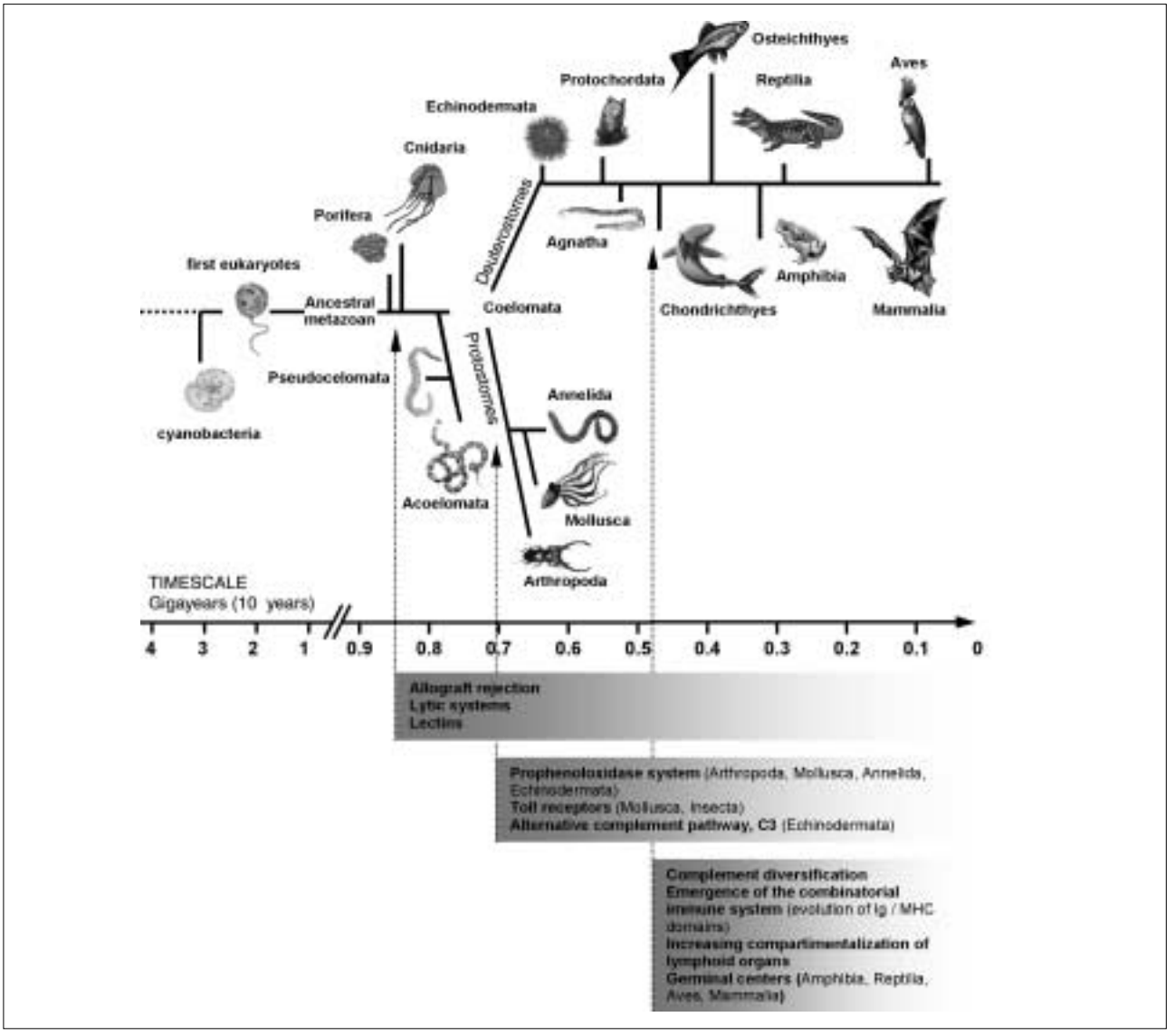


Figure 1. *Evolutive emergence of immune mechanisms through the animal kingdom.*

It is known that evolutive mechanisms act on the existing material and are restricted to the limitations of both biotic and abiotic resources available in the environment. Animals evolved from small bodies to bigger bodies, from less isolation from environment to higher degree of isolation, from higher dependency of the environmental conditions to higher degree of self-regulation systems. Therefore, a progressively richer internal medium is increasingly found. The immune system of fishes is conditioned by the particular environment, but also by their poikilothermic condition. Most pathogenic bacteria are opportunistic microorganisms normally present in the aquatic microflora. The obligatory pathogens *Renibacterium* y *Mycobacterium*[4] are scarce, but their virulence depends on environmental factors such as thermic, ionic and osmotic changes, iron and oxygen availability, pollutants, eutrophia, etc. In fish, immunocompetence depends more on fish weight rather than on age, mainly due to the need of a minimum number of immunocompetent cells. Thus, it is known that some fish larvae have the ability to process antigens, even when they are still dependent on yolk resources[5].

THE INNATE IMMUNE SYSTEM

Over 98% of pluricellular organisms are able to maintain their integrity thanks to an innate immune system based on

TABLE I. Relevant immune differences between jawed fishes and mammals

	Jawed Fishes	Mammals
Biotic constrictions		
Temperature range	-2 to 35°C	36.5 to 37.5°C
Primary environment	Water	Air
Metabolism	Poikilothermia Endothermia (eg. bluefin tuna and some pelagic fishes)	Homeothermia
External interfaces	Mucous skin, gills	Respiratory tree
Humoral diversity		
Ig isotypes	IgM, IgD? *(Teleostei)* IgM, IgX/IgR, IgW, NAR(C) *(Chondrichthyes)* IgM redox forms	IgM, IgA, IgD, IgE, IgG
Ig gene rearrangement	Multicluster *(Chondrichthyes* and some *Teleostei)*	Translocon
Non-specific diversity	Several C3 isoforms *(Teleostei)*	No C3 isoforms
Overall performance		
Antibody affinity	Low	High
Antibody response	Slow	Fast
Memory response	Weak	Strong
Affinity maturation	Low or absent	High
Low temperatures	High dependence, immunosuppressive response (only in poikilothermic fish)	Low dependence
Lymphoid organs		
Haematopoietic tissue	Head kidney *(Teleostei)* Epigonal and Leydig organs, meningeal tissue, orbital and subcranial hematopoietic tissue *(Chondrichthyes)*	Bone marrow
