Modulation of the IgM gene expression and IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (*Sparus aurata*) challenged with *Enteromyxum leei* (Myxozoa)

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ABSTRACT

The aim of the present work was to determine if a plant protein-based diet containing vegetable oils (VO) as the major lipid source could alter the distribution of IgM immunoreactive cells (IRCs) and the IgM expression pattern in the intestine and haematopoietic tissues of gilthead sea bream (GSB) (*Sparus aurata*) challenged with the myxosporean *Enteromyxum leei*. In a first trial (T1), GSB fed for 9 months either a fish oil (FO) diet or a blend of VO at 66% of replacement (66VO diet) were challenged by exposure to parasite-contaminated water effluent. All fish were periodically and non-lethally sampled to know their infection status. After 102 days of exposure, samples of intestine and head kidney were obtained for IgM expression and immunohistochemical detection (IHC). Additional samples of spleen were taken for IHC. Fish were categorized as control (C, not exposed), and early (E), or late (L) infected. The 66VO diet had no effect on the number of IgM-IRCs in any of the tissues or on IgM expression in C fish, whereas the infection with *E. leei* had a strong effect on the intestine. A combined time-diet effect was also observed, since the highest expression and IRCs values were registered in the posterior intestine (Pi) of E-66VO fish. A positive correlation was found between IgM expression and the presence of IgM-IRCs in the Pi. The effect of the time of infection was studied more in detail in a second trial (T2) in which samples of Pi were taken at 0, 24, 51, 91 and 133 days after exposure to the parasite. A significant increase of the IgM expression was detected only in parasitized fish, and very late after exposure. These results show that the duration of the exposure to the parasite is the most determinant factor for the observed intestinal IgM increased phenotype which gets magnified by the feeding of a high VO-based diet.

1. Introduction

Farmed fish are constantly exposed to different types of stressors such as high density culture conditions, inadequate diets or infections which might compromise their immune response. Besides viral and bacterial diseases, parasitoses have become one of the major threats for intensive fish aquaculture, as reported in recent cases of massive losses [1, 2].

Gilthead sea bream (GSB) (*Sparus aurata*) is the main cultured fish species in the Mediterranean, with a total annual production of more than 130,000 tonnes in 2010 [3]. *Enteromyxum leei* is a widespread enteric myxosporean parasite causing one of the most threatening parasitic diseases in Mediterranean sparid farms [4, 5]. In GSB, this parasite starts invading the posterior intestine (Pi) causing severe chronic enteritis with an intense inflammatory response [2]. The parasite spreads towards the anterior intestine (Ai), eventually occupying the entire intestinal tract [6-8]. During the slow progression of the infection, anorexia and cachexia are induced in the fish, leading to reduced growth performance and even death. *E. leei* outbreaks in high density stocking conditions lead to fatal consequences due to the direct fish-to-fish transmission of the parasite, either by cohabitation with infected fish or by exposure to a contaminated effluent [9, 10]. The lack of preventive and therapeutic measures to content this devastating enteromyxosis points out the urgent need for further understanding of the immune response of GSB to *E. leei*.

The increasing interest in replacing fish meal and fish oil by plant proteins and oils in aquafeeds has focused research on finding diets with optimum growth performance results without detrimental effects on fish immune status [11-14]. Previous studies on GSB with gradual levels of substitution of fish oil (FO) by vegetable oils (VO) in plant-protein based diets have demonstrated that it can be accomplished up to 66% without

any negative side-effects for the fish [15, 16]. Nevertheless, fish fed the 66% substitution diet (66VO) and challenged with *E. leei* showed a higher disease outcome than fish fed the FO diet [6]. In an effort to understand the possible underlying mechanisms involved in the greater progression of the infection in 66VO fish, we undertook a series of detailed studies of gut immunology and fish immune response in fish fed such diets and confronted with this myxosporean. Production of specific antibodies has been described for several myxosporean infections [17] and circulating antibodies against *Enteromyxum scophthalmi* produced by turbot [18] are involved in resistance to re-infection to survivor fish [19]. Furthermore, in *E. scophthalmi*-experimentally infected turbot, an increase of Ig+ cells occurred in the intestine, whereas a decrease was observed in lymphohaematopoietic tissues [20]. Protection against piscine parasitoses by generation of specific antibodies has been broadly documented [21]. Moreover, the expression and *in situ* localization of IgM in the intestine of rainbow trout suggests its important role interconnecting the humoral and local-mucosal immune responses [22].

The current work tries to decipher some of the above mentioned aspects for the GSB-*E. leei* model. We integrate the results of a first trial in which the combined effect of the myxosporean *E. leei* and the nutritional background of fish on IgM expression and localization in intestine and lymphohaematopoietic tissues was studied, with those of the kinetics of the expression of IgM in the intestine of GSB in response to the this parasite in a subsequent more focused trial.

2. Material and Methods

2.1 Experimental set up

Two experimental trials were undertaken in which naïve pathogen-free GSB were challenged by exposure to an *Enteromyxum leei*-contaminated effluent as previously described [10]. Fish were kept in 5 μm-filtered and UV-irradiated sea water (37.5‰ salinity), always at a temperature above 18 ºC. Details on water temperature of both trials can be found in Table 1. Before the experimental infections started, GSB were also checked for the absence of the parasite by non-lethal PCR diagnosis as described in Estensoro et al. (2011) [6] and fish were starved for two days before each sampling.

In the first trial (T1), samples were obtained as previously described [6]. Briefly, GSB were fed during 9 months either a FO based diet or a diet containing a blend of VOs at 66% replacement (66VO) (Supplementary Table 1, Supplementary Table 2) After this period, fish from both diet groups were exposed to *E. leei*-effluent (recipient group, R-T1, n = 30) or kept unexposed (control group, C-T1, n = 30). All fish were individually tagged with passive integrated transponders and non-lethally sampled at three consecutive times for parasite diagnosis. R-T1 fish were classified according to their first infection-timing in two categories: early infected (E), being infected at 32 or 53 days post exposure (p.e.) and late infected (L), being infected at 88 days p.e.. A final lethal sampling was performed 102 days p.e., and portions of Ai and Pi, head kidney (Hk) and spleen (Sp) were taken for immunohistochemistry. Pi and Hk samples were also immediately frozen in liquid nitrogen and stored at -80 ºC until further gene expression analyses. Parasite diagnosis was performed histologically from intestine samples.

In the second trial (T2), the control (C-T2, n = 50) group was also kept unexposed, and the recipient (R-T2, n = 40) group received the *E. leei*-effluent from a donor tank. Ten fish from each group were lethally sampled in four consecutive times, and an initial sampling of ten C-T2 fish was performed one day before the challenge. Tissue samples

of intestine were fixed in 10% buffered formalin for routine histological parasite diagnosis, and only Pi portions were collected in ice cold RNAlater solution (Ambion, TX, USA) in view of the results obtained in T1. They were kept for 24 h at 4 ºC and stored at -20 ºC until gene expression analysis was performed.

