Carbohydrate patterns in the digestive tract of *Sparus aurata* L. and *Psetta maxima* (L.) (Teleostei) parasitized by *Enteromyxum leei* and *E. scophthalm*i (Myxozoa)

María J. Redondo and Pilar Alvarez-Pellitero

Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Científicas, 12595 Ribera de Cabanes, Castellón, Spain

Corresponding author:

María J. Redondo

Instituto de Acuicultura de Torre de la Sal, Consejo Superior de Investigaciones Científicas, 12595 Ribera de Cabanes, Castellón, Spain.

E-mail: mjose@iats.csic.es

Tel: +34-964319500

ABSTRACT

The influence of *Enteromyxum* spp. infections on the carbohydrate patterns of the digestive tract of gilthead sea bream (GSB) *Sparus aurata* L. and turbot (TB) *Psetta maxima* (L.) has been studied. Histochemical stainings to differentiate the types of mucins and lectin-binding assays to detect terminal carbohydrate residues were applied to histological sections of GSB and TB uninfected or infected by *Enteromyxum leei* and *E. scophthalmi*, respectively. The number of intestinal GC decreased in severely infected fish in both parasitoses, though changes in mucin patterns were limited to the decrease in the staining intensity for acidic mucins in infected GSB. The TB stomach and intestine lacked histochemically detectable acidic mucins, or sialic acid detectable by SNA, in contrast with their abundance in GSB. Glucose/mannose, fucose and GlcNAc residues were less abundant in both infected hosts with respect to uninfected fish. In contrast, D-Gal and D-GalNAc moieties (detectable by BSL I) increased in most parts of *E. scophthalmi*-infected TB while decreasing (oesophagus) or remaining unchanged (intestine) in *E. leei*-infected GSB. The decreasing in the expression of acidic mucins and of sialic acid detectable by SNA in *E. leei*-infected GSB is remarkable. Differences in the carbohydrate patterns between both hosts could aid to explain the differences in the severity of both enteromyxoses. In addition, the changes induced by *Enteromyxum* spp. infections in the digestive tract of GSB and TB suggest a role of terminal carbohydrate residues in the parasite-host interaction.

*Key words***:** Myxosporea; lectins; mucins; histochemistry; turbot; gilhead sea bream

1. Introduction

The phylum Myxozoa includes a large number of species most of them parasites of fish. Relationships between these parasites and their hosts are often highly evolved and do not result in severe disease [1]. However, worldwide, a number of species cause disease and impact upon wild and farmed fish populations. Enteromyxoses, caused *Enteromyxum scophthalmi* and *E. leei* are among the most severe parasitic diseases in non-salmonid aquaculture, producing serious loses in turbot *Psetta maxima* (L.) and sparids, respectively [2–5]. Both myxozoans show a high affinity for the digestive tract, mainly for the intestine, the target organ.

Parasite infection of the gastrointestinal tract induces detrimental effects on host tissues and host physiology [6]. The mucin secretion can be an important mechanism of protection by eliminating the offending pathogens. The result often leads to expulsion of the pathogen but, in some instances, mucus depletion could be a deleterious side effect. In addition, enteric pathogens produce virulence factors that can disrupt the balance between mucin degradation and production/secretion [7–9]. Changes in mucin patterns have been reported for several helminth infections in mammals [10]. The process of glycosylation confers many of the general properties of mucins. Glycosylation changes of mucins also occur following some parasite infections, as in nematodiases. However, the interactions between parasites and gastrointestinal mucus or mucus glycoproteins have not been extensively studied [11]. Mucin patterns have also been reported to change in few piscine helminthiases [12,13], though no data on glycosylation changes are available.

Recognition, a central event in a variety of biological phenomena, is frequently mediated by carbohydrates and lectins. The lectin-carbohydrate residue interaction is frequently involved in the adhesion and penetration of parasites [14,15]. Previous studies on *E. scophthalmi* and *E. leei* suggest a role of some carbohydrate moieties in the interaction

with their hosts [16,17]. Changes in the number of goblet cells as a consequence of infection have been reported [18,19], but no information is available on the influence of the infections on the types of mucins and terminal carbohydrate residues present in the intestinal mucosa.

In the present work, the mucin and carbohydrate patterns of the digestive tract of gilthead sea bream *Sparus aurata* L. and turbot, and the changes induced by *E. leei* and *E. scophthalmi* infections, respectively, were studied. Histochemical techniques were applied to both uninfected and infected fish to detect the mucin types, and the distribution of several terminal carbohydrate residues was studied using plant lectin histochemistry.

2. Materials and methods

2.1. Fish infected by Enteromyxum leei or E. scophthtalmi

E. leei-infected gilthead sea bream (GSB) were obtained from experimental infections by cohabitation of recipient (R) and donor (D) fish and through waterborne contamination from the effluent of a tank containing infected fish [20]. Turbot (TB) were experimentally infected by feeding parasitized intestinal tissue from *E. scophtalmi* infected fish as detailed in Redondo et al. [21].

Experimental trials for both enteromyxoses were carried out at the facilities of the Instituto de Acuicultura Torre de la Sal (IATS) in Spain. Water was cooled or heated as necessary in order to maintain temperature below 20º C for TB or above 18º C for GSB. Water was 1-µm filtered and UV irradiated and salinity was 37’5 ‰. All fish were fed a commercial dry pellet diet at about 1% of body weight daily. Control groups consisted of naïve fish not exposed to the parasites.

In this study, GSB of two effluent experiments and TB of two oral infections were used. Five infected and 5 uninfected fish (150-200 g weight in all cases) from each fish species and experiment were selected for histochemical studies.

2.2. Sampling procedure

Fish were killed by overexposing to MS222 and bled from the caudal vein before the necropsies. Samples of the digestive tract (oesophagus, stomach, anterior, medium and posterior parts of the intestine) of uninfected control and infected fish were fixed in 10% buffered formalin and embedded in paraffin for histological processing, following standard histology procedures. To select the tissue samples for histochemical studies, Technovit 7100 resin (Kulzer, Heraeus, Germany) sections were also obtained and infection intensity was evaluated following a scale of 1+ to 6+, according to the relative numbers of parasites present in the studied tissues. Fish with infection intensity ≥ 3+ were selected for histochemical studies.

2.3. Mucin histochemistry

Paraffin sections (4 μm thick) were stained using the following histochemical techniques: periodic acid Schiff (PAS) to demonstrate neutral mucins (magenta-stained); alcian blue (AB) recognizing predominantly acidic mucins (blue-stained); AB pH 2.5 staining followed by PAS (PAS/AB) was performed to distinguish between neutral mucins (magenta-stained by PAS) and acidic mucins (blue-stained by AB); and aldehyde fuchsin-AB (AF-AA) for localisation of the carboxylated (purple-stained) and/or sulphated type (blue-stained) of acidic mucins. The staining intensities were evaluated in the sections with a scale of 0 to 3 (0

= no staining; 1 = weak; 2: moderate; 3 = strong) and the number of goblet cells was estimated semiquantitatively (+ to +++).

