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Piscidins in the intestine of European perch, *Perca fluviatilis*, naturally infected with an enteric worm

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ABSTRACT

This study set out to determine how an enteric parasite, the thorny headed worm *Acanthocephalus lucii*, affected the expression of antimicrobial peptides (piscidins) in its host population, the European perch (*Perca fluviatilis*) collected from Lake Piediluco in Central Italy. A total of 87 perch were examined; 44 (50.5%) were infected with *A. lucii* (1–18 worms fish^{-1}). Pathological changes and immune response were assessed using histological, ultrastructural and immunohistochemical techniques. The acanthocephalans only penetrated the surficial zone of the intestinal wall and induced only slight inflammation. The main damage was destruction of the mucosal epithelium covering the villi adjacent to the parasite's attachment site, and included necrosis and degeneration. Infected intestine had numerous mast cells (MCs), often in close proximity to, and within, the capillaries, and were associated with fibroblasts of the submucosal layer. Mast cells were irregular in shape with a cytoplasm filled by numerous electron-dense, membrane-bound granules. Immunostaining of intestine with antibodies against the antimicrobial peptides piscidin 3 and piscidin 4 showed subpopulations of MCs that were positive. Piscidin-positive MCs were mainly observed among the epithelial cells of the intestine, but also within the submucosa. In both uninfected and parasite-infected perch, the number of MCs positive for piscidin 4 was higher than those immunoreactive with piscidin 3 ($p < 0.05$). For both piscidins, there was no significant difference in the number of positive MCs between parasite-infected and uninfected intestine ($p > 0.05$). However, uninfected fish showed higher immunostaining intensity for piscidin 3 than infected conspecifics ($p < 0.05$).

1. Introduction

European perch, *Perca fluviatilis* (L.), is of great economic importance for the local fishery at Lake Piediluco, Italy. Unfortunately, the perch population there has declined drastically in recent years, apparently due to environmental deterioration caused by eutrophication and the introduction of exotic fish species that are potential competitors [1]. Due to its wide distribution in the Palaearctic region, European perch hosts numerous endoparasitic helminths [2]. For example, it is the principal definitive host for *Acanthocephalus lucii*, which is one of the most widely distributed acanthocephalans parasitizing freshwater fish throughout Europe [[3], current study].

In fish as in other vertebrates, the digestive tract is one of the primary routes of microbial and parasitic infection [4], and serves as a primary barrier limiting or preventing the entry of harmful organisms [5]. There are numerous reports on the effects of endoparasitic helminths on the alimentary canal and associated organs of fish; some accounts appear in Sharp et al. [6] and Dezfuli et al. [7]. 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 [8]. In contrast, "thorny-headed worms" (Phylum Acanthocephala) typically cause much more severe damage due to both high parasite density and aggressive penetration into the gut tissues [9].

In vertebrates, innate immunity is a complex system composed of cellular and humoral responses [10]. The innate immune system is the first line of defense against microbial and parasitic infections

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and is regarded as primeval and hence a universal form of host defense [11]. Under normal conditions, healthy fish can defend themselves against a broad spectrum of pathogens using a complex system of innate defense mechanisms [12,13]. Moreover, the alimentary canal has a series of well-developed chemical and physical barriers which cooperate with an efficient, local, mucosal immune system [14]. But, in a trade-off between parasite and host, many intestinal parasites have evolved mechanisms to evade their host's immune response, whilst the hosts have evolved a series of counter measures to deal with these [15]. As part of the infection process, certain intestinal worms induce structural modification to their host's tissues [7], and most likely are responsible for alterations to normal intestinal physiology [16].

In fish, the innate defenses responding to helminth infection are associated with an inflammatory reaction [12,17]; furthermore, enteric helminths elicit an increase in the number, migration and/or accumulation of certain types of host immune cell (*e.g.*, granulocytes) at the site of infection [6,7,18–20].

Innate immunity in teleosts involves a range of cell types, which commonly include eosinophilic granular cells (EGCs); this term was introduced by Roberts et al. [21] but, in recent years, there has been a tendency to use the more conventional term mast cells (MCs) as these cells have functional and morphological similarities to mammalian MCs [22]. MCs are probably present in all teleosts and are found in a variety of tissues, especially the gastrointestinal tract, skin and gills [14,18,23,24]. In all vertebrates, MCs are often strategically positioned at perivascular sites to regulate inflammatory responses [25]; indeed, these cells are motile [18,24,26]. Mast cells degranulate in response to exposure to a variety of pathogens [7,13] and known degranulating agents [23,27]. Indeed, MC degranulation has been shown to promote intestinal contraction in rainbow trout and gilthead seabream [respectively, [27,28]]. Interestingly, the MCs of perciform fish contain histamine, which can regulate the fish's inflammatory responses [28].

