Histochemical and immunohistochemical characterization of rodlet cells in the intestine of two teleosts, *Anguilla anguilla* and *Cyprinus carpio*

G Bosi[1], J A DePasquale[2], M Manera[3], G Castaldelli[4], L Giari[4], B Sayyaf Dezfuli[4*]

[1] Department of Health, Animal Science and Food Safety, Università degli Studi di Milano, Milan, Italy

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

[3] Faculty of Biosciences, Food and Environmental Technologies, University of Teramo, Teramo, Italy

[4] Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy

Running head: lectin- and immuno-histochemistry of rodlet cells

* **Corresponding author:** Bahram Sayyaf Dezfuli, Department of Life Sciences and Biotechnology, University of Ferrara, Borsari St. 46, 44121 Ferrara, Italy

Tel.: +39 - 0532 - 455701; Fax: +39 - 0532 - 455715

e-mail: dzb@unife.it

Abstract

RCs are characterized by a distinctive cell cortex and conspicuous inclusions named “rodlets”. These cells are particularly abundant and large in size in intestine of eels. Histochemical, immunohistochemical and ultrastructural investigations were carried out on European eel *Anguilla anguilla* and Common carp *Cyprinus carpio* from Northern Italy. Eight biotinylated lectins were used to probe for specific carbohydrate residues in deparaffinized, hydrated intestinal sections of eel and carp. Five antibodies were tested on intestinal sections of both fish species: inducible-nitric oxide synthase (i-NOS), leu-enkephalin, lysozyme, serotonin and tumor necrosis factor-α. Lectin histochemistry revealed rodlet cells (RCs) of the eel intestine to react with two of the eight lectins tested, specifically concanavalin A (ConA) and Sambucus nigra agglutinin (SNA). This contrasted to lectin staining of RCs in the intestine of common carp, where four of the eight lectins showed a positive reaction; Dolichos biflorus agglutinin (DBA), Wheat germ agglutinin (WGA), SNA and ConA. RCs in eel and carp intestine were immunoreactive with anti-bodies to lysozyme and i-NOS. The occurrence of the inflammatory peptides lysozyme and i-NOS in RCs of the eel and common carp pose in favor that these cells are involved in the mechanism of defense against pathogens.

Key words: rodlet cells, intestinal epithelium, eel, carp, lectin histochemistry, lysozyme

Introduction

In fish, the mucosal surfaces of skin, gills and digestive canal are the first line of defense acting as a physical barrier against pathogens and functioning as an active immune tissue (Parra, Reyes-Lopez & Tort 2015). This barrier is formed by the epithelial layer (Artis & Grancis 2008; Geppert, Sigg & Schirmer 2016) and by its components, namely mucous cells (Bosi *et al.* 2016), neuroendocrine cells (Bosi *et al.* 2015) and immune cells (Secombes & Ellis 2012; Gomez, Sunyer & Salinas 2013; Dezfuli *et al.* 2016a,b). In the gut, the different types of epithelial cells secrete several types of molecules such as proteins (hormones and enzymes), and simple and complex carbohydrates, the latter of which are often conjugated with peptides to form mucins, and biogenic amines (Schneeman 2002). Several accounts indicate that epithelial cells serve as the defensive front line of the mucosal immune system, by responding to signals from both apical (luminal) and basal (*lamina propria*) compartments (Moncada, Kammanadiminti & Chadee 2003). The innate immunity in teleosts relies on a range of cell types: the well known granulocytes (Secombes & Ellis 2012; Buchmann 2014; Dezfuli *et al.* 2016a), macrophage aggregates (MAs) (Agius & Roberts2003; Dezfuli, Manera & Giari 2015) and the less familiar rodlet cells (RCs) (Manera & Dezfuli 2004; Reite 2005; Reite & Evensen 2006). RCs are exclusive to fish and although two major hypotheses have emerged on their origin (see Manera & Dezfuli 2004), the prevalent view favours that they are endogenous fish cells. Although several functions have been assigned to them over the years, growing evidence suggests that the RCs have a role in the innate immune response of fish and that they most likely belong to the granulocytic line (Leino 1996; Manera & Dezfuli 2004; Reite 2005; Dezfuli, Giari & Shinn 2007; Manera, Giari & Dezfuli 2009; Dezfuli *et al.* 2016a).

The term "lectin" refers to a broad spectrum of glycoproteins and carbohydrate-binding proteins from all types of living organisms (Vasta & Ahmed 2008). Different biotinylated plant lectins are frequently used for histochemical studies of glycoconjugate patterns of expression in the alimentary canal of many fish species (Fiertak & Kilarski 2002; Domeneghini *et al.* 2005; Neuhaus *et al.* 2007a, 2007b; Schroers *et al.* 2009; van der Marel *et al.* 2014). RCs were probed with a panel of lectins in order to ascertain the specific carbohydrate content of these cells. Immunohistochemistry was used to evaluate intestinal RCs of eel and common carp with respect to messenger molecules and to lysozyme. Lysozyme is an antimicrobial enzyme which is believed to play an significant role in the innate immunity of fish (Lie *et al.* 1989; Siwicki *et al.* 1998; Ewart & Tsoi 2004; Magnadottir 2006). Inducible-nitric oxide synthase (i-NOS) is a member of the enzyme family involved in catalysis of L-arginine to produce nitric oxide (NO), an important cellular messenger molecule. NO acts as a messenger in several biological processes, particularly

inflammation, where NO production relies upon active i-NOS (Gookin *et al.* 2006; Keklikoglu *et al.* 2008; Heemskerk *et al.* 2009; Losada *et al.* 2012; Raposo *et al.* 2013). NO was originally thought to be solely a product of macrophages in response to inflammation but is now known to be produced by numerous cell types (Förstermann & Sessa 2012). The present study is the first to find that both lysozyme and i-NOS are present in the RCs of fish. The results further support a role for RCs in the innate immune response of fish.