2.4. Lectin histochemistry

Paraffin sections (4 μm thick) were collected on Super Frost-plus microscope slides (Menzel-Glaser, Germany) without additives and allowed to dry overnight. Slides were deparaffinised and hydrated and the endogenous peroxidase activity was blocked by incubation in hydrogen peroxide (0.3% (v/v) for 30 min). After rinsing with TTBS (20mM tris-HCl, 0.5M NaCl pH 7.2 containing 0.05% Tween 20), sections were incubated with biotinylated lectin solutions in TTBS, for 1 h at 20ºC. After rinsing, the sections were incubated with the avidin-biotin-peroxidase complex (ABC, Vector Laboratories, Burlingame, CA, USA) for 30 min at 20º C and bound peroxidase was finally revealed by adding DAB chromogen (3,3'-diaminobenzidine tetrahydrochloride) (Sigma, St Louis, MO, USA) for 5 min. The reaction was stopped with deionised water and the sections counterstained using Gill's haematoxylin, and finally mounted in di-*N*-butyl-phtalate in xilene. Incubation of ABC with the tissue sections alone served as control to discard the presence of endogenous biotin-binding proteins. Each lectin and its corresponding blocking sugar (0.2M) were incubated for 1 h at 20º C, before application to the sections as binding specificity controls. The concentrations of the lectins used in this study, their acronyms, major sugar specificities and blocking sugars used are listed in Table 1. They were obtained from Sigma (St Louis, MO, USA) or Vector (Vector Lab., Burlingame, CA, USA). The staining intensities were evaluated in the sections with a scale of 0 to 3 (0 = no staining; 1 = weak; 2: moderate; 3 = strong).

3. Results

3.1. Staining patterns in uninfected and Enteromyxum leei-infected gilthead sea bream (GSB) digestive tissue

3.1.1. Mucin histochemistry

The results on the detection of the different mucins in uninfected and infected GSB are summarized in Table 2. Uninfected fish showed abundant PAS positive carbohydrates in the brush border (BB) of the stomach, whereas a moderate amount was seen in the oesophagic (Fig. 1A) and intestinal goblet cells (GC) (Fig. 1B). The most evident change observed in parasitized fish with respect to uninfected fish was the dramatic decrease of the GC number (Fig. 1C), though changes in the intensity of PAS staining were not evident. Basal lamina was slightly PAS positive in both infected and uninfected fish.

Acidic mucins were scarce in the stomach BB and more abundant in the intestinal GC. The staining intensity was similar in uninfected (Fig. 1D) and infected fish, though the number of PAS/AB positive cells was lower in infected fish (Fig. 1E). Neutral mucins, present in the oesophagic GC and the stomach BB, were clearly less abundant in uninfected fish (Fig. 1F) than in parasitized fish (Fig. 1G). A higher number of intestinal GC containing carboxylated mucins than those with sulphated mucins (Fig. 1H) and some GC with both mucin types (Fig. 1I) were seen in uninfected fish. The scarce GC observed in infected fish were positive for carboxylated mucins (with a staining intensity similar to that of uninfected fish) and no cells with sulphated mucins were detected (Fig. 1J). The BB of the stomach epithelia showed slight staining for carboxylated mucins.

3.1.2. Lectin histochemistry

The lectin-binding patterns of the digestive tissues of uninfected and *E. leei*-infected GSB are summarized in Table 3. Con A stained the epithelial cells (except the BB) of uninfected fish, slightly in the oesophagus, anterior and medium intestine, and strongly in the posterior intestine (Fig. 2A). The staining pattern was similar in parasitized fish, except for the lower recognition in the posterior intestine (Fig. 2B). GC and rodlet cells (RC) were not stained in any fish.

UEA I lectin labelled the epithelial cells of the oesophagus (strongly) and the stomach (moderately) of both uninfected and infected fish. The epithelial cells of the three intestinal parts were intensely stained in uninfected fish (Figs. 2D, F), whereas in parasitized fish the recognition was also strong in the posterior part (Fig. 2C) but clearly lower in the anterior and medium intestine (Figs. 2E, G). GC (Fig. 2C) and RC were not recognised in any fish.

WGA stained moderately to strongly the apical part and BB of the epithelial cells in the oesophagus, stomach and the three parts of the intestine of uninfected fish (Figs. 2H, J). In infected fish, the recognition was also strong in the posterior intestine, in contrast to the very slight label in the anterior (Fig. 2I) and medium (Fig. 2K) parts. GC were variably labelled (Fig. 2J) and no stained RC was detected.

SBA recognised strongly the oesophagic (Fig. 2L) and stomach epithelia and the oesophagic GC (Fig. 2L) of both uninfected and infected fish. In contrast, intestinal epithelial cells were not labelled. Intestinal GC were variably stained and no differences were observed between uninfected (Fig. 2M) and infected (Fig. 2N) fish, apart from the lower number of GC in the latter. RC were clearly stained (Fig. 2O).

BSL I lectin stained mainly the GC (strongly in the oesophagus, Fig. 2P; variably in the intestine, Fig. 2Q), the RC (Fig. 2R) and the stomach BB of both uninfected and infected

fish. In addition, the oesophagic epithelial cells showed a slight label in uninfected fish (Fig. 2P), not found in infected fish.

SNA lectin did not label any structure of the oesophagus and stomach of both uninfected and infected fish. The apical part and BB of intestinal epithelial cells were not stained in the anterior part, and not or moderately labelled in the medium zone of both uninfected and infected fish. In contrast, in the posterior intestine, such structures were strongly stained in uninfected fish (Fig. 2S) and not or very slightly in infected fish (Fig. 2T). Staining intensity was also lower in the intestinal GC of parasitized fish with respect to uninfected fish.

Lectin staining was inhibited after blocking with each specific sugar in all cases.

3.2. Staining patterns of uninfected and E. scophthalmi-infected turbot (TB) digestive tissue

3.2.1. Mucin histochemistry

The results on the detection of the different mucins in uninfected and infected TB are summarized in Table 4. PAS positive carbohydrates were abundant in the stomach BB and intestinal GC of both non-parasitized (Fig. 3A) and parasitized fish (Figs. 3B-C), though the number of GC decreased drastically in the latter (Fig. 3B). The intestinal BB and basal lamina were also clearly stained by PAS.

Neutral mucins were abundant in the stomach BB and in the intestinal GC and BB (Fig. 3D) of uninfected fish, and in the scarce GC present in the parasitized intestine (Fig. 3E). No acidic mucins were detected in any part of the digestive tract. Consequently, staining for carboxylated and sulphated mucins was also negative.