Thymus	Involution species-dependent, influenced by seasonal changes and hormonal cycles	Involution with age
Lymphoid nodes	Absent	Present
Gut-associated lymphoid tissues	Not organized, lymphoid aggregates Leydig organ and spiral valve *(Chondrichthyes)*	Organized, Peyer patches
Germinal centres	Absent (melanomacrophage centres?), dendritic cells probably present	Present

cell phagocytosis and secretion of soluble antimicrobial molecules. Thus, the innate responses of insects and mammals involve antibacterial peptides and homologous receptors *(Toll* in insects and their TLR homologues in mammals) which use similar pathways of regulation and transcription[6-8].

The innate system is the earliest immune mechanism and it is characterised by being non-specific and therefore not depending upon previous recognition of the surface structures of the invader. It has also the advantage of being inducible by external molecules but at the same time is constitutive and reacts within a very short time scale. Although the ability to synthesize numerous unique antigen receptors is first found in jawed fishes, genes encoding for the immunoglobulin superfamily have been found much earlier[9], which shows the diverse potential of the innate immune system.

The innate response is the basis of immune defence of invertebrates and lower vertebrates. Its relevance may be assessed by taking into account the evolutive success of some invertebrates such as the arthropods, which are the major group of animals. Similarly, 40% of the species of vertebrates belong to fishes. Therefore their immune system should display a number of features that allow them to successfully maintain such a diversity, and their adaptation to a variety of environments, from deep sea to rivers or lakes of all climates and even environments combining dry and wet seasons.

Vertebrates show a moderated morphological diversity and about half of the 40,000 species develop their life cycle in water. All vertebrates gnatostomates share an essential immune structure characterized by: (i) highly conserved innate system and (ii) consistent development of a

combinatorial immune system, and (iii) bilateral communication between components of the innate and adaptive immunity. However, later in evolution the warm-blooded vertebrates arose and especially mammals have acquired a higher specialization in adaptive immunity. The acquisition of such adaptive immunity in vertebrates may have relieved some pressure on the innate mechanisms.

