Fish innate immunity against intestinal helminths

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Abstract

Most individual fish in farmed and wild populations are infected with parasites. Upon dissection of fish, helminths from gut are often easily visible. Enteric helminths include several species of digeneans, cestodes, acanthocephalans and nematodes. Some insights into biology, morphology and histopathological effects of the main fish enteric helminths taxa will be described here. The immune system of fish, as that of other vertebrates, can be subdivided into specific and aspecific types, which *in vivo* act in concert with each other and indeed are interdependent in many ways. Beyond the small number of well-described models that exist, research focusing on innate immunity in fish against parasitic infections is lacking. Enteric helminths frequently cause inflammation of the digestive tract, resulting in a series of chemical and morphological changes in the affected tissues and inducing leukocyte migration to the site of infection. This review provides an overview on the aspecific defence mechanisms of fish intestine against helminths. Emphasis will be placed on the immune cellular response involving mast cells, neutrophils, macrophages, rodlet cells and mucous cells against enteric helminths. Given the relative importance of innate immunity in fish, and the magnitude of economic loss in aquaculture as a consequence of disease, this area deserves considerable attention and support.

Key words: innate immune cells, metazoan parasites, mucus, mast cells, rodlet cells, phagocytes

1. Helminth intestinal parasites of fish

In fish, as in other vertebrates, the digestive tract is one of the primary routes of microbial and parasitic infections [1], and serves as a primary barrier limiting or preventing the entry of harmful organisms [2]. The intestinal canal affords a remarkably benign and rich environment for otherwise vulnerable enteric parasites, offering them protection and nutrients [3].

Helminths, also commonly known as parasitic worms, are multicellular organisms. There is no clear consensus on the taxonomy of helminths. Helminths include some turbellarians, ectoparasitic flukes (Monogenea), endoparasitic flukes (Aspidogastrea and Digenea), and Cestoda, all belonging to the phylum Plathyhelminthes; acanthocephalans, nemathelminths (nematodes), and hirudineas or leeches belonging to Anellida [4]. Helminth parasites are of considerable medical [5] and veterinary importance [6]. Parasitic helminths in natural habitats are able to reduce drastically their host fitness, which therefore have evolved powerful counter-measures to control infection [7]. Recently, Shinn et al. [8] provided estimates of economic loss associated with notable parasite infections in some of the world's leading finfish production industries. The successful infection of helminth largely depends on their capacity to evade and/or manipulate the generally efficient immune system of hosts [5,9,10]. Nevertheless, interaction between helminth and the piscine immune system are under-investigated [10].

There are numerous studies of the effects parasitic helminths have on the alimentary canal and associated organs of fish [11-18]. As part of the infection process, certain intestinal worms induce structural modification to their host's tissues (Tab. 1), and most likely are responsible for alterations to normal intestinal physiology [48]. Certain types of enteric helminths of fish (*e.g.,* digenetic trematodes, cestodes) usually do not cause severe, visible damage to the intestine, mainly due to their relatively superficial relationship with the host tissues [49] (Tab. 1a). In contrast other helminths such as acanthocephalans typically cause much more severe damage due to deep penetration of many species into the gut tissues [50] (Tab. 1b).

Parasitic helminths excrete or secrete (ES) a variety of molecules into their hosts. The ES products of trematodes, cestodes and nematodes contribute to immune evasion strategies of the parasites through different mechanisms [51]. Research into the ES substances produced by helminths infecting fish is still very much in its infancy with only a few scattered observations on nematode-fish models (see [6,52]) and a tapeworm-fish system [10,53]. From the earlier studies of fish-helminth conducted by the authors, no tegumental secretions packaged into extracellular vesicles were observed, however that does not exclude the possibility that a fraction of ES proteins, not packaged in vesicles, may be produced by parasite. A description of each enteric helminth taxon is provided below.

1.1. Digenean (endoparasitic flukes) (Tab. 1a, Fig. 1a)

The Digenean Flukes or digeneans (formerly digenetic trematodes) form a class of flatworms or platyhelminths. Flukes reproduce as adults and again as larvae, hence the name "di-genetic" or two births. Unlike generalized flatworms, most digeneans have two sucker-like holdfast organs. About 70 families with over 5000 species are known from all fishes. The digeneans that produce significant damage to their hosts as sexual adults are mainly those that occur in non-gut sites. With reference to parasitized intestine, the site of infection by most digeneans is restricted almost entirely to the paramucosal lumen, mucosa or epithelial tissues (Fig. 1a) [54]. Most intestinal digeneans probably feed by browsing on the mucosa or epithelial tissues, mucus, blood, products of host's digestion, and products of their own histolytic secretion [55]. Many digeneans with at least one sucker attach to the mucosal surface of the fish digestive tract (Fig. 1a) [22,56]. Therefore, the main damage is the destruction of the mucosal epithelium covering the villi, with subsequent necrosis and degeneration [54,56].

