Elsevier Editorial System(tm) for Fish and Shellfish Immunology
Manuscript Draft

Manuscript Number: FSIM-D-13-00481R1

Title: Interleukin gene expression is strongly modulated at the local level in a fish-parasite model

Article Type: Full Length Article

Keywords: Sparus aurata; Teleostei; Enteromyxum leei; Myxozoa; cytokines; gene expression; PCR-array; intestine

Corresponding Author: Dr. Ariadna Sitjà-Bobadilla, PhD

Corresponding Author's Institution: Instituto de Acuicultura de Torre de la Sal (CSIC)

First Author: Gregorio Pérez-Cordón, PhD

Order of Authors: Gregorio Pérez-Cordón, PhD; Itziar Estensoro, Ph D student; Laura Benedito-Palos, PhD; Josep Alvar Calduch-Giner, PhD; Ariadna Sitjà-Bobadilla, PhD; Jaume Pérez-Sánchez, Professor

Abstract: The goal of this work was to identify interleukin (IL)-related genes in the gilthead sea bream (GSB) (Sparus aurata L.) and how they are modulated by the parasite Enteromyxum leei, a myxozoan that causes severe enteritis with a strong inflammatory response. A Blast-X search of our transcriptomic GSB database (www.nutrigroup-iats.org/seabreamdb) identified 16 new sequences encompassing seven ILs (IL-7, IL-8, IL-10, IL-12β, IL-15, IL-18, and IL-34), the interleukin enhancer-binding factor 2 (ILF2), and eight IL receptors (IL-R); IL-R1, IL-6RA, IL-6RB, IL-8RA, IL-10RA, IL-10RB, IL-18R1, and IL-22R). Except for ILF2, their expression, plus that of IL-1β, IL-1R2, IL-6, and TNF-α (from public repositories), were analysed by 96-well PCR array of samples of blood, spleen, head kidney, and intestine of GSB that were anally intubated with E. leei (recipient group, RCPT). Only the expression profile of the intestine of RCPT fish showed significant difference as compared to samples from PBS-inoculated fish. At 17 days post inoculation (dpi), the expression of key pro-inflammatory ILs, such as IL-8, IL-8R, IL-12β, and TNFα was significantly up-regulated, whereas at 64 dpi, anti-inflammatory IL expression (IL-6, IL-6RB, IL-7, IL-10, IL-10RA, and IL-15) was predominant. These results indicate a modification of the IL expression at late times post infection, probably to protect the fish intestine from the parasite and damage inflicted by an excessive inflammatory response. Furthermore, the response is mainly mediated at the local level as no significant changes were detected in blood, spleen and head kidney.

Interleukin gene expression is strongly modulated at the local level in a fish-parasite model

Gregorio Pérez-Cordón[1], Itziar Estensoro[1], Laura Benedito-Palos[2], Josep Alvar Calduch-Giner[2], Ariadna Sitjà-Bobadilla*[1], Jaume Pérez-Sánchez[2]

[1]Fish Pathology Group, Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón, Spain

[2]Nutrigenomics and Fish Growth Endocrinology Group, Instituto de Acuicultura Torre de la Sal (IATS-CSIC), 12595 Ribera de Cabanes, Castellón, Spain

* Author for correspondence: Ariadna Sitjà-Bobadilla

e-mail: ariadna.sitja@csic.es

ABSTRACT

The goal of this work was to identify interleukin (IL)-related genes in the gilthead sea bream (GSB) (*Sparus aurata* L.) and how they are modulated by the parasite *Enteromyxum leei*, a myxozoan that causes severe enteritis with a strong inflammatory response. A Blast-X search of our transcriptomic GSB database (www.nutrigroup-iats.org/seabreamdb) identified 16 new sequences encompassing seven ILs (IL-7, IL-8, IL-10, IL-12β, IL-15, IL-18, and IL-34), the interleukin enhancer-binding factor 2 (ILF2), and eight IL receptors (IL-R); IL-R1, IL-6RA, IL-6RB, IL-8RA, IL-10RA, IL-10RB, IL-18R1, and IL-22R). Except for ILF2, their expression, plus that of IL-1β, IL-1R2, IL-6, and TNF-α (from public repositories), were analysed by 96-well PCR array of samples of blood, spleen, head kidney, and intestine of GSB that were anally intubated with *E. leei* (recipient group, RCPT). Only the expression profile of the intestine of RCPT fish showed significant difference as compared to samples from PBS-inoculated fish. At 17 days post inoculation (dpi), the expression of key pro-inflammatory ILs, such as IL-8, IL-8R, IL-12β, and TNFα was significantly up-regulated, whereas at 64 dpi, anti-inflammatory IL expression (IL-6, IL-6RB, IL-7, IL-10, IL-10RA, and IL-15) was predominant. These results indicate a modification of the IL expression at late times post infection, probably to protect the fish intestine from the parasite and damage inflicted by an excessive inflammatory response. Furthermore, the response is mainly mediated at the local level as no significant changes were detected in blood, spleen and head kidney.

Keywords: *Sparus aurata*; Teleostei; *Enteromyxum leei*; Myxozoa; cytokines; gene expression; PCR-array; intestine

1. Introduction

The gilthead seabream (GSB) (*Sparus aurata* L.), a teleostean sparid fish, is currently the main cultured species in the Mediterranean basin, with a total production increasing each year [1]. An understanding of the biology of fish species and of their immune system, in particular, is essential for improving fish health and wealth management [2], as well as for increasing fish farming production, which is currently the most important animal production industry capable of meeting the food demands of the constantly increasing human population. Intensive fish farming practices can also have an increasing effect on the evolution of pathogens, potentially contributing to the development of more virulent pathogens [3]. Among the different pathogens found in sparid fish, Myxozoa represent some of the most dangerous parasites threatening Mediterranean fish farms [4, 5]. *Enteromyxum leei*, in particular, dwells in the paracellular space in the intestine of several fish species, causing weight loss, delayed growth, reduced marketability, and even massive mortalities in some hosts [6].

Interleukins (ILs) are a group of cytokines that play a major regulatory role in the immune system. It was initially thought that ILs signalled only between leucocytes, but now it is known that they are produced by and target a wide variety of cells and comprise a complex system of cell-signalling within the immune system. Cytokines in general are produced at the site of entry of a pathogen and drive inflammatory signals that regulate the capacity of resident and newly arrived phagocytes to destroy the invading pathogen. They also regulate antigen presentation function in dendritic cells, and their migration to lymph nodes to initiate the adaptive immune response in mammals [7]. Since the first description of ILs more than 30 years ago [8], 37 ILs have been discovered. While most studies have been performed in mammals, they have

recently been extended to lower vertebrates, including fish. In fact, since the adaptive immune system developed in jawed vertebrates, studies of fish ILs may shed light on the evolution of these proteins and the immune system, in general [9]. The development of genomic and EST databases, and PCR-based homology cloning has recently led to the description of several key T cell markers, including CD4, CD8, CD3, CD28, and CTLA4, as well as important cytokines in fish, suggesting the existence of different T helper (Th) subtypes, similar to the mammalian Th1, Th2, and Th17 [10, 11]. These Th subsets are associated with certain specific cytokine profiles and differentiated functions. Currently, 20 genes encoding teleostean ILs have been identified and characterized [12-15]. However, global analyses of the dynamics of fish IL-related gene expression during parasitic infections are very scarce. To address this, we performed extensive Blast-X searches of the IATS updated transcriptomic GSB database (www.nutrigroup-iats.org/seabreamdb) and as result, we identified 16 new IL-related genes in GSB. This allowed us to develop an IL PCR-array of 19 genes, including tumour necrosis factor alpha (TNF-α), to measure the transcriptomic response of GSB exposed to *E. leei.*

2. Material and methods

2.1. Animal care, experimental design and sampling procedure

Clinically healthy juvenile GSB were obtained from a commercial fish hatchery. Upon arrival to the facilities of the Instituto de Acuicultura Torre de la Sal (IATS), they were checked for the absence of *E. leei* and grown in an open flow system with 5 μm-filtered and UV-irradiated sea water (37.5 ‰ salinity). Day length corresponded to the natural changes at our latitude (40º5´N; 0º10´E), and water temperature was maintained

between 18 ºC and 25 ºC. The oxygen content of water was kept above 85% saturation, and unionized ammonia remained below toxic levels (<0.02 mg/l). Fish were fed *ad libitum* a commercial diet (BioMar, Palencia, Spain).

