Bromodeoxyuridine DNA labelling reveals host and parasite proliferation in a fish-myxozoan model

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Bromodeoxyuridine DNA labelling reveals host and parasite proliferation in a fish-myxozoan model

Running title: Intestinal host and parasite proliferation

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Abstract

Enteromyxum leei is a myxozoan parasite responsible for enteritis in gilthead sea bream (*Sparus aurata*). The parasite proliferates in the paracellular space of the intestinal epithelium and induces an inflammatory reaction. In order to assess intestinal cell turnover and parasite proliferation, fish were infected with the parasite by anal intubation and after 17 and 64 days, the cell proliferative marker bromodeoxyuridine (BrdU) was administered and, after 24 hours, tissue samples were taken for immunohistochemical detection. Parasite exposure induced increased epithelial and immune cell proliferation in all intestinal segments at all time points, even before parasite establishment. This increased turnover was triggered early after intubation and mainly at a local level, as shown by an increased *proliferating cell nuclear antigen* (*pcna*) gene expression only at the posterior intestine after 17 days (not found in lymphohaematopoietic organs). Incorporation of BrdU in parasite secondary and tertiary daughter cells indicated that parasite endogeny is not by schizogonial division, which uses *de novo* synthesis pathway of pyrimidines. Altogether, BrdU immunolabelling and *pcna* gene expression showed the rapid proliferative response of the fish intestines upon a myxozoan infection and how this response is effectively triggered even before the parasite reaches or establishes in the site.

Keywords: *Enteromyxum leei*, *Sparus aurata*, *pcna*, immunohistochemistry, intestine

Introduction

The regenerative capacity of the intestinal epithelium, by means of a strict control of proliferation and programmed cell death, plays a central role in the maintenance of the intestinal homeostasis. The process of restoration of the epithelium is well documented in vertebrates, in which the mucosa is entirely regenerated through a continuous cycle of cell proliferation, migration and differentiation over the entire crypt-villus axis at an extraordinary high rate (Crosnier et al. 2006; Faro et al. 2009; Wallace et al. 2005; Wang et al. 2010). Epithelial renewal occurs through division of stem cells at the bottom of the intestinal folds, the inter-villus pocket region. From there, transit-amplifying and differentiated cells migrate towards the apical region of the folds, where apoptosis and shedding take place. The study of the intestinal epithelium turnover in the zebrafish model has demonstrated a great similarity between the teleostean and mammalian mucosal regeneration, the latter presenting a slower epithelial restitution in order to restrain excessive expansion and reduce susceptibility to carcinogenic transformations (Faro et al. 2009; Wang et al. 2010).

Fish intestinal remodelling can be affected by or can compensate the distress of the intestinal homeostasis provoked by diet interventions (Bakke-McKellep et al. 2007; Chikwati et al. 2013; Yoshida et al. 2016), radiation (Johnson et al. 1970), intestinal resection (Schall et al. 2015), adaptation to salinity changes (Takahashi et al. 2007; Takahashi et al. 2006), or pathogens (Dezfuli et al. 2012; Dezfuli et al. 2016b; Hemmer et al. 1998; Ronza et al. 2011). Inflammation is generally considered a beneficial response to tissue damage for its induction of a localized repair response. Such an epithelial repair and healing process involves local recruitment and/or proliferation of immune-relevant cells, controlled proliferation of stem cells in the fold base and

turnover of damaged epithelial cells, in order to restore the epithelial barrier integrity. The innate cellular immune response to parasites in fish intestines, whether or not leading to enteritis, mainly consists of an inflammatory reaction involving granular tissue formation and infiltration of leukocytes and macrophages, in microparasite infections (Sitjà-Bobadilla et al. 2016), as well as in helminthiasis (Dezfuli et al. 2016a). Cell proliferation in parasitized intestines plays a key role during the host immune response, as it may be decisive for the pathogenesis exerted by each parasite and, thus, be partially responsible for the success of either parasite establishment or its rejection.

Enteromyxum leei is a myxozoan parasite that infects the intestinal tract of gilthead sea bream, among many other species, causing chronic desquamative enteritis. Myxozoans are metazoan microscopic parasites, which constitute a clade of highly derived cnidarians (Fook and Siddall 2015). Enteromyxosis in this fish host eventually leads to emaciation, cachexia and immunosuppression, which enhances susceptibility to opportunistic pathogens and causes high mortality rates during outbreaks in aquaculture facilities (Davey et al. 2011; Palenzuela 2006; Sitjà-Bobadilla and Palenzuela 2012). The horizontal fish-to-fish transmission of *Enteromyxum* spp. and the lack of effective treatments pose a serious threat for fish farming, especially in the case of gilthead sea bream massively established in Mediterranean sea cages (Athanasopoulou et al. 2009; Colorni and Padrós 2011; Rigos and Katharios 2010; Sitjà-Bobadilla 2004; Sitjà-Bobadilla and Palenzuela 2012). *E. leei* stages reach the fish intestine through the digestive tract and penetrate the epithelium, where proliferative and sporogenic stages multiply in the paracellular space (Diamant et al. 1994; Diamant and Wajsbrodt 1997). The parasite quickly multiplies and colonizes other sites of the intestinal tract or infects other fish hosts, using the desquamated epithelial material that withholds parasite stages for partial protection in the intestinal lumen first and finally in the seawater when

