Occurrence of immune cells in the intestinal wall of *Squalius cephalus* infected with *Pomphorhynchus laevis*

Bahram S. Dezfuli[a*], Maurizio Manera[b], Luisa Giari[a], Joseph A. DePasquale[c], Giampaolo Bosi[d]

[a] Department of Life Sciences & Biotechnology, University of Ferrara, St. Borsari 46, 44121 Ferrara, Italy

[b] Faculty of Biosciences, Agro-Alimentary and Environmental Technologies, University of Teramo, St. Crispi 212, I-64100 Teramo, Italy

[c] Morphogenyx Inc, PO Box 717, East Northport, NY 11731, USA

[d] Department of Veterinary Sciences and Technologies for Food Safety, Università degli Studi di Milano, St. Trentacoste 2, 20134 Milan, Italy

*Corresponding author:

Dr. Bahram S. Dezfuli, PhD

Department of Life Sciences & Biotechnology, University of Ferrara, St. Borsari 46, 44121 Ferrara, Italy

Phone: +39 0532 455701

Fax: +39 0532 455715

E-mail dzb@unife.it

Abstract

A sub-population of 34 specimens of chub, *Squalius cephalus*, was sampled from the River Brenta (Northern Italy) and examined for ecto- and endo-parasites. *Pomphorhynchus laevis* was the only enteric helminth encountered. Immunofluorescence and ultrastructural studies were conducted on the intestines of chub. Near the site of parasite's attachment, mucous cells, mast cells (MCs), neutrophils and rodlet cells (RCs) were found to co-occur within the intestinal epithelium. The numbers of mucous cells, MCs and neutrophils were significantly higher in infected fish (Mann-Whitney U test, $p < 0.05$). Dual immunofluorescence staining with the lectin Dolichos Biflorus Agglutinin (DBA) and the macrophage-specific MAC387 monoclonal antibody, with parallel transmission electron microscopy, revealed that epithelial MCs often made intimate contact with the mucous cells. Degranulation of a large number of MCs around the site of the acanthocephalan's attachment and in proximity to mucous cells was also documented. MCs and neutrophils were abundant in the submucosa. Immune cells of the intestinal epithelium have been described at the ultrastructural level and their possible functions and interactions are discussed.

Key words: fish, parasites, intestinal mucosa, immune cells, mucus

1. Introduction

The mucosal immune system of higher vertebrates has been the subject of intense investigation for several decades. In contrast few details are known regarding mucosal immunity in fish [1,2]. In all vertebrates, the digestive tract is a primary route of infection [3]; the intestinal canal affords a remarkably benign and rich environment for otherwise vulnerable enteric parasites, offering them protection and nutrients [4].

In fish, the innate immune system comprises: proteins that mediate the responses to pathogen infection (e.g., the complement system); cytotoxic (*i.e.* natural killer) or phagocytic (*i.e.* macrophages, granulocytes) cells; and physical (*e.g.* epithelial) and chemical (*e.g.* anti-microbial peptides) barriers to minimise the likelihood of parasitic infection [5]. Evidence for the involvement of mast cells (MCs) [6-8], macrophage aggregates (MAs) [9,10], neutrophils [11-13] and rodlet cells (RCs) [14-16] in the immune system of fish is growing where they have been reported to play a crucial role in the defence against pathogens [17,18].

Numerous helminth parasites are characterized by long lifespans and due to this longevity the parasites tend to provoke chronic infections. Indeed, the immune response that develops during helminth infection often proceeds to induce pathological alterations in gut tissue [19], alterations which themselves end up being the primary cause of disease [20]. In chronic helminthiases, the main concern for the host is to modulate its immune response adequately [20].

A close relationship between epithelial MC degranulation and excessive mucus secretion by mucous cells has been reported in several studies of mammal-intestinal helminth disease systems [21-26]. Nevertheless, similar studies on the occurrence of intestinal MCs in fish-helminth systems are infrequent [15,27,28] and even fewer studies have quantified the effects of intestinal helminth infection on the density of mucous cells and on the composition of the mucus [29,30]. Therefore the present study sought to help clarify the mechanism by which epithelial MCs might induce excessive mucus secretion in the intestines of fish naturally infected with the enteric helminth *Pomphorhynchus laevis*. Fluorescent probe labeling and ultrastructural analysis revealed an intimate

physical relationship between MCs and mucous cells, with MC degranulation occurring in close proximity to mucous cells. The possible functions and cooperation of MCs, neutrophils, RCs and mucous cells in the mucosal immune system in response to infection by this intestinal worm are discussed.

2. Materials & Methods

2.1. Histology

Thirty-four specimens of *Squalius cephalus* (average total length 29.4 ± 1.1 cm, mean ± SE, range 21-37 cm, n = 34) were sampled in July 2014 by electrofishing from the River Brenta, Northern Italy. The chub were anaesthetised with a 125 mg L^{-1} MS222 (tricaine methanesulphonate, Sandoz), followed by severing the spinal cord. After dissection, fresh smears of gills, stomach, intestine, liver, heart, gonads, spleen and kidney were screened microscopically for ecto- or endo-parasites. Then, several pieces of 15 x 15 mm of gills, stomach, liver, heart, gonads, spleen, kidney were excised and fixed in 10% neutral buffered formalin for 24 h, wax-embedded and cut with routine techniques for the presence of parasites. *P. laevis* was the only enteric helminth found in chub. The worm was visible with the naked eye during screening of the intestine. In all infected chub *P. laevis* was encountered only in the mid-gut. For acanthocephalans found still attached to the intestine, their position was registered and 15 × 15 mm pieces of the mid-gut were fixed in 10% neutral buffered formalin for 24 h, wax-embedded as mentioned above. Corresponding pieces of uninfected mid-gut were also processed so that a direct comparison with the infected material could be made. No other parasites were detected with the above techniques.

