A fish model for the study of the relationship between neuroendocrine and immune cells in the intestinal epithelium: *Silurus glanis* infected with a tapeworm

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Abstract

Immunohistochemical, immunofluorescence and ultrastructural studies were conducted on a sub-population of 20 wels catfish *Silurus glanis* from a tributary of the River Po (Northern Italy). Fish were examined for the presence of ecto- and endo-parasites; in the intestine of 5 fish, 11 specimens of cestode *Glanitaenia osculata* were noted and was the only helminth species encountered. The architecture of intestine and its cellular features were nearly identical in either the uninfected *S. glanis* or in those harboring *G. osculata.* Near the site of worm's attachment, mucous cells, several mast cells (MCs), few neutrophils and some endocrine cells (ECs) were found to co-occur within the intestinal epithelium. MCs and neutrophils were abundant also in the submucosa. Immunohistochemical staining revealed that enteric ECs were immunoreactive to met-enkephalin, galanin and serotonin anti-bodies. The numbers of ECs, mucous cells and MCs were significantly higher in infected wels catfish (Mann-Whitney U test, $p<0.05$). Dual immunofluorescence staining with the biotinylated lectin *Sambucus nigra* Agglutinin and the rabbit polyclonal anti-met- enkephalin or anti-serotonin, with parallel transmission electron microscopy, showed that ECs often made intimate contact with the mucous cells and epithelial MCs. The presence of numerous MCs in intestinal epithelium shows *S. glanis* to be an interesting model fish to study processes underlying intestinal inflammation elicited by an enteric worm. Immune cells, ECs and mucous cells of the intestinal epithelium have been described at the ultrastructural level and their possible functions and interactions together will be discussed.

Key words: gut inflammation, endocrine cells, mast cells, mucous cells, immunohistochemistry, ultrastructure

1. **Introduction**

In fish, the mucosal surfaces of gills, skin and gut are the first line of defense against infection by acting as a physical barrier to foreign entities and functioning as an active immune tissue [1]. This barrier is formed by epithelial cells, mucous cells, neuroendocrine cells and intrinsic immune system cells [2,3]. The mucus itself is an essential component of this barrier [2]. In the last two decades an increasing number of accounts described histopathology of helminth infections in fish [3-9]. Enteric helminths frequently provoke digestive canal inflammation and the occurrence of worms within the host induce the recruitment or formation of different types of immune cells [9-14]. Mast cells (MCs) and neutrophils are two type of granulocytes which are very active in the innate immune response against pathogens and parasites [8]. MCs and neutrophils are morphologically, histochemically and functionally similar to their mammalian counterparts (respectively [15] and [16]). The most common granulocyte type involved in endoparasitic helminth infection in fish is MC [6,8,15,17]. MCs are key regulatory cells and coordinate several functions of the innate immune system [6,8,14,18,19]. With regard to neutrophils, they are involved in the inflammatory process, often during the period of initial pathogen challenge by intense migration to the site of injury and/or parasitic infection [8,13,20]. Nonetheless, continue production as well as increase in the number of neutrophils must be tightly regulated [20].

Physiological control and correct function of vertebrate digestive tract during feeding and fasting are regulated by the neuroendocrine system [20,21]. Several cases reported that enteric helminths influence the fish gut neuroendocrine system [e.g. 3,23-27]. Many neuromodulators interact with mucous cells and may regulate intestinal mucus secretion [28]. Three such modulators were detected in intestine of wels catfish, namely serotonin (5-HT), galanin and met-enkephalin. The present study is the first immunohistochemistry and ultrastructure-based evaluation of the intimate relationship between MCs, endocrine cells (ECs) and mucous cells in intestinal epithelium of *S. glanis*. The European wels catfish, *Silurus glanis* (L.) is one of the largest freshwater fish worldwide [29]. *S. glanis* is native to rivers of Eastern Europe and Western Asia, but it's current diffusion throughout Europe and the world is due to popularity of this species among anglers and interest in it as a potential species for aquaculture [30]. *S. glanis* is an opportunistic predator and is considered a scavenger [31] and might be able to serve as host for numerous parasite species. There is only one available study in which the authors marginally examined histopathology in *Silurus glanis* due to *Proteocephalus osculatus* = *Glanitaenia osculata* [32]. The lack of detailed information on cells involved in the innate immune response against *G. osculata* was one of the reasons which prompted us to undertake this preliminary study.

Currently zebrafish, medaka, and rainbow trout are among the fish species proposed as suitable models for immunological investigations [33-36]. Interestingly, zebrafish was also proposed as a promising model for studying MCs [37]. The occurrence of high numbers of MCs in intestinal epithelium revealed that *S. glanis* is also a very interesting model for mucosal immunity investigations in teleosts. Along with other recent reviews on mucosal immunity, we hope to highlight exciting new advances in our understanding of fish immune mechanisms against enteric helminths.

2. Materials and Methods

2.1. Specimen collection and preparation

A sub-population of 20 specimens of *S. glanis* (26.82 ± 5.56 cm in total length, mean ± standard deviation S.D.) and from 31 to 274 g (130.30 ± 67.95, mean ± standard deviation S.D.) in weight were caught by gill net in one occasion in a small tributary of the River Po (Northern Italy) on the outskirts of the city of Ferrara (44°49'39"72 N; 11°37'18"12 E). Fish were anaesthetised in 125 mg L^{-1} MS222 (Sandoz, Basel, Switzerland) until opercular movement ceased and then their spinal cords were severed. Immediately after euthanasia, a complete necropsy was performed on each fish with particular interest paid to the gills, gonads, liver, kidney, spleen and alimentary canal for the presence of helminths. In the fish examined, only a single helminth species, *G. osculata,* was encountered. Samples (15 × 15 mm) of the above mentioned organs were taken from all 20 specimens including the intestinal sites at which the parasite was found attached. The tissues were fixed in 4°C Bouin's fluid for 8 h. Thereafter, the samples were dehydrated through an alcohol series and then paraffin wax embedded using a Shandon Citadel 2000 tissue processor. The absence of parasites in uninfected fish, established during the necropsy, was confirmed by examination of histological sections.