1.2. Cestoda (tapeworms) (Tab. 1a, Fig. 1 b,c)

Tapeworms or cestodes form a large class of the flatworms with more than 5000 species identified. The common name comes from the long series of body segments which resemble a tape measure. All tapeworms are permanent parasites. Adults cestodes inhabit the digestive tract or, occasionally, associated organs of vertebrates definitive hosts [57,58]. Tapeworms usually consist of a chain of segments (proglottids) each with a set of reproductive organs and lack a digestive system, absorbing nutrients through a specialized outer layer of the body. Eucestoda possess a distinct anterior holdfast organ called the scolex (Fig. 1b,c), which varies remarkably in shape among the 11 orders [57]. The extent of damage caused by cestodes as in other helminths is generally related to the intensity of infection and depth of parasite penetration within the host tissue (Tab. 1a). Most tapeworms generally do not induce severe damage to the fish digestive tract, provoking only destruction of the superficial layer of the intestinal wall at the point of scolex attachment (e.g., *Cyathocephalus truncatus* see Tab. 1a, Fig. 1b,c). More rarely tapeworms penetrate more deeply, closely approaching the muscular layer and inducing both a complete loss of the intestinal architecture and an enhanced inflammatory response (e.g., *Monobothrium waganeri* see Tab. 1a).

1.3. Acanthocephala (thorny headed worms) (Tab. 1b, Fig. 1d,e)

These worms form a small phylum in the Animal Kingdom. The name "acanthocephala" means thorny headed. Acanthocephalans are all permanent parasites in the intestine of most vertebrates.

More than 1500 species are known, the vast majority of which are parasites of fish and use crustaceans as intermediate hosts. Acanthocephalans attach in the gut of their host with a globular or cylindrical and retractable, thorny proboscis. In addition to the intensity of infection, the extent of damage caused by acanthocephalans is related to the depth of parasite penetration within the host tissue (Tab. 1b). Some acanthocephalan genera parasite of fish, including *Acanthocephalus* [50] and *Pomphorhynchus* (Tab. 1b) penetrate deeply through the intestinal wall and provoke extensive damage to the alimentary canal (Fig. 1d). At the site of attachment the acanthocephalan parasite destroys the mucosal folds (Tab. 1b, Fig. 1d,e) and the proboscis hooks penetrate into the epithelium of adjacent villi for secure anchoring. Folds more distant from the worms remained intact.

1.4. Nematoda (roundworms) (Tab. 1c, Fig. 1f)

The phylum Nematoda are comprised of 256 families and more than 40000 species [59]. Most free-living forms are small to microscopic, but parasitic forms are large. It is believed that there are 125 families of zooparasitic nematodes, including species that exploit both freshwater, marine and brackish water fishes. Roundworms, as the name implies, are circular in cross-section, and often take the form of an elongate cylinder, tapered at each extremity. The body of nematode is covered by a thick, elastic and tough cuticle, which could be smooth, or more generally bears fine transverse striations at regular intervals [60]. Most species are dioecious, sexually dimorphic and oviparous.

Due to the rapid development of marine aquaculture, the importance of nematodes as fish pathogens is increasing [60]. Most of our knowledge on nematodes as fish parasites is founded in the numerous papers and monographs of Moravec [see 60]. Nematode parasites harm their host in different ways, such as causing mechanical injury, atrophy of tissues, castration, and occlusions of the alimentary canal and blood vessels [61,62]. While most references on nematode parasites are concerned with the prevalence and intensity of infection, there are some studies on the pathogenecity of fish intestinal nematodes (Tab. 1c).

2. Fish immune system

The Latin word "*immunis*" means "exempt from", and the term immunology refers to the field of research on defence mechanisms against infectious diseases [63]. The immune system has evolved to discriminate between self (e.g., host tissue) and non-self (e.g., pathogens, foreign bodies). Defence mechanisms include two broad categories which differ between them mainly for the receptor types used to recognize pathogens [64]:

I) Specific (adaptive or acquired) immunity, which responds to an invading pathogen and then reacts in an appropriate manner to eliminate it. The adaptive immune recognition is mediated by antigen receptors with narrow specificities. Upon repeated exposure to the pathogen, the specific immune system produces a faster and more adequate response. Lymphocytes are the primary cell types involved in specific immune responses [63,65].

II) Aspecific (non-specific or innate) immunity, which provides an immediate response to an invading organism and is composed of physical barriers, cellular and humoral components [66]. The innate immune recognition is mediated by germline encoded receptors with a broad specificity [67]. The innate defence is the only mechanism available to invertebrates and is of primary importance in vertebrates, especially in fish where the acquired immune response is sluggish compared to the instant and relatively temperature-independent innate immune response [66,68]. Innate response generally precedes the adaptive one and also plays a fundamental role in the organization of the acquired immune response and the maintenance of homeostasis [69].