Material & Methods

Specimen collection and tissue preparation

A total of 17 silver European eel (*Anguilla anguilla* L.) were obtained by the Po Delta Park Administration from the Comacchio Lagoons (Northern Adriatic Sea, Italy, 44º 36′ N, 12º 10′ E) on three occasions during fall 2016. A total of 18 Common carp (*Cyprinus carpio* L.) were sampled from the Po River near Ferrara (55' 18,12" N 12° 13' 48,72" E) using sinking trammel nets (30 mm mesh, 50 m long and 2 m high) in collaboration with professional fishermen. Fish were brought live to the laboratory of the Department of Life Sciences and Biotechnology, University of Ferrara, and euthanised using a lethal dose of 300 mg L^{-1} MS222 (tricaine methanesulfonate, Sandoz, Basel, Switzerland) and pithing. Thereafter the fish were weighed (eels: 450-1273 g, min-max; 804.53 ± 245.50 g, mean ± SD; carp: 624-5000 g, min-max; 2262.44 ± 1284.31 g, mean ± SD) and measured (eels: 63-81 cm in total length, min-max; 72.38 ± 5.79 cm, mean ± SD; carp: 38-75 cm in total length, min-max; 52.25 ± 10.57 cm, mean ± SD). A complete necropsy was performed on each fish immediately after euthanasia to exclude gross pathology, with particular attention paid to the intestine.

Pieces of the middle intestine (15 × 15 mm) of all eel and carp were fixed in 10% neutral buffered formalin for 24 h or in Bouin's fluid for 8 h. All the samples were then rinsed in several changes of 4ºC 70% ethanol before being stored in the same medium until processed for histology. The fixed tissues were dehydrated through an alcohol series and then paraffin wax embedded using a Shandon Citadel 2000 Tissue Processor (Shandon, UK). After blocking out, 5 µm thick sections were taken from each tissue block, stained with either Haematoxylin and Eosin (H&E), alcian blue 8 GX pH 2.5 and periodic acid Schiff's (AB/PAS) or used for lectin histochemistry and immunohistochemistry, examined and photographed using a Nikon Microscope ECLIPSE 80i (Nikon, Tokyo, Japan).

Histochemistry

Slides were de-paraffinized, re-hydrated, and washed 2x5min in 0.05 M Tris-HCl, 0.15 M NaCl containing 0.1% Triton-X 100 (TBS-T). Sections were treated with 1% H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) in TBS, then newly washed 2x5min in TBS-T, and incubated in a humid chamber with 10 µg/ml biotinylated lectin in 10 mM HEPES pH 7.5, 0.15 M NaCl, 0.08% sodium azide, 0.1 mM $CaCl_2$, for 3 h at room temperature (R.T). The lectins used, their acronyms, sources and major sugar specificities are reported in Table 1. The negative control was obtained by: (a) an incubation step with the buffer without lectin; (b) treatment of sections with a solution of lectins and the relative specific sugars (Table 1) added at a concentration of 0.2M. All these controls remove the lectin reactivity (Supporting Figs. 1-4). After a 2x5 min washing step in TBS-T, slides were treated with the streptavidin-biotin/horseradish-peroxidase complex (Vectastain® ABC Kit, Vector Labs., Burlingame, CA, USA) for 1 h at R.T., and then washed 2x5 min in TBS-T. The lectin histochemical reaction was developed with a freshly prepared diaminobenzidine solution (DAB: 4 mg 3,3'-diaminobenzidine tetrahydrochloride, Sigma-Aldrich, USA, in 10 ml of a 0.5 M Tris buffer at pH 7.6 containing 0,1 ml of 3% H_2O_2), followed by a counterstain with Mayer's Haematoxylin. Finally, sections were de-hydrated, cleared in xylene and mounted with Eukitt (Sigma-Aldrich, USA).

Immunohistochemistry

Re-hydrated sections were washed twice in 0.05 M Tris-HCl, 0.15 M NaCl containing 0.1% Triton-X 100 (TBS-T) for 2x5 min, then treated with 1% H_2O_2 (Sigma-Aldrich, USA) in TBS for 20 min. After washing in TBS-T, slides were incubated in a humid chamber with 1:20 goat normal serum for 30 min to block non-specific staining. Then sections were treated with the polyclonal rabbit antibody for 24 h at R.T.. The antibodies tested, their sources, working dilution, and the possible use of the unmasking technique are reported in Table 2. The antibody was omitted on the sections used as negative control. Sections were then washed twice in TBS-T for 5min, and treated with biotinylated goat anti-rabbit immunoglobulins (Vector Lab., USA) diluted 1:200 in TBS for 60 min. Afterwards, slides were washed 2x5 min in TBS-T and treated with the streptavidin-biotin/horseradish-peroxidase complex (Vectastain® ABC Kit, Vector Labs., USA) for 60 min at R.T.. After a brief washing step in TBS, slides were incubated with a freshly prepared diaminobenzidine solution as noted above, and counterstained with Mayer's Haematoxylin. Slides were de-hydrated and mounted with Eukitt (Sigma-Aldrich, USA). Sections of mammal tissues (human, swine, dog) were used as positive controls, and they gave the expected results.

Ultrastructural investigations

For electron microscopy, representative pieces (7 × 7 mm) of intestine of eel and common carp 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, Sigma-Aldrich, Saint Louis, Mo). Semi-thin sections (*i.e.* 1.5 μm) were cut on a Reichert Om U 2 ultramicrotome (Reichert, Vienna, Austria) 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 examined using a Hitachi H-800 electron microscope (Hitachi Ltd, Tokyo, Japan).

Results

No micro- or macroparasites were found in the main organs. The absence of parasites was confirmed by examination of histological sections. A high number of RCs were observed in the intestinal epithelium of *A. anguilla* (see Supporting Fig. 5).

Histochemistry and immunohistochemistry

The reactivity of RCs to the different histochemical markers used in the *A. anguilla* and *C. carpio* intestine are reported in Table 3. A panel of eight lectins was tested in this study and two of them, namely SNA and ConA, bound to sugar residues in the cytoplasm of the intestinal RCs of the eel (Fig. 1a, b). In the common carp intestine, four out of the eight used lectins (ConA, SNA, WGA and DBA) showed a positive reaction in the RC cytoplasm (Fig. 1c, d, e, f). A weak positivity to the lectin SNA in RCs was noticed in the eel intestine fixed in Bouin's fluid and not when buffered formaldehyde was used as fixative. No differences in staining intensity were detected in the RCs of the carp intestine fixed with Bouin's fluid or buffered formaldehyde (Tab. 3). In contrast the lectins PNA, DSL, LPA, and UEA I were not bound to the carbohydrate components of the intestinal RCs in the two fish (Tab. 3).