2.2. Dual fluorescence staining

Sections of the mid-gut of chub were dewaxed and re-hydrated, then treated with 0.1% Trypsin in Tris-HCl buffer saline (TBS; 0.05 M, pH 7.4, 0.55 M NaCl) for 20 min at room temperature (RT) as antigen retrieval process. Slides were incubated with a mouse monoclonal antibody specific to macrophage, diluted 1:50 (clone MAC387, code ab22506, Abcam, Cambridge, UK) in TBS for 3

days at 4 °C, washed in TBS, and treated with the Biotin-Avidin Blocking Kit (Vector Lab., USA). Monoclonal mouse anti-human macrophage antibody has been previously shown to cross-react with chub mast cells [31]. Sections were then incubated with 10 μg/ml goat biotinylated anti-mouse IgG (Vector Lab.) in TBS-T for 4 h at RT. After rinsing in TBS, the sections were treated with 10 μg/ml Fluorescein Avidin D (Vector Lab.) in 0.1M $NaHCO_3$ pH 8.5 with 0.15M NaCl for 4 h at RT.

The slides previously incubated for the demonstration of the MAC387 antigen were then treated with 10 μg ml^{-1} biotinylated Dolichos Biflorus Agglutinin (DBA, Vector Lab.) in 10 mM HEPES pH 7.5, 0.15 M NaCl, 0.08% sodium azide, 0.1 mM $CaCl_2$ for 3 days at 4°C. DBA is a glycoprotein, isolated from the horse gram seed, with a high binding affinity for N-acetylgalactosamine residues that are widely present in mucins, and has been previously used as marker for the intestinal mucous cells of fish [32,33]. Afterwards sections were rinsed in TBS 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 mounted with Vectashield® mounting medium (Vector Lab.) and examined on a Zeiss 510 confocal laser scanning microscope (CLSM). Sections of rat spleen were used as positive controls for the anti-macrophage antibody, whereas negative controls were obtained by the omission of the primary antibody on representative sections from chub infected with the acanthocephalans. Both sets of controls gave the expected results.

2.3. Transmission Electron Microscopy

For TEM, 7 × 7 mm pieces of infected and uninfected intestinal tissues were fixed in chilled 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for 3 h. The fixed tissues were then post-fixed in 1 % osmium tetroxide for 2 h and then rinsed and stored in 0.1 M sodium cacodylate buffer containing 6% sucrose for 12 h. Thereafter, the pieces of tissue were dehydrated through a graded acetone series and embedded in epoxy resin (Durcupan ACM, Fluka). Semi thin sections (1.5 μm) were cut on a Reichert Om U 2 ultra microtome and stained with toluidine blue. Ultra-thin sections (90 nm) were stained with 4% uranyl acetate solution in 50% ethanol and Reynold's lead citrate and then examined using an Hitachi H-800 transmission electron microscope. The identification of MCs

and neutrophils by LM is not completely reliable because both cells are similar in shape and contain granules in the cytoplasm. Therefore, TEM was used for accurate identification of these granulocytes.

Using TEM, the numbers of mucous cells, RCs, MCs and neutrophils were counted on grids in two tissue areas measuring 1,800 μm^2 from each fish (for uninfected N. areas = 30; for infected N. areas = 38 in close proximity to the point of parasite attachment). The cell counting was done by TEM for better accuracy and also to rule out any error on the correct number of granulocytes. STATISTICA 7 was adopted as statistical software package. Because the data did not meet Gaussian distribution (normality), evaluated with Kolmogorov–Smirnov test, a nonparametric method was used: the Mann-Whitney U test was performed to compare the cell densities between uninfected and infected fish and the densities of MCs and neutrophils between the epithelium and the connective tissues of the lamina propria and submucosa. The level of significance selected was $p < 0.05$.

3. Results

No parasites were found in gills or other visceral organs, and *P. laevis* appeared to be the only helminth present. Nineteen fish (55.9 %) were infected with the acanthocephalan. The intensity of infection was 1-20 worms per host (12.2 ± 1.3, mean ± standard error SE, n = 19). Mid-gut was the only infected region, where *P. laevis* were found to penetrate deeply through all the layers of the intestinal wall by means of its long slender neck, bulb and proboscis. Thus at the site of attachment, the mucosa, submucosa and both muscle layers were completely disrupted by the acanthocephalan's neck. A schematic representation of chub intestinal mucosa and *P. laevis* penetration in the intestinal wall is shown in Figure 1.

3.1. Fluorescent labeling: spatial relationship of mast and mucous cells in infected intestine

Mucous cells were present at high density in the intestinal epithelium of the parasitized chub (Figs 2 and 3). Mucous cells were labelled positively with DBA, with the lectin exhibiting a variable staining intensity that ranged from moderate to strong (Figs 2b and 3b,c). Despite this variation in

intensity, mucous cells were nonetheless discretely stained by the lectin and mucous granules were clearly visible.

MCs were also present within the intestinal epithelium, situated mainly near the basal membrane and frequently in close vicinity or in contact with the mucous cells (Figs 2b and 3 and see further). Dual fluorescence staining demonstrated a positive reaction of the MCs to either the MAC387 antibody or to the lectin (Figs 2b and 3). MCs were highly concentrated in the lamina propria and in the submucosa of the area in close proximity to the site of acanthocephalan attachment (Fig. 2a). The number of MCs was lower in *tunica muscularis;* although staining showed considerable fluorescent material, this is most likely the product~~s~~ of MC~~s~~ degranulation adhering to the acanthocephalan's tegument (Fig. 2a).

3.2. Quantitative data and ultrastructural analysis

The aspect of uninfected intestines was documented in Fig. 4. In both healthy and infected fish the intestinal epithelium is a simple columnar type, made up mainly of enterocytes, mucous cells and a few RCs. The occurrence of other cell types, MCs and neutrophils, varied among uninfected and infected individuals. The densities of all these cells are reported in Table 1. Briefly, the numbers of mucous cells, MCs and neutrophils were significantly higher in infected intestines (see Tab. 1). TEM sections of infected intestines showed MCs (Fig. 5a) and neutrophils within the epithelium, in the lamina propria and in the submucosal layer (see Tab. 1). MCs and neutrophils were more abundant in the lamina propria and submucosa than in epithelium (see Tab. 1).

MCs were irregular in shape with an eccentric nucleus, and a cytoplasm characterised by numerous large, electron-dense, membrane-bounded granules (Fig. 5b). The cytoplasm typically contained two to three mitochondria and an inconspicuous Golgi apparatus. Contact between MCs and mucous cells plasmalemma was frequently observed (Fig. 5c).