All experiments were carried out in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and institutional regulations (CSIC, IATS Review Board) and the current European Union legislation on handling experimental animals. In all lethal samplings, fish were euthanized under benzocaine anaesthesia (3-aminobenzoic acid ethyl ester, 100 mg/ml) (Sigma, St. Louis, MO, USA). Details of both trials are summarised in Table 1.

2.2 Immunohistochemical detection of IgM – Trial 1

Samples of Ai, Pi, Sp and Hk from T1 fish (C-FO n = 6; C-66VO n = 7; R-FO n = 7; R-66VO n = 7) were fixed in Bouin for 24 h, dehydrated in a graded ethanol series and embedded in paraffin. Sections (4 µm-thick) were collected on Super-Frost-plus microscope slides (Menzel-Glaser, Germany) and allowed to dry overnight. Slides were deparaffinised, hydrated and the endogenous peroxidase activity of the tissues was quenched by incubating in 0.3% (v/v) hydrogen peroxide for 30 min. Incubations were performed in a humid chamber at room temperature and all washing procedures consisted of successive 5 min immersions in TTBS (20 mM Tris-HCl, 0.5 M NaCl, 0.05% Tween 20, pH 7.2) and TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2). After washing, slides were blocked for 30 min with 1.5% normal goat serum (VECTOR Laboratories, Burlingame, CA, USA) and washed again. Sections were then incubated with a rabbit Pab anti-GSB IgM (1:60,000) obtained by Palenzuela et al. (1996) [23] for 1 h and washed. A biotinylated secondary goat anti-rabbit IgG antibody (1:200) was applied for one further hour. After washing, slides were incubated with the avidin-

biotin-peroxidase complex (ABC, VECTOR Laboratories) for 1 h and washed. Finally, bound peroxidase was visualised by addition of DAB chromogen (3,3'-diaminobenzidine tetrahydrochloride) (Sigma) for 5 min. The reaction was stopped with deionised water, and the sections counterstained with Gill's haematoxylin, dehydrated and mounted in DPX (di-*N*-butyl-phthalate in xylene). Negative controls were carried out omitting the primary antibody, the secondary antibody and the avidin-biotin-peroxidase complex, respectively, and were found to be negative.

For the quantitative analysis of IgM IRCs, ten random digital fields from each tissue section were captured with an Olympus DP70 camera connected to a Leitz Dialux22 light microscope at x400 magnification. Immunoperoxidase stained cells were counted for each field.

2.3 IgM expression

IgM expression was measured from both Pi and Hk samples obtained in T1 and from Pi samples obtained in T2. Tissues from both trials were homogenized in guanidine-detergent lysis buffer at a 50 mg/ml concentration. After protease K digestion, total RNA extraction was carried out with the ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems, Foster City, CA, USA). The vacuum-based wash and elution steps were performed according to the manufacturer's instructions. The RNA yield was 123 µg with absorbance measures of ($A_{260/280}$) 2.05-2.15, which were determined by spectrophotometry (Nanodrop 2000c, Thermo Scientific, Wilmington, DE, USA).

Reverse transcription (RT) was performed with 500 ng of the purified RNA (T1 and T2) using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) in a final volume of 100 µl. RT reaction conditions were set to 25°C for 10 min,

173 followed by 37°C for 2 h and a final step of 5 sec at 85ºC to inactivate the reverse
174 transcriptase.

175 Real-time PCR assays were carried out to quantify the abundance of intestinal and
176 renal transcript levels of IgM, using an iCycler IQ Real-time Detection System (Bio-
177 Rad, Hercules, CA, USA). The total PCR reaction volume of 25 µl included 7.5 µl of
178 diluted cDNA and 17.5 µl of IQ SYBR Green Supermix (Bio-Rad) and specific primers
179 at a final concentration of 0.3-0.9 µM to obtain amplicons of 51-134 bp in length (Table
180 2). Primers for GSB IgM were designed from a 1464 nucleotide sequence found by
181 BLAST search on the transcriptome database of the Aquamax European Project
182 (www.sigenae.org/iats). It comprises the short sequence (136 nt) present in GenBank
183 (accession AM493677) and has been submitted to GenBank with accession JQ811851.
184 β-actin was chosen as reference gene, and the amplification efficiency of PCR reactions
185 of both β-actin and IgM genes varied between 95% and 98%, respectively.

186 The dynamic range of standard curves (serial dilutions of RT-PCR reactions)
187 spanned five orders of magnitude, and the amount of product in a particular sample was
188 determined by interpolation of the cycle threshold (Ct) value.

189 Three replicates of each reaction were performed and the fluorescence data obtained
190 during the extension phase were normalized to β-actin by the delta-delta method [24]. β-
191 actin expression did not change in response to dietary treatments or to infective status.

192 *2.4 Statistical analysis*

193 One-way analyses of variance (ANOVA-I) followed by Student-Newman-Keuls test
194 were performed to detect differences in GE and IHC values within each diet group in T1
195 and within each group at the different sampling times in T2. When the test of normality
196 or equal variance failed, a Kruskal-Wallis ANOVA-I on ranks followed by Dunn's

method was applied instead. Differences in GE between C and R fish within each sampling point in T2 and between FO and 66VO groups within each time of infection (C, E, L) in T1 were analysed via t-test. Two-way ANOVAs (ANOVA-II) were performed in order to check the significance of the two factors considered in T1, time of infection and diet, and for interactions between them. A Spearman correlation test was run aiming to detect a possible correlation between gene expression and immunohistochemistry data in Pi and Hk of T1, gathering the data from both diet groups. The significance level was set at $p < 0.05$ for all tests. The statistical analyses were performed using Sigma Stat software (SPSS Inc., IL).

3. Results

3.1 Intestinal and renal IgM expression

Fig. 1 shows the IgM expression pattern in Pi and Hk obtained from GSB in T1. In the Pi, C-T1 groups had the lowest IgM expression values and no effect of the diet was observed. In R-T1groups, L-infected fish had slightly increased levels which did not differ significantly from their corresponding C groups, whereas E-infected fish presented a clearly up-regulated IgM expression, though only statistically significant for fish fed the 66VO diet, whose levels were fifteen-fold higher than those of C-T1-66VO. A significant effect of the diet was detected in both R-T1 fish, as the relative IgM expression of R-T1-E fish in the 66VO group was 3.0 times higher than in the respective FO group, and the expression of R-T1-L fish was 2.4 times higher for 66VO than for FO.