THE EXCISED PHENOTYPE: FISH AND ITS IMMUNE SYSTEM

Fish is a heterogeneous group of organisms that include the agnathans (lampreys and myxines), condryctians (sharks and rays) and teleosteans (bony fish)[10]. As in all vertebrates, fish have cellular and humoral immune responses, and central organs whose the main function is involved in immune defence. Fish and mammals show some similarities and some differences regarding immune function (Table I). Taking into account differences due to body compartments and cell organization, most of the generative and secondary lymphoid organs present in mammals are also found in fish, except for the lymphatic nodules and the bone marrow[11]. Instead, the head kidney, aglomerular, assumes hemopoietic functions[12, 13], and unlike higher vertebrates is the principal immune organ responsible for phagocytosis[14], antigen processing[15,16] and formation of IgM and immune memory through melanomacrophagic centres[17, 18]. The kidney in fish is a disperse organ with a Y shape that is placed along the body axis (Fig. 2). The lower part is a long structure situated parallel to the vertebral column, most of which works as a renal system. The active immune part, the head kidney or pronephros, is formed by two Y arms, which penetrate underneath the gills. In fish, this structure has a unique feature: the head kidney is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones. In addition, it is a well innervated organ. Thus, the head kidney is an important organ with key regulatory functions and the central organ for immune-endocrine interactions and even neuroimmuno-endocrine connections.

The thymus, another lymphoid organ situated near the opercular cavity in teleosts (Fig. 2), produces T Lymphocytes involved in allograft rejection, stimulation of phagocytosis and antibody production by B cells[19, 20]. The involution of thymus in fish is more dependent on hormonal cycles and seasonal variations than on the age[11]. Blood filtration and erythrocytic destruction is performed by the melanomacrophagic centres, formed by accumulation of macrophages associated to elipsoid capillaries. These centres may retain antigens as immune complexes for long periods[21].

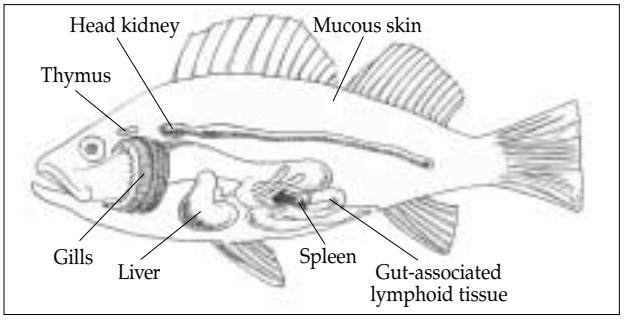


Figure 2. *Immune structures in teleost fish.*

Although it has been suggested that these centres would assume the functions of germinal centres, no evidence has been presented up to now[20, 22].

Fish immune cells show the same main features than that of other vertebrates, and lymphoid and myeloid cell families have been determined. The lymphoid system is a relatively recent evolutionary development since most animals prior to vertebrates rely on non-lymphoid cells or serum molecules. The existing functional analysis together with the reactivity of monoclonal antibodies suggest the presence of helper (Th) and cytotoxic (Tc) T lymphocytes and subpopulations of B cells (B1 y B2)[23-25]. The monocyte/macrophage cell lineage is the most studied in fish although no exact cell specific markers are available. Therefore, the term macrophage is often used as a loose definition for phagocytic cells independent of the differentiation status and the anatomical location. The majority of cytokines identified to date and of data concerning cytokine regulation has been obtained in monocyte/macrophage cell cultures[26].

External barriers and integumental mechanisms

In teleosts, the lymphoid tissue associated to teguments is distributed around the skin, gills and intestine, thus complementing the physical and chemical protection provided by the structure. Among the epidermal secretions, complement, antimicrobial proteins, lysozyme, phosphatases and trypsin are often found, although their amount and activity depend on the species[27]. As in other vertebrates the most exposed tissues are the gut, respiratory organs and the skin. This equipment is completed with immunocompetent cells such as leucocytes and intraepithelial plasmatic cells[28, 29].