3.2.2. Lectin histochemistry

The lectin-binding patterns of the digestive tissues of uninfected or *E. scophthalmi*-parasitized TB are summarized in Table 5. The stomach epithelium and RC of uninfected fish were strongly stained with Con A, except the apical part and BB (Fig. 3F). In contrast, label was lower in the epithelial cells of parasitized fish, though RC were similarly stained (Fig. 3G). Epithelial cells were slightly to moderately recognised (except the apical part and BB) in the anterior and medium parts of the intestine of uninfected and infected fish. The staining pattern was completely different in the posterior intestine. In uninfected fish, the whole epithelial cell was strongly stained (Fig. 3H), whereas in parasitized fish no label appeared in the apical part and BB and the recognition was slight in the remaining part of the cell (Fig. 3I). RC were clearly stained and GC were no labelled in any of the three intestinal parts.

UEA I stained strongly the stomach (Fig. 3J) and intestinal (Fig. 3K) epithelia of uninfected fish. In contrast, the label was clearly lower in infected fish, both in the stomach (Fig. 3L) and especially in the intestine, where the label was very slight (Fig. 3M). RC were stained occasionally and GC were not stained in any fish.

In uninfected fish, WGA stained intensely the apical part and BB of the stomach epithelial cells (Fig. 3N), and the intestinal BB (Fig. 3O) and GC. In infected fish, a similar label was observed in the intestinal BB and GC, whereas the recognition was clearly lower in the stomach epithelial cells (Fig. 3P). RC were not stained in any fish.

SBA labelled only and scantly few GC (Fig. 3Q) and RC (Fig. 3R) of both uninfected and infected fish. No other structure in any part of the digestive tract was recognised. RC was the only structure stained by BSL I in the digestive tract of uninfected fish (Fig. 3S). In contrast, a slight to moderate label was detected in the stomach (Fig. 3T) and intestinal (Fig. 3U) BB of parasitized fish. No structure in any part of the digestive tract from both infected and uninfected fish was stained with SNA.

Lectin staining was inhibited after blocking with each specific sugar in all cases.

4. Discussion

In the current work, the influence of enteromyxoses on the carbohydrate patterns of the digestive tract, the target organ of *Enteromyxum* spp., was demonstrated in both GSB and TB.

Mucins, the main structural component of the mucus layer covering the digestive epithelium, play a critical role in the maintenance of mucosal homeostasis and are responsible for the differential effector and regulatory responses against microorganisms, including commensals and pathogens [7–9]. The process of glycosylation confers many of the general properties of mucins including high-charge density from sialic acid and sulphate residues, protease resistance and water holding capacity [22]. The glycoproteins in fish mucus appear to be similar in make up to mammalian mucins (reviewed in [23]).

In the current work, only neutral mucins were detected in the digestive tract of TB, whereas acidic mucins, mainly carboxylated, were predominant in the intestine of GSB. These patterns were quite similar to those previously described in TB [24] and GSB [25,26]. The situation is variable in other fish, though both acidic and neutral mucins are frequently found, and carboxylated glycoconjugates are generally more abundant than sulphated ones [27–30].

Microbial products can alter the production of mucins by mucosal epithelial cells. The type of mucins can also influence the invasion of parasites and the interaction with the host [7,8]. Thus, sulphated glycoconjugates, either from mucosal mast cells or GC, prevent the mucosal invasion by *Strongyloides venezuelensis* in rats [31,32]. In the current work, few changes in mucin patterns were detected by histochemical staining in infected fish. In both

enteromixoses, the number of intestinal GC decreased in severe infections, thus confirming previous results in fish with medium and advanced infections [18,19]. It is also remarkable the decreasing in the expression of acidic carboxylated mucins in *E. leei*-infected GSB. Such results are in contrast with the increasing in acidic mucins frequently described in several mammalian nematodiases [30,33], though in rats infected with *Nippostrongylus brasiliensis*, an initial increase in sialomucin^{+} GC was followed by a gradual decrease [34]. A rise in acidic mucin secreting cells has also been reported in *Anguilla anguilla* infected by *Deropristis inflata* [12] and *Salmo trutta* naturally parasitized by the acanthocephalan *Pomphorhynchus laevis* and the cestode *Cyatocephalus truncatus* [13].

Adherence of pathogenic enteric organisms to specific receptors on mucosal surfaces is widely recognized as an important first step in the initiation of infectious diseases [35]. In their infection strategy, many microorganisms often use sugar-binding proteins, that is lectins and adhesins, to recognize and bind to host glycoconjugates [36]. The host glycosidic patterns can also change during mucosal infection/inflammation. This may be an important mechanism for unfavourably changing the niche occupied by mucosal pathogens or blocking the mechanisms that pathogens use to subvert the mucin barrier [8].

The main carbohydrate residues typically present in mammalian mucins, fucose, galactose, N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc) and neuraminic acid have been also observed in the digestive tract of several fish species, though patterns vary between them [25,26,28,37,38]. Most of such carbohydrates residues were also detected in the current study in uninfected GSB and TB, though some differences between both fish were observed. Remarkable is the absence of sialic acid (detectable by SNA staining, i.e. in alpha 2-6 linkage) in the TB stomach and intestine, in contrast with its abundance in the GSB intestine. This does not necessarily imply overall absence of sialic acid in TB GI tract, as SNA does not react with sialic acid in alpha2-3 linkage [39], and lectins in general may fail to

react with sialic acids that are O-acylatated, a modification observed in certain fish (salmon) glycoproteins [40]. In addition, Gal and GalNAc (as recognised by BSL I and SBA) were very scarce in TB, whereas they were expressed in several structures of GSB. Mannose residues were abundant in both fish but Con A recognized more structures in TB. Fucose expression (detectable by UEA-1) was quite similar in both fish species, though it was higher in GSB than in TB epithelial cells. GlcNAc and its polymers were abundant in the epithelial cells and the brush border of both fish. The observed GSB lectin binding pattern is similar to that previously described [25,26]. No previous information is available on the terminal carbohydrate residues of TB.

Changes in the glycosylation patterns as a consequence of infection were found in both GSB and TB, though differences were noticed according to the host/parasite model (Table 6). The dramatic decreasing of the expression of sialic acid (detectale by SNA lectin) in *E. leei*-infected GSB is remarkable. Glucose/mannose, fucose and GlcNAc were less abundant in both infected hosts with respect to uninfected fish. In contrast, D-Gal and D-GalNAc terminals (as recognised by BSL I) increased in most parts of *E. scophthalmi*-infected TB while decreasing (oesophagus) or remaining unchanged (intestine) in *E. leei*-infected GSB. Changes in the expression of mucins and carbohydrate residues as a consequence of intestinal infections have been reported in some host-parasite helminths models (reviewed in [10]). Thus, an increase in GC and in the expression of several carbohydrate residues could contribute to the expulsion *Echinostoma trivolvis* by mice [41,42] and *Nippostrongylus brasiliensis* by rats [43] with subsequent decrease of the infection damage. By contrast, in *Echinostoma caproni* infection, in which a loss of GC occurred and lectin binding to intestinal villi was reduced, mice were unable to expel the worms and showed damage to the intestinal mucosa [41]. This situation somewhat resembles that observed in enteromyxosis, as the expression of most carbohydrate residues decreased in

infected fish. The slight increase of Gal and GalNAc in TB, probably due to the unmasking of these residues, does not seem to influence the infection progress. The coccidian *Isospora sui*s also induced a decrease in the expression of some carbohydrate residues, such as α-GalNAc and fucose in the small intestine of infected piglets [44].