101 parasite stages are released with the fish faeces. Thus, epithelial cell proliferation,
102 turnover and shedding in the gilthead sea bream intestine are expected to be harshly
103 affected during *E. leei*-induced enteritis, but have not been previously evaluated.
104 Previous studies on gilthead sea bream have reported transcriptional modulation of
105 genes involved in the intestinal cell differentiation and proliferation, and epithelial
106 integrity, damage and repair during *E. leei*-induced enteritis (Calduch-Giner et al. 2012;
107 Davey et al. 2011; Pérez-Sánchez et al. 2013; Sitjà-Bobadilla et al. 2008).

108 This study is focused on the intestinal cell proliferation in a teleost species
109 during an enteric myxozoan experimental infection by means of 5-bromo-2'-
110 deoxyuridine (BrdU) *in vivo* labelling and by proliferating cell nuclear antigen (*pcna*)
111 gene expression at intestinal and systemic levels.

113 **Material and methods**

115 **Animal care, experimental design and sampling procedure**

117 Clinically healthy juvenile gilthead sea bream were obtained from a commercial fish
118 hatchery. Upon arrival to the facilities of the Instituto de Acuicultura Torre de la Sal
119 (IATS), they were checked to be SPF (*E. leei*-free) by routine molecular and
120 histological diagnostic techniques, and grown in an open flow system with 5 µm-filtered
121 and UV-irradiated seawater (37.5 ‰ salinity). Day length corresponded to the natural
122 changes at our latitude (40°5'N; 0°10'E), and water temperature was maintained
123 between 18 °C and 25 °C. The oxygen content of water was kept above 85% saturation,
124 and unionized ammonia remained below toxic levels (<0.02 mg/l). Fish were fed *ad*
125 *libitum* a commercial diet (BioMar, Palencia, Spain).

The parasite infection was performed by anal intubation as previously described (Estensoro et al. 2010). Briefly, 36 fish (average initial weight = 60.5 g) were intubated with 0.5 ml of *E. leei* infected-intestinal scrapings (recipient group, RCPT) and 36 fish (average initial weight = 58.7 g) were intubated with the same volume of PBS (control group, CTRL). At 17 (time point = t1) and 64 (time point = t2) days post intubation (dpi), 7 fish from both the CTRL and RCPT groups were intracoelomically injected with 5-bromo-2'-deoxyuridine (BrdU, Sigma, St. Louis, MO, USA) in Hank's Balance Salt Solution (Gibco, USA) / dimethyl sulfoxide (Sigma) (HBSS/DMSO,1:5) at a dose of 100 mg BrdU/kg fish weight.

Twenty four hours after BrdU injection, fish were sacrificed by overexposure to the anaesthetic MS-222 (Sigma, St. Louis, MO, USA) and tissue samples of anterior (AI, immediately after the pyloric caeca), middle (MI, equidistant from AI and PI), and posterior (PI, immediately before the rectum) intestine were taken, fixed in 4% paraformaldehyde and processed for paraffin embedment following routine histological procedures for parasite diagnosis and immunohistochemical localization of BrdU in 4 µm-thick tissue sections. Pieces of head kidney (Hk), spleen (Spl) and PI were excised from the same fish, rapidly frozen in liquid nitrogen and stored at -80°C until RNA extraction.

The experiment was carried out in accordance with the principles published in the European animal directive (86/609/EEC) for the protection of experimental animals, and was approved by the Consejo Superior de Investigaciones Científicas (CSIC) ethics committee and IATS Review Board, with permits associated to project AGL2009-13282-C02-01.

Diagnosis of the infection

Parasite diagnosis was performed on Giemsa stained AI, MI, and PI intestine segments. Infection intensity in each segment was semiquantitatively evaluated following a conventional scale from 1+ to 6+, with the following ranges: 1+ = 1–5; 2+ = 6–10; 3+ = 11–25; 4+ = 26–50; 5+ = 51–100; 6+ >100 parasite stages per microscope field observation at 120×. *E. leei* stages were classified as spores, sporoblasts and proliferative stages, the latter corresponding to stages one to three described in (Álvarez-Pellitero et al. 2008). A fish was considered positive for infection, when the parasite was found at least in one intestinal segment.