Vertebrates and invertebrates produce antimicrobial peptides (AMPs), which are a key factor in innate immunity [29]. One of the most common groups of AMPs in fish is the piscidins, a family of linear, amphipathic peptides [30]. Piscidins 1, 2, and 3 were first isolated from mast cells of the commercially cultured hybrid striped bass (*Morone chrysops* female × *Morone saxatilis* male) [30–32]. Piscidins have potent, broad-spectrum antimicrobial activity against viruses, bacteria, fungi, water molds and parasites [30,33–36]. Piscidin 4 (P4), isolated from MCs of hybrid striped bass, constitutes one of the most common antimicrobial peptides present in fish [31]. Moreover, recently piscidin 5 was reported from the above same fish species by Salger et al. [35].

We previously reported the occurrence of piscidin 3 (P3) in gill MCs of two perciforms, European sea bass *Dicentrarchus labrax* [37], and gilthead seabream, *Sparus aurata* [38], naturally infected with the monogenean *Diplectanum aequans* and the copepod *Ergasilus* respectively. Our present paper provides the first evidence of a response of piscidins (P3, P4) to an intestinal helminth infection in fish. Our histopathological and immunohistochemical methods will provide a basis for the future elucidation on piscine antihelminthic responses.

2. Material and methods

2.1. Animals

During 2011 and 2012, on three occasions, September 2011, April and September 2012, a total of 87 European perch (19.61 ± 7.23 cm, mean total length ± standard deviation; 163.58 ± 179.70 g, mean weight ± standard deviation) were obtained from Lake Piediluco (Province of Terni, Central Italy; 42° 31′ 01″ N; 12° 45′ 00″ E). The fish were caught by a gill net that was deployed on three occasions by professional fishermen belonging to the Piediluco Fish Consortium.

2.2. Histology and electron microscopy

Immediately upon landing, the fish were transferred alive to the Consortium's facility, where they were euthanised using an overdose of 125 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz, Basel, Switzerland). Afterwards, the spinal cord was severed and the fish measured and weighed. On post mortem, gills were examined for ectoparasites and were then sexed before the main visceral organs (i.e., digestive tract, liver, spleen, kidney, gonads) were removed and examined for helminths. For parasites found attached to the intestine, their exact position was recorded before a 15 × 15 mm piece of tissue that surrounded the site of attachment was excised and then fixed in chilled (4 °C) 10% neutral buffered formalin for 24 h. Thereafter, the fixed tissues were rinsed in several changes of (4 °C) 70% ethanol before being stored in the same medium until they were processed for histology. The tissues were dehydrated through an alcohol series and then paraffin wax embedded using a Shandon Citadel 2000 Tissue Processor (Shandon Citadel 2000, London, UK). After blocking out, 5 μm thick sections were taken from each tissue block and stained with hematoxylin and eosin (H&E), and/or Alcian blue 8 GX pH 2,5, combined with periodic acid Schiff's reagent (AB/PAS).

For light and electron microscopy, perch intestinal tissues (from all 44 infected fish and 20 healthy perch) measuring up to 7 × 7 mm in area were excised and fixed in chilled (4 °C) 2.5% glutaraldehyde solution, in 0.1 M sodium cacodylate buffer, pH 7.3. After 2.5 h, the tissues were rinsed for 12 h in 0.1 M sodium cacodylate buffer containing 6% sucrose. The specimens were then post-fixed in 1% osmium tetroxide in the same buffer for 3 h, dehydrated through a graded acetone series, and embedded in Epoxy resin (Durcupan ACM, Fluka, Buchs, Switzerland). Semi-thin sections (1.5 μm) were cut on a Reichert Om U 2 ultramicrotome using glass knives and then stained with toluidine blue. Ultra-thin sections (90 nm) were cut with diamond blade, stained with 4% uranyl acetate in 50% ethanol and Reynold's lead citrate and examined using a Hitachi H-800 electron microscope. For comparison, gut tissue from 10 uninfected perch were processed along with the parasitized samples.

2.3. Immunohistochemistry (IHC)

Formalin-fixed histological sections from 24 perch (10 healthy and 14 parasitized) were subjected to the indirect immunohistochemical method (peroxidase-anti-peroxidase immunocomplex) using anti-piscidin 3 (anti-HAGR) and anti-piscidin 4 (anti-5.3-02-3A) antibodies. The two primary antibodies were produced by a commercial laboratory (Bethyl Laboratories, Montgomery, Texas, USA) using the company's standard procedures detailed in Dezfuli et al. [37] and Corrales et al. [39]. Briefly, a synthetic peptide constituting the C-terminus of piscidin 3 (HAGRSIGRFLTG) was conjugated to keyhole limpet hemocyanin (KLH) and then injected into rabbits. The antiserum was then affinity purified by running over a column having a 12-mer piscidin fragment conjugated to cyanogen bromide-activated agarose as an immunosorbent. The resulting titer of the affinity-purified antibody was 1:13,000 via ELISA. The peptide specific antibody had less than 1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either free KLH, conjugated KLH, or free peptide that shares less than three amino acids in the sequence.