Most available information on the changes of mucins and carbohydrate residues in intestinal helminthiases indicate a primary protective role for acid glycosylation, and mainly of sialic acid. Terminal fucose residues are also considered to have a role in mucosal protection. In addition, sialic acids are intimately involved in recognition processes mediated by sialic acid-binding proteins [45], and fucosylated carbohydrate structures have also important roles in a variety of biological and pathological processes [46]. The absence of acidic mucins and sialic acid detectable by SNA (both present in GSB) and the low expression of other carbohydrate residues in TB could aid to explain the higher severity of enteromyxosis in this fish. GSB sialic acid could protect initially the intestine of this fish from invasion and dispersion of parasites. However, a dramatic decrease in the expression of NeuNAc occurred with the infection progress. In rats primary infected with *Trichinella spiralis*, intestinal mucus showed reduced glycosylation, particularly sialylation, whereas a later ejection response coincided with increasing in the contents of terminal GalNAc and sialic acid in secreted mucins (reviewed in [10]). Whether sialidases or the secretion of immature (underglycosylated mucins) could be involved in the changes observed in *E. leei*-infected GSB, as suggested in other parasitoses [10], remains to be investigated.

Lectin-binding patterns of rodlet cells did not change in infected fish with respect to uninfected fish, though the number of these cells was lower in the former. Glucose/mannose and fucose residues, detected in TB, were absent in GSB. The role of carbohydrate residues in this enigmatic cell type is unknown, though it could be related to their function. Rodlet cells are now considered a type of inflammatory cell [47] and thus they could participate in the

immune response as it occurs with mucins, which are one of the components involved in the relationship of the diverse elements of innate and adaptive immunity at the gut level [8].

The changes in the carbohydrate patterns of *Enteromyxum*-infected GSB and TB suggest an interaction of these myxozoans with terminal carbohydrate residues of their hosts. Such interaction could be mediated by parasite lectins as demonstrated in other host-parasite models, of which the GalNAc-binding lectin of *Entamoeba hystolytica* is a good example [48]. Among myxozoans, the lectin activity demonstrated in *Myxobolus cerebralis* is considered to play a role in the recognition and invasion of the host [49]. Thus, the presence of surface lectins and other virulence factors (such as proteases and glycosydases) in *Enteromyxum* spp. as well as their involvement in the interaction with host tissues deserves further investigations. Recent findings have highlighted the importance of carbohydrate interactions in bacterial infections of experimental animals and their potential use in anti-adhesion therapies [46,50]. The use of diets supplemented with specific carbohydrates that have the ability to influence gastrointestinal infections may have implications for parasite control [51]. This could be also a promising field in the treatment of piscine enteromyxoses. In addition, future studies should focus on the expression of intestinal mucin genes in response to the parasites, and its role in the outcome of infection as suggested for mammalian gastrointestinal parasites [7].

ACKNOWLEDGEMENTS

Funding for this work was obtained from the Spanish Ministry of Science and Education through the research project AGL2006-13158-C01. M. J. Redondo is recipient of a CSIC I3P contract funded by the European Social Fund. We are thankful to Mr. J. Monfort and L. Rodríguez for LM processing.

REFERENCES

[1] Lom J, Dyková I. Myxozoan genera: definition and notes on taxonomy, life-cycle terminology and pathogenic species. Folia Parasitol 2006;53:1–36.

[2] Branson E, Riaza A, Alvarez-Pellitero P. Myxosporean infection causing intestinal disease in farmed turbot, *Scophthalmus maximus* (L.), (Teleostei: Scophthalmidae). J Fish Dis 1999;22:395-399.

[3] Ghittino C, Latini M, Agnetti F, Panzieri C, Lauro L, Ciappelloni R. Emerging pathologies in aquaculture: effects on production and food safety. Vet Res Commun 2003;27:471-479.

[4] Palenzuela O, Redondo MJ, Alvarez-Pellitero P. Description of *Enteromyxum scophthalmi* gen. nov, sp. nov. (Myxozoa), an intestinal parasite of turbot (*Scophthalmus maximus* L.) using morphological and ribosomal RNA sequence data. Parasitology 2002;124:369-379.

[5] Quiroga MI, Redondo MJ, Sitjá-Bobadilla A, Palenzuela O, Riaza A, Macías A, Vázquez S, Pérez A, Nieto JM, Álvarez-Pellitero P. Risk factors associated with *Enteromyxum scophthalmi* (Myxozoa) infection in cultured turbot (*Scophthalmus maximus* L.). Parasitology 2006;133:433-442.

[6] Hoste H. Adaptive physiological processes in the host during gastrointestinal parasitism. Int J Parasitol 2001;31:231-244.

[7] Moncada DM, Kammanadiminti SJ, Chadee K. Mucin and toll-like receptors in host defense against intestinal parasites. Trends Parasitol 2003;19:306-311.

[8] Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA. Mucins in the mucosal barrier to infection. Mucosal Immunol 2008;1:183-197.

[9] Dharmani P, Srivastava V, Kissoon-Singh V, Chadee K. Role of intestinal mucins in innate host defense mechanisms against pathogens. J Innate Immun 2009;1:123-135.

[10] Theodoropoulos G, Hicks SJ, Corfield AP, Miller BG, Carrington SD. The role of mucins in host-parasite interactions: Part II-helminth parasites. Trends Parasitol 2001;17:130-135.

[11] Artis D, Grencis RK. The intestinal epithelium: sensors to effectors in nematode infection. Immunology 2008;1:252-264.

[12] Dezfuli BS, Manera M, Onestini S, Rossi R. Histopathology of the alimentary canal of *Anguilla anguilla* L. associated with digenetic trematodes: a light and electron microscopic study. J Fish Dis 1997;20:317-322.

[13] 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 Organ 2005;64:45-51.

[14] Jacobson RJ, Doyle RL. Lectin-Parasites Interactions. Parasitol Today 1996;12:55-61.

[15] Xu DH, Klesius PH, Shoemaker CA. Effect of lectins on the invasion on *Ichthyophthirius* theront to channel catfish tissue. Dis Aquat Org 2001;45:115-120.

[16] Redondo MJ, Cortadellas N, Palenzuela O, Alvarez-Pellitero P. Detection of carbohydrate terminals in the enteric parasite *Enteromyxum scophthalmi* (Myxozoa) and possible interactions with its fish host *Psetta maxima*. Parasitol Res 2008;102:1257-1267.

[17] Redondo MJ, Alvarez-Pellitero P. Lectinhistochemical detection of terminal carbohydrate residues in the enteric myxozoan *Enteromyxum leei* parasitizing gilthead seabream *Sparus aurata* (Pisces: Teleostei): a study using light and transmission electron microscopy. Folia Parasitol (in press).

[18] 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 Organ 2008;79:219-228.