The infection was performed by anal intubation as previously described [16]. Briefly, 36 GSB (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), seven fish from both the CTRL and RCPT group were sacrificed by overexposure to the anaesthetic MS-222 (Sigma, St. Louis, MO, USA) and pieces of head kidney (HK), spleen (SP), and posterior intestine (PI), were rapidly excised, frozen in liquid nitrogen and stored at -80 ºC. Tissue samples of anterior (AI), middle (MI), and posterior (PI) intestine were also taken for parasite diagnosis. Blood was collected from the caudal vein with EDTA-treated syringes and 150 µl were transferred to cooled eppendorf tubes with 500 µl lysis solution 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.

2.2. Diagnosis of the infection

Parasite diagnosis was performed on AI, MI, and PI intestine samples fixed in 4% paraformaldehyde, processed following routine histological procedures, embedded in paraffin, and stained with Giemsa. Infection intensity in each organ was

semiquantitatively evaluated in histological sections 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 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 [17]. A fish was considered positive for infection, when the parasite was found at least in one intestinal segment.

2.3. RNA extraction and reverse transcription

Total RNA from target tissues was extracted using the MagMAX™-96 total RNA isolation kit (Applied Biosystems, Foster City, CA, USA). RNA from blood samples was extracted and purified using the real total RNA spin blood kit (Durviz SL, Valencia, Spain). The RNA yield was 30−50 µg with absorbance measures (A260/280) of 1.9−2.1. Reverse transcription (RT) of 500 ng total RNA with random decamers was performed with the High-Capacity cDNA Archive kit (Applied Biosystems) and negative control reactions were run without reverse transcriptase.

2.4. Gene expression analysis

Real-time quantitative PCR was carried out with the CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), using a 96-well PCR array layout designed for simultaneously profiling a panel of 19 genes from 4 individuals under uniform cycling conditions. The array included 18 IL-related genes, the cytokine TNF-α, the peroxisome proliferator-activated receptor-gamma (PPARγ) as a standard gene, and β-actin as a housekeeping gene. The genes of interest included members of the IL-1, IL-6, IL-10, IL-12, common γ-chain cytokine, CXC chemokine, and other undetermined families, as well as some of their receptors. The liquid manipulations

148 required to perform the PCR array were performed by means of the EpMotion 5070
149 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, for each RT reaction,
150 660 pg of total input RNA was diluted to a 25 µl volume for each PCR reaction. PCR-
151 wells contained a 2x SYBR Green Master Mix (Bio-Rad) and specific primers at a final
152 concentration of 0.9 µM were used to obtain amplicons of 50−150 bp in length (Table
153 1). The PCR reaction was run under the following conditions: an initial denaturation
154 step was carried out at 95 ºC for 3 min, followed by 40 cycles of denaturation for 15 s at
155 95 ºC and annealing/extension for 60 s at 60 ºC. The efficiency of the PCR reactions
156 was always higher than 90% (amplification factor >1.90) and similar for all genes.
157 Negative controls without sample templates were routinely performed for each primer
158 set. The specificity of the reactions was verified by analysis of melting curves (ramping
159 rates of 0.5 ºC/10 s over a temperature range of 55-95 ºC), the linearity of serial
160 dilutions of RT reactions, and electrophoresis and sequencing of the amplified products.
161 Fluorescence data acquired during the PCR extension phase were normalized by the
162 delta-delta Ct method [18]. Inter-assay variation was corrected using the serial dilutions
163 of the standard gene as common reference values among plates. Technical replicates of
164 the samples were run initially to test the reproducibility of the method. As the obtained
165 data had a very high reproducibility score, technical replicates were finally omitted.
166
167 *2.5. Statistics*
168 Gene expression data are represented as the mean ± SEM of seven fish for each group
169 shown in Fig. 2 (intestine). In Supplementary Table 1 (HK, SP, blood) only the
170 averages are shown for visual simplification. For each gene from the different tissues,
171 the effects of pathogen exposure were analysed by the two-tailed Student´s *t* test. When
172 the test of normality or equal variance failed, a Mann-Whitney Rank Sum test was

applied instead. The significance level was set at $P < 0.05$. All statistical analyses were performed using Sigma Stat software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Molecular identity of interleukin and interleukin receptor genes

After an extensive search for annotated descriptions and sequence homologies in the IATS transcriptomic GSB database (www.nutrigroup-iats.org/seabreamdb), 16 new GSB gene sequences were identified and uploaded to GenBank under accessions JX976615-JX976630 (Table 2). Fourteen of the sequences encoded complete coding domains ranging from 288 nucleotides in length to more than 2 kb. Among them, six sequences were annotated as IL genes (IL-7, IL-8, IL-15, IL-18, IL-34, and ILF2; 2e-08> E-value >0) and the remaining were annotated as IL receptors (IL-1R1, IL-6RA, IL-6RB, IL-18RA, IL-10RA, IL-10RB, IL-18R1, and IL-22R; 2e-31> E-value >0). Additionally, two partial sequences encoding for ILs were unequivocally identified as IL-10 and IL-12β (E-value <2e-81).

3.2. Parasite infection

The prevalence and mean intensity of infection by *E. leei* were lower at 17 than at 64 dpi. At the second time point, the infection was not only established in the majority of the sampled animals, but also extended beyond the PI, reaching both AI and MI. Most parasite stages found at 17 dpi were proliferative cells, whereas at 64 dpi, sporoblasts and spores appeared (Fig. 1). No CTRL fish were infected.

198 *3.3. Interleukin expression after E. leei exposure*

199 The 19 genes analysed were expressed in the four studied tissues, but only the intestine
200 showed an mRNA expression profile with statistically significant differences between
201 CTRL and RCPT fish (Fig. 2). The expression of genes encoding several ILs was
202 significantly modulated by *E. leei* infection at both studied times after challenge. At 17
203 dpi, the IL-6 receptors (IL-6RA and IL-6RB), IL-8 and its receptor (IL-8RA), IL-10 and
204 one of its receptors (IL-10RA), IL-12β, and the cytokine TNF-α were significantly up-
205 regulated in RCPT fish (Fig. 2A). Although a few gene products showed up-regulation
206 at both sampling time points (IL-6RB, IL-10, IL-10RA, and TNF-α), others were
207 significantly up-regulated only at 64 dpi (IL-18R1, IL-6, IL-7, and IL-15) (Fig. 2B).
208 Interestingly, among all of them, IL-10 showed the highest statistical difference
209 between CTRL and RCPT samples ($P < 0.001$), and no gene was found to be
210 significantly down regulated in RCPT fish.

211 No statistically significant differences among IL-encoding genes were observed in HK,
212 SP, or blood samples (Supplementary Table 1). In HK, most genes, except for IL-1β at
213 64 dpi, showed a similar or decreased expression in RCPT fish. It must also be noted
214 that most ILs showed barely detectable mRNA expression levels at both 17 and 64 dpi,
215 but three genes (IL-6RA, IL-8RA and IL-22R) showed RNA expression levels that
216 stand out above the rest. In SP, the pattern was opposite to that observed in HK,
217 specifically at 17 dpi when many genes were up-regulated in RCPT fish, but not
218 significantly. At 64 dpi, only IL-6RA and IL-8RA were still up-regulated, whereas the
219 remaining ones were down-regulated. IL-6RA exhibited the highest expression values at
220 both sampling times. Similarly to what was observed in HK, some ILs genes (IL-1R2,
221 IL-18, IL-6, IL-12β, and TNF-α) showed a very low expression in SP. In blood, the

gene expression profile was very similar at both sampling times, but there was an overall decrease in expression at 64 dpi as compared to 17 dpi.

4. Discussion

Parasites are a major constraint on animal production throughout the world, and fish farming is not an exception. Myxozoa are responsible for substantial economic losses in aquaculture and fisheries [5, 19, 20]. Therefore, the study of the host immune response against them is of significant importance [21]. Furthermore, immunological studies are vital because immune function and the host-immune response parasites elicit are key factors for individual survival and for parasite evolution [22-24].