The purified, 44-mer synthetic piscidin 4 peptide, constituting the entire sequence of piscidin 4 was conjugated to KLH and then

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injected into rabbits. The antiserum was then affinity purified using a column having piscidin 4 conjugated to cyanogen bromide-activated agarose as an immunosorbent. The titer of this antibody was approximately 1:80,000 via ELISA. The peptide specific antibody had less than 1% cross-reactivity by ELISA, where 1% cross-reactivity is 100 times more antibody than is required to produce the same optical density with either free KLH, conjugated KLH, or free peptide that shares less than three amino acids in the sequence.

Sections (5 μm) were deparaffinised in xylene, rehydrated through a graded alcohol series, then endogenous peroxidase activity and non-specific staining were blocked in 3% H_2O_2 (10 min) and in normal goat serum (1:20, Elite Rabbit IgG Vectastain ABC Kit, Vector, Burlingame, USA) (30 min). After incubation with the primary antibodies (anti-HAGR diluted 1:400 and anti-5.3-02-3A 1:8000) for 3 h at room temperature, sections were incubated for 30 min with a biotinylated goat anti-rabbit serum (Elite Rabbit IgG Vectastain ABC Kit, Vector), and then for 30 min with avidin-conjugated horseradish peroxidase (Elite Rabbit IgG Vectastain ABC Kit, Vector). The sections were then developed using DAB (3,30-diaminobenzidine 0.04% w/v in TBS 0.05 M, pH 7.4) and H_2O_2 (0.005%), rinsed and counterstained with Alcian Blue and Harris's hematoxylin. Non-immune serum and diluent-only sections were used as negative controls. The positive control was intestine from hybrid striped bass (*M. saxatilis* × *M. chrysops*), which was known to be positive for piscidins 3 and 4, as the peptides were isolated from this fish. The specificity of the reaction was confirmed by pre-absorption of each antiserum with the corresponding antigen (the primary antibodies were preincubated with an equal volume of the specific blocking antigens for 1 h at 37 °C before applying onto tissue sections).

2.4. Statistical analysis

The number of MCs positive for piscidin 3 (P3) and piscidin 4 (P4) and their immunostaining intensity in the epithelium and submucosa were screened in intestinal sections of 24 perch (10 healthy and 14 parasitized) via light microscopy, using computerised image analysis software (Nis Elements AR 3.0). The infected fish have a range of intensity of infection between 5 and 12 worms In the sections taken from parasitized fish, the number of positive cells were counted in two zones: close to the point of parasite attachment ("close") and 1 cm away ("far").

The abundance of positive cells was evaluated at 400× magnification in ten areas between 10,000 and 30,000 μm^2 from one section for each fish; the numbers of cells counted in these ten fields were used as a single average value. Prior to analysis, the gaussian distribution (*i.e.*, normality) and the homogeneity of variances of the data were assessed, respectively, by means, with Shapiro–Wilk's Test and Levene's Test. The number of MCs positive for P4 were square root-transformed to meet these assumptions. A General Linear Model (ANOVA) was used to detect significant differences in the number of positive MCs between the uninfected and parasite-infected fish in both the "close" and "far" zones, as well as both epithelial and submucosal layers. In the model, the numbers of positive cells were entered as dependent variables, the intestine zones (healthy, "close" to parasite and "far" from parasite) as categorical predictors and the effective area of the measurement as a covariate. The level of significance selected was $p < 0{,}05$. Statistica 7 (StatSoft, Inc., Tulsa, OK) was used as the statistical package.

Immunostaining intensity was evaluated with a public domain Java image processing program (ImageJ 1.45s Java 1.6.0–20). The sections of each fish were photographed with the 40× objective of the optical microscope and the blue RGB stack images, which allowed the best immunostaining contrast with respect to the surrounding tissue. In each section, in the same tissue areas measured for cell counting, the values of mean 8 bits gray level of the cytoplasm of ten positive cells, both from the "close" zone and the "far" zone, were recorded: the minimum gray value corresponding to black was 0 and the maximum gray value corresponding to white was 255; thus, the highest immunostaining intensities corresponded to the lowest gray values. The ten measurements were used as a single average value. After the assessment of the gaussian distributions and the homogeneity of variances of the data, Statistica 7 was used for ANOVA one-way tests, with the same previously mentioned model (but covariate was not introduced) and level of significance.