The intestinal RCs of the eel and common carp were immunoreactive to anti-lysozyme (Fig. 2a, b) and to anti-i-NOS antibodies (Fig. 2c, d). The RCs of the two fish were not immunoreactive to anti-leu-enkephalin, -serotonin, and TNF-α antibodies (Tab. 3).

In Table 4 a positive reaction to the lectins and antibodies tested is reported according to the different cell types of the eel and carp intestine. Mucous cells reacted positively to DBA, PNA,

WGA and SNA in the two species, while in common carp ConA also showed a positive reaction in these cells. The brush border at the luminal surface of the intestinal enterocytes was reactive to the lectins DBA and WGA in the eel and in the common carp. SNA and ConA lectins also reacted positively in the epithelial brush border of *A. anguilla*. In the intestine of *C. carpio*, the apical cytoplasm of the enterocytes and the brush border showed a weak positive reaction to the lectin ConA. The lectin SNA reacted with mast cells (MCs) scattered in the *tunica propria-submucosa* of the eel intestine. In the common carp, MCs showed a reactivity to the lectins PNA, WGA, SNA, ConA.

MCs of intestine of eel and common carp were immunoreactive to anti-lysozyme and anti–i-NOS (Tab. 4), while serotonin and leu-enkephalin were detected in neuroendocrine cells of both fish. No cell types positive to DSL, LPA, and UEA I, or to anti -TNF-α (Tab. 4), were found in the intestinal wall of either *A. anguilla* or *C. carpio*.

Ultrastructural investigations

Transmission electron microscopy revealed the features of RCs in the intestine of the two fish species. In *C. carpio*, RCs were less numerous and were scattered among epithelial cells and mucous cells (Fig. 3a). The cytoplasm of RCs were filled with some rodlets (Fig. 3b). Most often the RCs were noticed near the apex of the epithelium and ready to expel their contents into the intestinal lumen (Fig. 3b). The thickness of the cell cortex ranged from 0.4 µm to 0.6 µm**.** The nuclei were round or oval with an irregular outline and were located in the basal portion of RCs. Heterochromatin was distributed randomly within the nuclei and was also found at the periphery. Few round small mitochondria were noticed in the apex of the cell. Many translucent vesicles were observed in the cytoplasm. Club-shaped rodlets were arranged such that the “head” of the club was oriented toward the basal nucleus, and the narrow part toward the apex of the cell (Fig. 3b).

In comparison to the RC of carp, in eel intestine, RCs were much more numerous and larger in size, and, in some parts of the intestine**,** the epithelium was composed of clusters of RCs (Fig. 3c). The thickness of the cell cortex ranged from 0.6 µm to 0.9 µm. The nuclei were round, oval, or U-shaped with an irregular outline and occupied the basal portion of the cell. Ultrastructural characterization of nuclei revealed randomly distributed zones of heterochromatin, as well as peripheral localization of heterochromatin. Mitochondria were often observed in the apex of the RCs. Also in the apex were some translucent vesicles. Club-shaped rodlets are arranged so that the “head” of the club is oriented toward the basal nucleus, and the tapered end toward an opening in the cytoplasmic border at the rodlet cell apex (Fig. 3c, e). The core extended along the full length of

the rodlet (Fig. 3c, e). Desmosomes between RCs and surrounding intestinal epithelial cells were frequently found (not shown). We observed several samples where the cell cortex of the RC was interrupted by an opening at the cell apex when the RC extended to the epithelial surface. The opening often had rodlet contents and microvilli extruding through it (not shown). In some sections of intestine, a few intact RCs were free in the lumen. Interestingly, unclassified bacteria (Fig. 3e) often made intimate contact with the plasma membrane of the latter (Fig. 3f). RCs in both the epithelium and in the lumen of eels were observed to be intact, showing no signs of vacuolization or organelle degeneration (Fig. 3c, d, e).

Discussion

Since the first description of RCs as parasites in 1892 by Thélohan fish histologists and pathologists have attempted to determine the origin and functions of these enigmatic cells. Contrasting points of view of the nature and function of RCs, along with several unresolved issues were reported in the review of Manera & Dezfuli (2004). Accounts on the role of RCs as immune effector cells have focused mainly on their recruitment and mobilization in response to virus (Sulimanovic *et al.* 1996), bacteria (Salinas *et al.* 2008; Ringø *et al.* 2010) and parasite infection (Leino 1996; Reite 2005; Dezfuli *et al.* 2007; Matisz, Goater & Bray 2010; Dezfuli *et al.* 2013, 2016a). Establishing the functional role of RCs depends upon identifying the molecular components of these cells, particularly those with known immune activity. Progress in this area has been rather limited and spaced over years. Nevertheless, immunohistochemical studies have revealed the presence of several immune-related peptides in RCs of different fish species (Mazon *et al.* 2007; Ronza *et al.* 2015). Moreover, the carbohydrate content in RCs of *Cyprinus carpio*, *Psetta maxima*, and *Sparus aurata* were demonstrated using lectin histochemistry (Redondo *et al.* 2008; Redondo & Alvarez-Pellitero 2010).

The RCs in the intestinal epithelium of eel were positively labeled by SNA and ConA, lectins that bind sialic acid and mannose/glucose residues, respectively. The sialic acid residues detected by SNA, especially in the terminal position of the carbohydrate backbone, confer a protection to the intestinal mucosa from pathogens (Fiertak & Kilarski 2002; Neuhaus *et al.* 2007a; Yashpal *et al.* 2014). ConA staining indicates a high quantity of mannose, which is associated with immature glycoconjugates (Schroers *et al.* 2009).

