Effects of *Enteromyxum* spp. (Myxozoa) infection in the regulation of intestinal E-cadherin: turbot against gilthead sea bream

Short title: **Intestinal E-cadherin regulation in enteromyxoses**

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Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

Abstract

Enteromyxoses are relevant diseases for turbot and gilthead sea bream aquaculture. The myxozoan parasites invade the intestinal mucosa, causing a cachectic syndrome associated to intestinal barrier alteration, nonetheless, their pathological impact is different. Turbot infected by *Enteromyxum scophthalmi* develop more severe intestinal lesions, reaching mortality rates of 100%, whereas in *E. leei*-infected gilthead sea bream, the disease progresses slowly, and mortality rates are lower. The mechanisms underlying the different pathogenesis are still unclear. We studied the distribution and expression changes of E-cadherin, a highly conserved protein of the adherens junctions, in the

intestine of both species by immunohistochemistry and quantitative PCR, using the same immunohistochemical protocol and common primers. The regular immunostaining pattern observed in control fish turned into markedly irregular in parasitized turbot, showing an intense immunoreaction at the host-parasite interface. Nevertheless, E-cadherin gene expression was not significantly modulated in this species. On the contrary, no evident changes in the protein distribution were noticed in gilthead sea bream, whereas a significant gene down-regulation occurred in advanced infection. The results contribute to the understanding of the different host-parasite interactions in enteromyxoses. Host and parasite cells appear to establish diverse relationships in these species, which could underlie the different pathological picture.

Keywords: Enteromyxosis, Myxosporea, cell junctions, intestinal barrier, host-parasite interaction

Introduction

Enteromyxoses caused by the intestinal myxozoan parasites *Enteromyxum scophthalmi* and *E. leei* pose a serious threat for marine aquaculture. These infections show fast spreading in productive units, given the direct fish-to-fish transmission of *Enteromyxum* spp., and provoke relevant economic losses for mortality and worsening of productive performances (Sitjà-Bobadilla & Palenzuela, 2012). The parasitic stages invade the digestive tract and then grow and proliferate in the lining epithelium, causing gut inflammation and impaired absorptive function (Bermúdez et al., 2010; Estensoro et al., 2011; Fleurance et al., 2008; Ronza, Robledo, et al., 2019).

E. scophthalmi mainly infects turbot (*Scophthalmus maximus*), though Senegalese sole (*Solea senegalensis*) grown in a turbot farm suffering an outbreak were also found to be infected by this myxozoan, although with no obvious disease signs until they were

examined (Palenzuela, Redondo, López, & Álvarez-Pellitero, 2007). Turbot is highly susceptible to enteromyxosis, showing serious lesions and high morbidity and mortality rates, to the extent that the disease can affect up to 100% of fish in a farming unit. The infection starts in pyloric caeca or anterior intestine and subsequently spreads along the entire gut (Redondo, Palenzuela, & Álvarez-Pellitero, 2004). Infected fish develop catarrhal enteritis of increasing severity throughout the disease (Bermúdez et al., 2010). On the other hand, *E. leei* affects a wide range of fish species, with different host susceptibility (Sitjà-Bobadilla & Palenzuela, 2012). Sharpsnout sea bream (*Diplodus puntazzo*) and tiger puffer (*Takifugu rubripes*) are among the most susceptible species, for which the disease constitutes a limiting factor and threatens the viability of their aquaculture (Rigos & Katharios, 2010). By contrast, enteromyxosis usually causes a subchronic disease in gilthead sea bream (GSB, *Sparus aurata*) with accumulated mortality below 20% (Sitjà-Bobadilla & Palenzuela, 2012). Unlike turbot, the intestinal epithelial integrity is always preserved in GSB, even in the most heavily infected cases (Fleurance et al., 2008). Field and experimental data indicate that the distal part of the intestine is the first and main target site of the parasite in this species (Estensoro, Redondo, Alvarez-Pellitero, & Sitjà-Bobadilla, 2010). In both species, turbot and GSB, the parasitization is associated to a cachectic syndrome characterized by weight loss, anorexia and amyotrophy, and no effective therapeutic options are currently available (Sitjà-Bobadilla & Palenzuela, 2012). The pathogenesis of the diseases involves the loss of the intestinal barrier function, although the mechanisms underlying the differences in the magnitude of the lesions are still unclear (Ronza, Robledo, et al., 2019; Sitjà-Bobadilla & Palenzuela, 2012).

E-cadherin is the main protein of the adherens junctions, one of the most important components for epithelial cell-cell junctional integrity. In mammals, its alteration has

been reported in a variety of conditions associated to gastrointestinal disorders and has been related to apoptosis, cell shedding and disturbances in secretory cells differentiation (Schneider et al., 2010). In fish, E–cadherin gene expression was modulated in the intestine of Atlantic salmon in response to an experimental dietary treatment affecting intestinal fluid permeability (Hu et al., 2016). It is an evolutionary conserved protein. In particular, the C–terminal region of the protein, corresponding to the cytoplasmic domain, remained quite conserved from Placozoa to human (Hulpiau & van Roy, 2011). Recently, an immunohistochemical technique for the detection of E-cadherin was set up in turbot using an anti-human E-cadherin commercial antibody (Ronza, Villamarín, et al., 2019).