[19] Bermúdez R, Losada AP, Vázquez S, Redondo MJ, Álvarez-Pellitero P, Quiroga M I.. Light and electron microscopic studies on turbot (*Psetta maxima* L.) infected with *Enteromyxum scophthalmi*: histopathology of turbot enteromyxosis. Dis Aquat Organ 2010;89:209-221.

[20] Sitjà-Bobadilla A, Diamant A, Palenzuela O, Alvarez-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.

[21] Redondo MJ, Palenzuela O, Riaza A, Macias A, Alvarez-Pellitero P. Experimental transmission of *Enteromyxum scophthalmi* (Myxozoa), an enteric parasite of turbot (*Scophthalmus maximus*). J Parasitol 2002;88:482-488.

[22] Moncada DM, Chadee K 2002. Production, structure, and function of gastrointestinal mucins: In: Blaser MJ (Ed): Infections of the Gastrointestinal Tract. Philadelphia, Lippincott, Williams & Wilkins, pp 57–79.

[23] Shephard KL. Functions for fish mucus. Rev Fish Biol Fisher 1994;4:401-429.

[24] Quintáns L. Análisis histológico, histoquímico y ultraestructural de la mucosa del canal alimentario del rodaballo (*Scophthalmus maximus*). Memoria de licenciatura. Universidad de Santiago de Compostela 2003.

[25] Domeneghini C, Pannelli Strain R, Veggetti A. Gut glycoconjugates in *Sparus aurata* L. (Pisces, Teleostei). A comparative histochemical study in larval and adult ages. Histol Histopathol 1998;13:359-372.

[26] Sarasquete C, Gisbert E, Ribeiro L, Vieira L, Dinis MT. Glycoconjugates in epidermal, branchial and digestive mucous cells and gastric glands of gilthead sea bream, *Sparus aurata* sole, *Solea senegalensis* and Siberian sturgeon, *Acipenser baeri* development. Eur J Histochem 2001;45:267-278.

[27] Scocco P, Menghi G, Ceccarelli P. Histochemical differentation of glycoconjugates occurring in the tilapine intestine. J Fish Biol 1997;51:848-857.

[28] Fiertak A, Kilarski WM. Glycoconjugates of the intestinal goblet cells of four cyprinids. Cell Mol Life Sci 2002;59:1724-1733.

[29] Park JY, Kim IS, Kim SY. Structure and mucous histochemistry of the intestinal respiratory tract of the mud loach, *Misgurnus anguillicaudatus* (Cantor). J Appl Ichthyol 2003;19:215-19.

[30] 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;106:477-487.

[31] Maruyama H, Yabu Y, Yoshida A, Nawa Y, Ohta N. A role of mast cell glycosaminoglycans for the immunological expulsion of intestinal nematode, *Strongyloides venezuelensis*. J Immunol 2000;164:3749-3754.

[32] Maruyama H, Hirabayashi Y, El-Malky M, Okamura S, Aoki M, Itagaki T, Nakamura-Uchiyama F, Nawa Y, Shimada S, Ohta N. *Strongyloides venezuelensis*: longitudinal distribution of adult worms in the host intestine is influenced by mucosal sulfated carbohydrates. Exp Parasitol 2002;100:179-185.

[33] Koninkx JF, Mirck MH, Hendriks HG, Mouwen JM, Van Dijk JE. *Nippostrongylus brasiliensis*: histochemical changes in the composition of mucins in goblet cells during infection in rats. Exp Parasitol 1988;65:84-90.

[34] Soga K, Yamauchi J, Kawai Y, Yamada M, Uchikawa R, Tegoshi T, Mitsufuji S, Yoshikawa T, Arizono N. Alteration of the expression profiles of acidic mucin, sialiltransferase, and sulfotransferases in the intestinal epithelium of rats infected with the nematode *Nippostronglus brasiliensis*. Parasitol Res 2008;103:1427-1434.

[35] Tse S-K, Chadee K. The interaction between intestinal mucus glycoproteins and enteric infections. Parasitol Today 1991;7:163-172.

[36] Imberty A, Varrot A. Microbial recognition of human cell surface glycoconjugates. Curr Opin Struct Biol 2008;18:567-576.

[37] Pedini V, Scocco P, Gargiulo AM, Ceccarelli P, Lorvik S. Glycoconjugate characterization in the intestine of *Umbrina cirrosa* by means of lectin histochemistry. J Fish Biol 2002;61:1363-1372.

[38] Pedini V, Dall'Aglio C, Parillo F, Scocco P. Glycoconjugate distribution in gastric fundic mucosa of *Umbrina cirrosa* L. revealed by lectin histochemistry. J Fish Biol 2005;66:222-229.

[39] Varki A, Schauer R.: Chapter 14: Sialic Acids, IN Essentials of Glycobiology. 2nd. Edition. Edited by Varki A., Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW and Etzler ME Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2009.

[40] Ylönen A, Kalkkinen N, Saarinen J, Bøgwald J, Helin J. Glycosylation analysis of two cysteine proteinase inhibitors from Atlantic salmon skin: di-O-acetylated sialic acids are the major sialic acid species on N-glycans. Glycobiology 2001;11:523-531.

[41] Fujino T, Fried B. *Echinostoma caproni* and *E. trivolvis* alter the binding of glycoconjugates in the intestinal mucosa of C3H mice as determined by lectin histochemistry. J Helminthol 1993;67:179–188.

[42] Fujino T, Fried B. The expulsion of *Echinostoma trivolvis* from C3H Mice: differences in glycoconjugates in mouse versus hamster small intestinal mucosa during infection. J Helminthol 1996;70:115-21.

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

[44] Choi B-Y, Sohn Y-S, Choi Ch, Chae Ch. Lectin histochemistry for glycoconjugates in the small intestines of piglets naturally infected with *Isospora suis*. J Vet Med Sci 2004;65:389-392.

[45] Varki NM, Varki A. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest 2007;87:851-57.

[46] Ma B, Simala-Grant JL, Taylor DE. Fucosylation in prokaryotes and eukaryotes. Glycobiology 2006;16:158R-184R.

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

[48] Frederick JR, Petri WA Jr. Roles of galactose/N-acetylgalactosamine-binding lectin of *Entamoeba* in parasite virulence and differentiation. Glycobiology 2005;15:53R-59R.

[49] Knaus M, El-Matbouli M. Characterisation of carbohydrate-binding sites in developmental stages of *Myxobolus cerebralis*. Parasitol Res 2005;97:505-514.

[50] Sharon N: Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochim Biophys Acta 2006;1760:527-537.

[51] Thomsen LE, Knudsen KEB, Hedemann MS, Roepstorff A. The effect of dietary carbohydrates and *Trichuris suis* infection on pig large intestine tissue structure, epithelial cell proliferation and mucin characteristics. Vet Parasitol 2006;142:112-22.

Table 1. Lectins used in this study: their acronyms, the concentration used and their sugar-binding specificities, and the corresponding blocking sugars and their concentrations.