Neutrophils were round to oval in shape with irregular borders, and contained a round nucleus and the typical rod-like electron dense granules (Fig. 5d). Fragments of rough endoplasmic reticulum were observed in the cytoplasm, whereas few mitochondria were present. RCs, in variable numbers,

were also observed among the epithelium of chub in infected fish (Fig. 6a), and were primarily located in zones in close proximity to the *P. laevis* site of attachment. RCs displayed typical cytological features such as a subplasmalemmar fibrillar capsule, an eccentric basally-placed nucleus and cytoplasmic inclusions called rodlets (Fig. 6a). Rodlets were located at the nuclear proximal end of the cell and their cores tips were oriented toward the apical end of the cell (Fig. 6a). Small vesicles were observed throughout the interior of the cell and mitochondria were not readily identified. In some cases expulsion of rodlets into the intestinal lumen was visible (Fig. 6b).

Morphological heterogeneity existed between adjacent mucous cells of the intestine, but the vast majority were pear-shaped cells while the remaining were slender, elongated cells. The nuclei of mucous cells were elongated and basally-placed, with the mucus granules occupying the entire supranuclear cytoplasm (Fig. 6c). A well developed rough endoplasmic reticulum, Golgi apparatus and numerous round-oval mitochondria were observed in the basal portion of the cell.

Mucus granules were spherical or polyhedral, surrounded by a single membrane, generally electron-dense, but some of them were electron-lucent (Fig. 6d). Discharge of mucous cells on the surface of epithelium (Fig. 6d) was more frequent in infected intestines than in uninfected ones.

4. Discussion

Fish which include over 27,000 species are, phylogenetically, the oldest vertebrate group representing more than one-half of the vertebrates on the planet [34]. Understanding the immune systems of fish, therefore, is of great relevance as it provides information on the evolution of immunity and the responses seen in fish against helminths will contribute to our core knowledge of immunological processes with potential relevance to other vertebrate classes [4,35].

The mucosal immune system plays a key role in the defence against pathogens and is therefore considered an important and highly active site of immune responses [36,37]. Healthy fish defend against pathogenic organisms using a complex system of innate and adaptive immune responses [2,7,38,39], and in helminth infection this response is associated with inflammatory

199 reactions [40,41]. The manner by which the host immune system deals with helminth infections is
200 very different from the way it copes with infection or invasion by microorganisms such as viruses,
201 bacteria, fungi and protozoa [42]. According to Buchmann [43], "…. a successful co-evolution of
202 the host and its parasite necessitates that the latter develop evading mechanisms in order to avoid
203 extinction."

204 In fish, the intestinal epithelial barrier is divided into the extrinsic, intrinsic and
205 immunological barriers [44]. According to Jutfelt [44], the intrinsic barrier is formed by
206 enterocytes, mucous cells, endocrine and immune cells (e.g., macrophages, neutrophils,
207 lymphocytes, natural killer-like cells and RCs). MCs are also included in immune cells [6,8,45, 46].
208 Notable morphological studies have been carried out on different types of fish immune cells.
209 Nonetheless, detailed data on fish immune cells were provided using transmission electron
210 microscopy and the interested reader is referred to excellent monographs by Zapata et al. [47] and
211 Ferguson [48].

212 The presence of *P. laevis* in chub induces an intense inflammatory response which results in
213 an increased number of neutrophils and MCs near the site of parasite attachment, both within the
214 epithelia, the lamina propria and the submucosa. Similar findings of an increase in granulocytes in
215 submucosa were reported in intestines of cestode-infected rainbow trout [15,49], and tench infected
216 with a cestode [50]. Recruitment of the MCs into intestine of fish infected with myxozoan parasite
217 *Enteromyxum leei* have been reported in several records [51-56] and the same phenomenon was
218 reported for other parasitic diseases (see review by Alvarez-Pellitero [57]).

219 In the current study of the chub-acanthocephalan system within the intestinal epithelium, a
220 close association or contact between RCs, neutrophils and MCs with mucous cells was documented.
221 We discuss further the role of these cells in fish-helminth systems, with emphasis on the eventual
222 effect MCs may have on mucous cell secretion.

223 RCs are unique to teleost fish tissues and are closely linked to other piscine inflammatory
224 cells such as MCs and epithelioid cells [45]. RCs are commonly associated with epithelia (e.g.,

gills, olfactory epithelium, kidney, intestine and skin) [14,58,59]. During the development of carp (*Cyprinus carpio)* RCs appear in the gills 14 days after fertilization, suggesting that this cell type is an innate constituent of fish tissues [60]. Consistent with the general belief that RCs have an important role in host defense against parasites [14,16,18,45,60-62], our study of infected intestinal epithelium finds RCs frequently in close association with mucous cells.

Neutrophils are among the first cell types to arrive at the site of insult and possess a formidable armamentarium in their response [11-13,63]. The involvement of neutrophils in fish in response to helminth infections is well documented [3,10,62], and their chemotaxis, phagocytosis and destruction of intracellular and extracellular pathogens demonstrate their important role in innate immunity [11,64]. In current study of parasitized intestine, neutrophils were observed to be in close contact with MCs in the epithelium, in lamina propria and in submucosa. This finding is consistent with degranulation of MCs in teleosts having a role in attracting granulocytes such as neutrophils to the site of infection [65].

MCs have been described in several teleost species and have been identified in digestive, circulatory, urinary, reproductive, tegumentary and respiratory tissues [66,67]. Close similarities in the tissue distribution, function, morphology and cytochemical properties have led to the suggestion that fish MCs are analogous to mammalian MCs [15,46,68]. MCs are motile [50,69,70] and often associated with bacteria [71,72] and parasitic infection [10,40,45,51-56,70,73]. The granules of fish MCs contain various phospholipids, acid mucopolysaccharides, alkaline phosphatases, acid phosphatases, arylsulphatase, 5-nucleotidase [74], lysozyme [75], serotonin, met-enkephalin [69], histamine [76] and the antimicrobial peptides named piscidins in species belonging to the order Perciformes [6,77,78]. In the current study, MC granules were reactive to the lectin DBA indicating the presence mucopolysaccharides with residues of α-N-acetyl-galactosamine. The degranulation of MCs in response to parasite presence has been reported in several recent studies, notably Dezfuli et al. [50], Rieger and Barreda [63] and Prykhozhi and Berman [7].