2.2. Dual histo- and immunohisto-chemical staining procedure

To investigate the morphology of the cellular components and their secretions at the site of parasite infection, a modification of the dual immuno- and histochemical staining procedure [3,38,39] was employed. Histological sections of the cestode-infected intestine were pre-treated as usual [see 23,24,27], then washed in Tris-buffered saline (TBS: 0.05 M Tris-HCl, 0.15 M NaCl) containing 0.1% Triton-X 100 (TBS-T), and incubated with the following anti-sera: a rabbit polyclonal anti-galanin 1:500 diluted in TBS (code AB5909, Millipore, USA), a rabbit polyclonal anti-met-enkephalin 1:100 diluted in TBS (code AB1975 Millipore, USA), and a rabbit polyclonal anti-serotonin 1:100 diluted in TBS (code AB938, Chemicon International Inc., Temecula, CA, USA)

(for details see [23,24,27]). The same sections were then stained with alcian blue (AB) 8GX pH 2.5 and periodic acid-Schiff (PAS) [40], to determine the glycoconjugates histochemistry of the mucous cells. Using the AB/PAS stain protocol, acidic glycoconjugates stain blue with AB, neutral glycoconjugates stain purple with PAS, while cells containing mixed glycoconjugates appear violet with the combined AB/PAS stain.

2.3. **Evaluation of mucous, mast and endocrine cells and data analysis**

All stained histological sections were examined and photographed using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan) and associated image analysis software (Nis Elements AR 3.0, Nikon). For quantitation of the histochemical and immunohistochemical preparations, the ECs that were immunoreactive to each primary antisera, and the mucous cells which stained either blue, purple or violet with the AB/PAS stain, and MCs were evaluated in four different microscopic areas on each histological section. A total of five sections were assessed from each fish (5 uninfected and 5 parasitized *S. glanis*). The mean number of ECs, mucous cells and mast cells per 100,000 μm^2 of epithelium in sections taken from uninfected and *G. osculata*-infected wels catfish were compared using the Mann-Whitney test. The level of significance was set at $p=0.05$.

2.4. **Immunofluorescence staining**

Sections (6-7 μm-thick) of the intestine of parasitised wels catfish were dewaxed, re-hydrated, and then rinsed in TBS-T. To inhibit non-specific reactions, the histology sections were treated with 1:20 normal goat serum in TBS for 60 min in a humid chamber; sections were then incubated with the primary anti-serum: 1:100 rabbit polyclonal anti-met-enkephalin in TBS for 24 h at room temperature (RT) or 1:100 rabbit polyclonal anti-serotonin in TBS for 24 h at RT. Slides were then washed in TBS-T before they were treated with the avidin-biotin blocking solutions as indicated in the manufacturer's guidelines (Vector Lab., USA). Thereafter, the sections were rinsed in TBS-T and incubated with 10 μg ml^{-1} goat biotinylated anti-rabbit IgG (Vector Lab.) in TBS for 2 h at RT. The sections were then rinsed twice in TBS-T and then treated with 10 μg ml^{-1} fluorescein avidin D (Vector Lab.) in 0.1 M $NaHCO_3$ pH 8.5 with 0.15 M NaCl for 2 h at RT.

The slides incubated for the demonstration of met-enkephalin-like or serotonin-like immunoreactivity were treated with 10 μg ml^{-1} biotinylated *Sambucus nigra* agglutinin (SNA, Vector Lab.) in 10 mM HEPES pH 7.5, 0.15 M NaCl, 0.08% sodium azide, 0.1 mM $CaCl_2$ for 3 h at RT. SNA is a lectin isolated from elderberry bark, with a preferential binding affinity to glycoconjugate residues of sialic acid attached to terminal galactose in α-2,6 and α-2,3. Previous trials in our lab have demonstrated that SNA is a good marker for the intestinal mucous cells of fish

[41,42]. After treatment, the sections were rinsed in TBS-T and then incubated with 10 μg ml^{-1} rhodamine avidin D (Vector Lab.) in 0.1 M $NaHCO_3$ pH 8.5 with 0.15 M NaCl for 2 h at RT. The stained tissue sections were then mounted with Vectashield® mounting medium (Vector Lab.) and examined on a Zeiss 510 confocal laser scanning microscope (CLSM). All sections were excited using multi-argon/helio-neon-green lasers with excitation and barrier filters set for fluorescein and rhodamine. Green and red fluorescent signals were obtained concurrently through alternate excitation (0.2 s^{-1}) at 488 nm and 540 nm, respectively. Under these viewing conditions, there was no cross-contamination of the two signals.

Sections of mammalian (*i.e.* swine and rat) tissues were used as positive controls, whereas negative controls were obtained by the omission of the primary antisera or the lectin on representative sections from infected and uninfected hosts. Both sets of controls gave the expected results.

2.5. **Transmission electron microscopy**

For electron microscopy, representative pieces (7 × 7 mm) *S. glanis* infected and uninfected intestines were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 3 h at 4°C before being post-fixed in 1% osmium tetroxide in the same buffer for 3 h. The samples were then dehydrated through a graded acetone series before being embedded in epoxy resin (Durcupan ACM, Fluka). Semi-thin sections (*i.e.* 1.5 μm) were cut on a Reichert Om U 2 ultramicrotome using glass knives and then stained with toluidine blue. Ultra-thin sections (*i.e.* 90 nm) were stained with a 4% uranyl acetate solution in 50% ethanol and Reynold's lead citrate and then were examined using a Hitachi H-800 electron microscope.

3. **Results**

Five (25%) of the 20 *S. glanis* were infected with *Glanitaenia osculata*, the intensity of infection ranged from 1 to 3 worms per host with an average worm burden of 2.20 ± 0.84 (mean number of worm per host ± SD). The totality of the worms were found in middle intestine.