Our understanding of the roles of ILs in the immune response has greatly increased since their discovery. In fish, the modulation of a few ILs by nutritional, anti-oxidant, and pathogenic factors has been reported [25-31], and a multiplex RT-PCR assay has recently been developed for the detection of cytokines (including 12 ILs) [13]. However, the current report is the widest analysis of IL-related genes by quantitative mRNA expression in a fish-metazoan parasite model, to our knowledge.

A Blast-X search of the IATS transcriptomic database identified 16 new IL-related genes, contributing significantly to the scarce number of IL sequences available for GSB in GenBank. For IL-8 and IL-10, the current sequences are longer than those previously available for IL-8 (AM765841) and for IL-10 (FG261948 and that reported by Pellizari et al. [32]). These genes included a broad set of both pro- and anti-inflammatory ILs and their receptors. Most of the obtained sequences were complete, and most genes were ubiquitously expressed, with substantial variation in their

246 expression depending on the tissue and sampling time. The current study is only the
247 second report on fish IL-34, which was just recently described for the first time in
248 several fish species [15]. Nevertheless, some ILs found in other fish species were not
249 found in GSB, such as IL-2, IL-4, IL-11, and IL-17. Further transcriptomic and genomic
250 studies will probably help to identify them. In any case, it is tempting to suggest that the
251 lack of some ILs could be due to overlapping functions, as described in mammals. It has
252 been shown that IL-2 function partially overlaps with IL-15 [33], and the function of IL-
253 11 overlaps with that of IL-6 [34]. Although we did not find IL-17, we identified the
254 receptor for IL-22, another IL that is also involved in the Th17 response and that
255 regulates intestinal and skin homeostasis [35]. Accordingly, the increase in IL-22R
256 expression in the intestine of RCPT fish (though not significant due to the high
257 individual variation), probably reflects the effort of the immune system to control the
258 intestinal homeostasis altered by the parasite.

259 The first notable effect of the parasite infection on the immune response is that
260 significant differences in gene expression were observed only in the intestine, the target
261 site of the parasite, and not in the lymphohaemopoietic tissues (HK and SP) or blood.
262 This highlights the importance of the local immune response in this host-parasite model,
263 and is in agreement with previous results for several fish parasites affecting skin and
264 gills [36-42]. Furthermore, the results reported here confirm the role of the gut
265 associated lymphoid tissue in the mucosal immune response [43, 44], and the cytokine
266 expression in the intestine of fish [45, 46].

267 The second important result is the shift in the type of immune response from
268 pro-inflammatory to anti-inflammatory, with the progression of the infection. In the
269 current host-parasite model, we observed an early and acute pro-inflammatory response
270 shortly after parasite inoculation (t1), as six IL-related genes and TNF-α were up-

271 regulated in RCPT fish. Most of these genes have a pro-inflammatory profile. Among
272 them, IL-12β showed the highest expression levels. IL-12 is a heterodimeric cytokine
273 comprised of covalently bound p35 (IL-12α) and p40 (IL-12β) subunits and a critical
274 regulator of Th1 type immunity. In mammals, IL-12 is secreted by antigen presenting
275 cells after activation by PAMPs and DAMPs and induces NK cells to produce IFN-γ
276 [47, 48]. In fish, the presence of multiple divergent p40 subunits means many IL-12
277 molecules may exist that could have different functions. In fact, in previous studies,
278 different p40 isoforms were also increased following injection with killed bacteria [49],
279 viral infection [50] or protozoan infection [51]. It is unknown whether p40c would drive
280 Th17 type responses and p40b would drive Th1 type responses in fish and the
281 evaluation of the bioactivities of each of these putative fish IL-12 isoforms is a
282 challenge to be deciphered in the future [11]. The GSB IL-12β clusters with fish p40a
283 and p40b in phylogenetic tree analysis (data not shown). Thus, the up-regulation of IL-
284 12β in parasitized GSB would engage the initial cellular response to *E. leei* through the
285 activation of T cells and non-specific cytotoxic cells (NCC; the evolutionary precursor
286 of mammalian NK cells) [52]. This is in accordance with the significantly higher
287 cytotoxic activity of HK leucocytes in GSB after 22 days of cohabitation with *E. leei*-
288 infected fish [53]. The activation of T cells by IL-12β in mammalian models also agrees
289 with the higher percentage of intestinal epithelial lymphocytes in turbot (*Psetta*
290 *maxima*) exposed to another *Enteromyxum* species, *E. scophthalmi* [54], and these
291 lymphocytes are largely considered to be T cells in teleosts.
292 Another important pro-inflammatory cytokine, IL-8, and its receptor, IL-8RA,
293 showed significant overexpression at t1. IL-8 induces chemotaxis in target cells, causing
294 them to migrate to the site of infection in mammals. Thus, GSB IL-8 could induce the
295 migration of granulocytes and macrophages to the site of infection, as already observed

296 in other fish species [55, 56]. This agrees with the increased number of mononuclear
297 and eosinophilic granular cells in the intestine of *E. leei*-infected GSB [16, 57]. The
298 third typically pro-inflammatory cytokine that was significantly up-regulated at t1 was
299 TNF-α, which is in agreement with its early overexpression in most fish-parasite models
300 [58]. In mammals, TNF-α plays an important role in bacterial, viral, and intracellular
301 parasite infections [59], but recent work seems to suggest that this may not be the case
302 in teleosts [60]. In our study, TNF-α was still up-regulated at t2, but in a previous study,
303 TNF-α was significantly down-regulated in the intestine of *E. leei*-infected GSB at 113
304 days after infection [61]. Thus, it is probable that *E. leei* infection requires longer
305 exposure times to induce a down-regulation of TNF-α expression.

306 Some IL receptors were also up-regulated at t1 in RCPT animals, such as IL-
307 6RA and IL-6RB (gp130), though the latter was predominant and remained high at t2.
308 This agrees with the fact that gp130 is ubiquitously expressed in all cell types in
309 mammalian models [62]. IL-6 is a multifunctional, pleiotropic cytokine, with both pro-
310 and anti-inflammatory functions, which regulates the Th17/Treg balance [63, 64], while
311 in some fish it induces Th2 cell differentiation and antibody production [65] and both
312 IL-6 receptors are expressed in IgM+ B lymphocytes of pufferfish (*Takifugu rubripes*)
313 [66]. Thus, the significant overexpression of IL-6RB and IL-6RA in RCPT intestine
314 could reflect the increase in the number of intestinal leukocytes responding to the
315 parasite and being stimulated by other pro-inflammatory ILs.

316 The anti-inflammatory profile at t2 was mainly represented by the significant up-
317 regulation of IL-10, IL-10RA, IL-7, IL-6, and IL-15. The highest fold change was seen
318 for IL-10, a central anti-inflammatory cytokine capable of inhibiting the expression of
319 many pro-inflammatory cytokines [67], and directly affecting T cell activation [68]. IL-
320 10 gene expression in several fish species is increased by LPS stimulation, bacterial and

parasitic infections and vaccination [31, 69-71]. The significant overexpression of IL-10 was coincident with a non-significant decreased expression of IL-1β and IL-8 in RCPT fish, which were no longer up-regulated at t2. Thus, IL-10 would play a key role in inducing down-regulation of different pro-inflammatory cytokines, as already suggested in other fish studies [72].

IL-6, IL-7, and IL-15 have pleiotropic functions, but their concurrent up-regulation at t2 implies that they play a role in the stimulation of B cell proliferation and differentiation, and the stimulation of IgM synthesis, as in mammalian models [73, 74]. This is in agreement with the significant increase of B cells, plasma cells, and IgM gene expression in the intestine of *E. leei*-infected GSB at late times after exposure [75]. In mammals, epithelial cells are one of the cell types that produce IL-7, and therefore, it is tempting to speculate that lymphopoiesis may be triggered locally from the intestinal tissue of GSB after exposure to the parasite.

In the second sampling (t2), however, two inflammatory cytokine-related genes (TNF-α and IL-18R1) were still up-regulated in the intestine of RCPT. IL-18R1 is the alpha subunit of the heterodimeric receptor for IL-18. Information on IL-18 in fish is very scarce and the data obtained from *in vitro* stimulated fish cells is variable [14, 76]. In mammalian models, this IL is generally considered to be pro-inflammatory, but it has the ability to enhance both Th1 and Th2 inflammation, depending on the circumstances under which it is produced. IL-18 induces IFN-γ synthesis in Th1 and NK cells in synergy with IL-12, whereas in the absence of IL-12, it induces NK and NKT cells to express Th2 cytokines (reviewed in [77]). Further studies are necessary to elucidate the biological consequences of the enhanced IL-18 expression in GSB, especially considering that IL-12β was no longer up-regulated at t2.