Host responses against invading pathogens are basic physiological reactions of all living organisms [3]. Fish include over 27,000 species and are, phylogenetically, the oldest vertebrate group representing more than one-half of the vertebrates on the planet [70]. Both wild and farmed fish suffer from a number of protozoan and metazoan parasites, fungi, bacteria and viruses. The importance of fish health is not limited to the professionals who work in fisheries science but extends to aquatic ecosystems where fish diseases play an important role. Understanding the immune system of fish is of great importance as it provides information on the evolution of immunity in vertebrates [71]. According to Whyte [72], teleost fish occupy a key evolutionary position in the development of the innate and adaptive immune responses in that they are the earliest class of vertebrates possessing the elements of both types of immunity. Parasitic infections in teleost fish are limited by constitutive innate defence mechanisms that render the host refractory or reduce the severity of infection [73]. Recent studies however, have begun to clarify the relative roles of innate and acquired immunity against parasitic infections in teleosts by recognizing the presence and significance of specific innate effector mechanisms [73].

2.1. Innate cellular response against intestinal helminths

The innate defences responding to infections are associated with an inflammatory reaction [3,6,9,63]. The attachment organs of endoparasitic helminths (Fig. 1) often provoke inflammation of the host gastrointestinal tract. Inflammation is a protective reaction of the host in response to injury, resulting in specific chemical and morphological alterations in cells and tissues [63,74]. In

teleosts, gills, skin, urogenital system and gut are the principal mucosal surfaces and represent the first line of defence [75]. The cellular involvement during the inflammatory response may be biphasic, beginning with an influx of neutrophils followed by the subsequent arrival of monocytes/macrophages [76]. Innate immunity in teleosts relies on a range of cell types [63], which are listed below. In turn we will examine each cell in detail with respect to morphology, function and involvement against helminth infections.

2.1.1. Mucous cells

Fish mucus is involved in a wide range of functions, including respiration, reproduction, excretion, feeding, ionic and osmotic regulation, and protection against, and resistance to, disease [77-80]. The first level of gastrointestinal defense consists of the substances secreted into the lumen, including mucus (Fig.1c,e), bicarbonate, nucleic acid, immunoglobulins, and other antibacterial and surface-active phospholipid materials [81]. Mucus is an essential component of mucosal innate immunity [75,77] with intestinal mucous cells playing a key role in controlling the inflammatory response [82,83]. In some fish species, mucous cells produce and release defensive materials [84,85]. Generally, the nucleus of the mucous cell is observed to be elongated and basally placed (Fig. 2a,b). Mucus granules occupy the entire supranuclear cytoplasm (Fig. 2a,b), appearing as spherules or polyhedrons surrounded by a single granule membrane. Within TEM sections, the mucus granules appeared electron-opaque and, in some instances, as electron-lucent granules (Fig. 2a,b).

Numerous studies of fish-helminth systems have demonstrated an increase in mucous cell abundance and/or mucus production (summarized in Table 1; examples shown in Fig. 1c,e). The intestines of brown trout, *Salmo trutta* and chub, *Squalius cephalus* infected with the acanthocephalan *Pomphorhyncus laevis* [16,32] as well as those of brown trout parasitized with *Echinorhynchus truttae* (Acanthocephala) and *Cyathocephalus truncatus* (Cestoda) [25] showed an hyperplastic response of mucous cells. Copious mucus secretion appeared as an adherent blanket around the worm body at the site of infection [16,25]. The increase in the number of mucous cells observed in the intestine of fish only in close proximity to helminths suggests that the parasite elicit a local rather than a general/diffuse response of the intestine [25,32].

Mucus qualitative changes have been reported in response to intestinal parasites of fish, possibly with a defensive significance [16,25,32,79,86,87]. In the infected intestine, mucous cells of several fish species secrete neutral and acidic glycoconjugates; the acidic types are mainly sialylated or non-sulphated glycoconjugates [e.g., 16,25,32]. Acid mucins enhance viscosity of

mucus secretion resulting in greater protection against pathogens [87]. In particular sialic acid-rich glycoconjugates inhibit bacterial adhesion to fish cells [79,88].

A close relationship between degranulation of mast cells (MCs) and excessive mucus secretion by mucous cells has been reported in several studies on mammal-intestinal helminth systems [89-91], while similar reports in fish-helminth systems are rare [49,92]. In a recent study of chub intestine naturally infected with an acanthocephalan, transmission electron microscopy and confocal microscopy revealed a close spatial relationship between intestinal mucous cells and MCs, and degranulation of MCs bordering the plasmalemma of mucous cells was frequently detected [33]. The same close relationship between MCs and mucous cells in the epithelium of infected intestine was reported in *Silurus glanis* (Fig. 2b). The possibility exists that in fish, as in mammals, epithelial MC degranulation induces excessive mucus discharge by mucous cells against an invading parasite.