3.1. **Histochemical and immunohistochemical staining of intestinal sections**

In infected intestine of wels catfish the total number of mucous cells increased significantly in comparison to uninfected conspecifics (Mann-Whitney U test, $p<0.05$; Table 1). Likewise, the number of mucous cells containing either acidic (*i.e.* staining blue) or neutral glycoconjugates (*i.e.* staining purple), also were found to have a highly significant increase (Mann-Whitney U test, $p<0.01$; Table 1). No difference was observed between the mucous cell communities secreting mixed

glycoconjugates (*i.e.* staining violet) in the uninfected compared with infected fish intestine (Table 1). The numbers of MCs were significantly higher in the intestinal epithelium of infected wels catfish 57.43 ± 4.97 mean number ± standard error of MCs per 100,000 μm^2) in comparison to uninfected ones (11.20 ± 1.71 mean number ± SE per 100,000 μm^2) ((Mann-Whitney U test, $p<0.01$).

In the sections taken from wels catfish harboring cestode, the number of ECs immunoreactive (IR) for each antibody (met-enkephalin, galanin and 5-HT) were significantly higher than those seen on sections from uninfected counterparts (Table 2). Contact between the IR ECs and the mucous cells (Fig. 1a, b, d, e, g, h), or single/grouped MCs (Fig. 1a, c, d, f, g, i) was observed in the wels catfish epithelium. The dual immunofluorescence protocol with the anti-met-enkephalin or -serotonin to mark the ECs in combination with the lectin SNA to stain the mucous cells confirms that there is a close relationship between these cell types in the intestine of *S. glanis* (Fig. 2a, b).

3.2. **Transmission electron microscopy**

In both groups of *S. glanis*, uninfected or harboring cestodes, the folds are comprised of a single layer of cylindrical epithelium that rests upon a connective tissue core. No alterations in ultrastructure and/or localization of the cellular components of the intestinal epithelium are noticed in infected catfish intestine (Fig. S1). Four principal cell types identified within the *S. glanis* intestinal epithelium are columnar-shaped enterocytes, mucous cells, ECs and immune cells. After enterocytes, mucous cells are the second most populous cell type observed. Mucous cells extend from the basal membrane toward the luminal side of the intestine (Fig. 3a) and possess elongated euchromatinic nuclei whereas the supernuclear portion commonly contains numerous mucus granules of differing sizes and electron-densities (Fig. 3a). Mucous cells display numerous round mitochondria, each with a moderate number of cristae, a well-developed rough endoplasmic reticulum and numerous free ribosomes (not shown). The mucous cells were seen to make tight connections with mast cells and endocrine cell (respectively, Fig. 3c, d, f).

In wels catfish intestine, ECs are characterised by the shape and electron density of their secretory granules (Fig. 3d). The endocrine cells with a basal euchromatinic nucleus were almost pyramidal. Most ECs though were seen lodged between the basal portion of some epithelial cells without a free border toward the gut lumen. The cytoplasm contained numerous round to oval shaped secretory granules (Fig. 3d). The secretory granules are similarly sized and filled with a fine electron-dense material. Often rough endoplasmic reticulum and free ribosomes are seen next to secretory granules (not shown), and indeed have few mitochondria and do not possess well- developed Golgi complexes. The basal part of ECs were observed to make several tight connections

with mucous cells. In several cases an EC is surrounded by epithelial MCs (Fig. 3d) and neutrophils.

Two types of immune cells are present in the epithelium of the *S. glanis*, MCs and neutrophils. Numerous MCs can be found in different levels of the intestinal folds, often appearing as a cluster in close proximity to the basal membrane (Fig. 3a). Somewhat surprisingly however, some MCs can be seen near the top of the fold (Fig. 3e). In epithelium frequent contact is observed between MCs and mucous cells (Fig. 3b) and between MCs and neutrophils (Fig. 3f). In submucosal sites, MCs are associated with fibroblasts and commonly in close vicinity to, and inside, the capillaries of the intestine (not shown). MCs are irregular in shape with an eccentric, polar nucleus, and a cytoplasm characterised by numerous large, electron-dense, membrane- bounded granules (Fig. S1, 3b, e). The cytoplasm typically contained two to three mitochondria and an inconspicuous Golgi apparatus. Neutrophils appear round to oval in shape although their outline is commonly irregular (Fig. 3f). These cells have a round nucleus and a cytoplasm that contains dark, elongated granules which are fibrous in appearance, and have very few mitochondria. In the submucosal layer, MCs and neutrophils co-occurred in some instances.

4. Discussion

S. glanis is now believed to have established self-sustaining populations in 80 % of the countries into which it has been introduced [43]. One of the most common cestode parasites of wels catfish in Europe is *Glanitaenia osculata*, and a recent paper describes its morphology and geographical distribution [44]. In Italy, little attention has been directed towards the parasites of this species except for sporadic reports [25,45]. We are aware of only a single record which marginally dealt with histopathology of *S. glanis* due to *Proteocephalus osculatus* = *Glanitaenia osculata* [32]. However no detailed information was provided on the type of cells involved in host reaction. Furthermore, there are no other studies on the catfish-cestode system as a model for studying fish immunology. The lack of such studies as well as those regarding the co-occurrence of numerous MCs, several endocrine cells and mucous cells in the epithelium were the main reasons for undertaking this preliminary study. Our early results suggest *S. glanis* to be suitable as a model for intestinal inflammation in a wild fish by an enteric helminth.