3. Results

3.1. Histology and electron microscopy

No parasites were found on or in the gills, liver, spleen, kidney, or gonads in a sample of 87 fish examined. Forty-four perch (50.57%) were infected with *A. lucii* in the intestine. Infection intensity ranged from 1 to 18 worms per host (3.55 ± 4.58; mean ± S.D.), Most worms were observed in the middle region of the intestine (Fig. 1a); thus, the results reported below referred mainly to this segment of the gastrointestinal tract. *A. lucii* contacted the mucosal folds, mainly causing damage by destroying the mucosal epithelium covering the villi next to the parasite's site of attachment (Fig. 1a). While villi more distant from the worm's body remained intact, increased numbers of rodlet cells and mucous cells were observed in the epithelium (not shown). In situ, infected areas of intestine were covered by a yellowish catarrh which appeared as a thick, adherent blanket of mucus that gave an intense positive signal when histological sections stained with alcian blue (Fig. 1b). These layers of catarrh covering the epithelium were most frequently observed in the intestines of infected fish in zones in close proximity (Fig. 1b) to the site of *A. lucii* attachment and adjacent to the body of the acanthocephalan. The majority of the parasite's body did not cross the submucosa (Fig. 1a), but in some cases, its proboscis penetrated very close to the muscularis layer (Fig. 1c). Within the submucosa, beneath the point of proboscis insertion, numerous MCs (Fig. 1c), fibroblast-like ensheathing cells, and collagen fibres were seen (Fig. 1d).

Transmission electron microscopy of infected intestine revealed the presence of MCs frequently surrounded by collagen fibres of the submucosa (Fig. 2a). In the submucosa and in the muscularis, numerous MCs were in contact with and/or inside the blood vessels (Fig. 2b and c). The MCs were irregular in shape, with an eccentric, polar nucleus, and cytoplasm having numerous large, electron-dense, membrane-bounded granules (Fig. 2d and e). The cytoplasm typically contained two to three mitochondria, a conspicuous Golgi apparatus (Fig. 2f) and a well-developed rough endoplasmic reticulum (Fig. 2f). Degranulation of MCs was rarely noticed in grids of both infected and uninfected intestines.

No differences in the response to the parasites were encountered between sexes.

3.2. IHC

Histological sections treated with anti-piscidin 3 (P3) and anti-piscidin 4 (P4) antibodies showed subpopulations of MCs that were positive among the epithelial cells of the intestine, in the submucosa and in the muscularis layer (Fig. 3a, b and e). Piscidin positive MCs were also adjacent to, and in some instances, inside the blood vessels. P4-positive MCs were more abundant than P3-positive cells in both unparasitized and parasitized fish (Fig. 3c–e). In the epithelial layer, the number of P4-positive MCs was

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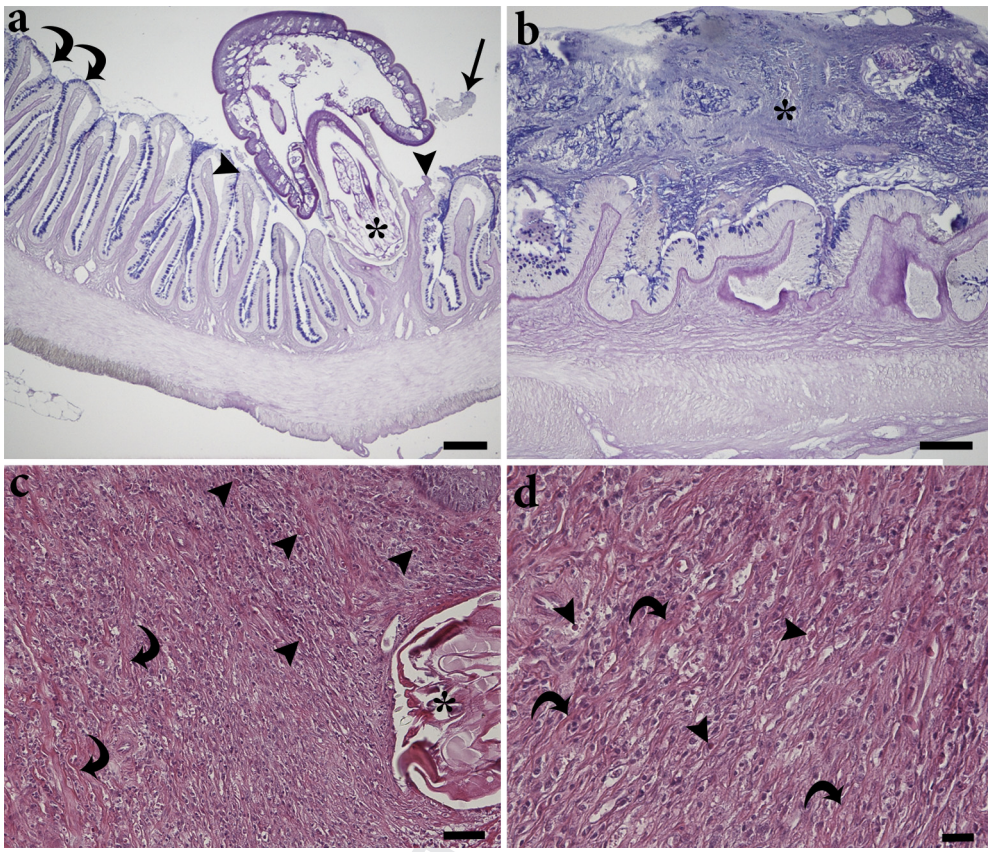