A positive reaction of carp RCs to DBA, WGA, SNA, and ConA indicates that glycoconjugates in this species also contains N-acetylglucosamine (NAcGlc) and N-acetylgalactosamine (NAcGal) residues, major glycoconjugates of a greater complexity than those detected in the same cells of *A.*

anguilla. Our findings confirm those reported by Imagawa *et al.* (1990) regarding the occurrence of DBA and WGA, as well as the negative reaction for the lectins PNA and UEA I, in RCs of the common carp. A previous lectin histochemical study showed positive staining of RCs by BSI, SBA and ConA in the intestines of gilthead sea bream and turbot parasitized by Myxozoa (Redondo *et al.* 2008; Redondo & Alvarez-Pellitero 2010). Comparing our data with those of Redondo and collaborators (2008), there are some differences: the RCs of the infected intestine of gilthead sea bream and turbot are negative to the lectins WGA and SNA, whereas RCs of common carp are positive to both WGA and SNA and eel RCs are positive to SNA.

The lectin DBA shows a binding specificity for α-linkage-D-NAcGal residues (Parillo, Fagioli & Ceccarelli 2002) in glycoconjugates that are actively involved in ion transportation (Spicer & Schulte 1988, 1992). The lectin WGA reacts preferentially with NAcGlc residues situated either internally or in a terminal position of the carbohydrate backbone. WGA also binds sialic acid residues but with lower affinity (Monsigny *et al.* 1980; Parillo *et al.* 2002). Since the RCs of the common carp were positive to both SNA (sialic acid) and WGA, it is likely that both lectins detect sialic acid residues as was suggested by Redondo *et al.* (2008).

The lectin reactivity of eel and carp RCs was also compared with respect to the two different fixatives used. Domeneghini *et al.* (2005) suggested that BG4 (6% mercuric chloride and 0.1% glutaraldehyde in 1% sodium acetate) is a better fixative for lectin histochemistry. Most studies on lectins (e. g., current study) however, used Bouin's fluid (Marchetti *et al.* 2006; Neuhaus *et al.* 2007a, 2007b; Diaz *et al.* 2008) and/or neutral formaldehyde (Fiertak & Kilarski 2002; Redondo & Alavarez-Pellitero 2010; Leknes 2014, 2015) as fixatives. It is well known that fixation can create or destroy chemical bonds that alter the normal configuration of carbohydrates and thus alter their reactivity to lectins. In the eel gut RCs reactive to ConA exhibit a more intense coloration in formaldehyde-fixed tissues, while RCs are marked by SNA only in Bouin's fluid-fixed tissues and not in formaldehyde-fixed tissues. With this in mind, we do agree with the suggestion that in experimental lectin histochemistry it is appropriate to have two sets of tissue samples, one fixed with Bouin's fluid and another sample set with neutral formaldehyde.

The RCs of the eel and common carp were immunoreactive to the polyclonal antibodies against i-NOS and lysozyme. The monoclonal antibody to acetyl-α-melanocytes stimulating hormone (MSH) has been proposed as a biochemical marker for RCs of the common carp (Ringø *et al.* 2010). In the turbot intestine, Vigliano *et al.* (2009) noticed immune-positive reaction of RCs to anti-S-100 antibody which recognizes a peptide involved in cytoskeletal dynamic processes. A recent study conducted on epidermal cells, explanted from *Lepomis macrochirus* and stained with

fluorescent phallotoxin and antibody to tubulin, showed that F-actin is a component of the fibrous capsule and that a microtubule network extends from the basal to apical ends of the RCs (DePasquale 2014). It is believed that fish RCs contain inflammatory substances like the cytokine TNF-α (Ronza *et al.* 2015), while on the other hand, monocytes/macrophages are the primary producers of TNF-α (Parameswaran & Patial 2010; Ronza *et al.* 2015). In many teleosts, TNF-α is involved in regulation of inflammatory mediators and migration of leucocytes to the site of infection (Zou *et al.* 2002; Mulero 2004; Grayfer, Walsh & Belosevic 2008; Ronza *et al.* 2015). In this study however, we did not find TNF-α-like molecules in the RCs or in other cell types of the *A. anguilla* and *C. carpio* intestinal epithelium.

Lysozyme is believed to play an important role in the innate immunity of fish (Ewart & Tsoi 2004; Magnadottir 2006). Lysozyme is an antimicrobial enzyme and has been found to be effective against Gram-positive and Gram-negative bacteria that infect wild and cultured salmonids. Moreover, lysozyme promotes phagocytosis of bacteria (Saurabh & Sahoo 2008) and has been detected in the intestinal mast cells of Atlantic salmon (Sveinbjørnsson, Olsen & Paulsen 1996). Several authors have reported that RCs secrete proteolytic enzymes (Iger & Abraham 1997; Abbate *et al.* 2006), apparently, RCs possess humoral bactericidal enzyme lysozyme, that has been detected in mucus, serum, and lymphatic organs of most teleosts (Magnadottir *et al.* 2005). The presence of lysozyme in RCs of both the eel and common carp strengthen the hypothesis for a defensive role of these cells against pathogenic microorganisms. Indeed, RCs contain i-NOS, one of the three types of nitric oxide synthase, an enzyme involved in the synthesis of nitric oxide that occurs during inflammation (Gookin *et al.* 2006; Keklikoglu *et al.* 2008; Losada *et al.* 2012). Moreover, the RCs of the eel swimbladder infected with the nematode *Anguillicoloides crassus* are immunopositive to NOS (data not shown). The i-NOS is produced and secreted mainly in phagocytes as macrophages and fish melano-macrophages (Zhao *et al.* 2010). Losada *et al.* (2012) reported that i-NOS occurs in many cell types of the turbot intestine but not in RCs, i-NOS positive mucous cells have also been reported in the gills of parasitized *Abramis brama* (Dezfuli *et al.* 2003). *De facto,* differences in the peptide and carbohydrate components are not only due to species-specific features, but also to individual factors (e.g. of genetic or environmental conditions), and/or processing tissue protocols.

In the intestinal epithelium of the eel, a positive reaction for DBA, PNA, WGA and SNA was observed in mucous cells. This was also true for common carp which were also reactive to ConA binding. Due to their high glycoconjugate content, mucous cells of the intestinal epithelium are the preferred cell type for lectin histochemical studies of intestinal carbohydrate expression patterns in fish (Fiertak & Kilarski 2002; Parillo *et al.* 2002; Domeneghini *et al.* 2005; Neuhaus *et*

al. 2007a, 2007b; Yashpal *et al.* 2014). Our data confirm in part the results reported by Domeneghini *et al.* (2005) for the intestinal epithelium of the eel, and information provided by Imagawa *et al.* (1990) and Fiertak & Kilarski (2002) for the intestinal mucous cells of the common carp. We do agree with their suggestions that the differences in lectin histochemistry reflect a diverse glycosylation pattern due to the alimentation (van der Marel *et al.* 2014), or to the presence of the pathogens (Guzman-Murillo, Merino-Contreras & Ascencio 2000; Neuhaus *et al.* 2007a; Schroers *et al.* 2009).