The digestive tract of vertebrates provides a favorable habitat for the establishment and growth of numerous pathogenic and parasitic organisms [46]. Numerous reports refer to harmful effects of helminths and intestinal worms often induce structural changes [3-5,8,9,13,27]. Helminths are a highly successful group of parasites that challenge the immune system in a manner different from rapidly replacing infectious agents [46]. Several helminth species induce inflammation to the

host tissue/organs [10,14]. Much attention has been paid to mucosal immunity in fish in the last decade [47,48]. Indeed, it is believed that mucosal immune responses in teleosts reveal the potential of this group of lower vertebrates as animal research models for study of human mucosal diseases [2,33]. The components of the mucosal immunity are epithelial cells, mucous cells, neuroendocrine cells and an intrinsic immune system [2,3]. Evidence has accumulated over the years which points to the essential role of enteric neuromodulators in inflammatory processes caused by helminths [3,27,49,50]. Interaction between immune, nervous and endocrine systems create a network where cytokines, hormones and neuropeptides collaborate with each other [21,22,51]. The intestinal nervous system, also known as "little brain of the gut", forms a complex and independent nervous system within the digestive tract [52]. The present study showed that in *G. osculata*-infected *S. glanis* intestine the number of enteric ECs immunoreactive to met-enkephalin, galanin and 5-HT was significantly higher than the number of the same cells counted in uninfected catfish. Similar findings on these and other neuromodulators in fish-helminth systems appeared in Dezfuli et al. [23,53] and Bosi et al. [3,26]. An increase in ECs immunoreactive to three neuropeptides in turbot digestive tract infected with myxozoan has appeared in Bermudez et al. [54].

As reported by Duffy-Whritenour and Zelikoff [55], 5-HT has an immunomodulatory role in fish. Among the functions of 5-HT are the regulation of epithelium and mucus secretion [28,56] and excitatory effects on gastrointestinal motility [21,57]. Met-enkephalin is an endogenous opioid peptide neurotransmitter, which occurs naturally in the brains of many animals and within the intestinal neuroendocrine system of teleosts [58,59]. It is thought that opioids regulate intestinal motility [54,59-61] and are involved in the discharge mechanism of mucus from mucous cells induced by luminal stimuli [62]. In the digestive tract of fish galanin acts as a cholinergic co- mediator [63,64] and the discharge of mucus from mucous cells is stimulated by cholinergic agonists [65]. Our data provide evidence for the role of the neuroendocrine system of *S. glanis* in the modulation of inflammatory responses to tapeworm *G. osculata*.

It has been proposed that amongst the innate defense mechanisms present at mucosal surfaces, the mucus itself is one of the most important [2,47]. Mucus is composed of a mixture of organic and inorganic molecules [66,67]. The number of mucous cells and mucus composition can vary in enteric helminth-infected fish [3,68,69]. As cell counting revealed in the wels catfish-*G. osculata* system, this worm induced an increase in the total number of mucous cells and the number of those containing acidic and neutral glycoconjugates in the intestinal epithelium. Recently we documented the strict contact between enteric mucous cells and ECs of fish *Squalius cephalus* harboring acanthocephalan *Pomphorhynchus laevis* and conspecifics with no worms [3]. Similar

contact between endocrine cells and intestinal mucous cells was also demonstrated here in uninfected/infected *S. glanis*.

Neutrophils and MCs are two type of granulocytes that play a role in intestine mucosal immunity and, in many instances both types of immune cell co-occurred in the epithelium of wels catfish intestine, although neutrophil counts were lower than MCs. In another fish-helminth system, *Tinca tinca* infected with *Monobothrium wageneri*, high numbers of neutrophils were found at sites of tapeworm attachment but in the submucosal layer and not in the epithelium [12]. Neutrophils are highly motile phagocytic cells that play a critical role in the immune response to infection [70,71] and not surprisingly are the first cells to migrate to a site of inflammation to help limit pathogen invasion [72]. In fish *Carassius auratus* it was shown that at the site of infection neutrophils change their phenotype throughout the acute inflammatory response and contribute to the induction and the resolution of inflammation [73].

MCs are normal residents of mucosal tissues and can bridge communication between the immune and nervous systems [74]. Very few studies are available regarding the presence of MCs in intestinal epithelium of fish [75,76]. The most important findings of the current study were the occurrence of a high number of MCs in intestinal epithelium of the wels catfish, and that in infected intestines epithelial MCs were often found in close proximity or contact with mucous cells and ECs. The granules of fish MCs contain a panel of inflammatory mediators [19] and several other molecules including serotonin, met-enkephalin [77], histamine [78], mucopolysaccharides with residues of α-N-acetyl-galactosamine [7] and piscidins [76,79-82]. MC degranulation near mucous cells was also observed in intestinal epithelium of *S. glanis*, consistent with degranulation of MCs, such degranulation was noticed in response to other pathogens and reported in [6,7,83]. Only three previous papers exist in which the authors observed MCs in intestinal epithelium of fish infected with helminths [7,8,84,current study]. Among several fish species intestines studied by us *S. glanis* is that with the most numerous and easily identified MCs in the epithelium.

All accounts of fish and mammals suggest that there is a cooperation between innate immune cells and mucous cells in the response of vertebrates tissues against helminths [85]. With reference to teleosts the mucosal immune system is a very active immunological site [86]. In this paper we propose *S. glanis* as a suitable species for mucosal immunity study where a close relationship clearly exists between innate immune cells, ECs and mucous cells. An increased focus on mucosal immunity in fish-helminth systems is likely to yield exciting new insights into the evolution of fish immunity and control mechanisms for inflammation.

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References

[1] D. Parra, F.E. Reyes-Lopez, L. Tort, Mucosal Immunity and B Cells in Teleosts: Effect of Vaccination and Stress, Front. Immunol. 6 (2015) 354.

[2] D. Gomez, J.O. Sunyer, I. Salinas, The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens, Fish Shellfish Immunol. 35 (2013) 1729-39.

[3] G. Bosi, A.P. Shinn, L. Giari, B.S. Dezfuli, Enteric neuromodulators and mucus discharge in a fish infected with the intestinal helminth *Pomphorhynchus laevis*, Parasite Vector 8 (2015) 359.

[4] H. Taraschewski, Host-parasite interactions in Acanthocephala: a morphological approach. Adv Parasitol. 46 (2000) 1-179.

[5] M. Santoro, S. Mattiucci, T. Work, R. Cimmaruta, V. Nardi, P. Ciprini, B. Bellisario, G. Nascetti, Parasitic infection by larval helminths in Antarctic fishes: pathological changes and impact on the host body condition index, Dis. Aquat. Organ. 105 (2013)139-48.