In this work, we aimed to advance our knowledge of the pathogenesis of the intestinal barrier dysfunction associated to enteromyxoses, by investigating the changes in the protein distribution and gene expression of E-cadherin in the digestive tract of *E. scophthalmi*-infected turbot and *E. leei*-infected GSB. In both species, the immunohistochemical protocol described by Ronza et al. (2019) together with quantitative PCR analysis using common primers was employed.

Materials and Methods

Experimental design and histopathology

The experimental infection of turbot with *E. scophthalmi* by oral intubation and of GSB with *E. leei* by effluent exposure were previously described (see Estensoro, Calduch-Giner, Kaushik, Pérez-Sánchez, & Sitjà-Bobadilla, 2012; Robledo, Ronza, et al., 2014). Briefly, for turbot, a group of naïve fish received an oral inoculum of infected turbot intestinal scrapings, whereas another group (CTRL) was intubated with PBS. For GSB, a group naïve fish received water effluent from a donor tank holding *E. leei*-infected GSB, whereas another was kept unexposed (CTRL).

120 In both trials, tissue samples were collected at different time points in Bouin's fluid and
121 in RNAlater for histopathological and molecular techniques, respectively. The status of
122 CTRL and experimentally infected fish was assessed by light microscopy on H&E,
123 toluidine blue and Giemsa stained sections. Challenged turbot were classified into three
124 groups (slightly, moderately and severely infected) according to the histopathological
125 grading described by Bermúdez et al. (2010). The pyloric caeca and posterior intestine
126 from 8 CTRL and 8 infected turbot at 24 and 42 days post-inoculation (DPI) were used.
127 In order to increase the uniformity of the samples, infected turbot at 24 DPI were chosen
128 among those graded as moderately infected and turbot at 42 DPI among those graded as
129 severely infected. Challenged GSB were classified into two groups: moderately and
130 severely infected according to a semiquantitative scale described in Picard-Sánchez et al.
131 (2019). Samples used in the study include anterior and posterior intestine of 6 CTRL and
132 10 infected fish at 51, 91 and 133 days post-exposure (DPE). For gene expression, only
133 posterior intestine was used, to have a representative number of fish in each grade.

134 Both infection trials were carried out at the facilities of the Institute of Aquaculture Torre
135 de la Sal (IATS) in accordance with national (Royal Decree RD1201/2005, for the
136 protection of animals used in scientific experiments) and institutional regulations (CSIC,
137 IATS Review Board) and the current European Union legislation on handling
138 experimental animals.

139 *Sequence comparison and primer design*

140 Available E-cadherin nucleotide and aminoacidic sequences from for turbot, human and
141 GSB (GenBank accession numbers MG137250, AB025106.1 and KF861995.1,
142 respectively) were compared using Clustal X 2.0 software, and BLASTN 2.9.0 and
143 BLASTP 2.6.1 online programs (Altschul et al., 1997, 2005; Larkin et al., 2007). Given
144 the protein and gene similarity of E-cadherin between turbot and GSB, common primers

were designed, based on the mRNA sequences. After analysis, common sequence regions between turbot and GSB were identified and primers were designed using *Primer Express* Software v2.0 (Applied Biosystems). Primer specificity was checked using NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Immunohistochemistry

Paraffin sections (3 µm thick) from Bouin's fixed tissue samples were dewaxed in xylene and rehydrated through a graded ethanol series. The assays were carried out with a previously developed protocol (Ronza, Villamarín, et al., 2019), using an automated stainer (Dako Autostainer, Dako, Glostrup, Denmark) after the antigen retrieval step, in order to standardize the immunostaining. Briefly, the primary antibody (1:50 working dilution, mouse monoclonal antibody to human E-cadherin, clone NCH–38, M3612, Dako) was incubated during 2 h at room temperature. After 30 min incubation with HRP-labelled secondary antibody, the peroxidase reaction was developed with a diaminobenzidine-positive chromogen (EnVision+ System-HRP kit, K 4011; Dako), achieving the desired signal after 1 min of incubation. The sections were washed three times for 5 min in 0.1 M phosphate buffered saline containing 0.05% Tween-20 between all subsequent steps. After counterstaining with haematoxylin, sections were unloaded by the Autostainer, dehydrated and coverslipped with DePeX mounting medium (Gurr®, BDH Prolabo, VWR International, Ltd. UK). In order to test the specificity of the immunoreaction, positive (human tissue) and negative (replacement of the primary antibody by PBS) controls were included.