Immunohistochemical detection of Bromodeoxyrudine (BrdU)

BrdU antigens in tissue sections on SuperfrostPlus slides (Menzel-Gläser) were retrieved by heating in a pressure-cooker for 15 min in 10 mM sodium citrate buffer (pH 6.0). After 30 min incubation in HCl 2N, slides were incubated for one hour with mouse Mab anti-BrdU clone BU-33 (1:500) (Sigma, St. Louis, MO, USA) at room temperature (RT). A secondary biotinylated antibody (1:200) (VECTOR Labs.) was added for one hour (RT) followed by incubation with avidin-biotin-peroxidase complex (VECTOR Labs.) for 30 min (RT). Bound peroxidase was visualized with 3,3'-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO, USA) and sections were counterstained with Gill's haematoxylin, dehydrated and mounted in DPX.

For each of the 7 fish per experimental group (CTRL, RCPT t1 and RCPT t2) and intestinal segment (AI, MI and PI observed at 500× magnification) ten random microscopical fields, regardless of the presence or absence of the parasite, were photographed with an Olympus DP70 camera (12.5 million pixels) connected to a Leitz

Dialux 22 light microscope. Immunoreactive cells were detected and quantified using ImageJ software (open-source Java-based imaging program). Labelled and non-labelled parasite stages were counted and their ratio calculated. Images were not manipulated.

Proliferating cell nuclear antigen (pcna) gene expression

Head kidney, spleen and posterior intestine RNA was extracted using the MagMAXTM-96 total RNA isolation kit (Applied Biosystems, Foster City, CA, USA). Reverse transcription was performed on 500 ng of total RNA using random decamers and the High-Capacity cDNA Archive kit (Applied Biosystems) following manufacturer's instructions. Negative control reactions without reverse transcriptase were included.

Real-time quantitative PCR was carried out with the CFX96 ConnectTM Real-Time PCR Detection System (BioRad, Hercules, CA, USA) using primers specific for gilthead sea bream *pcna* (GenBank accession number KF857335: 5'-CGTATCTGCCGTGACCTGT-3'/5'-AGAACTTGACTCCGTCCTTGG-3'). *β-actin* was included as a housekeeping gene (GeneBank accession number: X89920: 5'-TCCTGCGGAATCCATGAGA-3'/5'-GACGTCGCACTTCATGATGCT-3'). Each RT reaction of 20 µl contained 660 pg of total input RNA sample, 5 µl PyroTaq EvaGreen qPCR Mix Plus (Cultek Molecular Bioline) and specific primers at a final concentration of 0.45 µM. The PCR reaction conditions consisted of an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The specificity of reactions was verified by visual analysis of melting curves for each reaction performed. Fluorescence data acquired during the extension phase were normalized by the delta-delta Ct method (Livak and Schmittgen 2001).

Statistical analysis

The number of BrdU immunoreactive cells in the intestine of CTRL and RCPT groups was statistically analysed. For each intestinal segment, the differences between both groups and the differences between t1 and t2 for each group/intestinal segment were analysed by Student's *t* test. When the test of normality or equal variance failed, a Mann-Whitney U Sum test was applied instead. A one-way ANOVA followed by Student-Newman-Keuls was used to analyse the differences within each group for each time and the differences between each fish category for a given intestinal segment. Data that failed the normality or equal variance test, were analysed with Kruskal–Wallis one-way ANOVA on ranks followed by Dunn's method. The significance level was set at $P < 0.05$. All statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL, USA).

Results

Disease outcome

After 17 dpi (t1), *E. leei* was mainly detected at the PI with an infection prevalence of 42.9% (3 fish out of 7) and with a mean infection intensity of 2+. At the AI, prevalence of infection was 14.3% (1 fish out of 7, with 2+ intensity of infection) and no fish were infected at the MI at this time point. At 64 dpi (t2), prevalence of infection at the PI reached 85.7% (6 fish out of 7) with a mean infection intensity of 5.2+ and the parasite was widely extended in other intestinal segments. A 57.1% of prevalence of infection (4

fish out of 7) was found at the AI with a mean infection intensity of 3.3+, and a 14.3% of prevalence of infection (1 fish out of 7) was found at the MI with the maximum infection intensity. All fish with an established infection at the AI and/or MI, were also infected at the PI. Parasites found at 17 dpi were presporogonic proliferative stages, whereas at 64 dpi, sporoblasts and spores were also observed (Table 1, Fig. 1).