[6] B.S. Dezfuli, T. Bo, M. Lorenzoni, A.P. Shinn, L. Giari, Fine structure and cellular responses at the host-parasite interface in a range of fish-helminth systems, Vet. Parasitol. 208 (2015) 272-279.

[7] B.S. Dezfuli, M. Manera, L. Giari, J.A. DePasquale, G. Bosi, Occurrence of immune cells in the intestinal wall of *Squalius cephalus* infected with *Pomphorhynchus laevis*, Fish Shellfish Immunol. 47 (2015) 556-64.

[8] B.S. Dezfuli, G. Bosi, J.A. DePasquale, M. Manera, L. Giari, Fish innate immunity against intestinal helminths, Fish Shellfish Immunol. 50 (2016) 274-87.

[9] B.S. Dezfuli, M. Manera, G. Bosi, J.A. DePasquale, S. D'Amelio, G. Castaldelli, L. Giari, *Anguilla anguilla* intestinal immune response to natural infection with *Contracaecum rudolphii* A larvae, J. Fish Dis. 39 (2016) 1187-200.

[10] K. Buchmann, Fish immune responses against endoparasitic nematodes - experimental models, J. Fish Dis. 35 (2012) 623-35.

[11] B.S. Dezfuli, L. Giari, A. Lui, S. Squerzanti, G. Castaldelli, A.P. Shinn, M. Manera, M. Lorenzoni, Proliferative cell nuclear antigen (PCNA) expression in the intestine of *Salmo trutta trutta* naturally infected with an acanthocephalan, Parasit Vectors 5 (2012) 198.

[12] B.S. Dezfuli, A. Lui, L. Giari, G. Castaldelli, A.P. Shinn, M. Lorenzoni, Innate immune defence mechanisms of tench, *Tinca tinca* (L.), naturally infected with the tapeworm *Monobothrium wageneri*, Parasite Immunol. 34 (2012) 511-519.

[13] B.S. Dezfuli, A. Lui, F. Pironi, M. Manera, A.P. Shinn, M. Lorenzoni, Cell types and structures involved in tench, *Tinca tinca* (L.), defence mechanisms against a systemic digenean infection, J. Fish Dis. 36 (2013) 577–585.

[14] C.J. Secombes, A.E. Ellis, The Immunology of Teleosts, in: R.J. Roberts (Ed.), Fish Pathology, 4th edition, Blackwell Publishing, Chicester, 2012, pp. 144-166.

[15] O.B. Reite, Ø. Evensen, Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells, Fish Shellfish Immunol. 20 (2006) 192-208.

[16] M. Häger, J.B. Cowland, N. Borregaard, Neutrophil granules in health and disease, Intern. Med. J. 268 (2010) 25-34.

[17] B.S. Dezfuli, C.E. Fernandes, G.M. Galindo, G. Castaldelli, M. Manera, J.A. DePasquale, M. Lorenzoni, S. Bertin, L. Giari, Nematode infection in liver of the fish *Gymnotus inaequilabiatus* (Gymnotiformes: Gymnotidae) from the Pantanal Region in Brazil: pathobiology and inflammatory response, Parasit Vectors 9 (2016) 473.

[18] L.L. Reber, R. Sibilano, K. Mukai, S.J. Galli, Potential effector and immunoregulatory functions of mast cells in mucosal immunity, Mucosal Immunol. 8 (2015) 444-463.

[19] J. Galindo-Villegas, E. Garcia-Garcia, V. Mulero, Role of histamine in the regulation of intestinal immunity in fish, Dev. Comp. Immunol. 64 (2016) 178-186.

[20] J.J. Havixbeck, D.R. Barreda, Neutrophil development, migration, and function in Teleost Fish, Biology 4 (2015) 715-734.