Interestingly, macrophage-specific monoclonal antibody clone MAC387 recognises the L1 or Calprotectin molecule, an intracytoplasmic antigen comprising of a 12kD alpha chain and a 14kD beta chain. The protein is a member of the S100 family, and the subunits are termed S100A8 and S100A9. The antigen is expressed in granulocytes, blood monocytes, tissue histiocytes, squamous mucosal epithelia, and reactive epidermis. Variable results have been reported for [MAC387] staining in brain macrophages and microglia [79-83]. Interestingly, the antibody was properly clustered as anti-myeloid at the Third International Workshop and Conference on Human Leucocyte Differentiation Antigens [84]. Recently, a cell population termed basophil/MC common progenitor, has been described in the mouse spleen and is derived from granulocyte/macrophage progenitors in the bone marrow [85], supporting the hypothesis of a joint granulocyte/macrophage progenitor. This view is also supported by observation of Dobson et al. [86] in zebrafish MCs. MAC387 and other anti-macrophage antibodies have been used in order to test the hypothesis that human tissue mast cells are progeny of hemopoietic stem cells and are closely related to cells of the mononuclear phagocyte system. Although many antibodies showed cross reactivity with MCs (namely, KP1 [CD68], Ki-M1P, and PG-M1 [CD68]), MAC387 did not react with MCs in mammalian tissue [87].

Chub MCs have been previously shown to cross react with another anti-macrophage antibody (clone no. LN-5, code M-1919; Sigma, St Louis, MO, USA) and the data led the authors to propose a bone marrow-like origin (see [31]). The preceding conclusion was also based on comparisons between piscine MCs and mammalian mast cells [67], in addition to the fact that the antibody used in our previous investigation [31] also reacts with human bone marrow lymphoid and myeloid precursor cells [88]. The current account is the first description of the immune labeling of piscine MCs with MAC387 antibody. The apparent discrepancy with regard to mammalian MCs reactivity could be related to the retention in piscine MCs, of some myeloid characteristics which are lost, during maturation, in mammalian MCs. The possible phylogenetic implication of this result deserves further research.

The endoparasitic helminth attachment organ frequently induces inflammation of the host's gastrointestinal tract [19,89]. Inflammation is a protective reaction of the host in response to injury [83]. In the alimentary tract, the mucus barrier is an important part of the innate immune system which hydrates and protects the underlying epithelia. The main structural components of the mucus barrier are gel-forming mucins, secreted by epithelial mucous cells [90,91]. Fish mucus is involved in a wide range of functions, including ionic and osmotic regulation, feeding, respiration, reproduction, excretion and in the protection against, and resistance to, disease [30,92]. In some fish species mucous cells are able to produce and release defensive substances [93,94]. The few studies available that have quantified the effects of intestinal helminths on the density of mucous cells show an increase in the number of intestinal mucous cells of brown trout infected with acanthocephalans and/or cestodes [29,30]. The current study provides further evidence that helminths may elicit an hyperplastic response of mucous cells. As contrary, in the intestine of *Sparus auratus* infected with *E. leei* the number of goblet cells was considerably and significantly decreased in heavily infected fish [53]. There is no complete agreement on the role of excessive mucus secretion which, in the intestines of vertebrates infected with helminths, appears as an adherent blanket of mucus. It has been suggested that increased mucus production in mammals may facilitate the expulsion of intestinal nematodes [95]. However, all the *P. laevis* were found to be firmly attached to the host intestinal wall and no dislodged acanthocephalans were found at post-mortem. Thus we concur with the general suggestion that the main role of mucus is to protect the underlying intestinal mucosa as a physical barrier against the mechanical and biochemical damage induced by parasites [30,90].

There are numerous investigations on the presence and function of MCs in epithelia of mammals infected with enteric worms [21,23,24]. These studies indicate that in mammal-intestinal helminth systems there is a close relationship between MC degranulation and excessive mucus secretion by intestinal mucous cells [22,25,26]. However, few papers are available regarding the occurrence of MCs in intestinal epithelium of fish [15,27,77, current study]. Herein, in numerous grids especially of infected intestines, epithelial MCs were often found in contact with mucous

cells. In some cases MC degranulation near mucous cells plasmalemma was also noted. The relationship between the abundance of MCs in the pharyngeal mucous epithelium and propria-submucosa has been tentatively linked to the epithelial modifications (mainly hyperplasia) and to mucous-epithelial cysts formation observed in a branchial osteogenetic neoplasm in barbel *Barbus plebejus* [96]. Moreover, a distinct population of effector T cells, termed as Th9 cells, has been described in humans to have roles both in mucus production and in IL-9 secretion, functions which stimulate mast cells [97]. Indeed, NOS (inducible isoform) immunohistochemical reactivity in branchial mucous cells of *Abramis brama* infected with *Ergasilus sieboldi* (Copepoda) shows a massive presence of MCs in the primary lamellae tissue beneath the covering epithelia [98]. Similar findings on the presence of MCs and their relationship with mucous cells in the gills of different species of fish infected with crustaceans have been reported [99,100]. These accounts favour a cooperation between MCs and mucous cells in the immune response of fish tissues.

Currently our knowledge on mucosal immunity in fish-enteric helminth systems is too limited for definitive statements to be made. Nonetheless, it does not exclude the possibility that in fish as in mammals, epithelial MC degranulation induces excessive mucus discharge by mucous cells against a parasite. These results provide further evidence of the importance of epithelial MCs in the fish's defense mechanisms [40] and provide a foundation for future investigations into the role played by fish epithelial MCs in mucous cell secretion.

Acknowledgments

This study was supported by grants from the University of Ferrara (N° 2013).

Conflict of interest

The authors declare that there is no conflict of interest.

References

[1] Rombout JHWM, Abelli L, Picchietti S, Scapigliati G, Kiron V. Teleost intestinal immunology. Fish Shellfish Immunol 2011;31:616-26.

[2] Xu Z, Parra D, Gómez D, Salinas I, Zhang Y-A, Von Gersdorff Jørgensen L et al. Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. PNAS 2013;110:13097-102.

[3] Secombes CJ, Chappell LH. Fish immune responses to experimental and natural infection with helminth parasites. Annu Rev Fish Dis 1996;6:167–77.

[4] Buchmann K. Evolution of innate immunity: clues from invertebrates via fish to mammals. Front Immunol 2014;5:459.

[5] Dixon B, Stet RJ. The relationship between major histocompatibility receptors and innate immunity in teleost fish. Dev Comp Immunol 2001;25:683–99.