BrdU immunoreactivity

BrdU incorporation to nuclear DNA was successfully detected in samples taken 24 h after injection, mainly in enterocytes (Fig. 1, Fig. 2). Yet, distribution of immunoreactive epithelial cells was not only restricted to inter-villus pockets, as proliferating cells were detected along the entire mucosal folds in CTRL and RCPT fish, regardless of the intestinal section. In addition, abundant BrdU immunoreactive cells were detected in the lamina propria-submucosa of RCPT fish, even in non-infected intestinal sections. The size and morphology of the nuclei indicate that these cells are mainly granulocytes and some lymphocytes.

Mean counts of BrdU immunoreactive host cells at t1 were significantly higher in all intestinal segments of RCPT fish than in those of CTRL fish, and the greatest difference was found in the MI (Fig. 3). At t2, the number of BrdU immunoreactive cells was also higher in RCPT than in CTRL fish, though only statistically significant at the PI (Fig. 3). However, within each fish group, no significant differences were detected for the host cell counts among intestinal segments, nor between t1 and t2 within fish groups and intestinal segments (Fig. 3).

As cell proliferation in non-parasitized intestinal segments (AI and MI) seemed to be affected by the presence of the parasite at the PI, t1 and t2 data were merged for

each intestinal segment, and cell counts of CTRL fish were compared with those of three categories of RCPT fish: a) fish with non-parasitized AI/MI segments and also no parasite at the PI (NP), b) fish with non-parasitized AI/MI segments, but with parasitized PI (NP-PAR PI), and c) fish with parasitized AI/MI segments and also parasitized PI (PAR). As shown in Fig. 4, the number of BrdU labelled cells was significantly higher in parasitized PI and AI than in intestines from CTRL fish. However, no significant differences were found between CTRL intestines and non-parasitized segments of RCPT fish, even though they had higher number of BrdU labelled cells than CTRL fish. Furthermore, at the non-parasitized MI, cell counts were higher in fish in which the neighbouring PI segment was parasitized. Thus, an increasing trend was clear from CTRL to PAR fish, especially in the MI.

Moreover, BrdU labelling was also detected in proliferating parasite stages, mainly secondary and tertiary daughter cells (Fig. 1). The mean ratio of BrdU immunoreactive parasites/total parasites in the PI was 0.70 at t1 and 0.76 at t2, whereas in the AI and MI at t2 it was 0.70 and 0.80, respectively.

***pcna* gene expression**

At the PI segment, *pcna* gene expression was significantly higher in RCPT fish than in CTRL ones, only at t1. By contrast, no statistically significant differences were found for the *pcna* gene expression in the studied lymphohaematopoietic tissues (head kidney and spleen) (Fig. 5).

Discussion

Enteromyxum leei intestinal infection followed a postero-anterior progress, as previously described in gilthead sea bream, being the PI the main target site for the parasite and the MI the last and less infected intestinal portion (Cuadrado 2009; Estensoro et al. 2010; Fleurance et al. 2008). The per anal infection route provided a reliable source of infected fish, whose prevalence of infection at the given water temperature was similar to previous experimental infections by this same route (Estensoro et al. 2014; Estensoro et al. 2010; Pérez-Cordón et al. 2014). In the current experimental infection, the highest prevalences and intensities of infection were found at the PI at both samplings, indicating this was the first intestinal segment where the infection was established. At t2 (64 dpi), the parasite infection had not only extended to more fish along their intestinal segments, but also parasite sporogenesis had begun.

In order to assess intestinal cell turnover and parasite proliferation, the cell proliferative marker BrdU was administered. BrdU is incorporated into synthesizing nuclear DNA during the S-phase of the cell cycle and can be later immunohistochemically detected (Walsh and Eckert 2014). Since Gratzner (1982) described a monoclonal antibody to BrdU, this technique has proven to be very useful in many research fields to assess cell proliferation in normal tissues and tumours and to trace cells during development and differentiation (Goodlad 2017). This technique has been applied previously in teleost fish to evaluate the effects of gastrointestinal distress (Dezfuli et al. 2016b; Schall et al. 2015; Takahashi et al. 2007; Yoshida et al. 2016), but has not been used for sparids or for the study of host-parasite interactions involving myxozoans. Distribution of proliferating intestinal epithelial cells in gilthead sea bream was quite scattered throughout the mucosal folds, even in CTRL tissues, indicating elevated tissue renewal rates since no signs of hyperplasia were observed. *E. leei* infection clearly provoked a significant increase of intestinal epithelial cell proliferation