[21] A.E. Lomax, D.R. Linden, G.M. Mawe, K.A. Sharkey, Effects of gastrointestinal inflammation on enteroendocrine cells and enteric neural reflex circuits, Auton. Neurosci. 126-127 (2006) 250-257.
[22] P. Holzer, A. Farzi, Neuropeptides and the microbiota-gut-brain axis, Adv. Exp. Med. Biol. 817 (2014) 195-219.
[23] B.S. Dezfuli, S. Arrighi, C. Domeneghini, G. Bosi, Immunohistochemical detection of neuromodulators in the intestine of *Salmo trutta* L. naturally infected with *Cyathocephalus truncatus* Pallas (Cestoda), J. Fish Dis. 23 (2000) 265-273.
[24] B.S. Dezfuli, L. Giari, S. Arrighi, C. Domeneghini, G. Bosi, Influence of enteric helminths on the distribution of intestinal endocrine cells belonging to the diffuse endocrine system in brown trout, *Salmo trutta* L., J. Fish Dis. 26 (2003) 155-166.
[25] B.S. Dezfuli, G. Castaldelli, T. Bo, M. Lorenzoni, L. Giari, Intestinal immune response of sheatfish *Silurus glanis* and barbel *Barbus barbus* naturally infected with *Pomphorhynchus laevis* (Acanthocephala), Parasite Immunol. 33 (2011) 116-123.
[26] G. Bosi, C. Domeneghini, S. Arrighi, L. Giari, E. Simoni, B.S. Dezfuli, Response of the gut neuroendocrine system of *Leuciscus cephalus* (L.) to the presence of *Pomphorhynchus laevis* Muller, 1776 (Acanthocephala), Histol. Histopathol. 20 (2005) 509-518.
[27] G. Bosi, A.P. Shinn, L. Giari, E. Simoni, F. Pironi, B.S. Dezfuli, Changes in the neuromodulators of the diffuse endocrine system of the alimentary canal of farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum), naturally infected with *Eubothrium crassum* (Cestoda), J. Fish Dis. 28 (2005) 703-711.
[28] P. Plaisancié, A. Barcelo, F. Moro, J. Claustre, J.-A. Chayvialle, J.-C. Cuber, Effects of neurotransmitters, gut hormones, and inflammatory mediators on mucus discharge in rat colon, Am. J. Physiol. 275 (1998) G1073-G1084.
[29] Z.S. Hogan, Ecology and Conservation of Large-Bodied Freshwater Catfish: A Global Perspective, Am. Fish. Soc. Symp. 77 (2011) 39–53.
[30] A. Alp, C. Kara, F. Uckardes, J. Carol, E. Garcia-Berthou, Age and growth of the European catfish (*Silurus glanis*) in a Turkish Reservoir and comparison with introduced populations, Rev. Fish Biol. Fisheries 21 (2011) 283-294.
[31] P. Hickley, S. Chare, Fisheries for non-native species in England and Wales: angling or the environment? Fisheries Manag. Ecol. 11 (2004) 203–212.
[32] D. Azadikhah, A. Nekuie Fard, M. Seidgar, A. Hashempour, The infection rate and pathologic lesions induced by *Proteocephalus osculates* (Goeze, 1782) in European catfish (*Silurus glanis*) from North-west of Iran Bull. Env. Pharmacol. Life Sci. 3 (2014) 63-68.
[33] S. Brugman, The zebrafish as a model to study intestinal inflammation, Dev. Comp. Immunol. 64 (2016) 82-92.
[34] C. Grabher, A. Cliffe, K. Miura, J. Hayflick, R. Pepperkok, P. Rørth, J. Wittbrodt, Birth and life of tissue macrophages and their migration in embryogenesis and inflammation in medaka, J. Leukoc. Biol. 81 (2007) 263-271.
[35] T. Matsumoto, S. Terai, T. Oishi, S. Kuwashiro, K. Fujisawa, N. Yamamoto, Y. Fujita, Y. Hamamoto, M. Furutani-Seiki, H. Nishina, I. Sakaida, Medaka as a model for human nonalcoholic steatohepatitis, Dis. Mod. Mech. 3 (2010) 431-440.
[36] A.C. Mehinto, E.M. Hill, C.R. Tyler, Uptake and Biological Effects of Environmentally Relevant Concentrations of the Nonsteroidal Anti-inflammatory Pharmaceutical Diclofenac in Rainbow Trout (*Oncorhynchus mykiss*), Environ. Sci. Technol. 44 (2010) 2176–2182.
[37] S.V. Prykhozhi, J.N. Berman, The progress and promise of zebrafish as a model to study mast cells, Dev. Comp. Immunol. 46 (2014) 74-83.
[38] C. Domeneghini, G. Radaelli, G. Bosi, S. Arrighi, A. Di Giancamillo, M. Pazzaglia, F. Mascarello, Morphological and Histochemical Differences in the Structure of the Alimentary

Canal in Feeding and Runt (Feed Deprived) White Sturgeons (*Acipenser transmontanus*), J. Appl. Ichthyol. 18 (2002) 341-346.
[39] M.V. Gulubova, Y. Yovchev, T. Vlaykova, P. Hadjipetkov, D.K. Prangova, A. Popharitov, Application of light microscopical and ultrastructural immunohistochemistry in the study of goblet cell carcinoid in the appendix, World J. Surg. Oncol. 6 (2008) 15.
[40] R.W. Mowry, Special value of methods that colour both acidic and vicinal hydroxyl groups in the histochemical study of mucins. With revised directions for the colloidal iron stain, the use of Alcian Blue 8GX and their combination with the periodic acid-Schiff reaction, Ann. N.Y. Acad. Sci. 106 (1963) 402-443.
[41] C. Domeneghini, R. Pannelli Straini, A. Veggetti, Gut glycoconjugates in *Sparus aurata* L. (Pisces, Teleostei). A comparative histochemical study in larval and adult ages. Histol. Histopathol. 13 (1998) 359-372.
[42] C. Domeneghini, S. Arrighi, G. Radaelli, G. Bosi, A. Veggetti, Histochemical analysis of glycoconjugate secretion in the alimentary canal of *Anguilla anguilla* L, Acta Histochem. 106 (2005) 477-487.
[43] A.M. Cunico, J.R.S. Vitule, First records of the European catfish, *Silurus glanis* Linnaeus, 1758 in the Americas (Brazil), BioInvasions Records 3 (2014) 117-122.
[44] A. de Chambrier, T. Scholz, An emendation of the generic diagnosis of the monotypic *Glanitaenia* (Cestoda: Proteocephalidae), with notes on the geographical distribution of *G. osculata*, a parasite of invasive wels catfish, Revue suisse de Zoologie 123 (2016) 1-9.
[45] G. Paladini, A. Gustinelli, M.L. Fioravanti, D. Minardi, M. Prearo, Riedescription of *Thaparocleidos vistulensis* (Monogenea: Ancylodiscoididae) from Wels catfish (*Silurus glanis* L.) from Po river and taxonomic status of the genus, Ittiopatologia 5 (2008) 129-138.
[46] R.K. Grencis, Immunity to helminths: resistance, regulation, and susceptibility to gastrointestinal nematodes, Annu. Rev. Immunol. 33 (2015) 201-225.
[47] C.C. Lazado, C.M. Caipang, Mucosal immunity and probiotics in fish, Fish Shellfish Immunol. 39 (2014) 78-89.
[48] J.H. Rombout, G. Yang, V. Kiron, Adaptive immune responses at mucosal surfaces of teleost fish, Fish Shellfish Immunol. 40 (2014) 634-643.
[49] I. Fairweather Peptides: an emerging force in host response to parasitism, in: N.E. Beckage (Ed.), Parasites and pathogens: effects on host hormones and behaviour, Chapman & Hall, New York, 1997, pp. 113-119.
[50] M.C.M. Halliez, A.G. Buret, Gastrointestinal parasites and the neural control of gut functions, Front. Cell. Neurosci. 9 (2015) 452.
[51] M. Di Giovangiulio, S. Verheijden, G. Bosmans, N. Stakenborg, G.E. Boeckxstaens, G. Matteoli, The Neuromodulation of the Intestinal Immune System and Its Relevance in Inflammatory Bowel Disease, Front. Immunol. 6 (2015) 590.
[52] E.A. Mayer, Gut feelings: the emerging biology of gut-brain communication, Nat. Rev. Neurosci. 12 (2011) 453-466.
[53] B.S. Dezfuli, F. Pironi, L. Giari, C. Domeneghini, G. Bosi, Effect of *Pomphorhynchus laevis* (Acanthocephala) on putative neuromodulators in the intestine of naturally infected *Salmo trutta*, Dis. Aquat. Organ. 51 (2002) 27-35.
[54] R. Bermudez, F. Vigliano, M.I. Quiroga, J.M. Nieto, G. Bosi, C. Domeneghini, Immunohistochemical study on the neuroendocrine system of the digestive tract of turbot, *Scophthalmus maximus* (L.), infected by *Enteromyxum scophthalmi* (Myxozoa), Fish Shellfish Immunol. 22 (2007) 252-263.
[55] J.E. Duffy-Whritenour, J.T. Zelikoff, Relationship between serotonin and the immune system in a teleost model. Brain Behav. Immun. 22 (2008) 257-264.
[56] A.J. Kiliaan, G. Scholten, A. Groot, Exocytotic release of vasoactive intestinal polypeptide and serotonin from mucosal nerve fibres and endocrine cells of the intestine of the goldfish