Gene expression analysis

Tissue samples preserved in RNAlater were kept at 4ºC during 24 h and stored at -20ºC until RNA extraction. Total RNA was extracted from tissues of CTRL and challenged fish using TRIZOL Reagent (Life Technologies, Carlsbad, CA, USA) according to the

manufacturer's recommendations. RNA quality and quantity were evaluated using a Bioanalyzer (Agilent Technologies) and a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc), respectively. Good quality RNA (RIN > 7.5) was reverse transcribed (1 µg) into cDNA with random primers using AffinityScript Multiple Temperature cDNA Synthesis kit (Agilent Technologies) following the supplier's protocol. The qPCR analysis was carried out in a MX3005P thermocycler (Stratagene) using 2 µl of cDNA per reaction and 300 nM of each primer in a final volume of 20 µl according to the Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies) manufacturer's instructions. The constitutively expressed ribosomal protein S4 (RPS4), proved to be stably expressed in turbot (Robledo, Hernández-Urcera, et al., 2014), was chosen as the house-keeping gene for sample normalisation. Each sample was performed in triplicate for accuracy and error estimation including one reverse-transcription-negative control for each gene. Fluorescence readings at the end of each cycle were used to estimate threshold cycle values (Ct). Values were normalized to RPS4 and fold change at transcript level was determined with the relative quantitative method (ΔΔCt) (Livak & Schmittgen, 2001) using data from CTRL fish as reference values. Prior to quantitative analysis, a standard curve was constructed using six serial dilutions of cDNA (from 1,000 to 0.01 ng) and the efficiency of each primer set was determined. Each sample was analysed for primer–dimer, contamination, or mispriming by inspection of their dissociation curves.

Statistical analysis

The statistical analysis of gene expression in turbot was performed with SPSS Statistics 20.0 software (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean ± SEM, and significance of differences was determined by Student's *t*-test, after checking that

194 data follow a normal distribution using Shapiro-Wilk test. Results were considered
195 significant at $P < 0.05$.

196 In GSB, data on gene expression was analysed using SPSS 21.0. by one-way analysis of
197 variance (ANOVA) analysis followed by a Student-Newman-Keuls (SNK) post hoc test.
198 A P value < 0.05 was considered statistically significant.

199 ## Results

200 *Histopathology*

201 The most evident differences were noticed at an advanced stage of infection, when both
202 species showed a massive parasitic load in the lining epithelium of the entire intestinal
203 tract (Figure 1A-D). Inflammatory infiltration was observed in the lamina propria-
204 submucosa in both cases, although more severe in turbot. On the other hand, the epithelial
205 alterations were quite different: turbot intestine showed large areas of epithelial sloughing
206 and the characteristic "scalloped shape" of the remaining lining epithelium. As well,
207 enterocyte alterations consistent with necrotic and apoptotic changes were often observed
208 (Figure 1A, C).

209 *Sequence comparison and primer design*

210 The alignment performed between turbot and GSB E-cadherin resulted in 80% and 77%
211 identity scores of the nucleotide (Supporting file 1) and aminoacidic sequences,
212 respectively. In addition, the E-cadherin sequences of the two species showed similar
213 results when compared to human: the identity scores of the nucleotide and aminoacidic
214 sequences were 71% and 53%, respectively, in the turbot-human comparison, and 74%
215 and 50% in the case of GSB-human.

216 A set of common primers targeting the conserved regions of E-cadherin was designed
217 (E_CAD-F 5'GGAACGATGTGATGCCAAACT, E_CAD-R

CAGCTGCCTTCAGGTTGTCAT) to amplify a fragment of 105 bp in length of the E-cadherin gene in both species (Supporting file 1).

Immunohistochemistry

Homogeneous immunostaining of the lining epithelium located at the cell–cell junction area with a basolateral position was observed in CTRL (healthy) fish of both species (Figure 2A, B). The distribution pattern of E-cadherin in the intestinal mucosa did not substantially change in fish with moderate infection, but a more intense staining was noticed surrounding the parasitic structures in turbot (Figure 2C, D). The severely parasitized specimens of this species showed a clear alteration of the immunostaining, consisting in a disordered and irregular distribution pattern of E-cadherin in the epithelium, with areas of scant immunoreaction interspersed with other of intense staining, particularly at enterocyte-parasite interface (Figure 2E). On the contrary, the distribution pattern did not seem to be particularly affected by the massive presence of parasites in GSB (Figure 2F). Within each species, the immunohistochemical results were analogous between the anterior and posterior part of the intestine (Figure 3A-D).

Gene expression analysis

qPCR assays were developed to analyze the gene expression of E-cadherin in the digestive tract of parasitized turbot and GSB and their respective CTRL. Results showed one clear single peak in each case using the common primers designed, indicating the suitability of the developed method. The transcriptional levels indicated a significant ($P < 0.05$) down-regulation of E-cadherin in the posterior intestine of severely infected GSB, with a decreasing trend with disease worsening (Figure 4). On the contrary, no significant differences were found between CTRL and infected turbot, neither in anterior nor in the posterior region of the intestine, although both regions showed a trend to up-regulation during moderate infection (Figure 5A, B).

Discussion

In this work, the immunohistochemical distribution and gene expression of E-cadherin in the intestine of fish parasitized by *Enteromyxum* spp. were analysed. These myxozoan parasites colonize the lining epithelium causing a cachectic syndrome characterized by anorexia and weight loss. Nevertheless, the parasitosis shows a different course depending on the species affected; in particular, turbot shows high mortality rates associated to severe catarrhal enteritis while in GSB the intestinal lesions are milder and mortality rates lower. Understanding the pathophysiology of gut barrier in the pathogenesis of these diseases appears to be key to unravel the mechanisms underlying the different susceptibility.