The gills are a multifunctional wide surface regulating osmotic balance, excretion of part of the waste nitrogen compounds and gas, water and ion exchanges. As suggested by the named filter-feeding species, such as the sardine *(Sardina pilchardus)*, the gills may also perform feeding functions[30]. In addition to lymphocytes, the gill lamellae have pillar cells belonging to the reticuloendothelial system[31,

[32]. A relevant expression of the β form of the MHC II has been demonstrated in the gills of some salmonids[33].

As an aquatic organism, the fish body is subjected to continuous contact with many different types of microorganims. The first barrier against pathogens, the integumentary surface, is equipped with mechanisms protecting against pathogen entry. Surprisingly, some species are even resistant to immersion in water innoculated with the potent fish pathogen, *Aeromonas salmonicida*[34]. Among the important integumental mechanisms are mucus secretion, and a diverse group of antibacterial molecules. The latter are peptide-based molecules that act both directly and indirectly on components of the bacterial cell wall resulting in lysis.

Mucus is an important barrier in fish. Firstly because it provides the substrate in which antibacterial mechanisms may act. Secondly, in most fish species the mucus covers most of the external surfaces, and mainly the skin. Although this is a general trend, it is very clear that most freshwater species have a higher production of mucus compared with marine species. In addition, the production of mucus is significantly increased when subjected to stressing situations, such as chemical aggressions, which induce higher expression and activity of antibacterial agents[35]. In special cases, as in the Myxinoids, mucus production may be as high as 40% of the body weight, using this system for both immune and antipredator functions. Other surfaces such as gills have mucous cells juxtaposed between respiratory and osmoregulatory cells.

Antibacterial peptides have been found in the last years in fish surfaces. However, much work has to be done as important features regarding antibacterial activity and mechanisms of action have to be specifically determined[36]. Similarly, trypsin-like or cathepsin proteases have also been found[37]. Therefore, this is a promising field in fish immune therapy and probably a different trait compared to mammals in terms of the variety and sensitivity against microbial action.

Lysozyme is one of the most studied innate responses in fish. Lysozyme can act on the peptidoglycan layer of bacterial cell walls resulting in the lysis of the bacteria. Lysozyme has been found in mucus and ova[38], and serum lysozyme, probably coming from peritoneal macrophages and blood neutrophils, has been used as an indicator of non-specific immune response[39, 40]. The lysozyme response has been found to be variable in its potency depending on the species and the tissue location but is present in all species studied[41]. It appears that the lysozyme response in fish may be induced very rapidly and not only related to bacterial presence but also to other alarm situations such as after stress[35, 42]. Thus lysozyme in fish would be involved in the overall alarm response, acting as an acute-phase protein.

Lectins are important immune mediators in lower vertebrates and invertebrates. These proteins have the ability to bind carbohydrates, which are involved in the attachment to cell walls. Therefore lectins can block this attachment and subsequent invasion. Lectins are also involved in the induction of other immune mechanims such as activation of complement[43-45].

The complement system

Fish, as other vertebrates and invertebrates, activate their immune system after recognition of pathogen-associated molecular patterns (PAMPs) by specific receptors. These receptors act as soluble forms (LPS-binding protein, pentraxins, complement, collectins) or are associated to membranes of immune cells (epithelial, phagocytic, dendritic, granulocytic). The complement system appears to be one of the central immune responses in fish. The classic, alternative and possibly also the lectin pathways have been described in several teleost groups[46-48]. Moreover, it has been suggested that the lectin-mediated pathway may have preceded the immunoglobulin-mediated pathway[44]. Here, there are some relevant differences compared with mammals. The first is that fish possess multiple active isoforms of the key activation molecule C3. Mammals have one isoform of the C3 molecule whereas fish express several functionally active C3 isoforms, three in trout *(Oncorhynchus mykiss)* and medaka *(Oryzias latipes),* five in seabream (Sparus aurata) and carp *(Cyprinus carpio),* and three loci coding for three isoforms in zebrafish *(Danio rerio)*[49-55]. Teleost C3 share the two-chain structure although showing variations in the catalytic residues of the protein, they maintain a differential affinity for several substrates and they probably are products of several polymorphic genes[56, 57]. The biological meaning of the variability may be the specialization to bind specific surfaces and to increase efficiency to eliminate immunogens. This specialization remains, however, to be fully demonstrated. Similar to mammals in which Ig variability gives more efficiency in binding immunogens, the same variability process may have occurred in fish regarding the complement response[54]. Furthermore bacteriolytic and haemolytic activity of these C3 isoforms has been demonstrated to be higher than in mammals. This also would justify the preference for the alternative pathway response in fish[58].