In the Hk, no effect of the diet was observed on the relative IgM expression for any of the groups. There was no statistically significant difference between R-T1 and their

221 respective C-T1 group, though a slight but not significant IgM up-regulation was
222 detected in R-T1-L-66VO fish.

223 The ANOVA-II allowed the analysis of the relationship between the factors
224 involved in this study, *i.e.* the time of infection and the diet. In the Pi, both factors
225 affected the IgM expression significantly ($p < 0.001$) and furthermore, a significant
226 interaction between them was detected ($p < 0.001$). In the Hk, no significant effect of
227 infection time or diet was detected.

228 *3.2 Kinetics of IgM expression in intestine*

229 As the results of T1 showed that IgM expression in the intestine was more prone
230 than Hk to reflect changes induced by the infection and the diet, and that a time effect
231 was also detected, the kinetics of the IgM expression in intestine were studied more in
232 detail in T2 (Fig. 2). There was a gradual non-significant increase in the intestinal IgM
233 expression in R-T2 fish at 24, 51 and 91 days p.e., which corresponded to a gradual
234 increase in the percentage of fish with a fold change ≥ 1.5 in their transcription levels.
235 Thus, at 24 days p.e., 25% of R-T2 fish had reached this fold change in their intestinal
236 IgM expression, reaching 60 % at 91 days p.e.. However, the IgM transcription of R-T2
237 fish was significantly and clearly up regulated at 133 days p.e. ($p < 0.001$) *vs.* C-T2,
238 with values higher than those of R-T2 fish at 0, 24 and 51 days p.e.. There were no
239 statistically significant differences among C-T2 groups. When R-T2 fish were classified
240 in parasitized and non-parasitized fish, regardless of their sampling time, the up-
241 regulation of the IgM intestinal expression was statistically significant ($p < 0.05$) only
242 for parasitized fish, though an increasing trend was observed for non-parasitized fish
243 (inset, Fig. 2).

244 *3.3 Detection of intestinal, renal and splenic IgM-IRCs*

IRCs for the anti-GSB IgM antibody were found in Ai, Pi, Hk and Sp (Figs. 3, 4). Most positive cells exhibited the typical morphology of plasma cells with ovoid shape, a variable amount of cytoplasm depending on their degree of differentiation, usually voluminous, and a round or oval nucleus (large cytoplasm/nucleus ratio) (Fig.5). Cells morphologically resembling lymphocytes, round with small cytoplasm (high cytoplasm/nucleus ratio), were often present at the epithelial base and submucosa of R-T1 fish, though most of them were not immunoreactive (Figs.5E, 5F). The label obtained with the polyclonal anti-IgM antibody was strong and located in the cytoplasm and varying degrees of immunoreactivity were observed in positive cells (Figs. 5A, 5G). Another very scarce, morphological IgM-IRC type was detected in intestine as well as in lymphohaematopoietic tissues of both, C-T1 an R-T1 fish (Figs. 5I, 5K, 5L). These IgM-IRCs were round shaped, presented an eccentric nucleus and a voluminous cytoplasm with variable immunoreactivity, *i.e.* intense and homogeneous staining or weakly stained center with a peripheral strongly stained fringe.

In intestinal as well as in lymphohaematopoietic tissues, the C-T1 fish presented the lowest numbers of IgM-IRCs, regardless of the diet. No statistically significant differences occurred between any of the diet groups, though R-T1-66VO fish presented the highest counts of IgM-IRCs, with the only exception of R-T1-E in Hk for FO fish (Fig. 3).

In intestinal sections, IgM-IRCs were located mainly in the lamina propria and submucosa (Figs. 4B, 4D). Occasionally, IgM-IRCs were detected in close vicinity to blood vessels or to parasite stages in the epithelium and sporadically forming cell clusters (Figs. 5D, 5G, 5H, 5J). Infiltrated plasma cells between intestinal enterocytes were often observed in parasitized epithelia (Fig. 5G), showing in some occasions

cytoplasmatic protrusions (Fig. 5D) or being apparently in direct contact with the parasite even surrounding it (Figs. 5H, 5J).

At the Ai, R-T1-E fish presented the highest numbers of IgM-IRCs, regardless of the diet. Thus, the R-T1-E-66VO group exhibited a statistically significant increase of IRCs compared to the correspondent C-T1 group ($p < 0.05$) (Figs. 3A, 4C, 4D). A statistically significant effect of the time of infection on the IgM-IRC abundance ($p = 0.004$) was detected by the ANOVA-II in the Ai. At the Pi of R-T1, the main target site of *E. leei*, IgM-IRCs were more abundant than at the Ai. The same pattern of IRC distribution was observed, the number of IRCs being significantly higher in R-T1-E fish than in C-T1 in both dietary groups ($p < 0.001$ for FO; $p = 0.01$ for 66VO) (Figs. 3B, 4A, 4B). The time of infection had a statistically significant effect ($p < 0.001$) on the number of IRCs in the Pi.

IgM-IRCs in head kidney exhibited the morphology of plasma cells, as described above (Fig. 5B). They were scattered throughout the interstitial tissue, isolated or forming clusters, especially in the vicinity of ellipsoids. Occasionally, they were also found in close contact to melanomacrophage centers (MMCs). R-T1-E fish presented a statistically significant increase of IRCs compared to C-T1, only for animals fed the FO diet ($p < 0.05$) (Figs. 3C, 4E, 4F). A significant effect ($p < 0.009$) of the time of infection on the IRC abundance was detected by the ANOVA-II in this organ.

C-T1 and R-T1 fish presented IgM-IRCs dispersed throughout the spleen parenchyma, isolated as well as clustered (Figs. 4G, 4H, 5C). These positive cells exhibited also the typical plasma cell morphology and were often found around blood vessels, occasionally close to MMCs. A non significant increase of IRCs in the Sp of R-T1 was detected, which was stronger in the 66VO group (Fig. 3D). No statistically significant effect of the time of infection or the diet was observed.

No statistically significant interaction between both factors affecting the IgM-IRC abundance, *i.e.* the time of infection and the diet, was detected by the ANOVA-II in none of the analyzed tissues.

3.4 Correlation analysis: mRNA IgM transcrips vs. IgM-IRCs

The transcription of IgM in the Pi of T1 fish was positively correlated with the number of IgM-IRCs in this tissue ($p = 0.000$), with a correlation coefficient, $r_s = 0.612$. Thus, high IgM gene expression levels coincided with high amounts of IgM-IRCs in the Pi, regardless of the diet group or time of infection. In Hk, IgM transcription and the number of IgM-IRCs were not significantly correlated ($p = 0.194$).