The immunohistochemical results indicate a change in the distribution pattern of E-cadherin in turbot, with loss of homogeneity and enhanced presence at the enterocyte-parasite interface. In other parasitosis leading to intestinal barrier dysfunction, the pathogenesis involves the disassembly of cell-cell junctions and cytoskeleton, including the relocation of junctional proteins (Di Genova & Tonelli, 2016). As well, oxidative stress was demonstrated to induce redistribution of E-cadherin and other junctional proteins, causing an increment in intestinal permeability in mice (Rao, Basuroy, Rao, Karnaky, & Gupta, 2002). In turbot enteromyxosis, evidences of enhanced nitric oxide production and occurrence of oxidative stress have been highlighted (Losada, Bermúdez, Faílde, & Quiroga, 2012; Robledo, Ronza, et al., 2014). The relocation of E-cadherin may play a role in the pathogenesis of the characteristic severe epithelial desquamation observed in this species. It was demonstrated that the loss of E-cadherin from cell-cell junctions is related to anoikis (Fouquet et al., 2004), a form of apoptosis induced by loss of anchorage to the extracellular matrix. The occurrence of anoikis in turbot enteromyxosis was previously hypothesized because of the increasing number of

apoptotic enterocytes observed during the infection by histology (Bermúdez et al., 2010) and immunochemistry to active caspase-3 (Losada, Bermúdez, Faílde, de Ocenda, & Quiroga, 2014). Moreover, RNA-seq analysis of the intestine of *E. scophthalmi*-infected turbot also highlighted the up-regulation of different pro-apoptotic genes (Robledo, Ronza, et al., 2014).

On the contrary, the presence of *E. leei*, even in the case of severe parasitization, affected to a lesser extent the distribution of E-cadherin in GSB. The labelling pattern did not appear particularly altered compared to CTRL fish and there was no E-cadherin accumulation surrounding the parasite plasmodia. This suggests a different interaction between fish and myxozoan cells in this host-parasite scenario.

Once penetrating the host lining epithelium, both *Enteromyxum* species dwell, grow and proliferate in the paracellular space between enterocytes (Cuadrado et al., 2008; Redondo, Quiroga, Palenzuela, Nieto, & Álvarez-Pellitero, 2003). The intricacies of the relationship between host and myxozoan cells and their interaction during the infection are still far to be fully understood. In GSB and turbot enteromyxoses, Redondo & Álvarez-Pellitero (2009, 2010b) demonstrated the involvement of lectin/carbohydrate interactions between host and parasite cells in the mechanisms of adhesion and invasion. These authors suggested that the differences observed between the two fish species could aid to explain the differences in the severity of the related diseases (Redondo & Álvarez-Pellitero, 2010a). Nevertheless, the existence, nature and role of cell-cell junctions between these myxozoans and their hosts is an aspect to be elucidated. Myxozoa, as members of the Cnidarian phylum, might theoretically present all ancestral eumetazoan types of cell junctions. However, it is difficult to identify them because they are often atypical or inconspicuous (Gruhl & Okamura, 2015). In addition, it is still unclear how junctions can be formed between cells of different species, given the complexity and specificity of the

293 formation of cell junctions in an organism (Cavey & Lecuit, 2009; Shao et al., 2017).
294 Furthermore, in an infection scenario, the parasite might exploit the host cells' capabilities
295 to form junctions to subvert the defence response or simply to gain purchase of the host,
296 as well as the host may alter the structure of its cell junctions to prevent formation of
297 junctions with parasite cells (Gruhl & Okamura, 2015). Structures resembling gap
298 junctions have been described between plasmodia of *E. scophthalmi* and host intestinal
299 epithelium (Redondo et al., 2003), while close interdigitating membranes of primary cells
300 of *E. leei* with host cells have been observed at TEM (authors' unpublished data). The
301 genomic and transcriptomic characterization of *Enteromyxum* spp. would greatly help to
302 elucidate this aspect, and recently there have been promising progresses on this front
303 (Chang et al., 2015; A. Picard-Sánchez et al., 2017; Shpirer et al., 2014).

304 The scant or even absent development of mature spores of *E. scophthalmi* observed in
305 turbot, both in spontaneous and experimental infections, together with the particular
306 virulence of the parasite in this species has led to the hypothesis that turbot might
307 constitute an accidental host (Bermúdez et al., 2006; Redondo et al., 2004; Redondo,
308 Palenzuela, Riaza, Macías, & Álvarez-Pellitero, 2002). This can suggest an atypical
309 behaviour of *E. scophthalmi* during the infection, and the strong interaction with turbot
310 E-cadherin might also represent a strategy to gain purchase of the host and ensure its
311 survival once the epithelium starts to desquamate. In this sense, the presence of parasitic
312 stages covered by mucosal remnants/detached cells in the intestinal lumen was often
313 reported, and it has been interpreted as a strategy to get protection from external
314 conditions until the invasion of a new host (Bermúdez et al., 2010; Losada et al., 2014;
315 Redondo et al., 2003).