The initial adaptive response: Antibodies

Several phyla use proteins belonging to the superfamily of immunoglobulins (Ig)[59], but only 1.5% of metazoans are equipped with the genetic machinery associated to an

adaptive or combinatorial system, characterized by the presence of Ig, T cell receptors (TCR) and products of the MHC that allow clonal selection of B and T cells. The absence of such components in invertebrates and agnathans suggest the «explosion» of adaptive immunity 450 Millions of years ago, in a short period between the surge of jawed vertebrates from their ancestors, ostracoderms[60-62].

The teleost Ig are limited to mainly a IgM tetramer of approximately 800 kD and no definitive evidence of Ig diversity has been demonstrated in fish. It has been long considered that fish Ig have a restricted profile of isotopes as a result of a phylogenetic simplicity. This seems consistent with evolutionary data, as fish is the first group that shows antibody activity and therefore a simple profile of one Ig appears in this group. Later on, evolution resulted in the generation of complexity and a increasing number of Ig isotypes in amphibians, reptiles and birds, being maximal in the higher vertebrates, i.e., mammals. It is then assumed that the response of fish to immune challenge is mainly based on the innate immune response.

Nevertheless, in the last years another approach to determine the Ig variability in fish has been taken by a number of researchers[63]. This approach is based on the fact that only one gene can generate as many as six structural isoforms and, therefore, diversity would be the result of structural organisation rather than genetic variability. The tetramer structure of fish Ig is composed of four monomeric units, two identical heavy (H) chains (70 kD) and two light (L) chains (25 kD). Disulphide bonds and non-covalent combinations of units may coordinate the assembly of the subunits. Thus, in salmonids, the basic covalent subunit is a monomer but the final combination for the tetramer may be a fully crosslinked tetramer or combinations of monomers, dimers or trimers[64, 65]. A similar structure has been found in the toadfish and the sheepshead[66, 67]. Therefore, a tetramer composed by non-covalent associated monomers would have the flexibility to reach distant epitopes, compared to a rigid form. Similarly, this flexibility may serve to increase the avidity by binding more epitopes and to increase the possibilities for antibody attachment. Recent studies[64, 68] suggest that the number of final forms (up to six) observed in fish would be based on a kinetic model in which the disulfide bonds would accumulate and this polymerisation process would take place in intracellular compartments late in the secretory process.

Moreover, following what is found in higher vertebrates, the research on fish antibody response and its efficiency has been focused on the antibody titre using techniques such as agglutination, precipitation or ELISA. However, this approach and these indicators may be less relevant than in other vertebrates for measuring health status. For instance, if a particular redox form is required to react with a pathogen, a high antibody titre may not be a good indicator of antibody efficiency if this form is not serologically distinguishable from other forms. On the other hand an adequate form may give high efficiency even if it occurs at low titres. Future works on the specific redox forms will help to ascertain or modify the initial idea about the antibody function in fish[68].

Cytokines in fish

Cytokines as modulators of the immune response have been little studied in fish. A significant number of cytokines are functionally active in teleosts[69], but little data are available compared to mammals. Interleukin-1β, mainly produced by macrophages, has been characterised in bony fish and cartilaginous fish as well as in different animal groups including birds, amphibians, and mammals[26]. It is an important mediator of inflammation in response to infection and recently it has been reported in the trout to directly affect hypothalamic-pituitary-interrenal axis function, stimulating cortisol secretion[70]. This suggests the possibility of autocrine regulatory circuits within the composite immunoendocrine organ, the head kidney, which may affect immune function and haematopoiesis, apart from affecting cortisol secretion.