MCs of the common carp intestine were positive to PNA, WGA, SNA and ConA, while MCs of the eel intestine showed a positive reaction only with SNA. With reference to the *C. carpio* intestine, glycoconjugate components observed in these two inflammatory cells showed a different pattern with respect to those reported for the same cells by Imagawa *et al.* (1990). Indeed, in common carp a weakly positive reaction of macrophages to PHA-L, PSA, and LCA was also noticed (Imagawa *et al.* 1990). The finding of lysozyme in MCs of the eel and common carp intestines is consistent with that reported elsewhere (Sveinbjørnsson *et al.* 1996; Reite & Evensen 2006). Surprisingly, MCs of intestine in the fish species of this study were not positively stained with Serotonin antibody, whereas many epithelial endocrine cells contained leu-enkephalin- or serotonin-like molecules. The presence of these two neuromodulators in endocrine cells of the intestine of some fish species infected with helminths has previously been reported by our group (Dezfuli *et al.* 2002; Bosi *et al.* 2015).

Mayberry *et al.* (1979) reported the presence of intact *Rhabdospora thelohani (*rodlet cells) in host intestinal lumen and Reite (1997) demonstrated free RCs in intestinal lumen, near a parasite body. Our results document the presence of free, intact RCs in the intestinal lumen of eels, especially in portions of lumen with bacteria. In some instances, unclassified bacteria adhered to the RC plasma membrane. We do not have direct evidence on how this attachment is mediated, nevertheless possible involvement of membrane glycoconjugates and bacterial lectins may be inferred (Gilboa-Garber *et al.* 2011). Lectinophagocytosis has been reported to support the phagocytosis of bacteria by animal cells by one of two proposed modes (Ofek & Sharon 1988). In the first mode, bacteria superficial lectins bind to the complementary membrane glycoconjugates on the surface of immune cells. The second mode is the converse of the first; immune cell membrane lectins bind to glycoconjugates on bacterial cell surfaces (Ofek & Sharon 1988; Almkvist & Karlsson 2004). This survey on the nature of the glucidic residues and the presence of lysozyme in RCs reinforces the suggestion that RCs are a type of innate immune cell that can act against pathogens. Indeed, the involvement of RCs in fish bacterial intestinal infection has been reported in Salinas *et al.* (2008)

and Sitja-Bobadilla *et al.* (2007). Experimentally infected *Dentex dentex* display pathogenic bacteria in the intestinal lumen, parallel with elevated numbers of epithelial RCs, suggesting RCs participate in the cellular reaction against pathogenic challenge (Sitja-Bobadilla *et al.* 2007). Our findings are consistent with those of Sitja-Bobadilla *et al.* (2007) as high numbers of RCs were found in the intestinal epithelium of the eel, and free RCs were also located in the lumen where the bacteria were present. Nevertheless, further studies are required to understand the specific basis for contact between RCs and bacteria and to establish the pathogenicity of these bacteria. It is thus necessary to intensify immunohistochemical investigations on the contents of RCs, which will lead to an understanding of the role of these cells in fish innate immunity.

The main results of this investigation support the following conclusions: (a) in the eel intestine, RCs present a glycoconjugate pattern with mannose, glucose and sialic acid as predominant sugar residues. The carbohydrates rich in sialic acid residues are likely involved in a trapping process directed against luminal potential pathogens, preventing their adherence to the mucosa; (b) different fish species show a diverse lectin-binding pattern of RCs. Thus, lectin histochemistry is useful in providing a carbohydrate expression profile of these cells in different fish species; (c) the RCs of the eel and common carp contain lysozyme and i-NOS, two inflammatory peptides with anti-microbial function. It is likely the substances detected in RCs are involved in the mechanism of defense against pathogenic microorganisms of the luminal environment.

Acknowledgements

We thank Dr. M. Lanzoni from University of Ferrara for providing us the fish. This work was supported by local grants from the University of Ferrara to B.S.D (N° 2016).

References

Abbate F., German G.P., De Carlos F., Montalbano G., Laur R., Levanti M.B. & German A. (2006) The oral cavity of the adult zebrafish (*Danio rerio*). *Anatomy, Histology and Embryology* **35**, 299-304.

Agius C. & Roberts R.J. (2003) Melano-macrophage centres and their role in fish pathology. *Journal of Fish Diseases* **26**, 499-509.

Almkvist J. & Karlsson A. (2004) Galectins as inflammatory mediators. *Glycoconjugate Journal* **19**, 575-581.

Artis D. & Grancis R.K. (2008) The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunology* **1**, 252-264.

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

Bosi G., Giari L., DePasquale J.A., Carosi A., Lorenzoni M. & Dezfuli B.S. (2017) Protective responses of intestinal mucous cells in a range of fish–helminth systems. *Journal of Fish Diseases* **40**, 1001-1014.

Buchmann K. (2014) Evolution of innate immunity: clues from invertebrates via fish to mammals. *Frontiers in Immunology* **5**, 459.

DePasquale J.A. (2014) Rodlet cells in epidermal explant cultures of *Lepomis macrochirus*. *Acta Zoologica* **95**, 144–154.

Dezfuli B.S., Pironi F., Giari L., Domeneghini C. & Bosi G. (2002) Effect of *Pomphorhynchus laevis* (Acanthocephala) on putative neuromodulators in the intestine of naturally infected *Salmo trutta*. *Diseases of Aquatic Organisms* **51**, 27-35.

Dezfuli B.S., Giari L., Konecny R., Jaeger P. & Manera M. (2003) Immunohistochemistry, ultrastructure and pathology of gills of *Abramis brama* from Lake Mondsee, Austria, infected with *Ergasilus sieboldi* (Copepoda). *Diseases of Aquatic Organisms* **53**, 257–262.