316 The two host-parasite scenarios analysed also showed different results concerning the E-
317 cadherin gene expression. In the present work, a qPCR method has been developed for

318 the amplification of a fragment of E-cadherin gene using common primers for both
319 species. The optimized assay showed good specificity and sensitivity for both fish
320 species. Intestinal E-cadherin down-regulation is usually observed in mammal's diseases
321 characterized by high levels of pro-inflammatory molecules, such is the case of
322 inflammatory bowel disease, where it has been related with the weakening of cell-cell
323 adhesion in the lining epithelium (Arijs et al., 2011; Sanders, 2005). At this purpose, the
324 down-regulation of E-cadherin gene expression found in severely-parasitized GSB, where
325 a strong local immune response has been described (Pérez-Cordón et al., 2014), was the
326 expected result and might underlie the development of intestinal barrier dysfunction in
327 advanced infection. In addition, important changes in goblet cell composition and
328 distribution, intestinal mucin expression and mucus glycoprotein profile have been
329 described in *E. leei*–infected GSB (Estensoro et al., 2013; Estensoro, Redondo, et al.,
330 2012; Pérez-Sánchez et al., 2013), and maturation and positioning of goblet cells was
331 demonstrated to be impaired in mice with the E-cadherin gene inactivated (Schneider et
332 al., 2010). In previous studies in this species, the intestinal gene expression of E-cadherin
333 was also found to be modulated by some dietary interventions. In particular, it was
334 significantly up-regulated in GSB fed a diet low in fish meal and fish oil, and it was
335 restored when sodium butyrate was added (Estensoro et al., 2016). However, no changes
336 were detected when fed with Next Enhance®150 (Pérez-Sánchez et al., 2015) or with
337 olive oil bioactive compounds (Gisbert et al., 2017), and a lower expression was found in
338 the anterior intestine of fish fed DICOSAN or probiotics (Simó-Mirabet et al., 2017).

339 Why the down-regulation of E-cadherin gene expression detected in this study was not
340 accompanied by an observable change in immunostaining in GSB needs to be further
341 analyzed. The analysis of a greater number of time points might help to detect a possible
342 later immunostaining alteration. However, changes in gene expression levels are

343 frequently not reflected at the protein level (Liu, Beyer, & Aebersold, 2016; Vogel &
344 Marcotte, 2012), as well as it is possible that a hypothetical small reduction in E-cadherin
345 synthesis may not be detected by immunohistochemistry. Nevertheless, the distinctive
346 immunolabelling in the parasitized fish of the two species and the different modulation
347 of the gene expression are worthy of further investigation.

348 Remarkably, despite the large body of evidence reporting an exacerbated intestinal
349 immune response in turbot enteromyxosis (Ronza, Robledo, et al., 2019), we did not find
350 a significant modulation of E-cadherin gene expression. A trend of up-regulation was
351 noticed in moderate infection, a stage that, in this species, is characterized by cellular
352 infiltrates in the lining epithelium, consistent with intraepithelial lymphocytes (Bermúdez
353 et al., 2010). An intriguing hypothesis to be further investigated is the possible role of
354 these cells in attempting to preserve the integrity of the intestinal mucosa. Intestinal
355 intraepithelial lymphocytes express junctional molecules, including E-cadherin, and are
356 thought to play a main role in sustaining the epithelial barrier function in mice parasitized
357 by *Eimeria vermiformis* (Inagaki-Ohara et al., 2006).

358 Concluding, this work showed interesting changes of intestinal E-cadherin in both host-
359 parasite scenarios, combining the use of morphopathological and molecular techniques.
360 This is highly recommended in pathogenesis studies to get more exhaustive information,
361 which can also be useful to glimpse the future direction of a research line. The loss of the
362 physiological distribution of E-cadherin and its relocation at the enterocyte-parasite
363 interface in parasitized turbot, whereas no evident changes were noticed in GSB,
364 constitutes a remarkable finding that indicates diverse relationships with the parasite cells
365 according to the host. This might contribute to explain the different host-parasite
366 interactions and disease pathogenicity and deserves further attention. As well, more
367 studies are needed to fully understand the gene expression dynamics of junctional proteins

during the diseases. Along with E-cadherin as the main component of the adherens junctions, other proteins constituting the desmosomes and tight junctions (e.g. desmoglein 2, claudins, occludin, zonula occludens) have a relevant role in maintaining the gut barrier integrity, and several works in mammals reported their susceptibility to intestinal inflammation and/or as a target for intestinal parasites (Chelakkot, Ghim, & Ryu, 2018; Di Genova & Tonelli, 2016; Kamekura et al., 2015). To deepen our knowledge of the pathogenesis of barrier dysfunction during enteromyxoses, the exploration of possible perturbances in other junctional complexes should be included, ideally, combining the histopathological observations with transcriptomic and proteomic approaches. Understanding the relative weight of each factor (parasite, host immune response, oxidative stress) in the pathogenesis is crucial towards the design of control and therapeutic measures adapted to the singular fish-parasite model.