4. Discussion

There is a growing demand for new approaches combining nutritional and immunological studies to improve health management and animal welfare in aquaculture. The current study is the first one in focusing on the modulation of IgM by a myxosporean infection and by the nutritional background of GSB at both molecular and cellular levels, aiming to understand the local-systemic interaction. Immunohistochemistry demonstrated the presence of IgM-IRCs in all examined tissues, even in non-exposed fish, but IgM-IRCs were more abundant in the intestine than in lymphohaematopoietic organs in exposed fish. Most detected IgM-IRCs were compatible with plasma cells, followed by small amounts of IR-B-cells. Both IgM-bearing cell types had already been described in salmonids [25] and were also involved in the immune response of turbot to *E. scophthalmi* [20]. The IgM-IR macrophages containing strongly positive phagosomal-like structures observed in the latter infection model were not observed in the present study. Other Ig-containing cell types such as macrophages, neutrophils and non-specific cytotoxic cells have been found in several

fish species [26, 27], probably due to Ig-binding to Fc receptors or due to phagocytized immune complexes or plasma cells. Additionally, a scarce and unidentified IgM-IRC type with a variable but consistent cytoplasmatic staining was observed, suggesting variable stages of differentiation. To our knowledge, this unidentified IgM-IRC type has only been described in another enteromyxosis [20], but its size in GSB was not even half as large (4.5 - 5 μm) as in turbot (11 - 13 μm). IgM negative lymphocyte-like cells were often detected at the epithelial base and submucosa of the R-T1 (parasitized fish) intestines. These were also described in *E. scophthalmi* infected turbot and interpreted as T-cells [20].

IgM-IRCs were scattered in the lymphohaematopoietic tissues of C-T1 (unexposed fish), being the basal levels higher in Hk than in the remaining studied organs. By contrast, IgM-IRCs formed outstanding clusters around blood vessels in R-T1 fish, sometimes in close contact to MMCs. The connective tissue surrounding arterioles in these organs might be the equivalent to peri-arteriolar lymphoid sheaths (PALS) of mammals, which are poorly developed and not always present in teleost fish [28-31]. In PALS, lymphocytes and plasma cells accumulate, and are related to antigen trapping and lymphocyte stimulation. Furthermore, plasma cell clusters are considered primitive germinal centres, especially those close to antigen retaining MMCs [20, 27, 31-33]. Thus, the higher clustering of IgM-IRCs in Hk and Sp of infected fish, together with the higher values in E-infected fish, would indicate a higher cell stimulation and differentiation and the key role of these tissues in the initiation of an adaptive immune response, for antigen trapping and presentation functions. Parasitized fish also exhibited hypertrophied submucosal intestinal areas with high density of plasma cells, in comparison with the low numbers of C-T1. This increase indicates clearly a mucosal immune response, either produced locally or derived from blood or other organs. These

observations agree with the dispersed GALT structure of teleosts and the existence of a mucosal immune system responsible for the local synthesis of mucosal Ig in secretions [34].

The intestine of GSB, the target site for the parasite, also underwent the highest IgM up-regulation during the infection, reaching fifteen-fold and six-fold increases of IgM mRNA expression in the Pi of R-T1 and R-T2 fish, respectively. This increase correlated strongly with the highest levels of IgM-IRCs in this tissue. By contrast, no significant changes were observed in the expression levels in Hk, and therefore no correlation was found between IgM transcripts and IgM-IRCs. Studies integrating gene expression and *in situ* detection of Igs in fish are scarce, and they depict a great variability among fish species and pathogens. In Atlantic halibut lymphoid organs, a positive correlation was observed between IgM expression and ISH-IgM+ cells in Hk and Sp [35], whereas in European sea bass experimentally infected with betanodavirus, up-regulation of IgM in Hk occurred weeks before the number of B-cells from blood increased [36]. The ciliate *Ichthyophthirius multifilis* increased the IgM expression in the gills of rainbow trout, which was related to an active efflux of IgM-IRCs through the lamellar capillaries towards the site of infection [22]. In our study, no IgM-IRCs were detected in blood vessels, but they were found in high densities around them as a sign of influx and efflux from the blood stream. Thus, in the GSB-*E. leei* model, IgM also seems to be linking the systemic humoral immunity and the local response. The current results are in accordance with the central role of IgM in gut mucosal immune reactions [33, 37-39] in response to threatening situations (pathogens [36, 40-42], or environmental stress [43-45]). However, we cannot discard the possible action of another Ig isotype, IgT/IgZ, which seems to act exclusively in mucosal areas, and has

been described in very few fish species, with outstanding results in a myxosporean infection [40, 42, 46, 47], but not yet found in GSB.

The intense IgM synthesis/mobilization accounted for plasma cells and B-cells, did not however, result in a substantial level of protection against *E. leei* infection, as a high prevalence and intensity of infection was achieved in both trials. Several examples exist of adaptive humoral immune responses that do not result in a substantial level of protection [37, 48]. Failure of the IgM defence against *E. leei* might be attributed to resistance mechanisms described for other parasites, such as suppression of antibody function or avoidance of recognition [49]. In fact, specific antibodies against *E. leei* in GSB were detected from 50 days on after exposure in a low number of fish, and high antibody titres were found only in fish from long-lasting exposures or survivors of epizootics [50]. In turbot, specific antibodies against *E. scophthalmi* also appeared late after infection and antibody mediated resistance to this parasite only occurs in some previously exposed fish [18, 19]. Similarly, binding of rainbow trout antibodies to *Ichthyophthirius multifilis* was observed in gills and contributed to the exit of the parasite [40].

Previous studies revealed that the administration of the 66VO diet did not entail any harmful consequences for the fish growth or gut tissue integrity [15, 51-53], but was a predisposing factor that worsens the progression and severity of the disease in experimentally infected fish [6]. In the present study, the diet did not account for any detectable differences among the C-T1 fish, neither for the IgM mRNA expression nor for the IgM-IRC abundance. This agrees with previous data obtained from another trial in which no differences in total serum IgM were detected between FO and 66VO fish (unpublished results). The diet effect was only observed in the intestine when fish were exposed to the parasite (R-T1), as 66VO infected fish had increased intestinal IgM

392 expression. Furthermore, 66VO fish bearing the infection for a longer time period (R-
393 T1-E-66VO) underwent the strongest up-regulation. The effect of the diet on IgM was
394 also evident at cellular level in both intestine sections, as the highest numbers of IgM-
395 IRCs were observed in R-T1-66VO fish. Although modulation of immune related
396 factors/genes by dietary factors is well recognised, understanding the underlying
397 mechanisms of action is still a broad challenge. The 66VO diet contains lower levels of
398 *n*-3 long-chain polyunsaturated fatty acids than the control FO diet (supplementary
399 Table 2) and this could enhance the synthesis of pro-inflammatory products *via* a high
400 arachidonic:eicosapentanoic acid ratio [54]. This diet effect on the IgM profile only at
401 the intestinal level points to a direct local action. Some mechanisms affecting membrane
402 fluidity and therefore permeability [34, 55, 56] of the epithelial barrier might be
403 responsible for the severe infection in 66VO fish, which in turn would induce an up-
404 regulation of immune relevant genes and a stronger immune response.