Dezfuli B.S., Giari L. & Shinn A.P. (2007) The role of rodlet cells in the inflammatory response in *Phoxinus phoxinus* brains infected with *Diplostomum*. *Fish and Shellfish Immunology* **23**, 300-304.

Dezfuli B.S., Lui A., Pironi F., Manera M., Shinn A.P. & Lorenzoni M. (2013) Cell types and structures involved in tench, *Tinca tinca* (L.), defence mechanisms against a systemic digenean infection. *Journal of Fish Diseases* **36**, 577-585.

Dezfuli B.S., Manera M. & Giari L. (2015) Ultrastructural assessment of granulomas in the liver of perch (*Perca fluviatilis*) infected by tapeworm. *Journal of Comparative Pathology* **152**, 97–102.

Dezfuli B.S., Bosi G., DePasquale J.A., Manera M. & Giari L. (2016a) Fish innate immunity against intestinal helminths. *Fish and Shellfish Immunology* **50**, 274-287.

Dezfuli B.S., Manera M., Bosi G., DePasquale J.A., D'Amelio S., Castaldelli G. & Giari L. (2016b) *Anguilla anguilla* intestinal immune response to natural infection with *Contracaecum rudolphii* A larvae. *Journal of Fish Diseases* **39**, 1187-1200.

Diaz A.O., García A.M. & Goldemberg L. (2008) Glycoconjugates in the mucosa of the digestive tract of *Cynoscion guatucupa*: A histochemical study. *Acta histochemica* **110**, 76-85.

Domeneghini C., Arrighi S., Radaelli G., Bosi G. & Veggetti A. (2005) Histochemical analysis of glycoconjugate secretion in the alimentary canal of *Anguilla anguilla* L.. *Acta Histochemica* **106**, 477-487.

Ewart K.V. & Tsoi S.C.M. (2004) Innate immune recognition of pathogens in teleost fish. In: *Molecular Aspects of Fish and Marine Biology Vol. 3. Current Trends in the Study of Bacterial Viral Fish and Shrimp Diseases* (ed. by K. Y. Leung), pp. 82–116. World Scientific, Singapore.

Fiertak A. & Kilarski W.M. (2002) Glycoconjugates of the intestinal mucous cells of four cyprinids. *Cellular and Molecular Life Sciences* **59**, 1724–1733.

Förstermann U. & Sessa W.C. (2012) Nitric oxide synthases: regulation and function. *European Heart Journal* **33**, 829–837.

Geppert M., Sigg L. & Schirmer K. (2016) A novel two-compartment barrier model for investigating nanoparticle transport in fish intestinal epithelial cells. *Environmental Science: Nano* **3**, 388-395.

Gilboa-Garber N., Zinger-Yosovich K.D., Sudakevitz D., Lerrer B., Imberty A., Wimmerova M., Wu A.M. & Garber N.C. (2011) The five bacterial lectins (PA-IL, PA-IIL, RSL, RS-IIL, and CV-IIL): Interactions with diverse animal cells and glycoproteins. In: *Advances in Experimental Medicine and Biology*, pp. 155–211. Springer, Boston.

Gomez D., Sunyer J.O. & Salinas I. (2013) The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. *Fish and Shellfish Immunology* **35**, 1729-1739.

Gookin J.L., Chiang S., Allen J., Armstrong M.U., Stauffer S.H., Finnegan C. & Murtaugh M.P. (2006) NFkappaB-mediated expression of iNOS promotes epithelial defense against infection by *Cryptosporidium parvum* in neonatal piglets. *American Journal of Physiology. Gastrointestinal and liver physiology* **290**, G164-174.

Grayfer L., Walsh J.G. & Belosevic M. (2008) Characterization and functional analysis of goldfish (*Carassius auratus* L.) tumor necrosis factor-alpha. *Developmental and Comparative Immunology* **32**, 532-543.

Guzman-Murillo M.A., Merino-Contreras M.L. & Ascencio F. (2000) Interaction between *Aeromonas veronii* and epithelial cells of spotted sand bass (*Paralabrax maculatofasciatus*) in culture. *Journal of Applied Microbiology* **88**, 897–906.

Heemskerk S., Masereeuw R., Russel F.G.M. & Pickkers P. (2009) Selective iNOS inhibition for the treatment of sepsis-induced acute kidney injury. *Nature Reviews Nephrology* **5**, 629–640.

Iger Y. & Abraham M. (1997) Rodlet cells in the epidermis of fish exposed to stressors. *Tissue and Cell* **29**, 431-438.

Imagawa L.T., Hashimoto Y., Kon Y. & Sugimura M. (1990) Lectin histochemistry as special markers for rodlet cells in carp, *Cyprinus carpio*. *Journal of Fish Diseases* **13**, 537-540.

Keklikoglu N., Koray M., Kocaelli H. & Akinci S. (2008) iNOS expression in oral and gastrointestinal tract mucosa. *Digestive Disease and Science* **53**, 1437-1442.

Leino R.L. (1996) Reaction of rodlet cells to a myxosporean infection in kidney of the bluegill, *Lepomis macrochirus*. *Canadian Journal of Zoology* **74**, 217-225.

Leknes I.L. (2014) Goblet cell types in intestine of tiger barb and black tetra (Cyprinidae, Characidae: Teleostei). *Anatomia Histologia Embryologia* **43**, 352-360.

Leknes I.L. (2015) Goblet cells and mucus types in the digestive intestine and respiratory intestine in bronze corydoras (Callichthyidae: Teleostei). Anatomia Histologia Embryologia. **44**, 321-327.

Lie O., Evensen O., Sorensen A. & Froysadal E. (1989) Study on lysozyme activity in some fish species. *Diseases of Aquatic Organisms* **6**, 1–5.

Losada A.P., Bermúdez R., Faílde L.D. & Quiroga M.I. (2012) Quantitative and qualitative evaluation of iNOS expression in turbot (*Psetta maxima*) infected with *Enteromyxum scophthalmi*. *Fish and Shellfish Immunology* **32**, 243-248.

Magnadottir B. (2006) Innate immunity of fish (overview). *Fish and Shellfish Immunology* **20**, 137-151.