Acronym	Lectin source	Specificities	Lectin concentration (µg/ml)	Blocking sugars	Sugar concen-tration (M)
Con A	*Canavalia ensiformis*	Manα-1>Glcα-1> GlcNAcα-1	2	methyl-α-D-Man + methyl-α-D-Glc	0.2
UEA I	*Ulex europeaus*	L-Fucα1,2Galβ1,4 GlcNAcβ1,6	20	L-Fuc α	0.2
WGA	*Triticum vulgaris*	GlcNAc (ß1,4GlcNAc)$_{1-2}$> ß1,4GlcNAc> NeuNAc	10	GlcNAcα	0.5/0.2
SBA	*Glycine max*	Terminal α,ßGalNAc>α,ßGal	5	GalNAcα	0.2
BSL I	*Griffonia simplicifolia*	D-Gal>D-GalNAc	5	Gal + GalNAcα	0.2
SNA	*Sambucus nigra*	NeuAcα2,6Gal= NeuAcα2,6GalNAc	20	NeuNAc	0.2

Abbreviations: L-Fuc: L-fucose; GalNAc: N-acetylgalactosamine; Gal: galactose; Glc: D-glucose; GlcNAc: N-acetylglucosamine; Man: mannose; NeuNAc: N-acetylneuraminic acid.

Table 2. Mucin staining patterns of the digestive tract of *Sparus aurata*. GC: goblet cell. BB: epithelial brush border.

GILTHEAD SEA BREAM	Cells or structures	Oesophagus	Stomach	Intestine
PAS + structures	GC	1 /+++	-	1 /++ (**I:1/+**)
	BB	0	2	0
Acidic mucins	GC	0	-	2/++ (**I:1/+**)
	BB	0	1	0
Neutral mucins	GC	1/+++ (**I:3/+++**)	-	0
	BB	0	1 (**I:3**)	0
Carboxylated mucins	GC	0	-	3/++ (**I: 3/+**)
	BB	0	1	0
Sulphated mucins	GC	0	-	3/+ (**I: 0/+**)
	BB	0	0	0

Changes (bold-lined boxes) in *Enteromyxum leei*-infected fish (I) with respect to uninfected fish are indicated in parenthesis in bold letter. Numbers (0-3) indicate the staining intensity; + to +++ represent the abundance of GC showing each type of mucin estimated semiquantitatively (- = no GC).

Table 3. Lectin staining patterns of the digestive tract of *Sparus aurata*.

Lectin	Cells or structures	OE	ST	AI	MI	PI
Con A	EC	1	0	1	1	3 (**I:2**)
	GC	0	n.a.	0	0	0
	RC	0	0	0	0	0
UEA I	EC	3	2	3 (**I:0/1**)	3 (**I:0/1**)	3
	GC	0	n.a.	0	0	0
	RC	0	0	0	0	0
WGA	EC					
	BB	3	2	2 (**I:0/2**)	2 (**I:0/2**)	3
	A	2	0	0	2 (**I:0**)	3
	MB	0/1	0/1	0	0	0/1
	GC	2	n.a.	0/2	0/2	0/2
	RC	0	0	0	0	0
SBA	EC	3	3	0	0	0
	GC	3	n.a.	0/3	0/3	0/3
	RC	1/3	1/3	1/3	1/3	1/3
BSL I	EC					
	BB	0	2	0	0	0
	A	0	0	0	0	0
	MB	0/1 (**I:0**)	0	0	0	0
	GC	3	n.a.	0/2	0/2	0/2
	RC	3	3	3	3	3
SNA	EC					
	BB	0	0	0	0/2	3 (**I:0/1**)
	A	0	0	0	0/2	3 (**I:0/1**)
	MB	0	0	0	0	0
	GC	0	n.a.	0/3 (**I:0/1**)	0/3 (**I:0/1**)	0/3 (**I:0/1**)
	RC	0	0	0	0	0

Changes (bold-lined boxes) in fish infected by *Enteromyxum leei* (I) with respect to uninfected fish are indicated in parenthesis in bold letter. OE: oesophagus; ST: stomach; AI: anterior intestine; MI: medium intestine; PI: posterior intestine; EC: epithelial cell; BB: brush border; A: apical part; MB: medium and basal parts; goblet cell: GC; RC: rodlet cell. Numbers (0-3) indicate the staining intensity.

Table 4. Mucin staining patterns of the digestive tract of *Psetta maxima*. GC: goblet cell, BB: epithelial brush border.

TURBOT	Cells or structures	Stomach	Intestine
PAS + structures	GC	-	3/++ (**I:3/+**)
	BB	3	2
Acidic mucins	GC	-	0
	BB	0	0
Neutral mucins	GC	-	3/++ (**I:3/+**)
	BB	2	2

Changes (bold-lined boxes) in *Enteromyxum scophthalmi*-infected fish (I) with respect to uninfected fish are indicated in parenthesis in bold letter. Numbers (0-3) indicate the staining intensity; + to +++ represent the abundance of GC showing each type of mucin estimated semiquantitatively (- = no GC).

Table 5. Lectin staining patterns of the digestive tract of *Psetta maxima*

Lectin	Cells or structures	OE	ST	AI	MI	PI
Con A	EC					
	BB	0	0	0	0	3 (**I:0**)
	A	0	0	0/1	0/1	3 (**I:0**)
	MB	2	3 (**I: 1**)	1/2	1/2	2/3 (**I:1/2**)
	GC	0	n.a.	0	0	0
	RC	0	3	3	3	3
UEA I	EC	1	3 (**I:1**)	2 (**I: 0/1**)	2 (**I: 0/1**)	2 (**I: 0/1**)
	GC	0	n,a,	0	0	0 (I: n.o.)
	RC	0	0/2	0/2	0/2	0/2
WGA	EC					
	BB	0	3 (**I: 1/2**)	2	2	2
	A	0	3 (**I: 1/2**)	0	0	0
	MB	0	1	1	1	1
	GC	2	n.a.	2	2	2
	RC	0	0	0	0	0
SBA	EC	0	0	0	0	0
	GC	0	n.a.	1	1	n.o.
	RC	0	1	1	1	1
BSL I	EC					
	BB	0	0 (**I: 1/2**)	0 (**I: 1/2**)	0 (**I: 1/2**)	0 (**I: 1/2**)
	A	0	0	0	0	0
	MB	0	0	0	0	0
	GC	1	n.a.	0	0	0
	RC	0	2	2	2	2
SNA	EC	0	0	0	0	0
	GC	0	n.a.	0	0	0
	RC	0	0	0	0	0

Changes (bold-lined boxes) in fish infected by *Enteromyxum scophthalmi* (I) with respect to uninfected fish are indicated in parenthesis in bold letter; OE: oesophagus; ST: stomach; AI: anterior intestine; MI: medium intestine; PI: posterior intestine; EC: epithelial cell; BB: brush border; A: apical part; MB: medium and basal parts; GC: goblet cell; RC: rodlet cell. Numbers (0-3) indicate the staining intensity.

Table 6. Lectin staining patterns of *Sparus aurata* and *Psetta maxima* parasitized by *Enteromyxum leei* and *E. scophthalmi*, respectively.