405 The time of infection was a predominant factor in IgM dynamics, as evidenced
406 by IgM expression in Pi and IgM-IRC abundance in Ai, Pi and even Hk, in T1. R-T1-E
407 fish (early infected, and therefore with a well established infection) had increased
408 numbers of IgM-IRCs in these three organs, but these differences were only significant
409 at the Pi for both diet groups. The lower values of R-T1-E in Ai than in Pi were
410 probably due to the also lower prevalence of infection at the Ai [6]. Thus, a pattern of
411 IgM-IRC richness (E > L > C) can be drawn for intestinal segments and also for Hk of
412 FO fish, which differed from that observed in the Hk and Sp of 66VO (L > E > C). In
413 fact, the Hk of R-T1-L-66VO presented the highest number of IgM-IRCs among the
414 organs of recently infected fish. This decreasing trend in the lymphohaematopoietic
415 organs of early infected fish could indicate the beginning of plasma cell/B-cell
416 depletion, but cannot be considered a significant depletion of Ig-IRCs, as occurred in *E.*

scophthalmi-infected turbot [20], which could be explained by the higher pathogenicity of *E. scophthalmi* [57] that could account for a higher immunosuppressive effect.

In T2, the effect of the time of infection on IgM expression was confirmed. A progressive but slight increase was detected from 24 to 91 days p.e., which ended up with a very strong up-regulation of the IgM expression in the Pi of R-T2 fish at 133 days p.e., which coincided with a high prevalence and intensity of infection. Interestingly, non-parasitized R-T2 fish experienced a slight up-regulation, suggesting the on-set of a local immune response to confront the parasitic challenge, even before the parasite settlement takes place. Thus, a true parasite invasion was the main triggering factor of IgM mRNA expression in the Pi. Similar up-regulation of IgM has been documented in other pathogen (parasites, bacteria or virus) models, but with substantial differences in the timing [36, 37, 41]. It seems that both local and systemic adaptive immune responses are triggered against such pathogens, but in the GSB-*E. leei* model the local immune response seems to prevail and the increased IgM profile (expression and IRC) occurs remarkably later (>100 days p.e) than in the aforementioned studies. In fact, the higher IgM expression values of Pi in R-T2 fish (six-fold up-regulation, 133 days p.e.) than those of R-T1-E-FO fish (five-fold up-regulation, 102 days p.e.) could be due to the 31 days longer exposure in T2.

In conclusion, both the immunohistochemical and the gene expression studies showed that the increase of IgM is more pronounced at the local level where the parasite proliferates (intestine) than in lymphohaemopoietic organs. The time of exposure to the parasite (which determines the infection level) is the most determinant factor for the observed intestinal IgM increased phenotype, but gets magnified by long term feeding of a high VO diet which however did not affect growth or nutrient utilization.

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FIGURE CAPTIONS

646 **Fig. 1** IgM transcript levels in posterior intestine (Pi) (**A**) and head kidney (Hk) (**B**)
647 during Trial 1 (T1). Data of C-FO fish were used as arbitrary reference values in the
648 normalization procedure (values > 1 or < 1 indicate decrease or increase according to
649 the reference values). Upper and lower case letters indicate statistically significant
650 differences between C, L and E fish of the FO and the 66VO diet, respectively ($p <$
651 0.05). Statistically significant differences between diet groups are indicated by * ($p <$
652 0.05).
653
654 **Fig. 2** IgM transcript levels in the posterior intestine (Pi) of gilthead sea bream exposed
655 to *Enteromyxum leei* by effluent in Trial 2 (T2) at different sampling times. Data of C
656 fish at 0 days post exposure (p.e.) were used as arbitrary reference values in the
657 normalization procedure (values > 1 or < 1 indicate decrease or increase according to
658 the reference values). Numbers above each bar of R fish indicate the percentage of fish
659 with a fold change ≥ 1.5. The inset graph shows the expression values after grouping all
660 the sampling days and splitting the values of R fish in parasitized (PAR) and non-
661 parasitized (NON-PAR) fish. Upper and lower case letters indicate statistically
662 significant differences ($p < 0.05$) at different times among C and R groups, respectively.
663 Significant differences between C and R groups are indicated by ** ($p < 0.001$).
664
665 **Fig. 3** Average counts of IgM+ cells in anterior intestine (Ai) (**A**), posterior intestine
666 (Pi) (**B**), head kidney (Hk) (**C**) and spleen (Sp) (**D**) of gilthead sea bream in Trial 1 (T1).
667 Upper and lower case letters indicate statistically significant differences between C, L
668 and E fish of the FO and the 66VO diet, respectively ($p < 0.05$).
669
670 **Fig. 4** Photomicrographs of immunohistochemistry for IgM in control unexposed (A, C,
671 E, G) and recipient (B, D, F, H) gilthead sea bream paraffin sections counterstained with

heamatoxylin. **A-B**, Posterior intestine: A, fish oil diet (FO) fish; B, early infected, 66% replacement vegetable diet (66VO) fish. Note the epithelial parasite stages (arrows) and the abundant immunoreactive (IR) plasma cells in the submucosa. **C-D,** Anterior intestine: C, 66VO fish; D, early infected, 66VO fish. Note the epithelial parasite stages (arrows) and the abundant IR plasma cells in the submucosa. **E-F,** Head kidney: E, FO fish; F, early infected, FO fish. Note the gathering of IR plasma cells around blood ellipsoids (asterisks) in recipient fish. **G-H,** Spleen: G, 66VO fish; late infected 66VO fish. Note the gathering of IR plasma cells around blood vessels (asterisks) in recipient fish. Scale bar = 20 µm.