in RCPT compared to CTRL fish. This increased proliferation was more pronounced at early infection times (17 dpi). The intestinal epithelium exhibits the fastest renewal rate of all vertebrate tissues in order to preserve its essential barrier function. More specifically, in teleosts, enterocyte turnover rates are the highest, favouring a rapid repair and restitution of the epithelium in the pathogen-rich water environment (Wang et al. 2010). Epithelial turnover (proliferation, differentiation and apoptosis) ensures the anatomical barrier integrity, and can be considered part of the innate immunity since pathogens attached to the lining epithelium get shed and are eliminated with surface cells prior to their penetration and invasion (Kim et al. 2010; Liempi et al. 2016). This protective mechanism, already reported in mammals, is engaged for example upon infection, since the acceleration of the epithelial turnover helps to expel the parasitic nematode *Trichuris trichuria* from murine intestines (Cliffe et al. 2005), or pathogenic *Escherichia coli* from human urothelium (Mysorekar et al. 2002), or *Trypanosoma cruzi* from human placental trophoblasts (Droguett et al. 2017). Increased intestinal epithelial turnover has also been described in several protozoan infections (Buret et al. 1990) and basal and luminal epithelial proliferation of mouse prostates is induced by *Toxoplasma gondii* infection (Colinot et al. 2017). These mechanisms are poorly documented in teleosts and no reports exist in this regard about myxozoan infections. During common carp intestinal infection with the coccidian *Goussia carpelli*, an increase in the epithelial turnover rate occurred in order to counteract the epithelial destruction exerted by the parasite and regenerate the tissue (Hemmer et al. 1998). At initial phases of *Enteromyxum scophthalmi* infection in turbot, an increase in epithelial apoptosis prior to desquamation was observed, which was attributed either to the direct damage on the enterocytes by the parasite and/or its secretions, or to an increase in epithelial cell turnover in order to expel the parasite (Losada et al. 2014). Turbot enteromyxosis ends

up in lethal catarrhal enteritis and the parasite is much more pathogenic in this host than *E. leei* in gilthead sea bream. Transcriptomic analyses of turbot enteromyxosis revealed a complex cell death/proliferation balance, but in the pyloric caeca of infected turbot, tissue repair and cell proliferation genes (including *pcna*) were significantly up-regulated (Robledo et al. 2014). Similarly, a complex interplay between host and/or parasite mediated apoptosis and cell proliferation was reported for the gene expression in *E. leei*-infected PI of gilthead sea bream, in which the regenerative action was evidenced by the up-regulation of genes involved in cell proliferation (Calduch-Giner et al. 2012; Davey et al. 2011).

Interestingly, BrdU immunoreactive cells were most abundant at the MI of RCPT, compared to CTRL fish, at the early time after exposure to the parasite. MI was the only non-parasitized intestinal section of all the sampled fish at t1, when total prevalence of infection reached 42.9%. Intestines of fish histologically diagnosed as non-infected, and non-infected MI adjacent to parasitized segments, even presented significantly increased numbers of proliferating cells. Thus, exposure to *E. leei* seemed to trigger epithelial cell renewal in the intestine, even before the parasite attached and penetrated the lining epithelium or tissue damage was observed. This became more evident by merging t1 and t2 data (Fig. 4). The highest differences in the number of BrdU immunoreactive cells were found between CTRL and parasitized RCPT intestinal segments. However, an increase in immunoreactive cells was detected in non-parasitized intestinal segments of RCPT fish, which was even higher in non-parasitized AI and MI when their adjacent PI was parasitized.

Hemmer et al. (1998) reported increased epithelial cell proliferation in common carp only associated to damaged intestinal epithelia by *G. goussia* infection and proliferation was highest in severely infected intestine portions compared to acutely

affected ones. Cell proliferation in teleost intestines during the immune response to helminths is restricted to wounds and parasite attachment sites (Dezfuli et al. 2016a). Nevertheless, the prominent enterocyte renewal in gilthead sea bream was not restricted to injured and parasitized sites, pointing towards an early parasite-induced immunoregulation, in line with the observed cell proliferation among inflammatory infiltrates of RCPT fish. Of note, the systemic recruitment of migrating immune cell populations is locally complemented by *in situ* cell proliferation at the infection site. Previous studies demonstrated an early expression of pro-inflammatory cytokines at the PI occurring simultaneously with the current early cell proliferation (Pérez-Cordón et al. 2014). Regulation of epithelial cell proliferation in response to macroparasite infections is associated to a pro-inflammatory immune response in murine intestines (Cliffe et al. 2005) and in teleost skin (Kania et al. 2010), involving cytokines such as TNF α and IL-1 β . Furthermore, studies relate regulation of epithelial cell proliferation to IFN- γ production, which in turn modulates interleukin synthesis by macrophages (Artis et al. 1999; Cliffe et al. 2005), and IFN-related pathways were up-regulated and played a main role at systemic as well as intestinal level during enteromyxosis in gilthead sea bream (Davey et al. 2011) and turbot (Ronza et al. 2016). Previous studies found a local shift towards an anti-inflammatory cytokine response at 64 dpi (Pérez-Cordón et al. 2014), supporting the gene expression reported for 113-day chronic exposure (Sitjà-Bobadilla et al. 2008). This shift, in order to protect the fish intestine from an excessive inflammatory response, was currently reflected by means of BrdU immunohistochemistry as a slight decrease of the intestinal epithelial cell proliferation in t2, at the AI and MI.