An additional and crucial issue that has yet to be resolved is the role of the overproduction of mucus in the alimentary canal of fish infected with enteric helminths. It has been proposed that excessive mucus may contribute to the elimination of parasites from mammal intestine [93], yet in over three decades the authors of this review have never seen the expulsion of worms from the host gastrointestinal tract, despite having examined several fish-helminth systems i*n situ*. Thus, we believe that the lack of expulsion of many acanthocephalan and cestode species from host intestine could be due to the deep penetration of proboscis and scolex in the fish intestinal tissue. The function of blanket of mucus is mainly to protect the intestinal mucosa as a physical barrier against the mechanical and biochemical damages induced by parasites (Fig. 1c,e), as suggested in several studies [25,32,79,86,94].

Intestinal helminths are known to alter intestinal physiology, permeability, mucus secretion [16,25,83,95] and production of antimicrobial peptides [42], all of which may impact on bacterial survival and spatial organization [83]. Several peptides involved in the regulation of intestinal mucus secretion are released during inflammation [17,94-99]. Some opioid peptides like leu- and met-enkephalin are commonly found within the gut neuroendocrine system of teleosts [100]. Opioid peptides play an important role in the discharge mechanism of mucus from mucous cells induced by luminal stimuli [101]. Within the fish's intestine, galanin acts as a cholinergic co-mediator [102,103], and the release of mucus from mucous cells is stimulated by cholinergic agonists [104]. Serotonin is involved in mucus secretion [97]. A plethora of recent studies have shown that helminths can induce a marked change in the presence of certain neuromodulators in the intestine of their fish host (Figs. 3, 4a) [17,18,95,98,99,105,106]. Bosi et al. [95] demonstrated that chub intestine infected with an acanthocephalan shows an increase in the number of endocrine cells

releasing leu- and met-enkephalin, galanin, and serotonin, and suggest that these regulatory substances may be involved in the hyperplasia of mucous cells and in enhanced mucus discharge.

2.1.2. Mast cells (MCs)

Granulocytes, which are identified by distinctive cytoplasmic granules, are subdivided into neutrophils, eosinophils and basophils which are found in peripheral blood and some organs of fish [107]. The term "eosinophilic granule cells" was introduced by Roberts et al. [108] to indicate mononuclear granule-containing cells which were found distributed in the connective tissues of teleosts. In recent years there has been a tendency to use the more conventional term "mast cells" as these cells have functional and morphological similarities to mammalian MCs [109]. Fish MCs are irregular in shape with an eccentric, polar nucleus, and a cytoplasm characterised by numerous large, electron-dense, membrane-bounded granules (Fig. 2b-f). Piscine MCs constitute a heterogeneous cell population [76,109]. One of the most controversial aspects of this heterogeneity is the staining properties of the cytoplasmic granules [see 109].

MCs are important as initiators and effectors of innate immunity and regulators of the adaptive immune response. MCs exist in all classes of vertebrates, sharing both a similar morphology and, most likely, function [110]. MCs are probably present in all teleosts and are found in a variety of tissues and organs, especially the gastrointestinal tract (Fig. 4b), skin and gills [41,76,111]. MCs are motile [41,76,112] and are often strategically positioned at perivascular sites to regulate inflammation, thus placing them in a unique position to encounter invading organisms and to orchestrate a response [28,41,90,91,113].

MCs in non-mammalian vertebrates contain a wide range of compounds (i.e. heparin, neuropeptides, proteases) and also, in bony fishes, antimicrobial peptides (AMPs) [114,115]. Only the MCs of perciform fish contain histamine [110]; this biogenic amine regulates the inflammatory response [116] by acting on professional phagocytic granulocytes [110]. The occurrence of histamine in the gastric mucosa, as a regulatory molecule of acid gastric secretion, is a general feature in all vertebrates [117]. Both vertebrates and invertebrates produce AMPs, which are a key factor in innate immunity [118,119]. One of the most common groups of AMPs in fish are the piscidins, a family of linear, amphipathic peptides [120-122]. Piscidins have potent, broad-spectrum antimicrobial activity against viruses, bacteria, fungi, water molds and metazoan parasites [42,120,123,124].

MCs degranulate in response to exposure to a variety of pathogens [28,41,68] and known degranulating agents [109,111,125]. In turn MC degranulation can promote other events, for example intestinal contraction in gilthead seabream and rainbow trout [110,125]. Degranulation of

fish MCs close to the tegument of helminths in intestine (Fig. 2e) and other organs was reported in some studies of the present authors [e.g., 13,27,28]. Only the MCs in association with the extra-intestinal infections of the acanthocephalan *P. laevis* in *Gasterosteus aculeatus,* were observed lying on the surface of the parasite or the granules had penetrated the tegument [28].

Changes in the production of proliferating cell nuclear antigen (PCNA) within intestinal mucosa can provide an early indication of deviations to normal gut function and PCNA analysis has recently been applied to the field of aquatic parasitology [39,124]. Changes in PCNA expression can be determined through immunohistochemistry and marked increases in the rate of cellular division in intestinal tissue have been reported using this approach [126]. Infection of the acanthocephalan *Dentitruncus truttae* within the intestinal tract of *S. trutta* elicited a significant increase in the number of PCNA positive MCs at the site of parasite attachment when compared to the number found in both uninfected conspecifics and in tissue zones away from the point of parasite attachment [39].