[6] Silphaduang U, Noga EJ. Peptide antibiotics in mast cells of fish. Nature 2001;414:268-9.

[7] Prykhozhi SV, Berman JN. The progress and promise of zebrafish as a model to study mast cells. Dev Comp Immunol 2014;46:74–83.

[8] Dezfuli BS, Bo T, Lorenzoni M, Shinn AP, Giari L. Fine structure and cellular responses at the host-parasite interface in a range of fish-helminth systems. Vet Parasitol 2015;208:272-9.

[9] Agius C, Roberts RJ. Melano-macrophage centres and their role in fish pathology. J Fish Dis 2003;26:499-509.

[10] Dezfuli BS, Manera M, Lorenzoni M, Pironi F, Shinn AP, Giari L. Histopathology and the inflammatory response of European perch, *Perca fluviatilis* muscle infected with *Eustrongylides* sp. (Nematoda). Parasit Vectors 2015;8:227.

[11] Katzenback BA, Belosevic M. Characterization of granulocytecolony stimulating factor receptor of the goldfish (*Carassius auratus* L.). Dev Comp Immunol 2012;36:199–207.

[12] Brogden G, Krimmling T, Adamek M, Naim HY, Steinhagen D, von Kockritz-Blickwede M. The effect of b-glucan on formation and functionality of neutrophil extracellular traps in carp (*Cyprinus carpio* L.). Dev Comp Immunol 2014;44:280–5.

[13] Pijanowski L, Scheer M, Verbug-van Kemenade BML, Chadzinska M. Production of inflammatory mediators and extracellular traps by carp macrophages and neutrophils in response to lipopolysaccharide and/or interferon-γ2. Fish Shellfish Immunol 2015;42:473-82.

[14] Manera M, Dezfuli BS. Rodlet cells in teleosts: a new insight into their nature and functions. J Fish Biol 2004;65:597–619.

[15] Reite OB. 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 2005;19:253-67.

[16] Palenzuela O, Redondo MJ, Cali A, Takvorian PM, Alonso-Naveiro M, Alvarez-Pellittero A, Sitja-Bobadilla A. A new intranuclear microsporidium, *Enterospora nucleophila* n. sp., causing

an emaciative syndrome in a piscine host (*Sparus aurata*), prompts the redescription of the family Enterocytozoonidae. Int J Parasitol 2014;44:189-203.

[17] Jones SRM. The occurrence and mechanisms of innate immunity against parasites in fish. Dev Comp Immunol 2001;25:841-52.

[18] Matisz CE, Goater CP, Bray D. Density and maturation of rodlet cells in brain tissue of fathead minnows (*Pimephales promelas*) exposed to trematode cercariae. Int J Parasitol 2010;40:307–12.

[19] Wanstall ST, Robotham PW, Thomas JS. Pathological changes induced by *Pomphorhyncus laevis* Muller (Acanthocephala) in the gut of rainbow trout, *Salmo gairdneri* Richardson. Z Parasitenkd 1986;72:105-114.

[20] MacDonald AS, Araujo MI, Pearce EJ. Immunology of parasitic helminth infections. Infection and Immunity 2002;70:427–33.

[21] Anthony RM, Rutitzky LI, Urban JF, Stadecker MJ, Gause WC. Protective immune mechanisms in helminth infection. Nat Rev Immunol 2007;7:975–87.

[22] Allen JE, Maizels RM. Diversity and dialogue in immunity to helminths. Nat Rev Immunol 2011;11:375-88.

[23] Hepworth MR, Daniłowicz-Luebert E, Rausch S, Metz M, Klotz C, Maurer M et al. Mast cells orchestrate type 2 immunity to helminths through regulation of tissue-derived cytokines. PNAS 2012;109:6644-9.

[24] St. John AL, Abraham SN. Innate immunity and its regulation by mast cells. J Immunol 2013;190:4458-63.

[25] Voehringer D. Protective and pathological roles of mast cells and basophils. Nat Rev Immunol 2013;13:362-75.

[26] Montaner S, Galiano A, Trelis M, Martin-Jaular L, del Portillo HA, Bernal D, Marcilla A. The role of extracellular vesicles in modulating the host immune response during parasitic infections. Front Immunol 2014;5:433.

[27] Silphaduang U, Colorni A, Noga EJ. Evidence for widespread distribution of piscidin antimicrobial peptides in teleost fish. Dis Aquat Org 2006;72:241-52.

[28] Hellberg HBI, Vågnes ØB, Noga EJ. Mast cells in common wolfish *Anarhichas lupus* L.: ontogeny, distribution and association with lymphatic vessels. Fish Shellfish Immunol 2013;35:1769-78.

[29] Bosi G, Arrighi S, Di Giancamillo A, Domeneghini C. Histochemistry of glycoconjugates in mucous cells of *Salmo trutta* uninfected and naturally parasitized with intestinal helminths. Dis Aquat Org 2005;64:45–51.

[30] Dezfuli BS, Pironi F, Campisi M, Shinn AP, Giari L. The response of intestinal mucous cells to the presence of enteric helminths: their distribution, histochemistry and fine structure. J Fish Dis 2010;33:481-8.

[31] Dezfuli BS, Giari L, Simoni E, Bosi G, Manera M. Histopathology, immunohistochemistry and ultrastructure of the intestine of *Leuciscus cephalus* (L.) naturally infected with *Pomphorhynchus laevis* (Acanthocephala). J Fish Dis 2002;25:7-14.

[32] Domeneghini C, Pannelli Straini R, Veggetti A. Gut glycoconjugates in *Sparus aurata* L. (Pisces, Teleostei). A comparative histochemical study in larval and adult ages. Histol Histopathol 1998;13:359-72.

[33] Domeneghini C, Arrighi S, Radaelli G, Bosi G, Veggetti A. Histochemical analysis of glycoconjugate secretion in the alimentary canal of *Anguilla anguilla* L. Acta Histochem 2005;106:477-87.

[34] Toledo-Ibarra GA, Rojas-Mayorquín AE, Girón-Pérez MI. Influence of the cholinergic system on the immune response of teleost fishes: Potential model in biomedical research. Clin Dev Immunol 2013;Article ID 536534.

[35] Buchmann K. Fish immune responses against endoparasitic nematodes - experimental models. J Fish Dis 2012;35:623–35.