Fig. 1. Parasitized intestine of European perch. (a) Sagittal section through the middle region of intestine with attached *Acanthocephalus lucii*. There is contact between the parasite's trunk and damaged mucosal epithelium (arrows). Note the lack of intestinal folds at the site of attachment of the proboscis (asterisk). Intact epithelium (curved arrows) has numerous mucous cells distant from the parasite's attachment, AB/PAS, bar = 200 μm (b). Section of infected intestine stained with AB/PAS, in proximity to the parasite's site of attachment, with excessive catarrh layers (asterisk) covering the mucosal epithelium, bar = 100 μm (c). *A. lucii* proboscis embedded in the submucosa and penetrating near the muscularis layer; numerous mast cells (arrow heads) among collagenous fibres (arrows) are evident, H&E, bar = 50 μm (d). High magnification of the intestine beneath the proboscis; mast cells (arrow heads) and collagenous fibres (arrows) are visible, H&E, bar = 50 μm.

significantly higher than that of P3-positive MCs in both uninfected and parasitized fish, in both the "far" and "close" zones ($p < 0.05$); the same results were obtained with regard to the abundance of positive cells in submucosa ($p < 0.05$).

3.2.1. Piscidin 3

The number of P3-positive MCs was not significantly different in uninfected versus infected fish ($p > 0.05$) but immunostaining intensity was stronger in uninfected tissue in comparison to parasitized tissue ($p < 0.05$).

In both epithelial and submucosal layers, there were no significant differences in the number of immunoreactive MCs between healthy and parasitized intestine either close to or far from the worm's site of attachment ($p > 0.05$). The abundance of P3-positive cells in the submucosa was similar to that in the epithelial layer ($p > 0.05$).

P3-positive MCs in the epithelium, close to the site of acanthocephalan attachment, had lower immunostaining intensity than tissues of uninfected individuals ($p < 0.05$); there were no significant differences in immunostaining intensity between uninfected and infected tissues far from the parasite ($p > 0.05$); there was also no difference between "close" and "far" zones in the parasitized tissue ($p > 0.05$).

The immunostaining intensity of P3-positive MCs in the submucosa was similar in uninfected and infected tissues at both distances from the site of parasite attachment ($p > 0.05$). No differences in immunostaining intensity were seen between positive cells of the epithelium versus the submucosa ($p > 0.05$).

3.2.2. Piscidin 4

The number of P4-positive MCs in uninfected intestine was not significantly different from that of infected tissue ($p > 0.05$); nor was the immunostaining intensity different between uninfected versus parasitized tissues ($p > 0.05$).

In both the epithelial and submucosal layers, there were no significant differences in abundance of P4-positive cells or their immunostaining intensity between healthy and parasitized tissues in either the "far" or "close" zones ($p > 0.05$).

.In uninfected tissue, P4-positive cells were more abundant in the epithelium compared to the submucosa ($p < 0.05$), whilst in parasitized tissue, there was no difference ($p > 0.05$). No significant differences in the immunostaining intensity of the P4-positive cells were detected between epithelial and submucosal layers ($p > 0.05$).

4. Discussion

While immune responses in fish against microparasites have received considerable attention in recent years, resulting in numerous reports, there is relatively little knowledge about fish immunity to endoparasitic helminths [17]. *A. lucii* is a common, widely distributed, intestinal helminth that infects many freshwater fish in the western Palaearctic region [40]. While it can infect