However, since *E. leei* was directly inoculated into the fish intestine by anal route, we cannot discard that innate mucosal mechanisms were initiated before parasite

establishment, shortly after inoculation, which might eventually lead to the increase of cell proliferation detected at 17 dpi. Induction of urothelial cell proliferation was triggered in mice by pathogenic bacteria attachment and recognition prior to tissue damage, together with promotion of an early pro-inflammatory immune response (Mysorekar et al. 2002).

Notably, no differences in epithelial cell proliferation were found among intestinal segments within any of the experimental groups at any of the sampling points, though the parasite affected segments with different intensity and prevalence of infection. The PI was the segment with the highest abundance of BrdU immunoreactive cells in RCPT compared to CTRL fish and with the highest prevalence of infection at 64 dpi. In any case, the role of epithelial cell renewal in expelling *E. leei* from the fish intestine was quite limited and ineffective, since all fish except one were parasitized at t2, and PI epithelia were massively invaded by the myxozoan. Susceptibility to dietary induced enteritis in salmonids has been associated to slower enterocyte turnover rates, as in the Atlantic salmon PI (Chikwati et al. 2013) or in the rainbow trout PI (Yoshida et al. 2016). Both fish species counteracted tissue damage at the PI by accelerating enterocyte turnover rates. In the present study, fish were unable to elicit a protective response, though parasite exposure seemed to arouse increased numbers of proliferating intestinal cells. Thus, a parasite-driven modulation process to achieve or maintain a more suitable niche for parasite dwelling (immature enterocytes, modified mucus secretion and microbiota), should not be discarded. This parasite-driven process could be a strategy to increase infectivity by shedding viable parasite stages, which would thereafter infect further intestinal segments and hosts. Immature epithelial cells with impaired absorptive functions spread along the surface of the mucosal folds after the increase of the cell turnover rate during carp coccidiosis (Hemmer et al. 1998), which

may also not be immunologically competent and thus impair barrier function, antigen presentation or expression of antimicrobial peptides, pathogen recognizing receptors and cytokines (Álvarez-Pellitero 2011). An intestinal epithelial barrier mainly consisting of undifferentiated, immature enterocytes was also observed in salmonids affected by dietary induced enteritis (Bakke-McKellep et al. 2007; Chikwati et al. 2013; Krogh et al. 2003). Additionally, modulation of the mucin products in *E. leei* infected gilthead sea bream intestines was reported (Estensoro et al. 2012; Pérez-Sánchez et al. 2013), which probably involves a shift of the resident and transient microbiota as observed by the decreased bacterial adhesion to the secreted mucus mucins (Estensoro et al. 2013). Eventually, the microbiota might also affect the transit rate of the intestinal epithelial cells, as described in mice (Savage et al. 1981).

The present study also aimed to clarify if the parasite-induced modulation of the intestinal cell proliferation extended further than the parasite's target tissue and affected lymphohaematopoietic organs. Gene expression of *pcna*, which is necessary for DNA synthesis, both in replication and repair and rises during G1/S phase (Mailand et al. 2013), was analysed. Our results revealed a higher *pcna* gene expression in RCPT than in CTRL fish only at the intestinal level (PI) in t1, indicating that enhanced cell renewal occurred mainly locally, most likely in an attempt to substitute the infected epithelia and expel the parasite. This local response occurred with higher intensity at the early time after parasite exposure (t1), supporting BrdU immunohistochemical observations and correlating with the shift towards an anti-inflammatory immune response (Pérez-Cordón et al. 2014) restraining cell proliferation in t2. Though non-significantly, the opposite trend of decreased cell proliferation at t1 and increased proliferation at t2 was detected in both, Spl and Hk. This would be coherent with an early acute response mainly focused on the innate defence in the intestine, the infection site, whereas adaptive

immune mechanisms are launched at systemic level, later during the lasting chronic infection. Hence, the haematopoietic proliferation would indicate the onset of the maturation and initiation of the humoral and cellular adaptive response after antigen presentation, according to the massive leukocyte increase (plasma cells/B cells and mast cells) reported in Spl and Hk of *E. leei*-infected gilthead sea bream after 40 dpi (Estensoro et al. 2014) and to the reported activation of IFN- γ pathway, which promotes antigen presentation through activation of *mhc* gene expression (Davey et al. 2011).