Lectin	*Sparus aurata*	*Psetta maxima*
Con A	↓ PI	↓ ST ↓↓↓ PI
UEA I	↓↓ AI, MI	↓↓ ST ↓ AI, MI, PI
WGA	↓ AI, MI	↓ ST
SBA	↔	↔
BSL I	↓ ST	↑ ST, AI, MI, PI
SNA	↓↓↓ AI, MI, PI	Not present

Higher (↑), lower (↓ to ↓↓↓) or similar (↔) staining intensities with respect to uninfected fish in each part of the digestive tract are indicated. ST: stomach; AI: anterior intestine; MI: medium intestine; PI: posterior intestine.

FIGURE LEGENDS

Fig. 1. Mucin histochemistry on paraffin sections of the digestive tract of uninfected (UI) and *E. leei*-infected (I) gilthead sea bream. **A-C.** PAS. **A-B:** GC are moderately PAS^+ in the oesophagus (**A**) and intestine (**B**) of UI fish. **C:** Parasitized intestine showing abundant *E. leei* stages and very few GC. **D-G:** PAS/AB. Acidic mucins in GC of UI (**D**) and parasitized intestine (**E**). Notice the abundant *E. leei* stages. **F-G:** Neutral mucins in the oesophagic GC are less abundant in UI fish (**F**) than in I fish (**G**). **H-J:** AF/AB. **H:** Carboxylated (arrowheads) and sulphated (*) mucins in intestinal GC of UI fish. **I:** Detail of an intestinal GC of UI fish with both mucin types. **J:** Carboxylated mucins in GC of parasitized intestine. PAS/AB: PAS/alcian blue; AF/AB: aldehyde fuchsin/alcian blue; GC: goblet cells; IN: intestine; OE: oesophagus; STG: parasite stages. Scale bars: Figs. A-C, F-H, J = 40µm; Figs. D, E, I = 20µm.

Fig. 2. Lectin histochemistry on paraffin sections of the digestive tract of uninfected (UI) and *E. leei*-infected (I) gilthead sea bream. **A-B:** Con A lectin staining of posterior intestine. The staining intensity was clearly higher in UI (A) than in I (B) fish. No staining was detected in GC. **C-G:** UEA I lectin. Anterior (**D**) and medium (**F**) intestine of UI fish. Epithelial cells are intensely labelled. **C, E, G**: Intestine of I fish: Epithelial cells are strongly stained in the posterior intestine (**C**), in contrast to the slight label in the anterior (**E**) and medium (**G**) intestinal parts. See unlabelled GC and partially stained *E. leei* stages. **H-K:** WGA lectin. Strong label in the apical part and brush border of anterior (**H**) and medium (**J**) intestine of UI fish. In I fish, slight staining in the anterior (**I**) and medium (**K**) intestine. **L-O:** SBA lectin. **L:** Intense staining in the oesophagic epithelia and GC of UI fish. GC were variably stained in the anterior intestine of UI fish (**M**) and in the medium intestine of I fish (**N)**. **O:** Intense staining of RC in the anterior intestine of UI fish. **P-R:** BSL-I lectin. Clearly stained oesophagic GC (**P**) and intestinal RC (**R**) of UI fish. **Q:** Variable label in intestinal GC of I

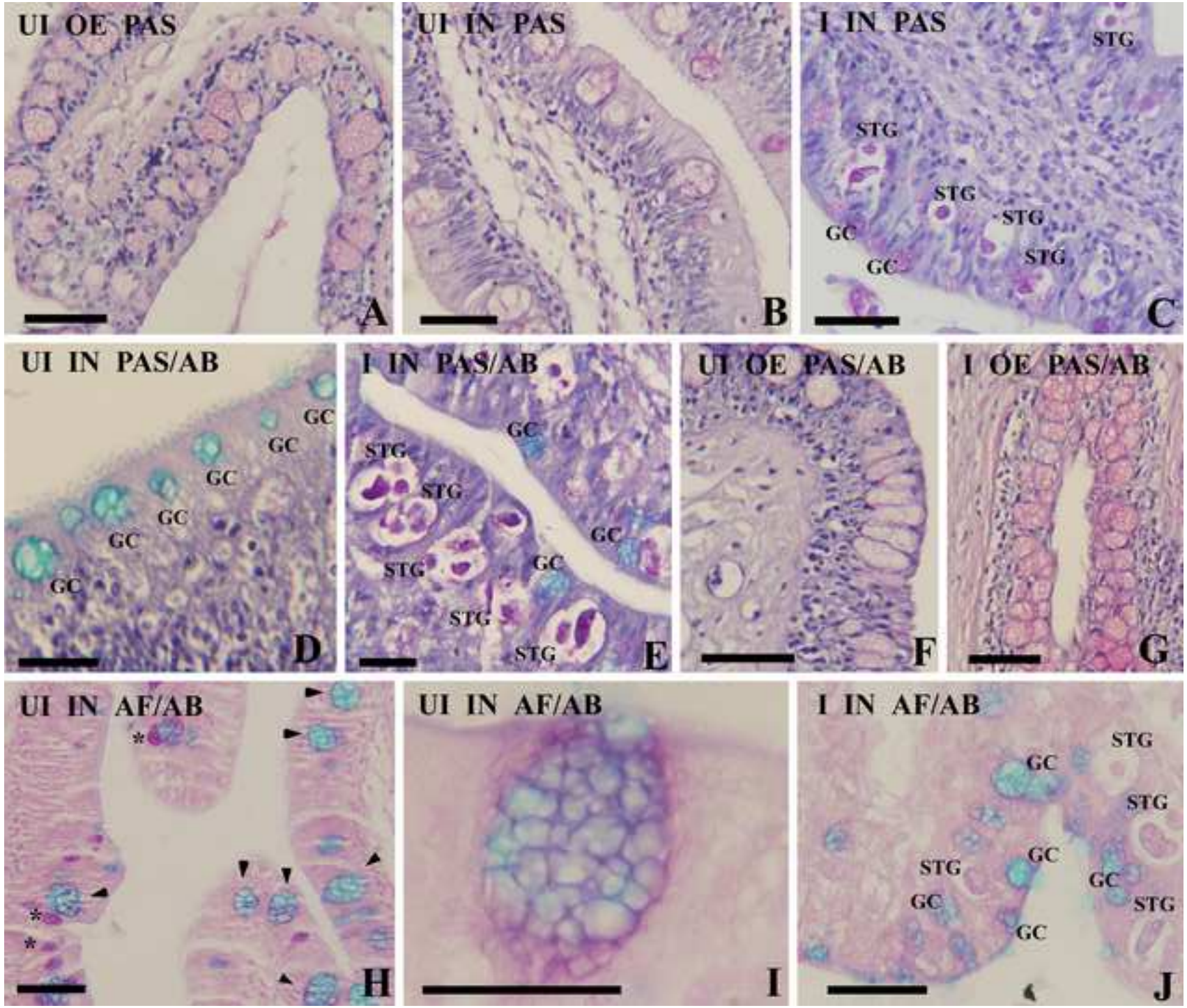
fish. **S-T:** SNA lectin. The apical part and BB of the posterior intestine epithelium are strongly stained in UI fish (**S**) and very slightly labelled in I fish (**T**). GC and *E. leei* stages are not stained. GC: goblet cells; RC: rodlet cell; IN: intestine; STG: parasite stages; AI: anterior intestine; MI: medium intestine; PI: posterior intestine. Scale bars: Figs. A-C, K-L, O-S = 20µm; Figs. E-G, I-J, M-N, T= 40µm; Figs. D, H= 50µm.