(*Carassius auratus*) and the tilapia (*Oreochromis mossambicus*): an ultrastructural study, Histochem. J. 29 (1997) 45–51.
[57] C. Olsson, S. Holmgren, The control of gut motility, Comp. Biochem. Physiol. A 128 (2001) 481-503.
[58] C. Domeneghini, G. Radaelli, S. Arrighi, F. Mascarello, A. Veggetti, Neurotransmitters and putative neuromodulators in the gut of *Anguilla anguilla* (L.). Localizations in the enteric nervous and endocrine systems, Eur. J. Histochem. 44 (2000) 295–306.
[59] A.P. Losada, R. Bermúdez, L.D. Faílde, A. Di Giancamillo, C. Domeneghini, M.I. Quiroga, Effects of *Enteromyxum scophthalmi* experimental infection on the neuroendocrine system of turbot, *Scophthalmus maximus* (L.), Fish Shellfish Immunol. 40 (2014) 577-583.
[60] J.R. Grider, Interplay of somatostatin, opioid, and GABA neurons in the regulation of the peristaltic reflex, Am. J. Physiol. 267 (1994) G696-G701.
[61] P. Holzer, Opioid receptors in the gastrointestinal tract, Regul. Pept. 155 (2009) 11-17.
[62] S. Zoghbi, A. Trompette, J. Claustre, M. El Homsi, J. Garzón, G. Jourdan, J.Y. Scoazec, P. Plaisancié, beta-Casomorphin-7 regulates the secretion and expression of gastrointestinal mucins through a mu-opioid pathway, Am. J. Physiol. 290 (2006) G1105-G1113.
[63] G. Sarnelli, P. Vanden Berghe, P. Raeymaekers, J. Janssens, J. Tack, Inhibitory effects of galanin on evoked [Ca^{2+}]i responses in cultured myenteric neurons, Am. J. Physiol. 286 (2004) G1009-G1014.
[64] G. Bosi, R. Bermùdez, C. Domeneghini, The galaninergic enteric nervous system of pleuronectiformes (Pisces, Osteichthyes): An immunohistochemical and confocal laser scanning immunofluorescence study, Gen. Comp. Endocrinol. 152 (2007) 22–29.
[65] D.R. Halm, S.T. Halm, Secretagogue response of goblet cells and columnar cells in human colonic crypts, *Am. J. Physiol. Cell Physiol.* 278 (2000) C212-C233.
[66] K.L. Shephard, Function for fish mucus, Rev. Fish Biol. Fish. 4 (1994) 401-429.
[67] M.G. Smirnova, L. Guo, J.P. Birchall, J.P. Pearson, LPS-up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells, Cell Immunol. 221 (2003) 42-49.
[68] B.S. Dezfuli, F. Pironi, M. Campisi, A.P. Shinn, L. Giari, The response of intestinal mucous cells to the presence of enteric helminths: their distribution, histochemistry and fine structure, J. Fish Dis. 33 (2010) 481-488.
[69] G. Bosi, B.S. Dezfuli, Responses of *Squalius cephalus* intestinal mucous cells to *Pomphorhynchus laevis*, Parasitol. Int. 64 (2015) 167-172.
[70] B.A. Katzenback, M. Belosevic, Characterization of granulocyte colony stimulating factor receptor of the goldfish (*Carassius auratus* L.), Dev. Comp. Immunol. 36 (2012) 199-207.
[71] E.A. Harvie, A. Huttenlocher, Neutrophils in host defense: new insights from zebrafish, J. Leukoc. Biol. 98 (2015) 523-537.
[72] C. Haslett, Granulocyte apoptosis and its role in the resolution and control of lung inflammation, Am. J. Respir. Crit. Care. Med. 160 (1999) S5–S11.
[73] J.J. Havixbeck, A.M. Riger, M.E. Wong, J.W. Hodgkinson, D.R. Barreda, Neutrophil contributions to the induction and regulation of the acute inflammatory response in teleost fish, J. Leukoc. Biol. 99 (2016) 241-252.
[74] A.L. St. John, S.N. Abraham, Innate immunity and its regulation by mast cells, J. Immunol. 190 (2013) 4458-4463.
[75] A.E. Ellis, Eosinophilic granular cells (EGC) and histamine responses to *Aeromonas salmonicida* toxins in rainbow trout, Dev. Comp. Immunol. 9 (1985) 251-260.
[76] U. Silphaduang, A. Colorni, E.J. Noga, Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish, Dis. Aquat. Org. 72 (2006) 241-252.
[77] P. Alvarez-Pellitero, Fish immunity and parasite infections: from innate immunity to immunoprophylactic prospects, Vet. Immunol. Immunopathol. 126 (2008) 171–198.