Fig. 5 Photomicrographs of immunohistochemistry for IgM in recipient gilthead sea bream intestine (Fig. A, D, E, F, G, H, I, J, K), head kidney (Fig. B) and spleen (Fig. C, L) paraffin sections counterstained with heamatoxylin. Epithelial parasite stages are indicated with arrows. **A**, immunoreactive (IR) plasma cells presenting variable differentiation degrees in the submucosa. **B**, IR plasma cells forming a cluster close to a blood ellipsoid (asterisk). **C**, scattered IR plasma cell. **D**, IR plasma cell infiltrating in the epithelium. Note its cytoplasmatic protrusions. **E**, IR B-cell (arrowhead). **F**, IR plasma cells in the submucosa and numerous lymphocyte-like IgM negative cells (arrowheads). **G**, IR plasma cells infiltrated in the epithelial base. **H**, IR plasma cell (arrowhead) adjacent to an epithelial parasite stage. **I**, unidentified epithelial IR cell with a strong homogeneous staining in the cytoplasm (arrowhead) and goblet cells (GC). **J**, IR plasma cell surrounding parasite stage. **K**, unidentified epithelial IR cell (arrowhead) presenting a weakly stained central cytoplasm with a strongly stained external fringe and a peripheral nucleus. **L**, unidentified splenic IR cell with a strong homogeneous staining in the cytoplasm.

Figures 1,2,3

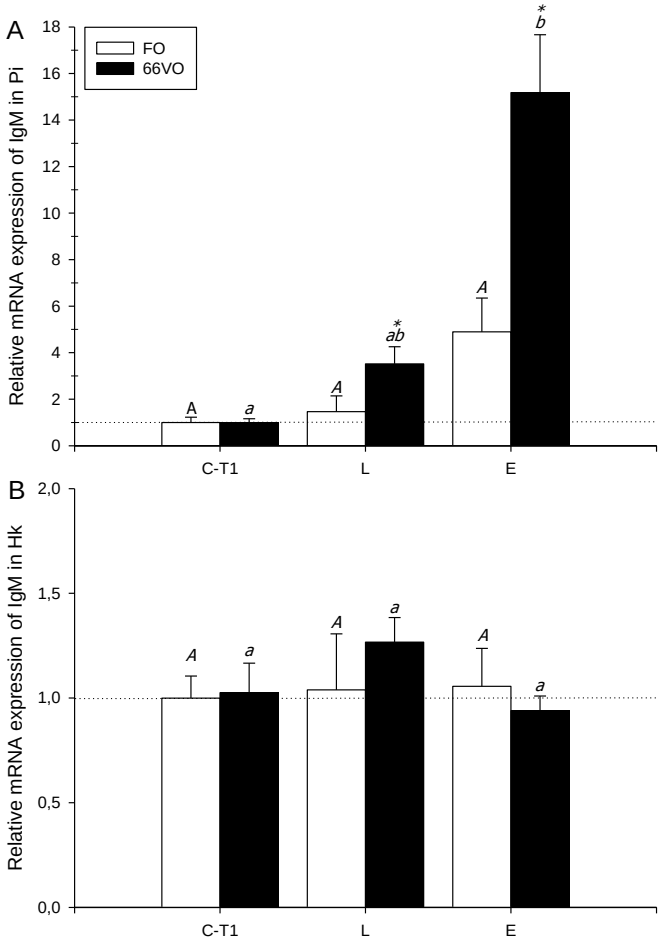


Figure 1

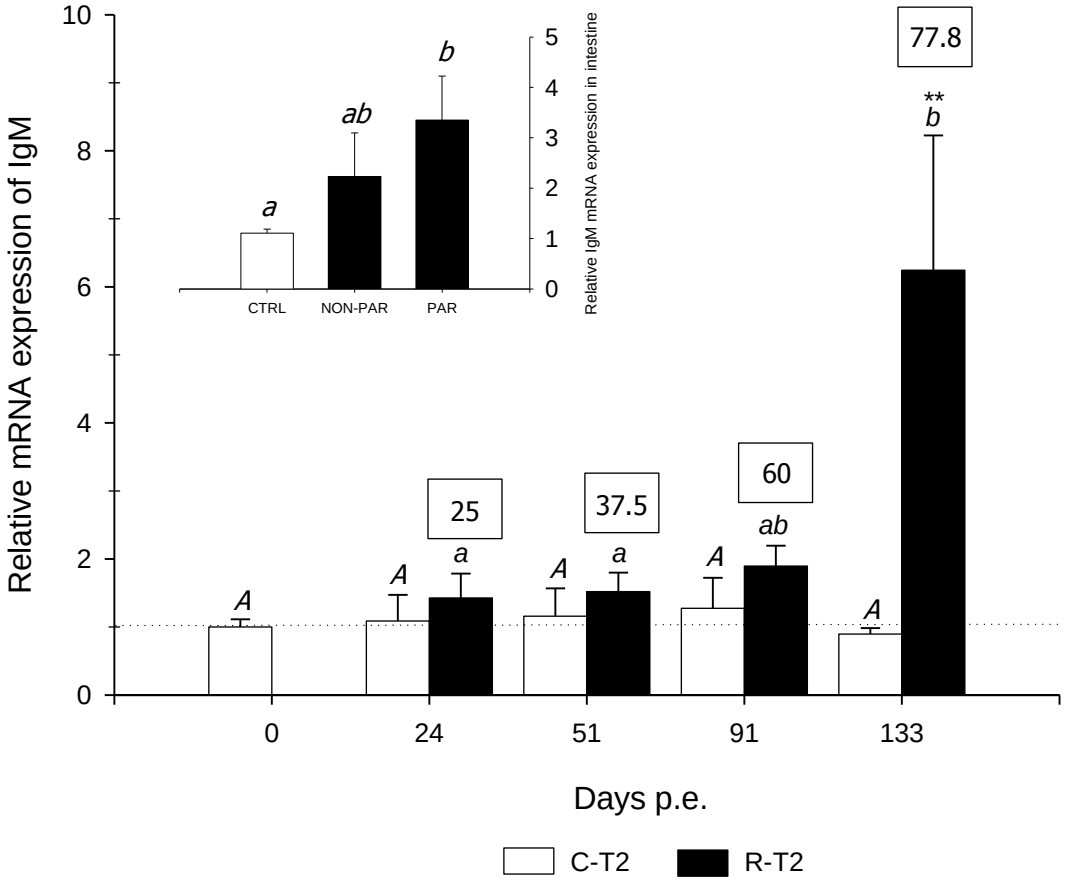
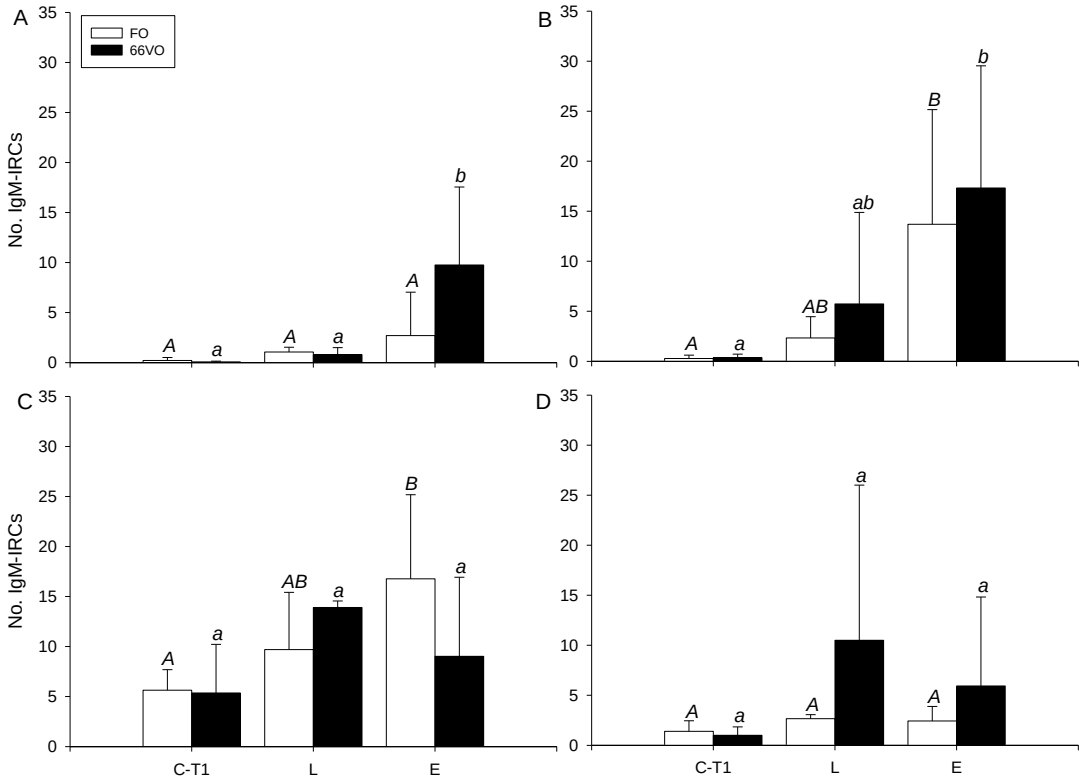