Another potentially important cytokine, Tumour Necrosis Factor alpha (TNF-α), has also been cloned in various fish species[71-74]. Furthermore, TNF-like protein activity has been shown to induce apoptosis, and to enhance neutrophil migration and macrophage respiratory burst activity[75]. Due to the diversity of functions that have been reported in mammalian vertebrates it is likely that TNF-α will also prove to be a key player in neuroimmunoendocrine responses in fish.

Other cytokines and regulatory molecules reported in fish include a number of interleukins (IL-6, IL-8, IL-10, IL-12) (Goetz FW, personal communication) and Tumor Growth Factor (TGF)-β[76-79], and interferon regulatory factor (IRF)-1[80]. A recent study identified an interferon-like peptide in the zebrafish[81]. It becomes apparent that the relevant cytokines have been conserved in vertebrates and most of these molecules show similar roles in all animal groups.

CONCLUSION: THE DIVERSITY AFFAIR

As a group, fish are situated at a crossroads that helps to understand the evolutive history of the immune system (Fig. 1). They are in the baseline of the vertebrate «radiation», the so-called Big Bang of the combinatorial immune response[62]. Moreover, some traits of the fish immune system

anticipate mammalian sophistication. For instance, the melanomacrophagic centres in fish anticipate the function of the mammalian lymphatic nodules. The haemopoietic function of the head kidney is also found in some amphibians (Protidae and apoda), which represent a continuity in the vertebrate immune pattern[20]. On the other hand, characteristics of fish non-specific immune defence depict a divergent evolutionary pathway respect to the higher vertebrates, based on the specialisation and reinforcement of some crucial components of the inflammatory response[54].

A second consideration regarding poikilothermic vertebrates is that temperature thresholds limit immunocompetence. Thus, below certain temperatures or after thermal shock Ig production and T cell responses are reduced[82]. So, although fish may have a higher tolerance to low temperatures, a severe temperature reduction will result in immunosuppression, even for complement and acute phase proteins[83]. If this change is accompanied with seasonal and environmental ethologies (migrations, spawning), the effects can be severely aggravated due to the rise of cortisol and sexual steroids[84].

There is a third trait concerning the differential reproductive strategies between warm and cold-blooded vertebrates. Opposite to the elevated investment of birds and mammals to the offspring, the strategy in most fish consists in producing a high offspring number with very limited parental care. Therefore, the equipment included with the egg is the only source for energy, defence and protection. Thus, the presence of lectins, bactericidal proteins and Ig have been described in fish eggs[38], together with a protecting microflora in the larvae which may influence further contacts with potential pathogens[4].

Once the evolutionary scenario is known, a question arises to whether the diversification of innate immunity compensates for the weak specific immunity. It seems clear that not all the machinery of the somatic reorganization can be compensated with the genesis of functionally diverse isoforms of proinflammatory proteins. But, is such compensation really necessary? The evolutive answer is probably *no*. For example, one of the marine invertebrate groups that is characterized by its resilience, longevity and diversity is the mollusk group in which the capacity of allorejection is very poor[85]. Later studies on the evolution of the immune system create another potential point of discussion: the acquisition of recombination activation genes (RAG) may have coincided with the elaboration of the neural crest and its corresponding tissues. It should be remembered that the nervous and the immune system share properties of specific recognition, memory and ability to target and build aggressive responses. Therefore, the acquisition of such capabilities in jawed vertebrates may facilitate the development of such diversity in different directions, and eventually the interaction between the systems that share such capabilities, as occurs with some cytokines[9, 86].

It has been suggested that the main adaptive value of an immune system is not to provide a finished/closed system, but the potential to generate a somatic repertoire of receptors or a diversity of relevant inflammatory proteins[59]. If the degree of adaptation of a group of organisms to the environment and the variety of the related species in the fossil records are used as indicators of evolutive quality of a particular morphological and physiological design, fish still represent the most ancient and numerous group of vertebrates, from their explosive radiation in the Devonic period. Their persistence in the aquatic environment is surely related to the success of their immune system.

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