[78] I. Mulero, M.P. Sepulcre, J. Meseguer, A. Garcia-Ayala, V. Mulero, Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response, PNAS 104 (2007) 19434-19439.
[79] J. Corrales, W.L. Gordon, E.J. Noga, Development of an ELISA for quantification of the antimicrobial peptide piscidin 4 and its application to assess stress in fish, Fish Shellfish Immunol. 27 (2009) 154–163.
[80] B.S. Dezfuli, F. Pironi, L. Giari, E.J. Noga, Immunocytochemical localization of piscidin in mast cells of infected sea bass gill, Fish Shellfish Immunol. 28 (2010) 476-482.
[81] B.S. Dezfuli, L. Giari, M. Lorenzoni, M. Manera, E.J. Noga, Perch liver reaction to *Triaenophorus nodulosus* plerocercoids with an emphasis on piscidins 3, 4 and proliferative cell nuclear antigen (PCNA) expression, Vet. Parasitol. 200 (2014) 104-110.
[82] S.A. Salger, B.J. Reading D.A. Baltzegar, C.V. Sullivan, E.J. Noga, Molecular characterization of two isoforms of piscidin 4 from the hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), Fish Shellfish Immunol. 30 (2011) 420-424.
[83] A.E. Ellis, Innate host defense mechanisms of fish against viruses and bacteria, Dev. Comp.
[84] O.B. Reite, The rodlet cells of teleostean fish: their potential role in host defence in relation to the role of mast cells/eosinophilic granule cells, Fish Shellfish Immunol. 19 (2005) 253-267.
[85] H.V. Simpson, S. Umair, V.C. Hoang, M.S. Savoian, Histochemical study of the effects on abomasal mucins of *Haemonchus contortus* or *Teladorsagia circumcincta* infection in lambs, Exp. Parasitol. 130 (2016) 209-217.
[86] I. Salinas, The mucosal immune system of Teleost fish, Biology 4 (2015) 525-539.

Figure captions

Fig. 1 Histological sections from the intestine of *Silurus glanis* infected with the cestode *Glanitaenia osculata* and incubated with either the galanin (**a-c**), the met-enkephalin (**d-f**), or the serotonin (**g-i**) antibody followed by a subsequent AB/PAS stain for mucous cells. Micrographs **a**, **d**, and **g** show the general distribution of the mucous (thick arrows), endocrine (thin arrows) and mast cells (arrowheads) in the intestinal mucosa. The close contact between the immunoreactive endocrine epithelial cells (thin arrows) and the mucous cells (thick arrows) can be observed in micrographs **b**, **e**, and **h**. The micrographs **c**, **f**, and **i** revealed the close contact between the endocrine cells (thin arrows) immunoreactive to the three neurosubstances and the mast cells (arrowheads) placed at the epithelial base. Scale bars: a, d, g: 50 μm; b, c, e, f, h, i: 20 μm.

Fig 2 In the intestinal epithelium of infected *S. glanis*, the endocrine cells immunofluororeactive to the antibody anti-serotonin (**a**, thin arrows) and met-enkephalin (**b**, thin arrows) are observed in close proximity to the SNA-positive mucous cells (thick arrows). Scale bars: 20 μm.

Fig 3 Transmission electron micrographs of *Silurus glanis* intestine infected with tapeworm *Glanitaenia osculata* **(a)** Mucous cells (white arrows) near the apex of the intestinal epithelium, mast cells (curved arrows) in deeper part of epithelium are visible; scale bar = 4 μm. **(b)** Close contact between mucous cells (white arrow) and mast cells (curved arrow) can be seen; scale bar = 0.7 μm. **(c)** Tight connection between a mucous cell (white arrow) and an endocrine cell (arrow) is visible, the cytoplasm of endocrine cell is filled with electron-dense granules, scale bar = 0.3 μm. **(d)** Close vicinity of a mast cell (curved arrow) and endocrine cell (arrow) within the epithelium, scale bar = 0.5 μm. **(e)** Two mast cells (curved arrows) near the apex of the intestinal epithelium, the cytoplasm of both cells are filled with numerous electron-dense granules, scale bar = 2.5 μm. **(f)** Basal part of the epithelium**;** co-presence of mast cells (curved arrows), neutrophils (arrow heads) and endocrine cell (arrow) is evident, scale bar = 2 μm.

Table 1. Mean number ± standard error of mucous cells per 100,000 μm^2 in the uninfected wels catfish compared with conspecific parasitized with *Glanitaenia osculata* (Cestoda). Mucous cells were considered for their staining affinity to Alcian Blue (AB, acidic gliconjugates), Periodic Acid Schiff (PAS, neutral glycoconjugates), and to AB/PAS (mixed glycoconjugates). Counts and the statistical Mann-Whitney test were performed as reported in the text. Significance was set at $p<0.05$.

Mucous cells positive to:	Uninfected *Silurus glanis*	*S. glanis* parasitized with *G. osculata*	U	z	p
AB	113.90 ± 3.70	138.47 ± 3.84	3009	-4.864	<0.01
PAS	52.44 ± 2.57	40.15 ± 1.44	3436	-3.820	<0.01
AB/PAS	131.75 ± 2.60	128.97 ± 2.84	4729	-0.662	0.51
Total mucous cells	297.86 ± 5.11	319.69 ± 6.49	4161	-2.049	<0.05

Table 2. Mean number ± standard error of endocrine cells per 100,000 μm^2 in the uninfected wels catfish compared with conspecific parasitized with *Glanitaenia osculata* (Cestoda). Endocrine cells were considered for their binding affinity to the rabbit polyclonal anti-Galanin, -Met-enkephalin, -Serotonin. Counts and the statistical Mann-Whitney test were performed as reported in the text. Significance was set at $p< 0.05$.

Endocrine cells positive to:	Uninfected *Silurus glanis*	*S. glanis* parasitized with *G. osculata*	U	z	p
Galanin	3.61 ± 0.37	21.05 ± 1.86	2358	-6.553	<0.01
Met-Enkephalin	5.72 ± 0.40	19.36 ± 0.86	594	-10.770	<0.01
Serotonin	4.97 ± 0.37	14.72 ± 0.91	1583	-8.355	<0.01