[36] Pérez T, Balcázar JL, Ruiz-Zarzuela I, Halaihel N, Vendrell D, De Blas I et al. Host-microbiota interactions within the fish intestinal ecosystem. Mucosal Immunol 2010;3:355-60.

[37] Salinas I, Zhang Y-A, Sunyer JO. Mucosal immunoglobulins and B cells of teleost fish. Dev Comp Immunol 2011;35:1346-65.

[38] Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Dev Comp Immunol 2001;25:827-39.

[39] Zhu LY, Nie L, Zhu G, Xiang LX, Shao JZ. Advances in research of fish immune-relevant genes: A comparative overview of innate and adaptive immunity in teleosts. Dev Comp Immunol 2013;39:39-62.

[40] Alvarez-Pellitero P. Mucosal intestinal immunity & response to parasite infections in ectothermic vertebrates. Immunology and immune system disorders. New York: Nova Science Publishers; 2011.

[41] Bahlool QZM, Skovgaard A, Kania PW, Buchmann K. Effects of excretory/secretory products from *Anisakis simplex* (Nematoda) on immune gene expression in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 2013;35:734–9.

[42] Salgame P, Yap GS, Gause WC. Effect of helminth-induced immunity on infections with microbial pathogens. Nat Immunol 2013;14:1118–26.

[43] Buchmann K. Antiparasitic Immune Mechanisms in teleost Fish: A Two-Edged Sword? Bull Eur Ass Fish Pathol 2000;20:48.

[44] Jutfelt F. Barrier function of the gut. In: Farrell AP, Editor, Encyclopedia of Fish Physiology: From Genome to Environment, Vol. 2, San Diego: Academic Press; 2011, p. 1322–31.

[45] Reite OB, Evensen Ø. Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. Fish Shellfish Immunol 2006;20:192-208.

[46] Crivellato E, Ribatti D. The mast cell: an evolutionary perspective. Biol Rev 2010;85:347–60.

[47] Zapata AG, Chiba A, Varas A. Cells and Tissue of the Immune System of Fish. In: Iwama G, Nakanishi T, Editors, The Fish Immune System, San Diego: Academic Press; 1996, p. 1-53.

[48] Ferguson HW. A text and atlas of normal tissues in teleosts and their responses in disease. In: Ferguson HW, Editor, Systemic Pathology of Fish, London: Scotian Press; 2006, p. 10-23.

[49] Sharp GJE, Pike AW, Secombes CJ. The immune response of wild rainbow trout *Salmo gairdneri* Richardson to naturally acquired plerocercoid infections of *Diphyllobothrium dendriticum* (Nitzsch 1824) and *D. ditremum* (Creplin 1825). J Fish Biol 1989;35:781-94.

[50] Dezfuli BS, Giari L, Squerzanti S, Lui A, Lorenzoni M, Sakalli S et al. Histological damage and inflammatory response elicited by *Monobothrium wageneri* (Cestoda) in the intestine of *Tinca tinca* (Cyprinidae). Parasit Vectors 2011;4:225.

[51] Sitjà-Bobadilla A, Diamant A, Palenzuela O, Álvarez-Pellitero P. Effect of host factors and experimental conditions on the horizontal transmission of *Enteromyxum leei* (Myxozoa) to gilthead sea bream, *Sparus aurata* L., and European sea bass, *Dicentrarchus labrax* (L.). J Fish Dis 2007;30:243–250.

[52] Álvarez-Pellitero P, Palenzuela O, Sitjà-Bobadilla A. Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). Parasitol Int 2008;57:110–120.

[53] Fleurance R, Sauvegrain C, Marques A, Le Breton A, Guereaud C, Cherel Y, Wyers M. Histopathological changes caused by *Enteromyxum leei* infection in farmed sea bream *Sparus aurata*. Dis Aquat Org 2008;79:219–228.

[54] Estensoro I, Redondo MJ, Álvarez-Pellitero P, Sitjà-Bobadilla A. Novel horizontal transmission route for *Enteromyxum leei* (Myxozoa) by anal intubation of gilthead sea bream *Sparus aurata*. Dis Aquat Org 2010;92:51–58.

[55] Estensoro I, Mulero I, Redondo MJ, Álvarez-Pellitero P, Mulero V., Sitjà-Bobadilla A. Modulation of leukocytic populations of gilthead sea bream (*Sparus aurata*) by the intestinal parasite *Enteromyxum leei* (Myxozoa: Myxosporea). Parasitology 2014;141:425-440.

461 [56] Katharios P, Rigos G, Divanach P. Enteromyxum leei (Myxozoa), a lethal intruder of tropical
462 pet fish: first case in humphead wrasse, *Cheilinus undulatus* (Ruppell, 1835). J Exotic Pet Med
463 2011;20:138–143.
464 [57] Álvarez-Pellitero P. Fish immunity and parasite infections: from innate immunity to
465 immunoprophylactic prospects. Vet Immunol Immunopathol 2008;126:171–198.
466 [58] DePasquale JA. Rodlet cells in epidermal explant cultures of *Lepomis macrochirus*. Acta Zool
467 2014;95:144-54.
468 [59] DePasquale JA. Tyrosine phosphatase inhibitor triggers rodlet cell discharge in sunfish scale
469 epidermis cultures. Acta Zool 2014;95:209-19.
470 [60] Mazon AF, Huising MO, Taverne-Thiele AJ, Bastiaans J, Verburg-van Kemenade BML. The
471 first appearance of rodlet cells in carp (*Cyprinus carpio* L.) ontogeny and their possible roles
472 during stress and parasite infection. Fish Shellfish Immunol 2007;22:27–37.
473 [61] Vigliano FA, Bermúdez R, Nieto JM, Quiroga MI. Development of rodlet cells in the gut of
474 turbot (*Psetta maxima* L.): relationship between their morphology and S100 protein
475 immunoreactivity. Fish Shellfish Immunol 2009;26:146-53.
476 [62] Dezfuli BS, Lui A, Pironi F, Manera M, Shinn AP, Lorenzoni M. Cell types and structures
477 involved in tench, *Tinca tinca* (L.), defence mechanisms against a systemic digenean infection.
478 J Fish Dis 2013;36:577–85.
479 [63] Rieger AM, Barreda DR. Antimicrobial mechanisms of fish leukocytes. Dev Comp Immunol
480 2011;35:1238–45.
481 [64] Henry KM, Loynes CA, Whyte MK, Renshaw SA. Zebrafish as a model for the study of
482 neutrophil biology. J Leukoc Biol 2013;94:633–42.
483 [65] Matsuyama T, Iida T. Tilapia mast cell lysates enhance neutrophil adhesion to cultured
484 vascular endothelial cells. Fish Shellfish Immunol 2002;13:243-50.
485 [66] Ellis AE. Eosinophilic granular cells (EGC) and histamine responses to *Aeromonas*
486 *salmonicida* toxins in rainbow trout. Dev Comp Immunol 1985;9:251-60.
487 [67] Reite OB. Mast cells eosinophilic granule cells of teleostean fish: a review focusing on
488 staining properties and functional responses. Fish Shellfish Immunol 1998;8:489–513.
489 [68] Dobson JT, Seibert J, Teh EM, Da'as S, Fraser RB, Paw BH et al. Carboxypeptidase A5
490 identifies a novel mast cell lineagein the zebrafish providing new insight into mast cell fate
491 determination. Blood 2008;112:2969–72.
492 [69] Dezfuli BS, Giovinazzo G, Lui A, Giari L. Inflammatory response to *Dentitruncus truttae*
493 (Acanthocephala) in the intestine of brown trout. Fish Shellfish Immunol 2008; 24:726–33.