Although no significant changes were detected in HK, SP, or the blood of RCPT fish, a global expression pattern can be described. In HK the majority of the studied IL-related genes showed low and similar expression values in RCPT and CTRL fish, at both time points. However, three genes (IL-6RA, IL-8RB and IL-22) showed high constitutive expression levels that surprisingly were down-regulated in RCPT fish at both time points. This down-regulation may be indicative of the impairment of the hematopoietic function of HK with a specifically negative effect on T cells since this type of lymphocytes is a common cellular target for IL-6, IL-8 and IL-22 [78]. By contrast, in SP, most of the genes had higher values in RCPT than in CTRL fish at t1, whereas a mixed pattern was found at t2. The slightly higher expression level of IL-related genes in the blood of RCPT fish at t1 when compared to t2 would indicate an early mobilization of immune cells from the lymphohaemopoietic organs to the site of the infection, but not enough to explain the high levels in the intestine. In parasite-infected common carp (*Cyprinus carpio*), the up-regulation of immune genes was more moderate and earlier in the blood than in the skin [37]. Thus, it is probable that changes could be greater in GSB blood at earlier times, which would enable to use the blood as a non-lethal surrogate for tissue samples.

The different expression levels of IL-related genes in the tissues may shed light on the T cell composition of GSB. Indeed, it is remarkable how low the expression levels of the majority of the ILs analysed in this study were in SP, in contrast with the high and constitutive expression levels of receptors for IL-1β, IL-18, IL-6, IL-10, and IL-22. This would indicate that, as a whole, SP cells are a target for both pro- and anti-inflammatory cytokines. The GSB data in HK suggest an important role for a subset of ILs in lymphopoiesis in this teleost and may just reflect the cell type composition of this organ, which is considered the equivalent of the mammalian bone marrow [79, 80].

In conclusion, the intestinal IL gene expression of GSB after infection by *E. leei* was characterized by an early pro-inflammatory profile, which later switched to an anti-inflammatory pattern. Further studies are necessary to characterize the mechanisms by which this myxozoan parasite modulates this immune response, and the possible role of this cytokine shift in parasite clearance or immunopathological effects. For such a purpose, the development of antibodies that recognize different T cell subsets is crucial. Finally, the different expression patterns of IL-related genes in the lymphohaemopoietic tissues of GSB contributes to our knowledge of the different leukocyte types, receptors, and functions, as a complement to the information available for model fish species [81, 82]. Further studies on the biological function of these cytokines and the different cell types involved in their production are to come.

Acknowledgements

This work was funded by the EU through project ARRAINA (KBBE-2011-288925), by MICINN through project AGL2009-13282-C02-01, and by the "Generalitat Valenciana" (projects PROMETEO 2010/006 and ISIC 2012/003). I. E. received a Ph D FPI fellowship and G. P-C was contracted under the "Juan de la Cierva" program (MICINN). The authors thank J. Monfort and L. Rodríguez for histological processing and M.A. González for technical assistance during gene expression analyses.

References

[1] APROMAR. La Acuicultura Marina en España. http://www.apromar.es/informes.asp 2012.

[2] Segner H, Sundh H, Buchmann K, Douxfils J, Sundell K, Mathieu C, et al. Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. Fish Physiol Biochem 2012;38:85-105.

[3] Pulkkinen K, Suomalainen LR, Read AF, Ebert D, Rintamaki P, Valtonen ET. Intensive fish farming and the evolution of pathogen virulence: the case of columnaris disease in Finland. Proc R Soc B 2010;277:593-600.

[4] Golomazou E, Karagouni E, Athanassopoulou F. The most important myxosporean parasite species affecting cultured Mediterranean fish. J Hell Vet Med Soc 2004;55:342-52.

[5] Palenzuela O. Myxozoan infections in Mediterranean mariculture. Parassitologia 2006;48:27-9.

[6] Sitjà-Bobadilla A, Palenzuela O. *Enteromyxum* Species. In: Woo PTK & Buchmann K, editors. Fish Parasites: Pathology and Protection, Oxfordshire UK: CAB International; 2009, p. 163-76.

[7] Chabalgoity, JA, Baz A, Rial A, Grille S. The relevance of cytokines for development of protective immunity and rational design of vaccines. Cytokine Growth Factor Rev 2007;18:195-207.

[8] Gery I, Gershon RK, Waksman BH. Potentiation of T-lymphocyte response to mitogens 1. The responding cell. J Exp Med 1972;136:128-42.

[9] Mulero V, García Ayala A, Meseguer J. What can we learn from fish? An evolutionary perspective of pro-inflammatory cytokines. Inmunología 2005;24:17-22.

414 [10] Castro R, Bernard D, Lefranc MP, Six A, Benmansour A, Boudinot R. T cell
415 diversity and TcR repertoires in teleost fish. Fish Shellfish Immunol 2011;31:644-
416 54.
417 [11] Wang T, Secombes CJ. The cytokine networks of adaptive immunity in fish. Fish
418 Shellfish Immunol 2013. Fish Shellfish Immunol 2013;35:1703-18.
419 [12] Secombes CJ, Wang T, Bird S. The interleukins of fish. Dev Comp Immunol
420 2011;35:1336-45.
421 [13] Kono T, Takayama H, Nagamine R, Korenaga H, Sakai M. Establishment of a
422 multiplex RT-PCR assay for the rapid detection of fish cytokines. Vet Immunol
423 Immunopathol 2013;151:90-101.
424 [14] Pereiro P, Balseiro P, Romero A, Dios S, Forn-Cuni G, Fuste B, et al. High-
425 throughput sequence analysis of turbot (*Scophthalmus maximus*) transcriptome
426 using 454-pyrosequencing for the discovery of antiviral immune genes. PLoS
427 ONE 2012;7.
428 [15] Wang TH, Kono T, Monte MM, Kuse H, Costa MM, Korenaga H, et al.
429 Identification of IL-34 in teleost fish: Differential expression of rainbow trout IL-
430 34, MCSF1 and MCSF2, ligands of the MCSF receptor. Mol Immunol
431 2013;53:398-409.
432 [16] Estensoro I, Redondo MJ, Álvarez-Pellitero P, Sitjà-Bobadilla A. Novel
433 horizontal transmission route for *Enteromyxum leei* (Myxozoa) by anal intubation
434 of gilthead sea bream *Sparus aurata*. Dis Aquat Org 2010;92:51-8.
435 [17] Álvarez-Pellitero P, Palenzuela O, Sitjà-Bobadilla A. Histopathology and cellular
436 response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo*
437 (Teleostei). Parasitol Int 2008;57:110-20.

438 [18] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-
439 time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 2001;25:402-8.
440 [19] Sitjà-Bobadilla A. Can Myxosporean parasites compromise fish and amphibian
441 reproduction? Proc R Soc B 2009;276:2861-70.
442 [20] Rigos G, Katharios P. Pathological obstacles of newly-introduced fish species in
443 Mediterranean mariculture: a review. Rev Fish Biol Fisher 2010;20:47-70.
444 [21] Sitjà-Bobadilla A. Fish immune response to Myxozoan parasites. Parasite
445 2008;15:420-5.
446 [22] Downs CJ, Brown JL, Wone B, Donovan ER, Hunter K, Hayes JP. Selection for
447 increased mass-independent maximal metabolic rate suppresses innate but not
448 adaptive immune function. Proc R Soc B 2013;280. DOI.10.1098/rspb.2012.2636.
449 [23] Scharsack JP, Koch K, Hammerschmidt K. Who is in control of the stickleback
450 immune system: interactions between *Schistocephalus solidus* and its specific
451 vertebrate host. Proc R Soc B 2007;274:3151-8.
452 [24] Best A, Long G, White A, Boots M. The implications of immunopathology for
453 parasite evolution. Proc R Soc B 2012;279:3234-40.
454 [25] Kim DH, Austin B. Cytokine expression in leucocytes and gut cells of rainbow
455 trout, *Oncorhynchus mykiss* Walbaum, induced by probiotics. Vet Immunol
456 Immunopathol 2006;114:297-304.
457 [26] Patel N, Kreider T, Urban, Jr., Gause WC. Characterisation of effector
458 mechanisms at the host: parasite interface during the immune response to tissue-
459 dwelling intestinal nematode parasites. Int J Parasitol 2009;39:13-21.
460 [27] Montero D, Mathlouthi F, Tort L, Afonso JM, Torrecillas S, Fernández-Vaquero
461 A, et al. Replacement of dietary fish oil by vegetable oils affects humoral