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Supporting information

(Additional supporting information may be found online in the Supporting Information section)

Supporting File 1. Alignment of E-cadherin nucleotide sequences of turbot (MG137250.1) and gilthead sea bream (KF861995.1). The locations of the primer recognition sites are indicated by arrows.

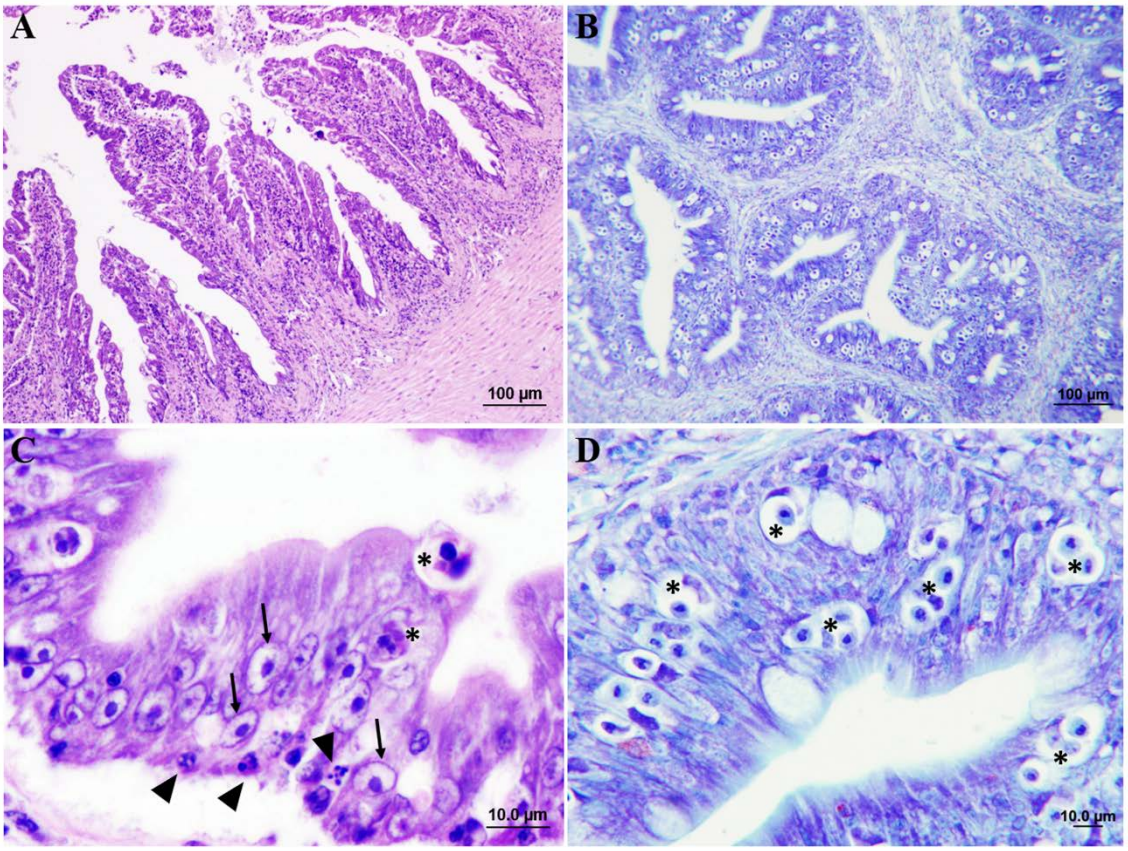

Figure 1. Comparative photomicrographs of turbot pyloric caeca (A, C) and gilthead sea bream posterior intestine (B, D) with a severe parasitization by *Enteromyxum scophthalmi* and *E. leei*, respectively. Both species present a high parasite load, but lesions are more serious in turbot, with a marked inflammatory infiltration in the lamina propria, alteration of the mucosal architecture and epithelial detachment (A). At higher magnification (C), apoptotic bodies (arrowheads) and enterocytes with nuclear hypertrophy and central migration of chromatin (arrows) can be noticed in turbot, associated to the presence of two parasitic forms (*). By contrast, despite the elevated number of parasites (*), there are no major alterations in the lining epithelium of gilthead sea bream (D). Haematoxylin & eosin (A, C) and Giemsa (B, D).