[70] Dezfuli BS, Giari L, Lui A, Squerzanti S, Castaldelli G, Shinn AP et al. Proliferative cell nuclear antigen (PCNA) expression in the intestine of *Salmo trutta trutta* naturally infected with an acanthocephalan. Parasit Vectors 2012;5:198.

[71] Vallejo AN, Ellis AE. Ultrastructural study of the response of eosinophilic granule cells to *Aeromonas salmonicida* extracellular products and histamine liberators in rainbow trout, *Salmo gairdneri* Richardson. Dev Comp Immunol 1989;13:133-48.

[72] Lamas J, Bruno DW, Santos Y, Anadon R, Ellis AE. Eosinophilic granular cell response to intraperitoneal injection with *Vibrio anguillarum* and its extracellular products in rainbow trout, *Oncorhynchus mykiss*. Fish Shellfish Immunol 1991;1:187–94.

[73] Dezfuli BS, Squerzanti S, Fabbri S, Castaldelli G, Giari L. Cellular response in semi-intensively cultured sea bream gills to *Ergasilus sieboldi* (Copepoda) with emphasis on the distribution, histochemistry and fine structure of mucous cells. Vet Parasitol 2010;174:359-65.

[74] Ezeasor DN, Stokoe WM. A cytochemical, light and electron microscopic study of the eosinophilic granule cells in the gut of the rainbow trout, *Salmo gairdneri* Richardson. J Fish Biol 1980;17:619-34.

[75] Sveinbjornsson B, Olsen R, Paulsen S. Immunocytochemical localizationof lysozyme in intestinal eosinophilic granule cells (EGCs) of Atlantic salmon: *Salmo salar* L. J Fish Dis 1996;19:349–55.

[76] Mulero I, Sepulcre MP, Meseguer J, Garcia-Ayala A, Mulero V. Histamine is stored in mast cells of most evolutionarily advanced fish and regulates the fish inflammatory response. PNAS 2007;104:19434-9.

[77] Dezfuli BS, Lui A, Giari L, Pironi F, Manera M, Lorenzoni M et al. Piscidins in the intestine of European perch, *Perca fluviatilis*, naturally infected with an enteric worm. Fish Shellfish Immunol 2013;35:1539–46.

[78] Masso-Silva JA, Diamond G. Antimicrobial peptides from fish. Pharmaceuticals 2014;7:265–310.

[79] Flavell DJ, Jones DB, Wright DH. Identification of tissue histiocytes on paraffin sections by a new monoclonal antibody. J Histochem Cytochem 1987; 35:1217-26.

[80] Brandtzaeg P, Jones DB, Flavell DJ, Fagerhol MK. Mac 387 antibody and detection of formalin resistant myelomonocytic L1 antigen. J Clin Pathol 1988;41:963-70.

[81] Fagerhol MK. Nomenclature for proteins: is calprotectin a proper name for the elusive myelomonocytic protein? J Clin Pathol: Mol Pathol 1996;49:M74-9.

[82] Schäfer BW, Heizmann CW. The S100 family of EF-hand calciumbinding proteins: functions and pathology. Trends Biochem Sci 1996;21:134-40.

528 [83] Gutierrez M, Forster FI, McConnell SA, Cassidy JP, Pollock JM, Bryson DG. The detection of
529 CD2+, CD4+, CD8+, and WC1+ T lymphocytes, B cells and macrophages in fixed and paraffin
530 embedded bovine tissue using a range of antigen recovery and signal amplification techniques.
531 Vet Immunol Immunopathol 1999;71:321-34.
532 [84] Jones DB, Flavell DJ, Wright DH. M4.12. Tissue reactivity of a monoclonal antibody directed
533 against cells of the human monocyte-macrophage lineage in formalin-fixed, paraffin-embedded
534 tissue. In: McMichael AJ, Beverley PCL, Cobbold S, Crumpton MJ, Gilks W, Gotch FM, et al.,
535 editors. Leucocyte typing III. White cell differentiation antigens. Proceedings of the 3rd
536 International Workshop and Conference, 1984.
537 [85] Arinobu, Y., Iwasaki, H., Gurish, M.F., Mizuno, S., Shigematsu, H., Ozawa, H., Tenen, D.G.,
538 Austen, K. F. & Akashi, K. Developmental checkpoints of the basophil/mast cell lineages in
539 adult murine haematopoiesis. PNAS 2005;102:18105-10.
540 [86] Dobson J.T, Seibert J, The EM, Da'as S, Fraser RB, Paw BH, Lin TJ, Berman JN.
541 Carboxypeptidase A5 identified a novel mast cell lineage in the zebrafish providing new insight
542 into mast cell fate determination. Blood 2008;112:2969-72.
543 [87] Horny HP, Ruck P, Xiao JC, Kaiserling E. Immunoreactivity of normal and neoplastic human
544 tissue mast cells with macrophage-associated antibodies, with special reference to the recently
545 developed monoclonal antibody PG-M1. Human Pathol 1993;24:355-8.
546 [88] Shin SS, Sheibani K, Kezirian J, Nademanee A, Forman SJ, Lee SK, Winberg CD.
547 Immunoarchitecture of normal human bone marrow: a study of frozen and fixed tissue sections.
548 Human Pathol 1992;23:686-94.
549 [89] Sears BF, Rohr JR, Allen JE, Martin LB. The economy of inflammation: when is less more?
550 Trends Parasitol 2011;27:382–7.
551 [90] Schroers V, van der Marel M, Neuhaus H, Steinhagen D. Changes of intestinal mucus
552 glycoproteins after preoral application of *Aeromonas hydrophila* to common carp (*Cyprinus*
553 *carpio*). Aquaculture 2009;288:184–9.
554 [91] Shephard KL. Function for fish mucus. Rev Fish Biol Fish 1994;4:401–29.
555 [92] Mowry RW. Special value of methods that colour both acidic and vicinal hydroxyl groups in
556 the histochemical study of mucins. With revised directions for the colloidal iron stain, the use
557 of Alcian Blue 8GX and their combination with the periodic acid-Schiff reaction. Ann NY
558 Acad Sci 1963;106:402–43.
559 [93] Cho JK, Park IY, Kim HS, Lee WT, Kim MS, Kim SC. Cathepsin D produces antimicrobial
560 peptide parasin I from histone H2A in the skin mucosa of fish. FASEB J 2002;16:429–31.