462 immunity and expression of pro-inflammatory cytokines genes in gilthead sea
463 bream *Sparus aurata*. Fish Shellfish Immunol 2010;29:1073-81.
464 [28] Domínguez B, Pardo BG, Noia M, Millan A, Gómez-Tato A, Martínez P, et al.
465 Microarray analysis of the inflammatory and immune responses in head kidney
466 turbot leucocytes treated with resveratrol. Int Immunopharmacol 2013;15:588-96.
467 [29] Uran PA, Goncalves AA, Taverne-Thiele JJ, Schrama JW, Verreth JAJ, Rombout
468 JHWM. Soybean meal induces intestinal inflammation in common carp (*Cyprinus*
469 *carpio* L.). Fish Shellfish Immunol 2008;25:751-60.
470 [30] Sepulcre MP, Saffopoulou E, Kotoulas G, Meseguer J, Mulero V. *Vibrio*
471 *anguillarum* evades the immune response of the bony fish sea bass
472 (*Dicentrarchus labrax* L.) through the inhibition of leukocyte respiratory burst
473 and down-regulation of apoptotic caspases. Mol Immunol 2007;44:3751-7.
474 [31] Chettri JK, Kuhn JA, Jaafar RM, Kania PW, Møller OS, Buchmann K. Epidermal
475 response of rainbow trout to *Ichthyobodo necator*: immunohistochemical and
476 gene expression studies indicate a Th1-/Th2-like switch. J Fish Dis 2013. DOI:
477 10.1111/jfd.12169.
478 [32] Pellizzari C, Krasnov A, Afanasyev S, Vitulo N, Franch R, Pegolo S, Patarnello
479 T, Bargelloni L. High mortality of juvenile gilthead sea bream (*Sparus aurata*)
480 from photobacteriosis is associated with alternative macrophage activation and
481 anti-inflammatory response: Results of gene expression profiling of early
482 responses in the head kidney. Fish Shellfish Immunol 2013;34:1269-78.
483 [33] Becknell B, Caligiuri MA. Interleukin-2, interleukin-15, and their roles in human
484 natural killer cells. In: Alt FW, editor. Advances in Immunology Vol 86, San
485 Diego: Elsevier Academic Press Inc; 2005, p. 209-39.

[34] Paul SR, Bennett F, Calvetti JA, Kelleher K, Wood CR, Ohara RM, et al. Molecular-cloning of a cDNA-encoding interleukin-11, a stromal cell-derived lymphopoietic and hematopoietic cytokine. Proc Natl Acad Sci USA 1990;87:7512-6.

[35] Blaschitz C, Raffatellu M. Th17 cytokines and the gut mucosal barrier. J Clin Immunol 2010;30:196-203.

[36] Lindenstrom T, Buchmann K, Secombes CJ. *Gyrodactylus derjavini* infection elicits IL-1 beta expression in rainbow trout skin. Fish Shellfish Immunol 2003;15:107-15.

[37] Gonzalez SF, Buchmann K, Nielsen ME. Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: Inflammatory responses caused by the ectoparasite *Ichthyophthirius multifiliis*. Fish Shellfish Immunol 2007;22:641-50.

[38] Morrison RN, Zou J, Secombes CJ, Scapigliati G, Adams MB, Nowak BF. Molecular cloning and expression analysis of tumour necrosis factor-α in amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immunol 2007;23:1015-31.

[39] Forlenza M, Walker PD, de Vries BJ, Wendelaar Bonga SE, Wiegertjes GF. Transcriptional analysis of the common carp (*Cyprinus carpio* L.) immune response to the fish louse *Argulus japonicus* Thiele (Crustacea: Branchiura). Fish Shellfish Immunol 2008;25:76-83.

[40] Olsen MM, Kania PW, Heinecke RD, Skjoedt K, Rasmussen KJ, Buchmann K. Cellular and humoral factors involved in the response of rainbow trout gills to *Ichthyophthirius multifiliis* infections: Molecular and immunohistochemical studies. Fish Shellfish Immunol 2011;30:859-69.

[41] Khoo CK, Abdul-Murad AM, Kua BC, Mohd-Adnan A. *Cryptocaryon irritans* infection induces the acute phase response in *Lates calcarifer* : A transcriptomic perspective. Fish Shellfish Immunol 2012;33:788-94.

[42] Fast MD, Muise DM, Easy RE, Ross NW, Johnson SC. The effects of *Lepeophtheirus salmonis* infections on the stress response and immunological status of Atlantic salmon (*Salmo salar*). Fish Shellfish Immunol 2006;21:228-41.

[43] Rombout JHWM, Abelli L, Picchietti S, Scapigliati G, Kiron V. Teleost intestinal immunology. Fish Shellfish Immunol 2011;31:616-26.

[44] Salinas I, Zhang Y-A, Sunyer JO. Mucosal immunoglobulins and B cells of teleost fish. Dev Comp Immunol 2011;35:1346-65.

[45] Mulder IE, Wadsworth S, Secombes CJ. Cytokine expression in the intestine of rainbow trout (*Oncorhynchus mykiss*) during infection with *Aeromonas salmonicida*. Fish Shellfish Immunol 2007;23:747-59.

[46] Inami M, Taverne-Thiele AJ, Schroder MB, Kiron V, Rombout JHWM. Immunological differences in intestine and rectum of Atlantic cod (*Gadus morhua* L.). Fish Shellfish Immunol 2009;26:751-9.

[47] Vignali DA, Kuchroo VK. IL-12 family cytokines: immunological playmakers. Nat Immunol 2012;13:722-8.

[48] Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol 2003;3:133-46.

[49] Nascimento DS, do Vale A, Tomas AM, Zou J, Secombes CJ, dos Santos NMS. Cloning, promoter analysis and expression in response to bacterial exposure of sea bass (*Dicentrarchus labrax* L.) interleukin-12 p40 and p35 subunits. Mol Immunol 2007;44:2277-91.

534 [50] Forlenza M, de Carvalho Dias JD, Vesely T, Pokorova D, Savelkoul HF,
535 Wiegertjes GF. Transcription of signal-3 cytokines, IL-12 and IFN, coincides with
536 the timing of CD8 up-regulation during viral infection of common carp (*Cyprinus*
537 *carpio* L.). Mol Immunol 2008;45:1531-47.
538 [51] Grayfer L, Hanington PC, Belosevic M. Macrophage colony-stimulating factor
539 (CSF-1) induces pro-inflammatory gene expression and enhances antimicrobial
540 responses of goldfish (*Carassius auratus* L.) macrophages. Fish Shellfish
541 Immunol 2009;26:406-13.
542 [52] Ristow S, Evans DL, Jaso-Friedmann L. Analyzing nonspecific cytotoxic cells in
543 fish. In: Campbell KS, Colonna M, editors. Methods in molecular biology:
544 Natural killer cell protocolls Vol. 121, Clifton, NJ: Humana Press; 2000, p. 347-
545 57.
546 [53] Cuesta A, Salinas I, Rodríguez A, Muñoz P, Sitjà-Bobadilla A, Álvarez-Pellitero
547 P, et al. Cell-mediated cytotoxicity is the main innate immune mechanism
548 involved in the cellular defence of gilthead seabream (Teleostei: Sparidae) against
549 *Enteromyxum leei* (Myxozoa). Parasite Immunol 2006;28:657-65.
550 [54] Sitjà-Bobadilla A, Redondo MJ, Bermúdez R, Palenzuela O, Ferreiro I, Riaza A,
551 et al. Innate and adaptive immune responses of turbot, *Scophthalmus maximus*
552 (L.), following experimental infection with *Enteromyxum scophthalmi*
553 (Myxosporea : Myxozoa). Fish Shellfish Immunol 2006;21:485-500.
554 [55] Huising MO, Stolte E, Flik G, Savelkoul HFJ, Verburg-van Kemenade BML.
555 CXC chemokines and leukocyte chemotaxis in common carp (*Cyprinus carpio*
556 L.). Dev Comp Immunol 2003;27:875-88.