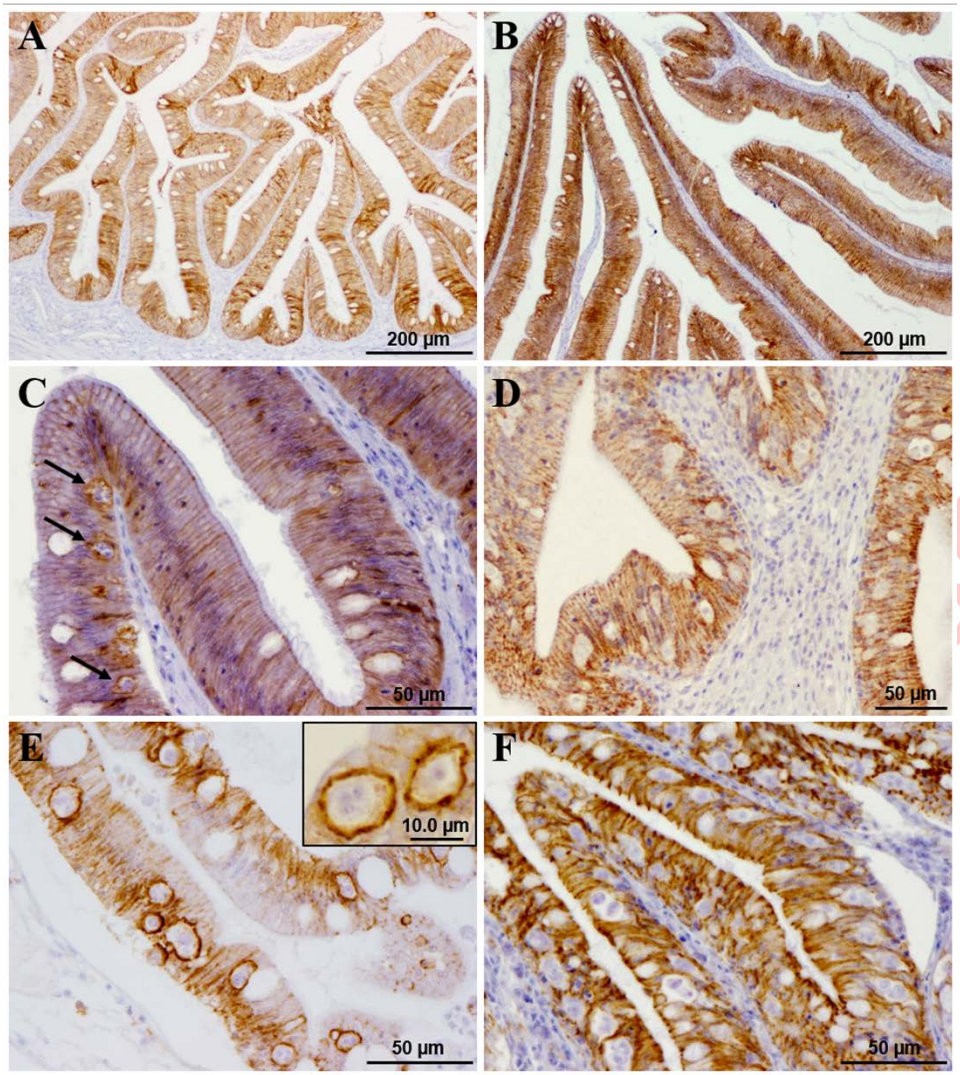

Figure 2. Immunohistochemical detection of E-cadherin in turbot (A, C, E) and gilthead sea bream (B, D, F) intestine. A, B = Control fish; C, D = *Enteromyxum*-infected fish with moderate infection; E, F = *Enteromyxum*-infected fish with severe infection. A, B) E-cadherin immunostaining in control fish showed a regular distribution with immunolocalization at cell-cell contacts in the intestinal mucosa. Bars = 200 µm. C) In moderately infected turbot the general distribution pattern of E-cadherin was conserved, but a more intense immunoreaction was noticed surrounding the parasitic forms (arrows). Bar = 50 µm. D) No outstanding changes in E-cadherin distribution were noticed in gilthead sea bream with moderate infection. Bar = 50 µm. E) The immunostaining was markedly altered in severely parasitized turbot, with areas of scant immunoreaction and a strong label intensity at the contact areas between host and parasites (see inset). Bar = 50 µm; Inset bar = 20 µm. F) Even in case of advanced enteromyxosis with a massive parasite load in the epithelium, E-cadherin immunostaining was not significantly affected in gilthead sea bream sections. Bar = 50 µm.