MCs play an important role in responding to inflammation and their number increases in allergic reactions in mammals [113] and as a consequence of helminth infection (Tab. 1; Figs 2c, 4b). Evidence of MC migration has been found in gut tissues of salmonids [127,128]. Within the intestines of infected fish, numerous MCs were observed to be in close contact with capillaries and the outer layer of the endothelia as well as within the lumen of the blood vessels [13,28,41,42]. This close association with the endothelial cells of capillaries suggests that MCs migrate across the endothelium [30,41]. Indeed, the occurrence of the MCs throughout the propria-submucosa of intestine [12,39,42,129] suggests that there is a resident population of these cells. Based on a considerable body of descriptive data, it is reasonable to presume that fish have two populations of MCs, a circulating and a resident population, and the presence of parasites may induce recruitment of MCs to the site(s) of infection [80] and proliferation *in loco* of MCs of the resident population [39,124]. Accordingly, acute MC activation is a feature of many types of tissue injury; experimental studies have demonstrated that pathogen products can activate MCs [130].

From *in vivo* infection experiments, it has become evident that the tapeworm *Schistocephalus solidus* is capable of substantial manipulation of cellular immune responses of its second intermediate host, the three-spined stickleback *Gasterosteus aculeatus* [131]. Accordingly, an initial increase of granulocytes in the blood and head kidney of infected fish was observed; only after 63 day post-infection, the proportions of granulocytes started to decrease at both sites, while lymphocytes were increasing. This might reflect the ability of *S. solidus* to impair the cellular response of its host [131]. Scharsack et al. [53] were the first to investigate *in vitro* the effects of helminth excretory/secretory (ES) products on piscine leukocytes. Unfortunately, our current

knowledge on the ES substances produced by fish helminths and their effects on their host's immune systems are too limited for conclusions to be made at this time [52].

2.1.3. Phagocytes: neutrophils and macrophages

Phagocytosis is a well-conserved innate defense mechanism that has served as a robust platform for incorporation of novel layers of immunological control [132]. Phagocytes contribute to both pro-inflammatory and anti-inflammatory (resolution) responses at infectious foci [132,133]. In fish, two major professional phagocyte populations have been described: granulocytes (particularly neutrophils) and mononuclear phagocytes (circulating monocytes and tissue macrophages) [107]. Furthermore, B cells (a type of lymphocyte) have been shown to have phagocytic capacities in teleost [134]. The role of piscine neutrophils and macrophages in inflammatory regulation and pathogen killing has not yet been studied in detail [135]. Nevertheless, phagocytosis and production of oxygen radicals/reactive oxygen species (ROS), nitric oxide (NO) and reactive nitrogen species (RNS) are known to occur in both fish phagocytes [136,137]. Moreover, they can synthesize cytokines/chemokines [66,138]. Monocytes and macrophages release ROS and RNS predominantly intracellularly, while neutrophils do so both intracellularly and extracellularly [139]. The extracellular release of these reactive oxygen and nitrogen species provide a defense against pathogens that have escaped internalization or are too large to be internalized [139]. In mammals NO, RNS, ROS exert biocide action, contributing to the control of bacteria, parasites and tumoral cells, and recent evidence suggests additional functions in innate and adaptive immunity such as cytokine response and immune cell apoptosis modulation [140]. Among its numerous functions, NO exhibits potent toxic and antimicrobial effects against different fish pathogens [139].

Neutrophils are involved in the inflammatory process, especially during the period of initial pathogen challenge, migrating to and accumulating at the site of parasitic infection or injury [9,19,139,141-143]. Fish neutrophils present morphological and histochemical staining properties very similar to their counterparts in mammals [144], indeed, they can be recognized by the presence of myeloperoxidase in their cytoplasmic granules [63]. Neutrophils appear round to oval in shape though their outline was commonly irregular (Fig. 2d,e). These cells have a round nucleus and a cytoplasm that contains dark, elongated granules which are lamellar in appearance (Fig. 2d,e). Neutrophils can be found in spleen, kidney and blood and in inflammatory lesions [145,146]. The continuous production of neutrophils, and the capacity of the immune system to respond to pathogens by increasing neutrophil numbers, must be tightly regulated [142]. In response to inflammatory stimuli, neutrophils migrate from the circulating blood to infected sites; whereupon they efficiently bind, engulf, and kill bacteria by proteolytic enzymes, antimicrobial proteins, and

reactive oxygen species [139,147]. The receptors and growth factors that are involved in neutrophil development, as well as the overall development process itself, have remained largely unexamined in teleosts [142] but some data are provided in a recent review by Havixbeck and Barreda [139].