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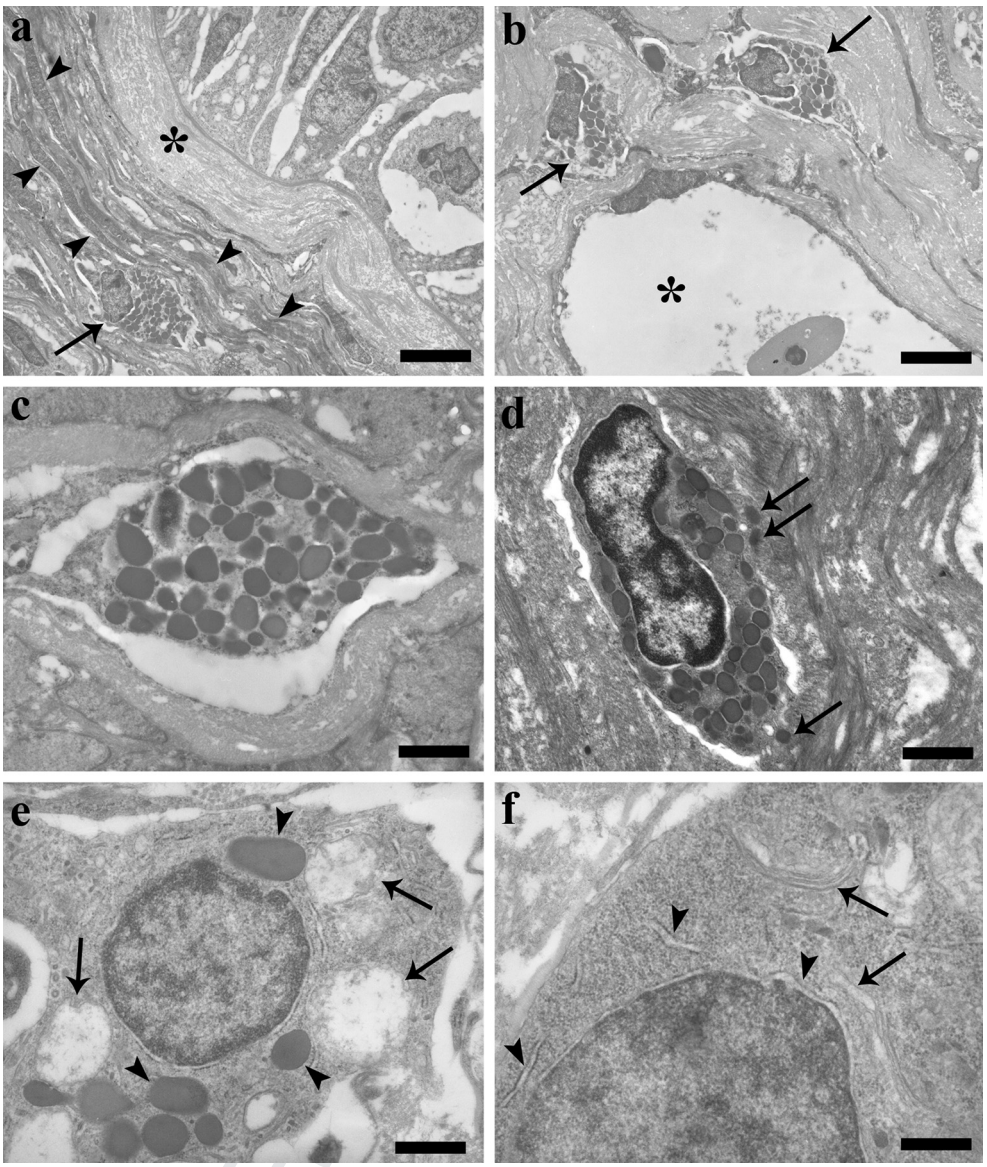

Fig. 2. Electron micrographs of parasitized intestine of European perch. (a) A mast cell (arrow) encircled by fibroblast-like ensheathing cells (arrow heads) inside the submucosa; note the basal membrane (asterisk), bar = 3.1 μm (b). Two mast cells (arrows) surrounding a blood vessel (asterisk) in the submucosa, bar = 3.0 μm (c). High magnification of an intact mast cell inside a blood vessel; numerous electron-dense, membrane-bounded granules are visible, bar = 1.2 μm (d). A mast cell with an eccentric, polar nucleus; some granules (arrows) seem to leave the cytoplasm, bar = 1.0 μm (e). Close to the mast cell nucleus, some mitochondria (arrows) and electron-dense granules (arrow heads) are evident, bar = 0.6 μm (f). The cytoplasm is filled with numerous free ribosomes, a conspicuous Golgi apparatus (arrows) and a well-developed rough endoplasmic reticulum (arrow heads) are visible, bar = 0.4 μm.

36 species, its principal definitive host is the European perch [3]. Among numerous records on the effects of endoparasitic helminths on the alimentary canal and associated organs of fish, some recent accounts appeared in Reite [8], Dezfuli et al. [20], and Snail et al. [41]. Due to their relatively weak attachment to the gut, many enteric helminths do not severely damage the intestine [8,22]. However, Acanthocephala are especially aggressive in their attachment, with their pathogenicity related to the depth of proboscis penetration into the host tissue [9,20]. *A. lucii* as well as a few other species of the phylum (*e.g.*, *Telosentis exiguus*, *Neoechinorhynchus rutili*, *Paratenuisentis ambigus*) are short neck species [9] which have a relatively shallow attachment, with penetration limited to the submucosa [[9] and current study]; thus, host inflammation is typically less intense compared to long neck species.