Relative mRNA expression of IgM
10
8
6
4
2
0
0
24
51
91
133
Days p.e.
A
A
a
A
a
A
ab
A
b
**
25
37.5
60
77.8
C-T2
R-T2
Relative IgM mRNA expression in intestine
5
4
3
2
1
0
a
ab
b
CTRL
NON-PAR
PAR

Figure 2


A
B
C
D
FO
66VO
No. IgM-IRCs
No. IgM-IRCs
0
5
10
15
20
25
30
35
A
a
A
a
A
b
A
a
AB
ab
B
b
A
a
AB
a
B
a
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a
A
a
A
a
C-T1
L
E
C-T1
L
E

Figure 3

Figure 4

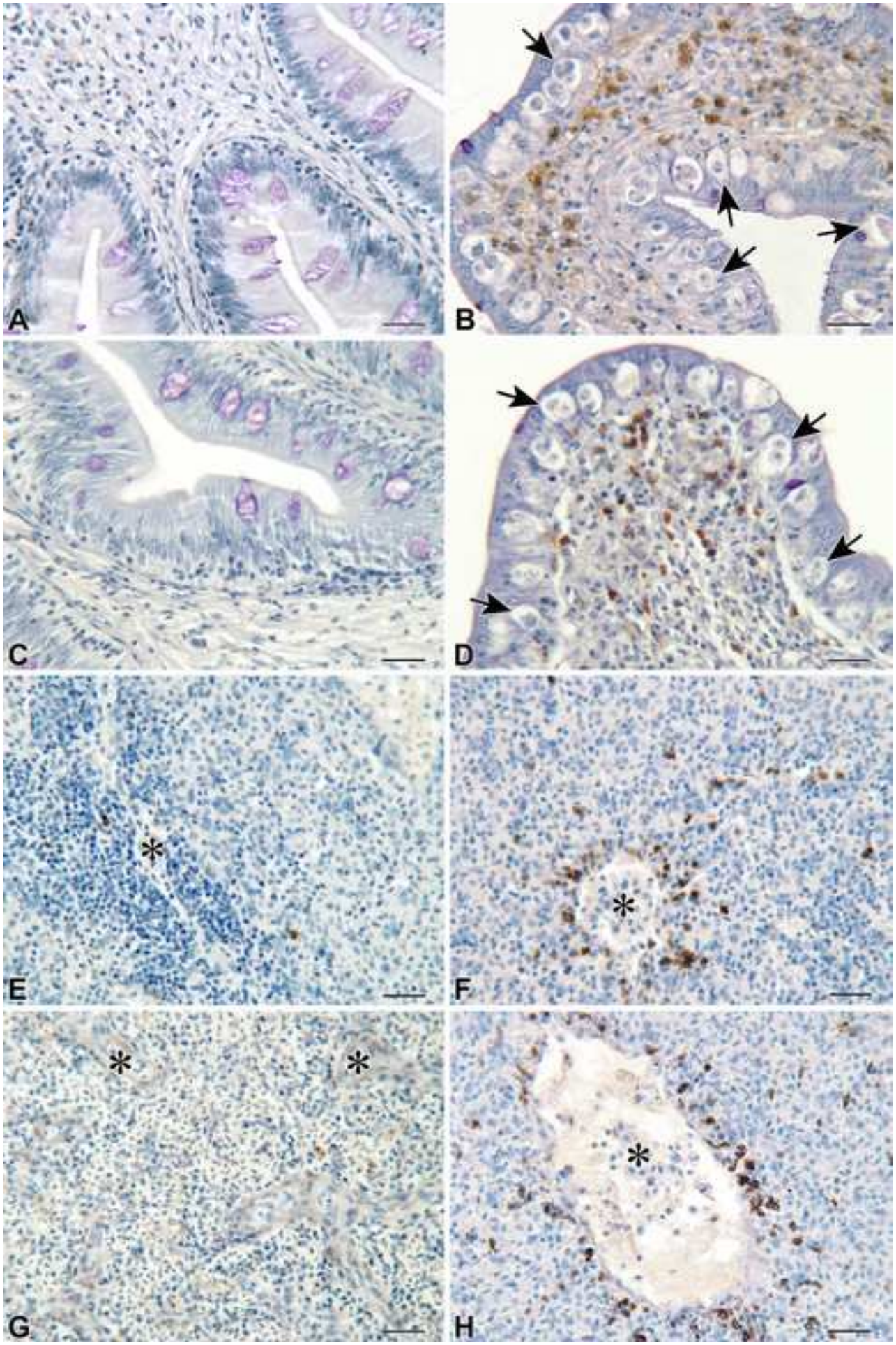

Figure 5

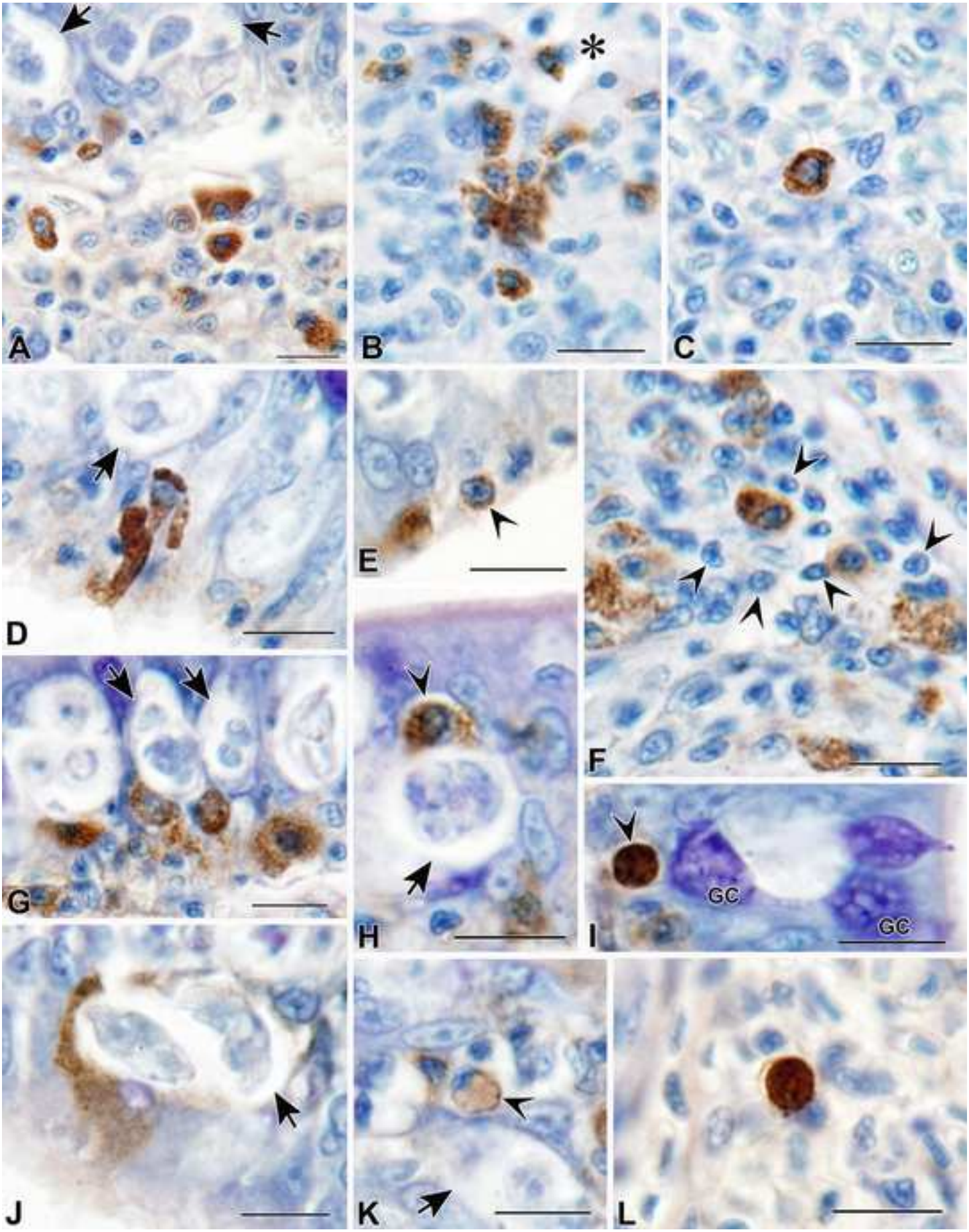

Table 1 Experimental and sampling details of the effluent transmission of *Enteromyxum leei* to *Sparus aurata* in two trials. Experimental groups are control unexposed (C) and exposed recipient (R) fish. In trial 1 fish were fed either the fish oil based diet (FO) or the 66% vegetable oil substitution diet (66VO). Parasite diagnosis was performed by non-lethal PCR (NL-PCR) or histology (HIS), the number between brackets indicates the number of fish examined in each sampling post exposure (p.e.). For each sampling, it is indicated which tissue samples (Ai = anterior intestine, Pi = posterior intestine, Hk = head kidney, Sp = spleen) were taken for which type of analysis (GE = gene expression, IHC = immunohistochemistry), and the number of fish analyzed between brackets.

Trial	1			2				
Groups	C-FO, C-66VO, R-FO, R-66VO			C, R				
Initial weight	224 g			214 g				