Under some "emergency" conditions, localized infection can induce long-range recruitment of neutrophils from hematopoietic tissue [145], including the bone marrow in humans and the CHT (caudal hematopoietic tissue) in zebrafish larvae, which are the primary sites of neutrophil production [146]. Mobilization and activation of granulocytes has been considered a significant part of the immune response to helminth parasites by rainbow trout [141], roach and carp [148,149] and three spined-sticklebacks [131]. Fish neutrophils have also been shown to phagocytise small foreign particles [65,142], and upon degranulation release the granule contents in close proximity to parasites (Fig. 2e) [19,27,150]. Neutrophils commonly co-occur with macrophages that readily engulf small extracellular pathogens, such as viruses and bacteria [68]. There are several records of mammals infected by helminths where the macrophages were able to kill trematode larvae [151] and∕or eosinophils and neutrophils were able to kill adult and larvae of nematodes [152,153]. The mechanism by which these cells mediate protection against helminth infection is through recruitment to the site of infection, surrounding the worm, and adhesion to the parasite's body. Eosinophils and neutrophils then degranulate on the cuticle of nematodes [152,153], while the macrophages penetrate the tegument of the trematode inflicting damage that ultimately results in the death of the parasite [151].

In the case of helminth-infected fish neutrophils have been observed in intestine of tench, *Tinca tinca* harboured metacercariae of a trematode [19] and in the intestine of tench infected with adult *Monobothrium wageneri* [27] (Fig. 2e). The tight clustering of this tapeworm and the deep penetration of their scolices inflict severe mechanical damage to the tench intestine and induce an intense inflammatory response, with the migration, recruitment and degranulation of neutrophils at the site of infection (Tab. 1; Fig. 2e). However, what is less clear is whether these phagocytes have the ability to directly kill helminths [9].

Teleosts macrophages are widespread in the gills and body cavity, but are mainly encountered as reticulo-endothelia cells (mononuclear phagocyte system) in the kidney and spleen [63]. Macrophages of adult fishes and their ontogeny have been reported in some teleost species using appropriate markers for specific cell populations [154]. The macrophages appeared large and contained vesicular structures with electron-opaque contents (dense bodies) and electron-lucent vesicles (Fig. 2f). In mammals, the macrophage colony-stimulating factor receptor (Mcsfr) has been used as a marker of macrophages, since its expression in embryonic and adult mice is largely

restricted to the monocyte/macrophage lineage [155,156]. Moreover, the Mcsfr gene is expressed in macrophages of zebrafish [157] and gilthead seabream [158].

Many macrophages of fish contain different types of pigments including melanin [63]. These groups of cells are termed macrophage aggregates (MAs) or melano-macrophage centres [159,160]. MAs may be found within tissue encapsulating many foreign bodies and parasites [160]. MAs functions have been reported to include the focal destruction, detoxification and recycling of endogenous and exogenous materials [161,162]. Fundamental to the protection offered by the phagocytes is their bactericidal activity, which is closely associated with the production of reactive oxygen and nitrogen intermediates [136].

In fish, the innate defences responding to helminth infection involve macrophages [163-165] and MAs [19,58,159,166]. Activity of macrophages from *Oncorhynchus mykiss* in response to diplostomules of the eye fluke *Diplostomum spathaceum* has been reported *in vitro* by Whyte et al. [163]. According to Roberts [167], when a common acute inflammatory response is elicited, it is characterized by the presence of neutrophils and monocytes in the blood and by an accumulation of neutrophils and macrophages at the site of infection (Fig. 4b). Within an inflammatory site, macrophages are exposed to both pro-inflammatory stimuli and dying cells [133].

Larval migration of digeneans, cestodes and nematodes and their subsequent encapsulation within the viscera and body tissues of fish, generally induces the development of fibro-granulomatous lesions [19,58,124,165,168]. Fish granulomas are inflammatory foci composed of concentric layers of epithelioid cells and they are very similar to mammalian granulomas [167,169]. Epithelioid cells are so-named because of their morphological similarity to epithelial cells [170]. It is believed that epithelioid cells are typically transformed macrophages, which have the principal role of engulfing foreign agents [63,160]. The formation of epithelioid and giant cells in fish is known to occur from a variety of insults and their origin from macrophages was observed *in vitro* [107]. Macrophages seem to play an important role in the immune response to helminth parasites in fish [9,163]. Nevertheless, the exact role of macrophages in the immunity against helminths has not yet been elucidated [171].

2.1.4. Rodlet cells

For over a century, fish histologists and pathologists have attempted to determine the origin and functions of the enigmatic rodlet cell (RC). RCs appear elongate in shape and are characterized by a distinctive cell cortex and club-shaped electron-dense inclusions, called rodlets (Fig. 2a,c), which accounts for their name. RCs have been encountered in a wide range of tissues of freshwater and marine teleosts [172-174]. RCs have been observed in many organs, most often associated with

epithelia, including the intestinal epithelium (Fig. 2a), and generically associated to mesothelia and endothelia [19,172,175-177].

Contrasting points of view of the nature of RCs have been proposed since their first description by Thélohan [178] who believed they were parasites. Several authors favoured the parasitic nature of RCs [179-183] but this remains questionable, with many of the unresolved issues enumerated in the review of Manera and Dezfuli [172].