557 [56] Harun NO, Zou J, Zhang YA, Nie P, Secombes CJ. The biological effects of
558 rainbow trout (*Oncorhynchus mykiss*) recombinant interleukin-8. Dev Comp
559 Immunol 2008;32:673-81.
560 [57] Fleurance R, Sauvegrain C, Marques A, Le Breton A, Guereaud C, Cherel Y, et
561 al. Histopathological changes caused by *Enteromyxum leei* infection in farmed sea
562 bream *Sparus aurata*. Dis Aquat Org 2008;79:219-28.
563 [58] Álvarez-Pellitero P. Fish immunity and parasite infections: from innate immunity
564 to immunoprophylactic prospects. Vet Immunol Immunopathol 2008;126:171-98.
565 [59] Körner H, McMorran B, Schluter D, Fromm P. The role of TNF in parasitic
566 diseases: Still more questions than answers. Int J Parasitol 2010;40:879-88.
567 [60] Roca FJ, Mulero I, López-Muñoz A, Sepulcre MP, Renshaw SA, Meseguer J, et
568 al. Evolution of the inflammatory response in vertebrates: Fish TNF-α is a
569 powerful activator of endothelial cells but hardly activates phagocytes. J Immunol
570 2008;181:5071-81.
571 [61] Sitjà-Bobadilla A, Calduch-Giner J, Saera-Vila A, Palenzuela O, Álvarez-Pellitero
572 P, Pérez-Sánchez J. Chronic exposure to the parasite *Enteromyxum leei*
573 (Myxozoa: Myxosporea) modulates the immune response and the expression of
574 growth, redox and immune relevant genes in gilthead sea bream, *Sparus aurata* L.
575 Fish Shellfish Immunol 2008;24:610-9.
576 [62] Taga T, Kishimoto T. gp130 and the interleukin-6 family of cytokines. Annu Rev
577 Immunol 1997;15:797-819.
578 [63] Eulenfeld R, Dittrich A, Khouri C, Mueller PJ, Muetze B, Wolf A, et al.
579 Interleukin-6 signalling: More than Jaks and STATs. Eur J Cell Biol 2012;91:486-
580 95.

581 [64] Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, et al. IL-6 is an
582 antiinflammatory cytokine required for controlling local or systemic acute
583 inflammatory responses. J Clin Invest 1998;101:311-20.
584 [65] Chen HH, Lin HT, Foung YF, Han-You Lin J. The bioactivity of teleost IL-6: IL-
585 6 protein in orange-spotted grouper (*Epinephelus coioides*) induces Th2 cell
586 differentiation pathway and antibody production. Dev Comp Immunol
587 2012;38:285-94.
588 [66] Kaneda M, Odaka T, Suetake H, Tahara D, Miyadai T. Teleost IL-6 promotes
589 antibody production through STAT3 signaling via IL-6R and gp130. Dev Comp
590 Immunol 2012;38:224-31.
591 [67] Malefyt RD, Abrams J, Bennett B, Figdor CG, Devries JE. Interleukin-10 (IL-10)
592 inhibits cytokine synthesis by human monocytes - an autoregulatory role of IL-10
593 produced by monocytes. J Exp Med 1991;174:1209-20.
594 [68] Taylor A, Akdis M, Joss A, Akkoc T, Wenig R, Colonna M, et al. IL-10 inhibits
595 CD28 and ICOS costimulations of T cells via src homology 2 domain-containing
596 protein tyrosine phosphatase 1. J Allergy Clin Immunol 2007;120:76-83.
597 [69] Inoue Y, Kamota S, Ito K, Yoshiura Y, Ototake M, Motitomo T, et al. Molecular
598 cloning and expression analysis of rainbow trout (*Oncorhynchus mykiss*)
599 interleukin-10 cDNAs. Fish Shellfish Immunol 2005;18:335-44.
600 [70] Zhang DC, Shao YQ, Huang YQ, Jiang SG. Cloning, characterization and
601 expression analysis of interleukin-10 from the zebrarish (*Danio rerio*). J Biochem
602 Mol Biol 2005;38:571-6.
603 [71] Kumari J, Bøgwald J, Dalmo RA. Vaccination of Atlantic salmon, *Salmo salar* L.,
604 with *Aeromonas salmonicida* and infectious pancreatic necrosis virus (IPNV)
605 showed a mixed Th1/Th2/Treg response. J Fish Dis 2013;36:881-6.

606 [72] Seppola M, Larsen AN, Steiro K, Robertsen B, Jensen I. Characterisation and
607 expression analysis of the interleukin genes, IL-1 beta, IL-8 and IL-10, in Atlantic
608 cod (*Gadus morhua* L.). Mol Immunol 2008;45:887-97.
609 [73] Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells
610 by γ_c family cytokines. Nat Rev Immunol 2009;9:480-90.
611 [74] Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-
612 inflammatory properties of the cytokine interleukin-6. BBA-Mol Cell Res
613 2011;1813:878-88.
614 [75] Estensoro I, Calduch-Giner JA, Kaushik S, Pérez-Sánchez J, Sitjà-Bobadilla A.
615 Modulation of the IgM gene expression and the IgM immunoreactive cell
616 distribution by the nutritional background in gilthead sea bream (*Sparus aurata*)
617 challenged with *Enteromyxum leei* (Myxozoa). Fish Shellfish Immunol
618 2012;33:401-10.
619 [76] Zou J, Bird S, Truckle J, Bols N, Horne M, Secombes C. Identification and
620 expression analysis of an IL-18 homologue and its alternatively spliced form in
621 rainbow trout (*Oncorhynchus mykiss*). Eur J Biochem 2004;271:1913-23.
622 [77] Smith DE. The biological paths of IL-1 family members IL-18 and IL-33. J
623 Leukocyte Biol 2011;89:383-92.
624 [78] Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E, et al.
625 Interleukins, from 1 to 37, and interferon-gamma: Receptors, functions, and roles
626 in diseases. J Allergy Clin Immunol. 2011 127:701-U317.
627 [79] Boehm T. Evolution of Vertebrate Immunity. Current Biol 2012;22:R722-R32.
628 [80] Zapata A, Diez B, Cejalvo T, Gutiérrez-de Frías C, Cortés A. Ontogeny of the
629 immune system of fish. Fish Shellfish Immunol 2006;20:126-36.

[81] Lieschke G. Zebrafish - An emerging genetic model for the study of cytokines and hematopoiesis in the era of functional genomics. Int J Hematol 2001;73:23-31.

[82] Savan R, Sakai M. Genomics of fish cytokines. Comp Biochem Phys D 2006;1:89-101.

635 **Figure captions**

636

637 **Figure 1.** Prevalence and mean intensity of infection of gilthead sea bream (*Sparus*
638 *aurata*) anally intubated with *Enteromyxum leei* at the three examined intestinal
639 segments at t1 and t2 (17 and 64 days post inoculation, respectively dpi). Note
640 the small number of proliferative stages (arrows) and the disorganized
641 architecture of the epithelium in t1, and the spores (arrows) and the empty
642 spaces generated by the parasite in t2 (*).

643 **Figure 2.** Relative expression (mean + SEM) of interleukin-related genes in the
644 posterior intestine of gilthead sea bream (*Sparus aurata*) after 17 (A, t1) and 64
645 (B, t2) days of intubation with *Enteromyxum leei*. Statistically significant
646 differences between control (CTRL) and intubated (RCPT) are indicated by * (P
647 < 0.05) or ** ($P < 0.001$). β-actin was used as a housekeeping gene in the
648 normalization procedure. Expression values were referred to those of IL-10RA
649 in CTRL fish at t1 (arbitrarily referred as 1).

650 **Table 1.** Forward and reverse primers for quantitative real-time PCR of interleukin-
651 related genes of gilthead sea bream (*Sparus aurata*).

652 **Table 2.** Characteristics of new assembled sequences of interleukin-related genes of
653 gilthead sea bream (*Sparus aurata*), according to BLAST searches.