Temperature[1]	21.3 ± 0.25 (18.5-26)			18.6 ± 0.16 (15- 21.5)				
	Parasite challenge		**Samples for**		**Parasite challenge**			**Samples for**
	Days p.e.	**Diagnosis**[2]	**GE**	**IHC**	**Days p.e.**	**Diagnosis**	**Prevalence**[3] **(%)**	**MI**[3]	**GE**
	0	NL-PCR	-	-	0	NL-PCR (n = 10 C)	0	-	Pi (n = 8 C)
	32	NL-PCR	-	-	24	HIS (n = 10 C; n = 10 R)	0	-	Pi (n = 8 C; n = 8 R)
	53	NL-PCR	-	-	51	HIS (n = 10 C; n = 10 R)	50	3.2	Pi (n = 8 C; n = 8 R)
	88	NL-PCR	-	-	91	HIS (n = 10 C; n = 10 R)	80	5.2	Pi (n = 8 C; n = 10 R)
	102	HIS	Pi, Hk (n = 19 C; n = 25 R)	Ai, Pi, Hk, Sp (n = 13 C; n = 14 R)	133	HIS (n = 10 C; n = 9 R)	55.6	5.8	Pi (n = 8 C; n = 9 R)

[1] Mean water temperature (ºC) ± standard error; maximum and minimum values are indicated between brackets.
[2] Infection values for Trial 1 are available in Estensoro et al. (2011).
[3] Prevalence of infection and mean intensity (MI) of infection were calculated from the posterior intestine of R fish.

Table 1 Forward and reverse primers for intestinal and renal real-time PCR assays of IgM and β-actin used for the transcriptional analysis in T2.

Gene	*GenBank accession*	*Primer sequence*	*Position*
IgM	JQ811851	F TCA GCG TCC TTC AGT GTT TAT GAT GCC	990-1019
		R CAG CGT CGTCGT CAA CAA GCC AAG C	1123-1099
β-actin	X89920	F TCC TGC GGA ATC CAT GAG A	811-829
		R GAC GTC GCA CTT CAT GAT GCT	861-841