In any case, it should not be disregarded, that both BrdU labelling and *pcna* expression do not only indicate cellular replication, but also DNA repair (Chikwati et al. 2013). In addition to the evident damage inflicted by the parasite to the epithelial integrity, cellular stress and damage were reported by means of up-regulated heat shock protein genes in *E. leei*-infected gilthead sea bream (Sitjà-Bobadilla et al. 2008). Thus, the immunoreactive cells detected in the epithelium might be considered as non-fully differentiated enterocytes, which still retain their replicative capacity, or as cells damaged by the direct effect of the parasite or by the host's inflammatory reaction, which are under repair process.

The life cycle of myxozoans in fish is dominated by proliferative stages composed of a single cell, containing one to several hundred cells within its cytoplasm. The outer cell is referred to as the primary cell or mother cell and the internal cells as secondary or daughter cells. Secondary cells can also harbour inner cells named tertiary cells (Feist et al. 2015). In the current study, BrdU incorporation was detected into the nucleus of a myxozoan species, particularly in *E. leei* young internal daughter cells (secondary and tertiary cells). The proliferative rate of the parasite presented scarce variation along time and segments. However, at the PI, parasite proliferation rate increased at t2, and MI was the segment with the highest rate, which would be in

agreement with the fact that MI is the last segment to be invaded by the parasite. Labelling with thymidine analogues has also been used to study the cell cycle of protozoan and metazoan parasites, such as *Schistosoma*, *Leishmania* or *Trypanosoma* (da Silva et al. 2017; Galanti et al. 2012; Mandell and Beverley 2017), and even to track the possible *in vitro* proliferation of another myxozoan, *Enteromyxum scophthalmi* (Redondo et al. 2003). However, it cannot be applied for those organisms in which replication does not occur mainly via canonical binary fission (but by schizogony), as for *Plasmodium* parasites. Schizogony is a common form of asexual division in the Apicomplexa, in which the nucleus divides several times, before the cytoplasm divides into smaller new multiple uninucleate cells (merozoites). These parasites do not incorporate BrdU because they rely only on *de novo* synthesis of pyrimidines and do not salvage thymidine analogues like BrdU for conversion into nucleotides (Merrick 2015). In most species, two metabolic routes contribute to the intracellular deoxyribonucleotides (dNTP) pool, the *de novo* and the salvage pathway (Reichard 1988). Within myxozoans, the process of formation of all internal cells has been controversial and referred as endogeny. Some authors have regarded that endogeny occurs through engulfment, followed by mitosis of the internalized cells and not through endogenous budding (Morris 2010). Endogenous budding, though presented as a dogma for many decades, has never been confirmed. In any case, schizogonial division would be discarded by the current results, since BrdU was clearly incorporated into *E. leei* secondary and tertiary daughter cells.

The present study highlights the intense activation of intestinal epithelial cell proliferation, suggesting the involvement of the local immune response, in contrast to the absence of cell proliferation at a lymphohaematopoietic level. This process appears

475 to be triggered by the exposure of the parasite, but further studies are required to unravel
476 the underlying immune-regulating mechanisms.

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Tables

Table 1 *Enteromyxum leei* infection in gilthead sea bream in the anterior (AI), middle (MI) and posterior (PI) intestinal segments at two sampling times: t1 at 17 days post intubation (dpi), and t2 at 64 dpi. Infection intensity is indicated with a semiquantitative conventional scale.

	t1 (n = 7)			t2 (n = 7)		
Intestinal segment	AI	MI	PI	AI	MI	PI
Prevalence of infection (%)	14.3	0	42.9	57.1	14.3	85.7
Mean intensity of infection	2+	-	2+	3.3+	6+	5.2+

Figure legends

Fig. 1 Giemsa staining (a-c) and BrdU immunodetection (d-g) in gilthead sea bream intestinal sections. Non-parasitized (d) and *Enteromyxum leei*-parasitized (a-c, e, g) recipient fish sections; control non-infected fish intestinal section (f). Proliferative parasite stages (white arrowheads) were detected in t1 (a) and disporoblasts with mature spores (black arrowheads) in t2 (b). Note the hyperplasia of the lamina propia-submucosa (c) and the presence of immunoreactive cells (d, e) and parasite stages (e, asterisks) in the epithelium. Immunoreactive epithelial cells reaching the mucosal fold tips (arrowheads) in a control intestinal section (f). *E. leei* stages (arrows) with immunoreactive daughter cells in the intestinal epithelium (g). Scale bars = 20 µm.

Fig. 2 BrdU immunodetection in the intestine of control uninfected (CTRL) and recipient (RCPT) gilthead sea bream experimentally infected with *Enteromyxum leei* at

17 (t1) and 64 (t2) days post intubation. Anterior (AI), middle (MI) and posterior (PI) intestinal sections are shown. Scale bars = 50 μ m.