Fig. 3. Mucin and lectin histochemistry on paraffin sections of the digestive tract of uninfected (UI) and *E.scophthalmi*-infected (I) turbot. **A-C:** PAS. PAS^+ GC are abundant in the intestine of UI fish (**A**) and very scarce in I fish (**B**, posterior intestine; **C**, anterior intestine). *E. scophthalmi* stages show some PAS^+ structures. **D-E:** PAS/AB. **D:** Neutral mucins in the GC and BB of the posterior intestine of UI (**D**) and in the scarce GC of parasitized intestine (**E**). **F-I:** Con A lectin. **F-G**. Stomach. The epithelium is intensely stained in UI fish (**F**) and very slightly labelled in I fish (**G**). RC (in **G**) are strongly labelled in both cases. **H-I.** Posterior intestine**.** Epithelial cells are strongly stained in UI fish (**H**) and slightly recognised in I fish (**I**). Notice the labelled RC and the unstained GC. Some structures in *E. scophthalmi* stages (in I) are also stained. **J-M:** UEA I lectin**.** Staining was strong in the stomach (**J**) and medium intestine (**K**) (except in GC) of UI fish, in contrast with the **s**light label in the stomach (**L**) and intestine (**M**) of parasitized fish. Some RC and *E. scophthalmi* stages are also labelled. **N-P:** WGA lectin. Staining was strong or moderate in the apical part and BB of stomach (**N**) and intestine (**O**) of UI fish, and slighter in the stomach of I fish (**P**). RC were not stained. Notice the intense label in intestinal GC (in **O**) and the variable staining in *E. scophthalmi* stages (in **P**). **Q-R:** SBA lectin. **Q:** Slight staining in intestinal GC (**Q**) and RC (**R**) in UI fish. **S-U:** BSL I lectin. **S:** Stained intestinal RC in UI fish. **T-U:** I fish. The BB of stomach (**T**) and intestine (**U**) are clearly labelled, in contrast with the absence of staining in UI fish (**S**). PAS/AB: PAS/alcian blue; GC: goblet cells; BB: brush border; RC: rodlet cell; IN: intestine; STG: parasite stages; AI: anterior intestine; MI: medium intestine; PI: posterior

intestine. Scale bars: Figs. A-H, L, M-P, Q, T-U, X, AA = 40µm; Figs. I-K, O, R, S, V, W, Y –Z = 20µm.

Figure

Click here to download high resolution image



Figure

Click here to download high resolution image

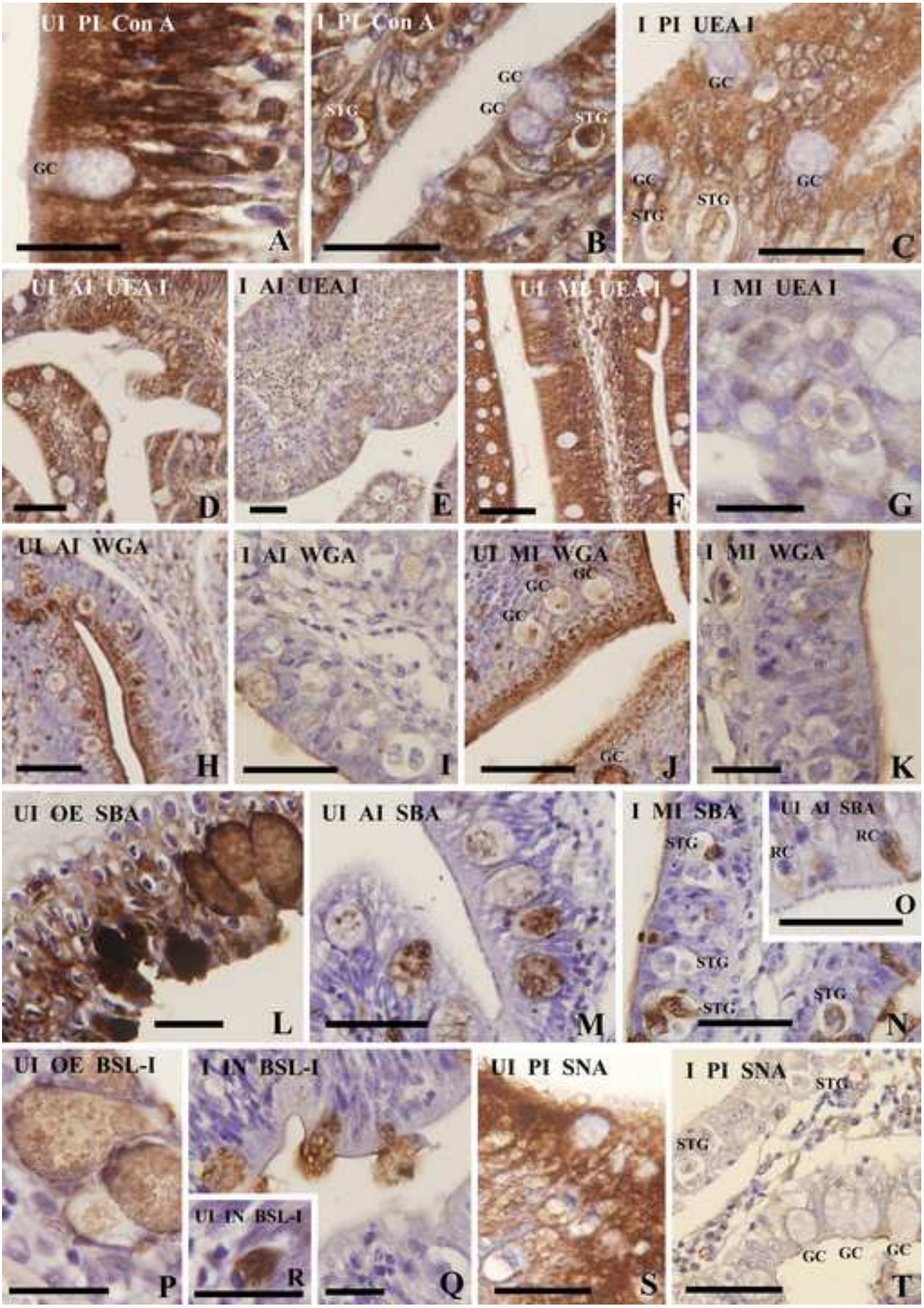


Figure
Click here to download high resolution image