[94] Liévin-Le Moal V, Servin AL. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. Clin Microbiol Rev 2006;19:315-37.

[95] Ishikawa N, Horii Y, Nawa Y. Immune-mediated alteration of the terminal sugars of goblet cells in the small intestine of *Nippostrongylus brasiliensis*-infected rats. Immunology 1993;78:303-307.

[96] Manera M, Biavati S. Branchial osteogenetic neoplasm in barbel *Barbus barbus plebejus*. Dis Aquat Org 1999;37:231-6.

[97] Ozdemir C, Akdis M, Akdis CA. T-Cell Response to Allergens. Chem Immunol Allergy 2010;95:22-44.

[98] Dezfuli BS, Giari L, Konecny R, Jaeger P, Manera M. Immunohistochemistry, ultrastructure and pathology of gills of *Abramis brama* from lake Mondsee, Austria, infected with *Ergasilus sieboldi* (Copepoda). Dis Aquat Org 2003;53:257-62.

[99] Andrews M, Battaglene S, Cobcroft J, Adams M, Noga E, Nowak B. Host response to the chondracanthid copepod *Chondracanthus goldsmidi*, a gill parasite of the striped trumpeter, *Latris lineata* (Forster), in Tasmania. J Fish Dis 2010;33:211-20.

[100] Dezfuli BS, Giari L, Lui A, Lorenzoni M, Noga EJ. Mast cell responses to *Ergasilus* (Copepoda), a gill ectoparasite of sea bream. Fish Shellfish Immunol 2011;30:1087-94.

Figure captions

Fig. 1. Schematic view of the intestinal wall of *Squalius cephalus*. (a) Uninfected intestine. (b) Infected intestine with attached acanthocephalan (asterisk). The intestinal mucosa of *S. cephalus* is lined by a simple columnar epithelium consisting of typical columnar epithelial cells with a sparse intermingling of mucous cells and different types of immune cells.

Fig. 2. (a) Laser scanning microscope image of the mid-gut section of the chub parasitised with the acanthocephalan *Pomphorhynchus laevis*. Mucous cells DBA-positive (thin arrows) are observed in the epithelium; several mast cells (MCs) immunofluororeactive to MAC387 monoclonal antibody are scattered in the lamina propria and in the submucosa, and their number decrease in *tunica muscularis* (TM). The thick arrows show positive fluorescence to MAC387 on the surface of the parasite integument, notably due to degranulation of MCs. Scale bars 100 µm. **(b)** Section of intestinal folds of the parasitised chub showing mucous cells positive to DBA (thin arrows). In the connective axis of the folds, several green-yellow MCs are observed: their staining depends on the co-localization of MAC387 and DBA (thick arrows). Some of them are located at the epithelial base (arrowheads), nearby the mucus vesicle of a mucous cells. Scale bar 50 µm.

Fig. 3. (a) Laser scanning microscope image of the intestinal epithelium of the parasitised chub at 488 nm with mast cells (MCs) immunofluororeactive to MAC387. **(b)** The same microscopic image acquired at 540 nm showing mucous cells and MCs positive to the lectin DBA. **(c).** Superimposition image of the previous with mucous cells marked with DBA (thin arrows), and MCs positive to both MAC387 and DBA (thick arrows). MCs are clearly observed at the epithelial base. Scale bars 20 µm.

Fig. 4. Transmission electron micrographs of intestinal epithelium from uninfected chub, *Squalius cephalus* (L.) **(a)** Numerous enterocytes with nuclei indicated by arrows; scale bar = 2.86 µm. **(b)** A mucous cell (arrow) scattered among the enterocytes; scale bar = 3.33 µm.

Fig. 5. Transmission electron micrographs of intestinal epithelium from chub infected with the acanthocephalan *Pomphorhynchus laevis*. **(a)** Mast cells (arrows) scattered among enterocytes within the intestinal epithelium; scale bar = 2.86 µm. **(b)** A mast cell (arrow): the cytoplasm is filled with the typical electron-dense granules, note the eccentric nucleus; scale bar = 1.17 µm. **(c)** A mast cell and a mucous cell are in close contact: MC granules (arrow head) in close proximity to mucous cell plasmalemma, mucous cell cytoplasm containing numerous mitochondria (arrows); scale bar = 0.83 µm. **(d)** Neutrophils (arrows) within the epithelium. Note the aspect of the dark, elongated granules inside the cytoplasm; scale bar = 1.53 µm.

Fig. 6. Transmission electron micrographs of the intestinal epithelium of *P. laevis*-infected fish. **(a)** A mucous cell (arrow) and a rodlet cell (curved arrow) within: both cells are discharging their contents in the intestinal lumen; scale bar = 2.98 µm. **(b)** One rodlet (arrow) is releasing from the apical part of a rodlet cell; scale bar = 0.69 µm. **(c)** A mucous cell (arrow) with basal nucleus (arrow head), note different electron-density of mucus granules, scale bar = 2.00 µm. **(d)** A mucous cell is discharging mucus granules on the surface of the epithelium; scale bar = 1.54 µm.