Inflammation comprises a complex series of homeostatic mechanisms involving the immune, nervous and circulatory systems in response to tissue injury or infection [42]. Parasitic infection of the alimentary canal can have detrimental effects on host digestive function [16] by inducing intestinal inflammation and immune reactions, mainly in gut-associated lymphoid tissue [12]. According to Rombout et al. [14], the digestive tract has a series of well-developed physical and chemical barriers which cooperate with an efficient local mucosal immune system. In fish, the innate defenses responding to enteric helminth infection are associated with an inflammatory reaction [6,12]; consequently, an increase in the migration and accumulation of certain types of host immune cells (*e.g.*, granulocytes) occur at the site of infection [7,43]. Immunocompetent cells have been reported in detail in the gut of a few species of teleosts; leucocytes are abundant in the epithelium

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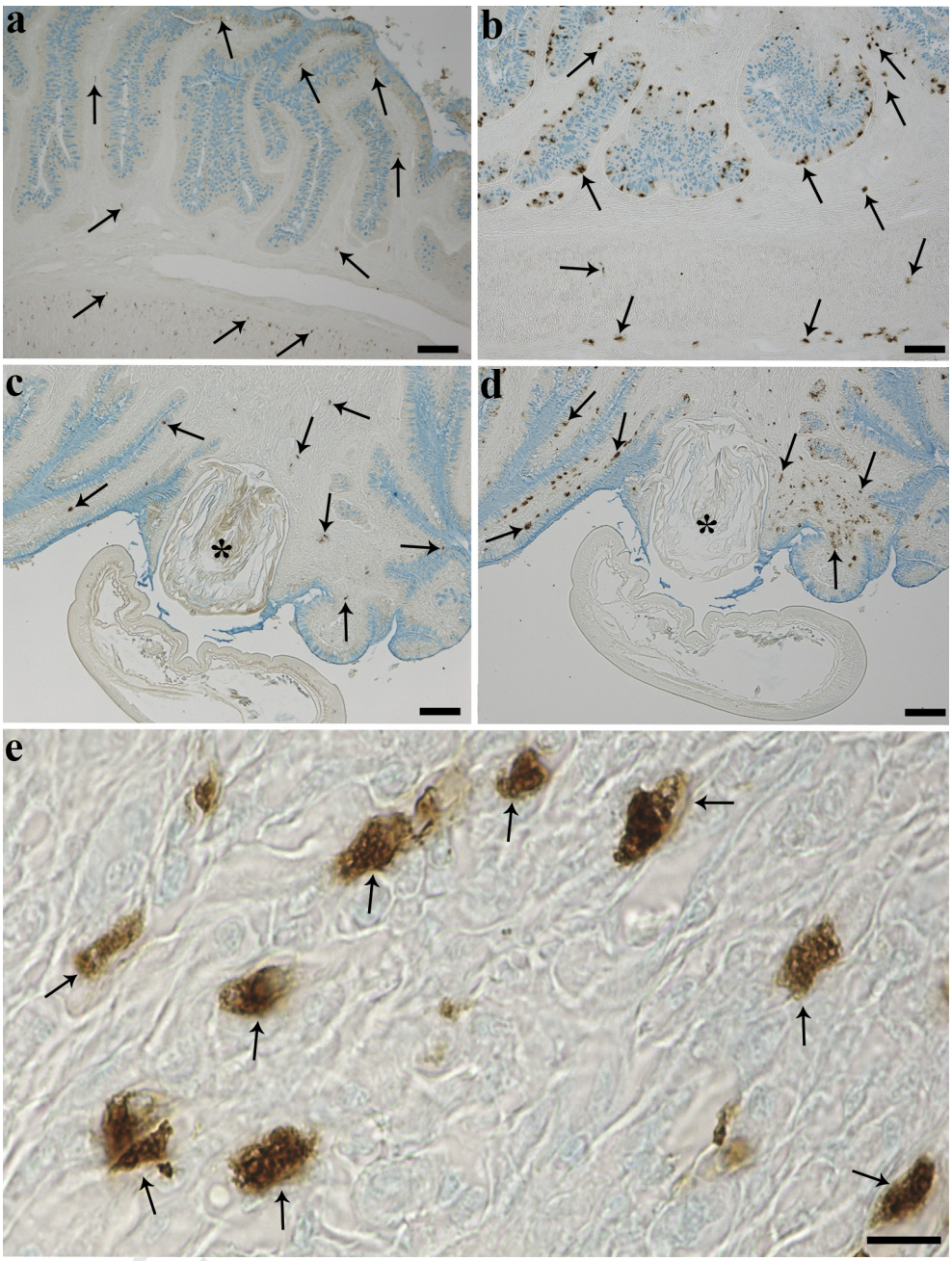

Fig. 3. Immunohistochemistry of European perch intestine. (a) Micrograph showing mast cells positive for piscidin 3 in epithelium, submucosa and muscle layers of unparasitized intestine, bar = 100 μm. (b) Numerous mast cells immunoreactive for piscidin 4 in epithelium, submucosa and muscle layers of unparasitized intestine, bar = 100 μm. (c) Parasite proboscis (asterisk) in the submucosa, very few cells (arrows) are positive for piscidin 3, bar = 100 μm. (d) Serial section treated with piscidin 4 antiserum, showing numerous positive mast cells (arrows) near the proboscis (asterisk), bar = 100 μm. (e) Several mast cells (arrows) appear positive for piscidin 4 in the submucosa of a parasitized intestine, bar = 10 μm.

and lamina propria (for review see Ref. [14]). Nevertheless, there have been few detailed records on the identification of immune cells involved in the response to enteric helminths [6,7,20,22].