654

655 **Supplementary Material**

656 **Supplementary Table 1**: Compilation of the gene expression profile of interleukin-
657 related genes in the head kidney, blood and spleen of gilthead sea bream (*Sparus*
658 *aurata*) intubated with *Enteromyxum leei* after 17 (t1) and 64 (t2) days. Data are
659 shown as the mean expression values for each gene in control (CTRL) and

660 intubated (RCPT) fish. No significant differences between CTRL and RCPT fish
661 were found in none of these tissues. The different intensity of the colours stands
662 for differences in relative expression units.
663

Table 1. Forward and reverse primers for quantitative real-time PCR of gilthead sea bream (*Sparus aurata*).

Gene name	Symbol	GenBank		Primer sequence
Interleukin-1 beta	IL-1β	CAD11603	F	GCG ACC TAC CTG CCA CCT ACA CC
			R	TCG TCC ACC GCC TCC AGA TGC
Interleukin-1 beta receptor 1	IL-1R1	JX976615	F	GAA GCT GTA CGA CGC CTA C
			R	CTC CAC TGC CTT ACT GTA TCC
Interleukin-1 beta receptor 2	IL-1R2	CAL30143	F	CCT GAC CTC TCC GTG ACC TCT AA
			R	TGG CTG CTG CTG CTG ATG A
Interleukin-18	IL-18	JX976626	F	CTG ACA ATA ACA GGA TTG CCT CGG TGA TG
			R	GGA CAA CCC CTC AAG ACC AGT TTC AAG
Interleukin-18 receptor 1	IL-18R1	JX976627	F	CGG AGA CCT GAA GAG AAG T
			R	CAC CAT GAT CGT ACT CGT AAC
Interleukin-6	IL-6	B6CKP4	F	TCT TGA AGG TGG TGC TGG AAG TG
			R	AAG GAC AAT CTG CTG GAA CTG AGG
Interleukin-6 receptor A	IL-6RA	JX976616	F	GCA GTG CTC GTA CTC TTC
			R	CTC CGC TCT TCC TCA TTG
Interleukin-6 receptor B	IL-6RB	JX976617	F	CAG TGT CGG AGT ATG TGG TTG AGT
			R	CCC TCT GCC AGT CTG TCC AA
Interleukin-7	IL-7	JX976618	F	CTA TCT CTG TCC CTG TCC TGT GA
			R	TGC GGA TGG TTG CCT TGT AAT
Interleukin-15	IL-15	JX99625	F	GAG ACC AGC GAG CGA AAG GCA TCC
			R	GCC AGA ACA GGT TCA AGG TTG ACA GGA A
Interleukin-8	IL-8	JX976619	F	CAG CAG AGT CTT CAT CGT CAC TAT TG
			R	AGG CTC GCT TCA CTG ATG G
Interleukin-8 receptor A	IL-8RA	JX976620	F	CTT GTT TCA TCT GAC GAT AG
			R	AAG AGG ATG CTT GTG TAG
Interleukin-10	IL-10	JX976621	F	AAC ATC CTG GGC TTC TAT CTG
			R	GTG TCC TCC GTC TCA TCT G
Interleukin-10 receptor A	IL-10RA	JX976622	F	GAG GAC AAT GAA GAG GAA GAC AGG AG
			R	TGT TCG TAG CGG AGT TGG ACT
Interleukin-10 receptor B	IL-10RB	JX976623	F	AGA CCC ACA GGC TTC AGA T
			R	GCA GCG TCA CCA GGT TAG
Interleukin-22 receptor	IL-22R	JX976628	F	TTA CGG CGA GCC TGA GTG
			R	GAG GTC ACA ATG CTG CTT CTG
Interleukin-12 subunit beta	IL-12β	JX976624	F	ATT CCC TGT GTG GTG GCT GCT
			R	GCT GGC ATC CTG GCA CTG AAT
Interleukin-34	IL-34	JX976629	F	TCT GTC TGC CTG CTG GTA G
			R	ATG CTG GCT GGT GTC TGG
Tumor necrosis factor alpha	TNF-α	AJ413189	F	CAG GCG TCG TTC AGA GTC TC
			R	CTG TGG CTG AGA GCT GTG AG
β-actin	ACTβ	X89920	F	TCC TGC GGA ATC CAT GAG A
			R	GAC GTC GCA CTT CAT GAT GCT
Peroxisome proliferator-activated receptor gamma	PPARγ	AY590304	F	CGC CGT GGA CCT GTC AGA GC
			R	GGA ATG GAT GGA GGA GGA GGA GAT GG

IL-I family and related receptors: IL-1β, IL-1R1, IL-1R2, IL-18, IL-18R1
IL-6 type family and related receptors: IL-6, IL-6RA, IL-6RB
Common γ-chain cytokine family: IL-7, IL-15
CXC chemokine family and related receptors: IL-8, IL8-RA
IL-10 family and related receptors: IL-10, IL-10RA, IL10RB, IL-22R
IL-12 family: IL12β
Other cytokines: IL34, TNF-α

Table 2. Characteristics of new assembled sequences of interleukin-related genes of gilthead sea bream (*Sparus aurata*) according to BLAST searches.

Contig(s)	F[a]	size (nt)	Annotation[b]	Best match[c]	E[d]	CDS[e]	GenBank
C2_638	396	2790	IL-1R1	XP_003454962	7e-141	56-1975	JX976615
C2_757	380	2395	IL-6RA	AAR25684	3e-139	121-1863	JX976616
C2_1057	251	2681	IL-10RA	XP_003450895	0.0	373-1921	JX976622
C2_1779	169	1319	IL-15	NP_001028220	7e-42	238-726	JX976625
C2_2614	226	1204	IL-22R	XP_003438468	2e-80	33-689	JX976628
C2_4733	132	2069	IL-8RA	ABM66445	3e-165	408-1484	JX976620
C2_6418	70	1789	IL-18R1	XP_003445783	2e-31	150-1748	JX976627
C2_7820	35	1160	IL-34	NP_001244230	7e-53	470-1126	JX976629
C2_8010	57	1443	ILF2	XP_003455300	0.0	74-1237	JX976630
C2_10984	66	3398	IL-7	CBN81835	6e-31	220-780	JX976618
C2_14280	17	575	IL-8	AAY18807	4e-55	193-480	JX976619
C2_17128	29	2531	IL-6RB	XP_003439693	0.0	186-2479	JX976617
C2_20061	25	1105	IL-12β	ACQ57941	1e-132	<103-948	JX976624
C2_22174	29	1727	IL-10RB	XP_003443306	3e-114	99-1133	JX976623
C2_30367	5	863	IL-18	DAA01562	2e-08	156-641	JX976626
C2_34356	15	631	IL-10	ABH09454	2e-81	92->631	JX976621

[a] Number of reads composing the assembled sequences.

[b] Gene identity determined through BLAST searches: IL-1R1, Interleukin-1 receptor 1; IL-6RA, Interleukin-6 receptor A; IL-10RA, Interleukin-10 receptor A; IL-15, Interleukin-15; IL-22R, Interleukin-22 receptor; IL-8RA, Interleukin-8 receptor A; IL-18R1, Interleukin-18 receptor 1; IL-34, Interleukin-34; ILF2, Interleukin enhancer-binding factor 2; IL-7, Interleukin-7; IL-8, Interleukin-8; IL-6RB, Interleukin-6 receptor B; IL-12β, Interleukin-12 subunit beta; IL-10RB, Interleukin-10 receptor B; IL-18, Interleukin-18; IL-10, Interleukin-10.

[c] Best BLAST-X protein sequence match.

[d] Expectation value.

[e] Codifying domain sequence.