The literature on RCs as endogenous fish cells is extensive [see 172,184]. RCs display intraspecific and interspecific variability in size. The topic has been previously and specifically addressed by Manera et al. [185] who concluded that size differences had to be attributed mainly to fish species. Nevertheless, tissue type (within the same species) contributed significantly as a source of difference, providing evidence for the presence of RC morphotypes [185]. Additional reports have also suggested the possible existence of RC morphotypes [186-188]. In particular, co-occurrence of two different types of mature RCs were reported in the kidney tubules of gilthead sea bream *Sparus aurata* L. [187]. Advances in molecular biology and immunological methods applied to RCs should enable us to elucidate whether different putative morphotypes may behave differently [185].

Several factors likely influence the variance in RC numbers: fish species, crowding season, the ionic concentration of the water and stressful stimuli including exposure to toxicants [189-193]. A correlation has been shown between the rising number of RCs and fish parasite infections [20,167,186,194-198]. RC numbers increase in fish infected with metazoans, especially at the site of infection or attachment [19-21,109,123,168,175,195-197]. Indeed, in the intestine of eel, *Anguilla anguilla,* naturally infected with digenean species, free RCs were observed in host lumen and bacteria were attached to them (Dezfuli personal observations).

Within the last decade, many authors favoured the hypothesis that RCs belong to the host defence system [19,76,172,175,186,194,199]. These suggestions were based primarily on common features between RCs and leucocytes (e.g. marginal location of such cells in blood vessels) [19,197] and aggregation of RCs at the site of parasite infection (see above). Occurrence of RCs in reactive foci as a result of infection with micro- and macro-parasites was reviewed by Manera and Dezfuli [172] and there have been further reports on this topic [19,21,27,168,175,184,197,200]. Data on numerous fish-helminth systems suggest that RCs represent an inflammatory cell type closely linked to other piscine immune cells (e.g. mast cells, epithelioid cells and mesothelial cells) [19,27,76,109,165].

3. **Conclusions: overview on fish response to helminths**

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Generally enteric helminths elicit an increase in the number, migration and/or accumulation of certain types of host immune cells at the site of infection (summarized in Table 1).

The innate immune responses of fish to infections share several similarities although each host-parasite system has its own peculiarity.

The main cell types involved and the extent of the fish defence response vary according to:

- the depth of penetration of the helminth: RCs and mucous cells occurred particularly when the helminth is attached to the intestinal epithelium (*e.g.,* eel-digeneans); granulocytes and macrophages increase especially in response to those worms which penetrate more deeply into the intestinal wall (*e.g.,* trout-acanthocephalans, tench-tapeworm);
- the dimension and morphological characteristic of the helminth and intensity of worm burden;
- phase of the infection: neutrophils are the first cell type recruited to the site of an acute inflammatory response; macrophages prevail during a chronic inflammation.

In most infections, both the histological damage and the inflammatory reaction are limited to the point of attachment of the parasite or near its body whilst the intestinal areas far from the helminths resemble uninfected intestines. Thus the elicited response appears local rather than general or diffuse. One of the main and more frequent reactions of the intestine against a worm is the hyperplasia of the mucous cells and secretion of excessive mucus around the parasite. When a helminth challenge occurs in the intestine both the density of mucous cells and the composition of their mucin granules could be modified.

The different host immune cells, which are typically able to phagocytise and/or secrete active compounds, often co-occur at sites of infection and cooperate to orchestrate an efficient and integrated defence response. Some examples are seen in the relationships between: MCs and endocrine cells; epithelial MCs and mucous cells for regulation of mucus discharge; and MCs and fibroblasts at sites of infection for host tissue remodelling.

4. Further research perspectives

In recent years there has been a renaissance in the study of piscine immune systems. These investigations have significantly expanded our knowledge of the evolution and diversification of vertebrate immune system [201]. This is exemplified by the recent publication of a number of review articles illustrating not only the growing scientific interest for this area but also emphasizing the series of notable advances made. Beyond the small number of well-described models that exist, research programs focusing on innate immunity in fish against parasitic infections are lacking. Part of this perhaps lies in the challenges in creating the different types of host-parasite models that are

necessary to address the range of responses observed in fish [6]. Given the relative importance of innate immunity in fish, and the magnitude of economic loss in aquaculture as a consequence of disease, then this area deserves considerable attention and support.

In recent years there have been significant advances in the understanding of the molecular mechanisms involved in the immunomodulation of various ES proteins and other products generated by mammalian helminths. Still, knowledge regarding the occurrence and effects of helminth ES proteins on the immune systems of fish is limited. Likewise, the mucosal immune system of higher vertebrates has been the subject of intense investigation for several decades but in marked contrast to this, only scant details regarding intestinal mucosal immunity in fish are known. For example, several AMPs and acute phase response related factors such as lysozyme, various anti-proteases or cytokines have been recorded in teleosts but their role in the host's response to parasitic infection is not completely known. Some insights on expression of pro-inflammatory cytokines in fish infected with ectoparasites are provided in [202,203].