Magnadottir B., Lange S., Gudmundsdottir S., Bogwald J. & Dalmo R.A. (2005) Ontogeny of humoral immune parameters in fish. *Fish and Shellfish Immunology* **19**, 429-439.

Manera M. & Dezfuli B.S. (2004) Rodlet cells in teleosts: a new insight into their nature and functions. *Journal of Fish Biology* **65**, 597-619.

Manera M., Giari L. & Dezfuli B.S. (2009) Rodlet cells biometry: interspecific and intraspecific variability. *Journal of Fish Biology* 74, 474-481.

Marchetti L., Capacchietti M., Sabbieti M.G., Accili D., Materazzi G. & Menghi G. (2006) Histology and carbohydrate histochemistry of the alimentary canal in the rainbow trout *Oncorhynchus mykiss*. *Journal of Fish Biology* **68**, 1808-1821.

Matisz C.E., Goater C.P. & Bray D. (2010) Density and maturation of rodlet cells in brain tissue of fathead minnows (*Pimephales promelas*) exposed to trematode cercariae. *International Journal for Parasitology* **40**, 307-312.

Mayberry L.F., Marchiondo A.A., Ubelaker J.E. & Kazic D. (1979) *Rhabdospora thelohani* Laguesse, 1895 (Apicomplexa): new host and geographic records with taxonomic consideration. *Journal of Protozoology* **26**, 168-178.

Mazon A.F., Huising M.O., Taverne-Thiele A.J., Bastiaans J. & Verburg-van Kemenade B.M.L. (2007) The first appearance of Rodlet cells in carp (*Cyprinus carpio* L.) ontogeny and their possible roles during stress and parasite infection. *Fish and Shellfish Immunology* **22**, 27-37.

Moncada D.M., Kammanadiminti S.J. & Chadee K. (2003) Mucin and Toll-like receptors in host defense against intestinal parasites. *Trends in Parasitology* **19**, 305-311.

Monsigny M., Roche A.C., Sene C., Maget-Dana R. & Delmotte F. (1980) Sugar-lectin interactions: how does wheatgerm agglutinin bind sialoglycoconjugates? *European Journal of Biochemistry* **204**, 147–153.

Mulero V. (2004) The tumor necrosis factor alpha of the bony fish seabream exhibits the in vivo proinflammatory and proliferative activities of its mammalian counterparts, yet it functions in a species-specific manner. *Cellular and Molecular Life Sciences* **61**, 1331-1340.

Neuhaus H., van der Marel M., Caspari N., Meyer W., Enss M.-L. & Steinhagen D. (2007a) Biochemical and histochemical effects of perorally applied endotoxin on intestinal mucin glycoproteins of the common carp *Cyprinus carpio*. *Diseases of Aquatic Organisms* **77**, 17-27.

Neuhaus H., van der Marel M., Caspari N., Meyer W., Enss M.-L. & Steinhagen D. (2007b) Biochemical and histochemical study on the intestinal mucosa of the common carp *Cyprinus carpio* L., with special consideration of mucin glycoproteins. *Journal of Fish Biology* **70**, 1523-1534.

Ofek I. & Sharon N. (1988) Lectinophagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. *Infection and Immunity* **56**, 539-547.

Parameswaran N. & Patial S. (2010) Tumor necrosis factor-alpha signaling in macrophages. *Critical Reviews in Eukaryotic Gene Expression* **20**, 87-103.

Parillo F., Fagioli O. & Ceccarelli P. (2002) Glucidic determinants expressed by the digestive apparatus of *Umbrina cirrosa* (L.) fries as revealed by lectin histochemistry. *Acta Histochemica* **104**, 209-215.

Parra D., Reyes-Lopez F.E. & Tort L. (2015) Mucosal Immunity and B Cells in Teleosts: Effect of Vaccination and Stress. *Frontiers in Immunology* **6**, 354.

Raposo C., Nunes A.K.D.S., Luna R.L.D.A., Araújo S.M.D.R., Da Cruz-Höfling M.A. & Peixoto C.A. (2013) Sildenafil (Viagra) protective effects on neuroinflammation: the role of iNOS/NO system in an inflammatory demyelination model. *Mediators of Inflammation* **2013**, 1- 11.

Redondo M.J. & Alvarez-Pellitero P. (2010) Carbohydrate patterns in the digestive tract of *Sparus aurata* L. and *Psetta maxima* (L.) (Teleostei) parasitized by *Enteromyxum leei* and *E. scophthalmi* (Myxozoa). *Parasitology International* **59**, 445–453.

Redondo M.J., Cortadellas N., Palenzuela O. & Alvarez-Pellitero P. (2008) Detection of carbohydrate terminals in the enteric parasite *Enteromyxum scophthalmi* (Myxozoa) and possible interactions with its fish host *Psetta maxima*. *Parasitology Research* **102**, 1257-1267.

Reite O.B. (1997) Mast cells/eosinophilic granule cells of salmonids: staining properties and responses to noxious agents. *Fish and Shellfish Immunology* **7**, 567-584.

Reite O.B. (2005) The rodlet cells of teleostean fish: their potential role in host defence in relation to the role of mast cells/eosinophilic granule cells. *Fish and Shellfish Immunology* **19**, 253-267.

Reite O.B. & Evensen O. (2006) Inflammatory cells of teleostean fish: A review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish and Shellfish Immunology* **20**, 192-208.

Ringø E., Lovmo L., Kristiansen M., Bakken Y., Salinas I., Myklebust R., Olsen R.E. & Mayhew T.M. (2010) Lactic acid bacteria vs. pathogens in the gastrointestinal tract of fish: a review. *Aquaculture Research* **41**, 451-467.

Ronza P., Losada A.P., Villamarín A., Bermúdez R. & Quiroga M.I. (2015) Immunolocalization of tumor necrosis factor alpha in turbot (*Scophthalmus maximus*, L.) tissues. *Fish and Shellfish Immunology* **45**, 470-476.

Salinas I., Myklebust R., Esteban M.A., Olsen R.E, Meseguer J. & Ringø E. (2008) In vitro studies of *Lactobacillus delbrueckii* subsp. *lactis* in Atlantic salmon (*Salmo salar* L.) foregut: tissue responses and evidence of protection against *Aeromonas salmonicida* subsp. *salmonicida* epithelial damage. *Veterinary Microbiology* **128**, 167-177.