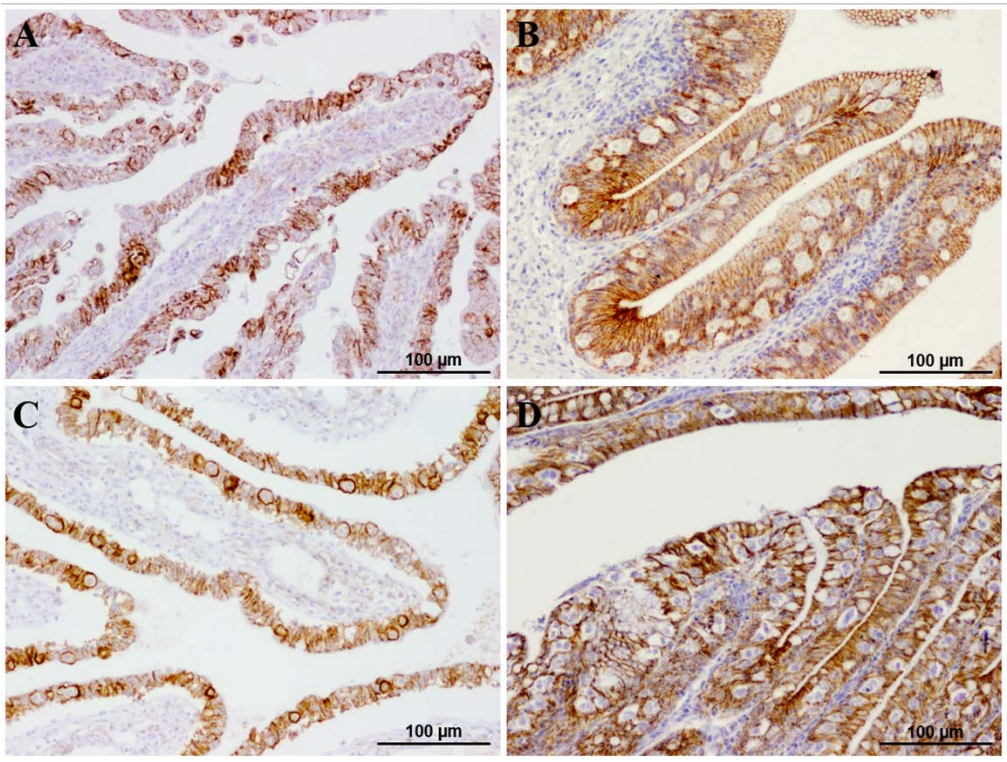

Figure 3. Comparative photomicrographs of E-cadherin immunostaining in the anterior (A, B) and posterior (C, D) intestinal regions of severely infected turbot (A, C) and gilthead sea bream (B, D). Bars = 100 µm.

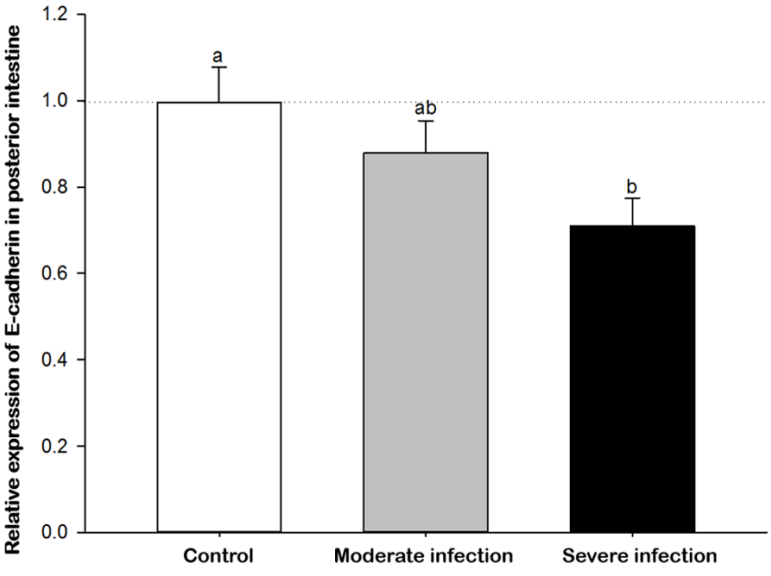

Figure 4. Mean and standard error of the mean of E-cadherin transcript levels in gilthead sea bream posterior intestine. Control, moderately and severely *Enteromyxum*-infected fish were analysed. The transcript levels in control fish were used as references values (values > 1 or <1 indicate increase or decrease with respect to the reference). Different letters indicate statistically significant differences ($P < 0.05$).

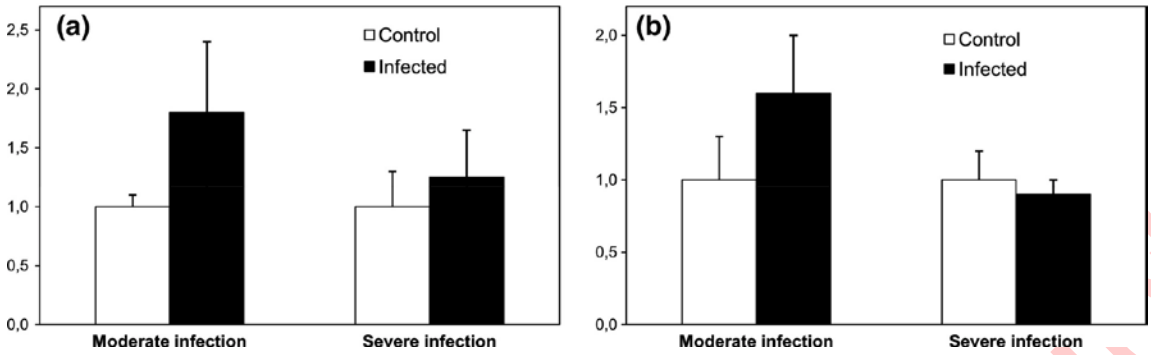

Figure 5. Mean and standard error of the mean of E-cadherin transcript levels in turbot pyloric caeca (A) and posterior intestine (B). Moderately and severely *Enteromyxum*-infected fish at 24 and 42 days post-infection, respectively, were analysed. The transcript levels in the respective control fish groups were used as references values (values > 1 or <1 indicate increase or decrease with respect to the reference).