Further studies are required on the relationship between fish immune systems and helminths. Indeed, it is necessary to intensify immunohistochemical investigations in search of a possible link between the different fish innate immune cells. Current advances in molecular biology should enable us to elucidate the nature of some of these cells (e.g. MCs and RCs), for which data is currently lacking. The application of molecular biological and immunopathological approaches to fish-helminth systems will expand our knowledge of fish pathology and lead to a greater understanding of the immune mechanisms in fish. Ultimately this might lead to the discovery of novel aspects in mammalian immunity.

Figure captions

Fig. 1. Histological sections showing intestine of fish infected with helminths belonging to different taxa. (**a**) Section of *A. anguilla* intestine with two digenean trematodes attached to the epithelium through the ventral suckers (arrows), Azan-Mallory stain, bar = 100 μm. (**b**) Intestine of brown trout *Salmo trutta* infected with the cestode *Cyatocephalus truncatus* (asterisk); the scolex (arrow) of the parasite erodes the epithelium, Azan-Mallory stain, bar = 200 μm. (**c**) Near the cestode (asterisk) attached by its scolex (arrow) a high number of mucous cells and a mucus layer (curved arrows) are clearly visible in the intestine of a brown trout, Alcian Blue/PAS, bar = 200 μm. (**d**) All the intestinal layers of the chub *Squalius cephalus* are disrupted by the acantocephalan *Acantocephalus anguillae* (asterisk); the hooked proboscis (arrow) of the parasite penetrates deeply into the intestinal wall, Azan-Mallory stain bar = 200 μm. (**e**) Abundant mucus secretion (curved arrow) surrounds the acantocephalan *Echinorhynchus truttae* (asterisk) infecting the intestine of *Salmo trutta*, Alcian Blue/PAS, bar = 200 μm. (**f**) A nematode (asterisk) is visible in the lumen of the intestine of brown trout, Haematoxylin and Eosin, bar = 100 μm.

Fig. 2. Transmission electron microscopy of fish immune cells in infected intestines. (**a**) *Anguilla anguilla* infected with trematodes: in close proximity to the epithelial surface two mucous cells (arrows) and one rodlet cell (arrow head) scattered among enterocytes are visible, bar = 4.2 μm. (**b**) Close contact between a mast cell (arrow) and a mucous cell (curved arrow) in the epithelial layer of the intestine of *Silurus glanis* infected with a cestode, bar = 3.0 μm. (**c**) Co-occurrence of rodlet cells (arrow heads) and numerous mast cells (arrows) in the sub-mucosa layer of tench infected with the tapeworm *Monobothrium wageneri*, bar = 3.3 μm. (**d**) Micrograph from *Squalius cephalus* intestine showing two mast cells (arrows) and one neutrophil (curved arrow) in the inflammatory tissue around the acanthocephalan *Pomphorhynchus laevis*, bar = 2.5 μm. (**e**) Several neutrophils (curved arrows) and mast cells (arrows) in degranulation in the vicinity of the scolex tegument of *M. wageneri* (asterisk), bar = 3.3 μm. (**f**) Presence of mast cells (arrows) and macrophages (curved arrow) in the intestinal granuloma encircling a larva of the nematode *Contracaecum rudolphii*, bar = 2.6 μm.

Fig. 3. Sections taken from the intestine of infected fish showing the immunoreactivity of the endocrine cells to some peptides involved in mucus secretion. (**a**) Several endocrine cells immunoreactive to the anti-galanin antibody scattered in the intestinal epithelium of rainbow trout, *Oncorhynchus mykiss* infected with the cestode *Eubothrium crassum* (asterisk). Some positive endocrine cells (arrows) are in contact with the mucous cells (arrow heads), bar = 50 μm. (**b**) The

image shows two endocrine cells positive to the anti-met-enkephalin antibody (arrows) in a mucous cells-rich epithelial region of the intestine of the brown trout *Salmo trutta* parasitized the acanthocephalan *Pomphorhynchus laevis*, bar = 10 µm. (**c**) In the intestinal epithelium of *Squalius cephalus* infected with *P. laevis*, anti-serotonin immunoreactive endocrine cells (arrows) and mucous cells (curved arrows) mainly containing acid glycoconjugates (Alcian Blue positive) are numerous, bar 20 µm.

Fig. 4. Confocal Laser Scanning Microscope images of the intestine of *Squalius cephalus* infected with the acanthocephalan *Pomphorhynchus laevis*. (**a**) Numerous endocrine cells immunoreactive to anti-met-enkephalin antibody (arrows) close to and in contact with mucous cells positive to the lectin DBA (arrow heads) in the intestinal epithelium, bar = 10 µm; (**b**) High number of mast cells and macrophages-like cells immunoreactive to the anti-macrophage (MAC387) antibody in the tunica propria-submucosa (arrows) and in the epithelium (curved arrows) near the mucous cells positive to the lectin DBA (arrow heads). Asterisk indicates the parasite's body, bar = 20 µm.

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