Saurabh S. & Sahoo P.K. (2008) Lysozyme: an important defence molecule of fish innate immune system. *Aquaculture Research* **39**, 223–239.

Schneeman B.O. (2002) Gastrointestinal physiology and functions. *British Journal of Nutrition* **88**, S159–S163.

Schroers V., van der Marel M., Neuhaus H. & Steinhagen D. (2009) Changes of intestinal mucus glycoproteins after peroral application of *Aeromonas hydrophila* to common carp (*Cyprinus carpio*). *Aquaculture* **288**, 184-189.

Secombes C.J. & Ellis A.E. (2012) The immunology of teleosts. In: *Fish Pathology*, fourth ed., (ed. by R. J. Roberts), pp. 144-166. Blackwell Publishing, Chicester.

Sitjà-Bobadilla A., Pujalte M.J., Bermejo A., Garay E., Alvarez-Pellitero P. & Pérez-Sánchez J. (2007) Bacteria associated with winter mortalities in laboratory-reared common dentex (*Dentex dentex* L.). *Aquaculture Research* **38**, 733-739.

Siwicki A.K., Klein P., Morand M., Kiczka W. & Studnicka M. (1998) Immunostimulatory effects of dimerized lysozyme (KLP-602) on the nonspecific defense mechanisms and protection against furunculosis in salmonids. *Veterinary Immunology and Immunopathology* **61**, 369–378.

Spicer S.S. & Schulte B.A. (1988) Detection and differentiation of glycoconjugates in various cell types by lectin histochemistry. *Basic and Applied Histochemistry* **32**, 307–320.

Spicer S.S. & Schulte B.A. (1992) Diversity of cell glycoconjugates shown histochemically: A perspective. *Journal of Histochemistry and Cytochemistry* **40**, 1–38.

Sulimanovic D., Curic S., Zeba L. & Berc A. (1996) The possible role of rodlet cells in the immune system of carp (*Cyprinus carpio* L.). *Veterinarski Arhiv* **66**, 103–109.

Sveinbjørnsson B., Olsen R. & Paulsen S. (1996) Immunocytochemical localization of lysozyme in intestinal eosinophilic granule cells (EGCs) of Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases* **19**, 349-355.

van der Marel M., Pröpsting M.J., Battermann F., Jung-Schroers V., Hübner A., Rombout J.H.W.M. & Steinhagen D. (2014) Differences between intestinal segments and soybean meal–induced changes in intestinal mucus composition of common carp *Cyprinus carpio* L. *Aquaculture Nutrition* **20**, 12-24.

Vasta G.R. & Ahmed H. (2008) Animal Lectins: A Functional View. CRC Press, Boca Raton.

Vigliano F.A., Bermúdez R., Nieto J.M. & Quiroga M.I. (2009) Development of rodlet cells in the gut of turbot (*Psetta maxima* L.): Relationship between their morphology and S100 protein immunoreactivity. *Fish and Shellfish Immunology* **26**, 146-153.

Yashpal M., Kumari U., Mittal S. & Mittal A.K. (2014) Glycoproteins in the Buccal Epithelium of a Carp, *Cirrhinus mrigala* (Pisces, Cyprinidae): A Histochemical Profile. *Anatomy, Histology and Embryology* **43**, 116-132.

Zhao K., Huang Z., Lu H., Zhou J. & Wei T. (2010) Induction of inducible nitric oxide synthase increases the production of reactive oxygen species in RAW264.7 macrophages. *Bioscience Reports* **30**, 233-241.

Zou J., Wang T., Hirono I., Aoki T., Inagawa H., Honda T., Soma G.I., Ototake M., Nakanishi T., Ellis A.E. & Secombes C.J. (2002) Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. *Developmental and Comparative Immunology* **26**, 161-172.

Figure captions

Figure 1. Sections of intestines of *Anguilla anguilla* (a and b) and *Cyprinus carpio* (c-f) showing the histochemical reactivity of rodlet cells (RCs) to different lectins. (a) several RCs (arrows) positive to ConA; (b) some RCs (arrows) positive to SNA; (c) a RC (arrow) positive to ConA; (d) a RC (arrow) reactive to SNA; (e) two RCs (arrows) and brush border positive to WGA; (f) a RC (arrow) weakly reactive to DBA. Scale bars: 20 µm.

Figure 2. Immunoreactivity of intestinal rodlet cells (RCs) of eel and carp to lysozyme and inducibile nitric oxide synthase (i-NOS) antibodies. (a) Two RCs positive to anti-lysozyme antibody at the superficial area of the eel intestinal epithelium (arrows); (b) Two RCs in the common carp intestine positive to anti-lysozyme (arrows); (c) A group of RCs, in longitudinal section (arrows) and transversal section (arrowheads) reactive to the anti-i-NOS antibody in the epithelium of the eel intestine; (d) In the intestinal epithelium of carp several RCs (arrows) reactive to i-NOS antibody are visible. Scale bars: 20 µm.

Figure 3. Transmission electron microscopy micrographs of intestine of common carp and European eel. (a) Portion of common carp intestine, three RCs (arrows) are scattered among epithelial and mucous cells, scale bar: 4.2 µm. (b) High magnification of a RC in common carp intestine, capsule (arrow), some rodlets (curved arrows) in cell cytoplasm near basal nucleus (white asterisk) are visible. RC with apical part opened into the brush border (arrowheads), scale bar: 1.4 µm. (c) A cluster of RCs (arrows) in **a** small portion of the eel intestinal epithelium. RCs are very close to the brush border, scale bar: 3.3 µm. (d) Two RCs (arrows) of eel intestine are scattered among two mucous cells (arrow heads). Note the presence of numerous unknown bacteria (curved arrows) within the lumen, scale bar: 3.3 µm. (e) Micrograph of an intact free RC (arrow) in eel intestinal lumen. RC is encircled by the bacteria (curved arrows) some of which are in close proximity to the RC capsule, scale bar: 2.0 µm. (f) High magnification of the tight contact between bacteria (curved arrow) and plasma membrane of the RC (arrowhead). At the point of contact, RC plasma membrane is indistinguishable from bacterial capsule, scale bar: 0.25 µm.