Fig. 3 Number of BrdU immunolabelled intestinal cells (mean + SEM, $n = 7$) at time 1 (t1, 17 dpi) and time 2 (t2, 64 dpi) in control (CTRL) and recipient (RCPT) gilthead sea bream experimentally infected with *Enteromyxum leei*. Equal upper or lower case letters indicate no statistical significant differences among intestinal segments (anterior (AI), middle (MI), posterior (PI)) within each group. Asterisks indicate statistical significant differences between CTRL and RCPT fish within each intestinal segment (* = $P < 0.05$; ** = $P < 0.001$).

Fig. 4 Number of BrdU immunolabelled intestinal cells (mean + SEM) at the anterior, middle and posterior intestinal segments for pooled data from t1 and t2. Four fish categories were created according to the presence of *Enteromyxum leei*: control (CTRL); RCPT fish non-parasitized at any segment (NP); RCPT fish non-parasitized at anterior or middle intestines, but parasitized at the posterior intestine (NP-PAR PI); RCPT fish parasitized AI/MI and also parasitized at PI (PAR). Different letters indicate statistically significant differences ($P < 0.05$) among fish categories within each intestinal segment. The red arrows above each segment indicate the mean fold increase between CTRL and PAR fish.

Fig. 5 *pcna* gene expression in the posterior intestine (PI), spleen (Spl) and head kidney (Hk) of recipient (RCPT) gilthead sea bream experimentally infected with *Enteromyxum leei*, at t1 and t2 samplings (mean + SEM, $n = 6$ fish). Fold changes are relative to each

770 time point control, represented by the dotted line at $y = 1$. Asterisk (*) indicates
771 statistically significant difference with the respective time point control ($P < 0.05$).

Fig. 1 Giemsa staining (a-c) and BrdU immunodetection (d-g) in gilthead sea bream intestinal sections. Non-parasitized (d) and *Enteromyxum leei*-parasitized (a-c, e, g) recipient fish sections; control non-infected fish intestinal section (f). Proliferative parasite stages (white arrowheads) were detected in t1 (a) and sporoblasts with mature spores (black arrowheads) in t2 (b). Note the hyperplasia of the lamina propria-submucosa (c) and the presence of immunoreactive cells (d, e) and parasite stages (e, asterisks) in the epithelium. Immunoreactive epithelial cells reaching the mucosal fold tips (arrowheads) in a control intestinal section (f). *E. leei* stages (arrows) with immunoreactive daughter cells in the intestinal epithelium (g). Scale bars = 20 μm .

173x185mm (300 x 300 DPI)

Fig. 2 BrdU immunodetection in the intestine of control uninfected (CTRL) and recipient (RCPT) gilthead sea bream experimentally infected with *Enteromyxum leei* at 17 (t1) and 64 (t2) days post intubation. Anterior (AI), middle (MI) and posterior (PI) intestinal sections are shown. Scale bars = 50 μ m.

173x142mm (300 x 300 DPI)

Fig. 3 Number of BrdU immunolabelled intestinal cells (mean + SEM, n = 7) at time 1 (t1, 17 dpi) and time 2 (t2, 64 dpi) in control (CTRL) and recipient (RCPT) gilthead sea bream experimentally infected with *Enteromyxum leei*. Equal upper or lower case letters indicate no statistical significant differences among intestinal segments (anterior (AI), middle (MI), posterior (PI)) within each group. Asterisks indicate statistical significant differences between CTRL and RCPT fish within each intestinal segment (* = P < 0.05; ** = P < 0.001).

130x113mm (300 x 300 DPI)

Fig. 4 Number of BrdU immunolabelled intestinal cells (mean + SEM) at the anterior, middle and posterior intestinal segments for pooled data from t1 and t2. Four fish categories were created according to the presence of *Enteromyxum leei*: control (CTRL); RCPT fish non-parasitized at any segment (NP); RCPT fish non-parasitized at anterior or middle intestines, but parasitized at the posterior intestine (NP-PAR PI); RCPT fish parasitized AI/MI and also parasitized at PI (PAR). Different letters indicate statistically significant differences ($P < 0.05$) among fish categories within each intestinal segment. The red arrows above each segment indicate the mean fold increase between CTRL and PAR fish.

296x104mm (300 x 300 DPI)

Fig. 5 *pcna* gene expression in the posterior intestine (PI), spleen (Spl) and head kidney (Hk) of recipient (RCPT) gilthead sea bream experimentally infected with *Enteromyxum leei*, at t1 and t2 samplings (mean + SEM, n = 6 fish). Fold changes are relative to each time point control, represented by the dotted line at y = 1. Asterisk (*) indicates statistically significant difference with the respective time point control (P < 0.05).

88x54mm (300 x 300 DPI)