Figure 1

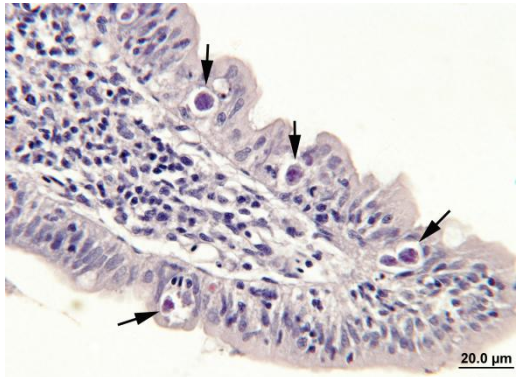
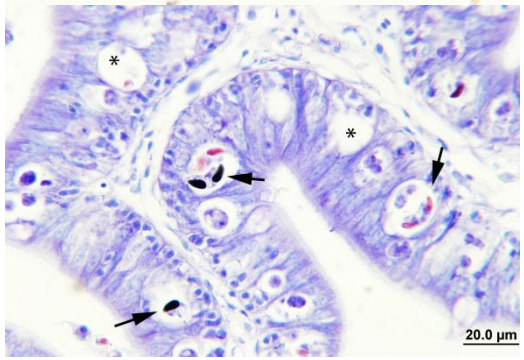
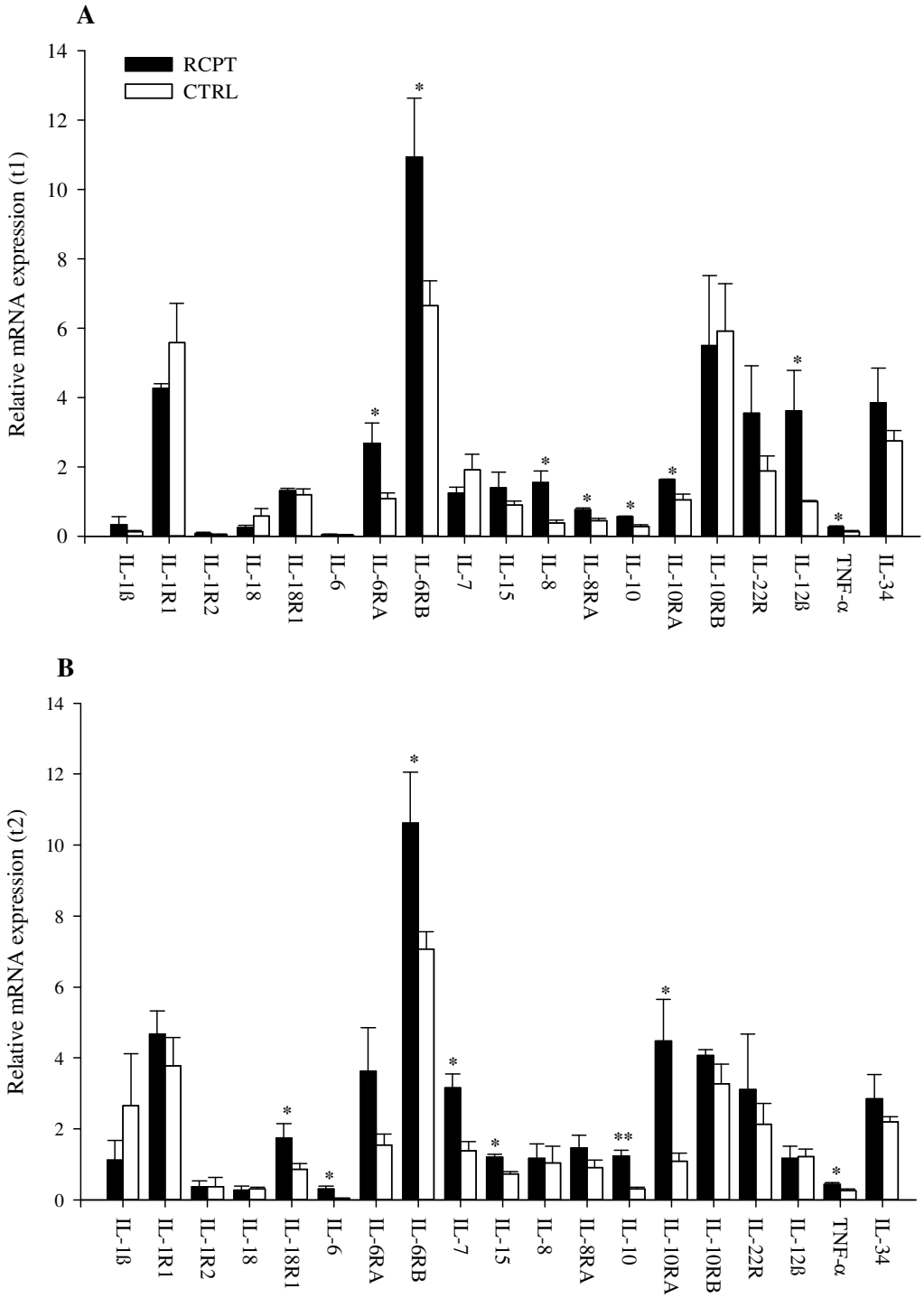
Time		**t1**			**t2**	
Intestinal segment	AI	MI	PI	AI	MI	PI
Prevalence (%)	0	0	42.8	42.8	14.3	85.7
Mean intensity	-	-	2+	4+	6+	5.2+
Histology t1						
Histology t2						

Figure 2

Fig. 2



Supplementary Table 1. Compilation of the gene expression profile of interleukin-related genes in the head kidney, blood and spleen of gilthead sea bream (*Sparus aurata*) intubated with *Enteromyxum leei* after 17 (t1) and 64 (t2) days. Data are shown as the mean expression values for each gene in control (CTRL) and recipient (RCPT) fish. No significant differences between CTRL and RCPT fish were found in none of these tissues. The different intensity of the colours stands for differences in relative expression units.

	Head Kidney				Blood				Spleen			
Gene	t1		t2		t1		t2		t1		t2	
	CTRL	RCPT	CTRL	RCPT	CTRL	RCPT	CTRL	RCPT	CTRL	RCPT	CTRL	RCPT
IL-1β	0.37	0.28	0.44	1.44	0.19	0.23	0.2	0.17	0.1	0.08	0.22	0.3
IL-1R1	2.39	2.48	1.93	1.45	1.2	1.36	1.09	0.74	0.78	0.96	0.64	0.64
IL-1R2	0.03	0.02	0.07	0.08	0.72	0.92	0.59	0.35	0.01	0.02	0.01	0.01
IL-18	0.08	0.09	0.05	0.05	0.89	1.11	0.81	0.47	0.02	0.02	0.02	0.02
IL-18R1	0.39	0.4	0.24	0.25	1.61	2.03	1.36	0.88	0.55	0.77	0.32	0.32
IL-6	0.03	0.02	0.02	0.03	1.64	1.87	1.7	1.23	0.02	0.04	0.02	0.02
IL-6RA	5.27	4.61	3.94	2.97	1.66	1.92	1.85	1.68	1.45	3.31	1.16	2.63
IL-6RB	0.96	0.85	0.74	0.75	1.15	1.31	1.17	0.79	0.98	0.84	0.82	0.71
IL-7	0.24	0.25	0.18	0.25	1.4	1.66	1.17	0.84	0.23	0.2	0.16	0.13
IL-15	0.04	0.03	0.03	0.03	0.02	0.03	0.14	0.11	0.09	0.11	0.07	0.05
IL-8	0.02	0.06	0.01	0.02	0.05	0.06	0.04	0.03	0.05	0.04	0.05	0.09
IL-8RA	5.78	4.89	3.57	2.93	1.89	2.02	2.28	2.05	0.5	1.13	1.09	1.6
IL-10	0.31	0.31	0.18	0.17	2.16	2.42	1.65	1.4	0.07	0.13	0.04	0.04
IL-10RA	1.05	0.8	1.1	0.97	1.25	1.31	1	0.62	1.05	1.04	1.22	0.6
IL-10RB	1.02	0.83	0.58	0.45	0.21	0.15	0.33	0.39	1.18	1.22	0.78	0.58
IL-22R	5.99	3.89	4.51	3.58	1.35	1.58	1.29	0.87	0.36	0.55	0.67	0.54
IL-12β	0.01	0.01	0.01	0.01	1.26	1.56	1.08	0.75	0.02	0.02	0.02	0.01
TNF-α	0.02	0.02	0.01	0.02	1.36	1.62	1.17	0.72	0.04	0.55	0.03	0.02
IL-34	1.04	1.04	0.77	0.78	0.02	0.04	0.14	0.15	0.81	0.85	0.71	0.52

Highlights

- 16 new IL-related sequences of gilthead sea bream were identified
- The expression of 19 IL-related genes was analysed in different tissues
- The expression in *E. leei* infected fish was statistically changed only at intestine
- An anti-inflammatory response shift at late times post infection was detected

Graphical Abstract