European perch infected with *A. lucii* had increased MCs within the submucosa; MCs were numerous at the site of infection but were also present in fewer numbers in uninfected intestine. In mammals, MC products are pivotal in mediating leukocyte recruitment into inflammatory sites [44]; this same function was reported in fish [26,45]. In all vertebrates, MCs are often strategically positioned at perivascular sites to regulate inflammatory responses [24,25]. In our current study, MCs were associated with capillaries and fibroblasts in the submucosa of both infected and uninfected perch. With reference to the mast cell–fibroblast association in fish [46,47], as in mammals [48], it was reported that fibroblasts can influence mast cell motility and proliferation. Also, several records suggest that MCs are involved in fibrotic processes and in tissue remodeling [49]. The occurrence of large numbers of both MCs and fibroblasts in infected perch intestine leads us to presume that this association might be necessary to mediate intestinal remodeling after tissue injury provoked by *A. lucii*.

Antimicrobial peptides are involved in innate immune defense against invading microorganisms [29]. A large variety of AMPs have

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been isolated from fish [30,32,50], one of the most common groups being the piscidins [30]. A plethora of studies in fish have shown that piscidins have potent, broad-spectrum antimicrobial activity against many microorganisms and parasites [30,32–38]. Several types of piscidins have been isolated from MCs of a wide range of teleost taxa (see Ref. [35]), P4 and P5 are the most recent members of the piscidin family discovered ([31] and [35], respectively). It is believed that piscidin 4 might be more likely to function extracellularly than other piscidins because it would presumably be less toxic to host cells [34].

We previously found that gills of European seabass and gilthead seabream infected with ectoparasites had larger numbers of P3-positive MCs compared to uninfected gills (see respectively [37,38]. In contrast, there was no significant difference in the number of P3- or P4-positive MCs in unparasitized versus parasitized intestine of European perch; nonetheless, there were more P4-positive MCs than P3-positive MCs. As mentioned, mammalian MCs are a heterogenous group comprised of populations that differ in biochemical, histochemical and functional features [51], but it is unknown if fish MCs are similarly heterogenous [52]. Fish MCs can vary in intensity of piscidin immunostaining [52], suggesting heterogeneity. In European perch, the signal intensity for P3 and P4 was not different in MCs of the submucosa versus the epithelium, in contrast to Silphaduang et al. [52], where, in several fish species, a more intense immunoreactivity to piscidins was seen in epithelial MCs of several organs (gills, stomach and intestine) in comparison to those in deeper tissues such as lamina propria and submucosa of the gut.

One of the most important results of our current study was that, while the density of MCs containing piscidins was the same in unparasitized and parasitized fish, piscidin 3 staining intensity was reduced adjacent to the parasite. Parasites by definition are organisms which invade the host, and survive in it until they are ready to penetrate the defensive barriers and avoid the immune attack of the host [53]. Parasitic worms have evolved strategies to manipulate the host immune system, some of which may lead to a reduction in inflammation [54]. Moreover, long-lived parasites such as the helminths are more remarkable for their ability to downregulate host immunity, protecting themselves from elimination and minimizing severe pathology in the host [55]. It is well-known that some parasites immunosuppress their host, reducing the likelihood of rejection [55,56]. A family of immunomodulatory proteins (helminth defense molecules [HDMs]) secreted by medically important parasitic helminths alter innate immune cell function, facilitating their survival [57]. Corrales et al. [58] observed that piscidin 4 in gill MCs of hybrid striped bass was significantly lower in ectoparasite-infected gill, suggesting that piscidin 4 is significantly down-regulated during this parasitosis. In the present study, piscidin 3 expression was reduced adjacent to *A. lucii*. These two studies suggest parasites might actively modulate AMP expression. Both piscidin 3 and piscidin 4 are known to have potent antiparasitic activity [30,33–36].

Our data are among the first to provide direct evidence for the presence of P4 in MCs of the alimentary canal of fish; indeed, this is the first record of an apparent response of MC piscidins to an intestinal helminth infection. Based on these results, it is reasonable to presume that in the intestine-helminth system, it is most likely that MCs containing piscidins behave differently compared with MCs of the gill-parasite system. Our data pave the way for future studies aimed at determining the role played by MCs in response to endoparasites of teleost fish.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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