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

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-R6RA, IL-R6RB, 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

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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](http://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 (40°5’ N; 0°10’ E), and water temperature was maintained
between 18 °C and 25 °C. 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 °C. 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
required to perform the PCR array were performed by means of the EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany). Briefly, for each RT reaction, 660 pg of total input RNA was diluted to a 25 μl volume for each PCR reaction. PCR-wells contained a 2x SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 μM were used to obtain amplicons of 50–150 bp in length (Table 1). The PCR reaction was run under the following conditions: an initial denaturation step was carried out at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The efficiency of the PCR reactions was always higher than 90% (amplification factor >1.90) and similar for all genes. Negative controls without sample templates were routinely performed for each primer set. The specificity of the reactions was verified by analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55-95 °C), the linearity of serial dilutions of RT reactions, and electrophoresis and sequencing of the amplified products. Fluorescence data acquired during the PCR extension phase were normalized by the delta-delta Ct method [18]. Inter-assay variation was corrected using the serial dilutions of the standard gene as common reference values among plates. Technical replicates of the samples were run initially to test the reproducibility of the method. As the obtained data had a very high reproducibility score, technical replicates were finally omitted.

2.5. Statistics
Gene expression data are represented as the mean ± SEM of seven fish for each group shown in Fig. 2 (intestine). In Supplementary Table 1 (HK, SP, blood) only the averages are shown for visual simplification. For each gene from the different tissues, the effects of pathogen exposure were analysed by the two-tailed Student’s t test. When 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.
3.3. Interleukin expression after E. leei exposure

The 19 genes analysed were expressed in the four studied tissues, but only the intestine showed an mRNA expression profile with statistically significant differences between CTRL and RCPT fish (Fig. 2). The expression of genes encoding several ILs was significantly modulated by E. leei infection at both studied times after challenge. At 17 dpi, the IL-6 receptors (IL-6RA and IL-6RB), IL-8 and its receptor (IL-8RA), IL-10 and one of its receptors (IL-10RA), IL-12β, and the cytokine TNF-α were significantly up-regulated in RCPT fish (Fig. 2A). Although a few gene products showed up-regulation at both sampling time points (IL-6RB, IL-10, IL-10RA, and TNF-α), others were significantly up-regulated only at 64 dpi (IL-18R1, IL-6, IL-7, and IL-15) (Fig. 2B).

Interestingly, among all of them, IL-10 showed the highest statistical difference between CTRL and RCPT samples ($P < 0.001$), and no gene was found to be significantly down regulated in RCPT fish.

No statistically significant differences among IL-encoding genes were observed in HK, SP, or blood samples (Supplementary Table 1). In HK, most genes, except for IL-1β at 64 dpi, showed a similar or decreased expression in RCPT fish. It must also be noted that most ILs showed barely detectable mRNA expression levels at both 17 and 64 dpi, but three genes (IL-6RA, IL-8RA and IL-22R) showed RNA expression levels that stand out above the rest. In SP, the pattern was opposite to that observed in HK, specifically at 17 dpi when many genes were up-regulated in RCPT fish, but not significantly. At 64 dpi, only IL-6RA and IL-8RA were still up-regulated, whereas the remaining ones were down-regulated. IL-6RA exhibited the highest expression values at both sampling times. Similarly to what was observed in HK, some ILs genes (IL-1R2, 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, antioxidant, 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
expression depending on the tissue and sampling time. The current study is only the
second report on fish IL-34, which was just recently described for the first time in
several fish species [15]. Nevertheless, some ILs found in other fish species were not
found in GSB, such as IL-2, IL-4, IL-11, and IL-17. Further transcriptomic and genomic
studies will probably help to identify them. In any case, it is tempting to suggest that the
lack of some ILs could be due to overlapping functions, as described in mammals. It has
been shown that IL-2 function partially overlaps with IL-15 [33], and the function of IL-
11 overlaps with that of IL-6 [34]. Although we did not find IL-17, we identified the
receptor for IL-22, another IL that is also involved in the Th17 response and that
regulates intestinal and skin homeostasis [35]. Accordingly, the increase in IL-22R
expression in the intestine of RCPT fish (though not significant due to the high
individual variation), probably reflects the effort of the immune system to control the
intestinal homeostasis altered by the parasite.

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

The second important result is the shift in the type of immune response from
pro-inflammatory to anti-inflammatory, with the progression of the infection. In the
current host-parasite model, we observed an early and acute pro-inflammatory response
shortly after parasite inoculation (t1), as six IL-related genes and TNF-α were up-
regulated in RCPT fish. Most of these genes have a pro-inflammatory profile. Among them, IL-12β showed the highest expression levels. IL-12 is a heterodimeric cytokine comprised of covalently bound p35 (IL-12α) and p40 (IL-12β) subunits and a critical regulator of Th1 type immunity. In mammals, IL-12 is secreted by antigen presenting cells after activation by PAMPs and DAMPs and induces NK cells to produce IFN-γ [47, 48]. In fish, the presence of multiple divergent p40 subunits means many IL-12 molecules may exist that could have different functions. In fact, in previous studies, different p40 isoforms were also increased following injection with killed bacteria [49], viral infection [50] or protozoan infection [51]. It is unknown whether p40c would drive Th17 type responses and p40b would drive Th1 type responses in fish and the evaluation of the bioactivities of each of these putative fish IL-12 isoforms is a challenge to be deciphered in the future [11]. The GSB IL-12β clusters with fish p40a and p40b in phylogenetic tree analysis (data not shown). Thus, the up-regulation of IL-12β in parasitized GSB would engage the initial cellular response to *E. leei* through the activation of T cells and non-specific cytotoxic cells (NCC; the evolutionary precursor of mammalian NK cells) [52]. This is in accordance with the significantly higher cytotoxic activity of HK leucocytes in GSB after 22 days of cohabitation with *E. leei*-infected fish [53]. The activation of T cells by IL-12β in mammalian models also agrees with the higher percentage of intestinal epithelial lymphocytes in turbot (*Psetta maxima*) exposed to another *Enteromyxum* species, *E. scophthalmi* [54], and these lymphocytes are largely considered to be T cells in teleosts.

Another important pro-inflammatory cytokine, IL-8, and its receptor, IL-8RA, showed significant overexpression at t1. IL-8 induces chemotaxis in target cells, causing them to migrate to the site of infection in mammals. Thus, GSB IL-8 could induce the migration of granulocytes and macrophages to the site of infection, as already observed
in other fish species [55, 56]. This agrees with the increased number of mononuclear and eosinophilic granular cells in the intestine of E. leei-infected GSB [16, 57]. The third typically pro-inflammatory cytokine that was significantly up-regulated at t1 was TNF-α, which is in agreement with its early overexpression in most fish-parasite models [58]. In mammals, TNF-α plays an important role in bacterial, viral, and intracellular parasite infections [59], but recent work seems to suggest that this may not be the case in teleosts [60]. In our study, TNF-α was still up-regulated at t2, but in a previous study, TNF-α was significantly down-regulated in the intestine of E. leei-infected GSB at 113 days after infection [61]. Thus, it is probable that E. leei infection requires longer exposure times to induce a down-regulation of TNF-α expression.

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

The anti-inflammatory profile at t2 was mainly represented by the significant up-regulation of IL-10, IL-10RA, IL-7, IL-6, and IL-15. The highest fold change was seen for IL-10, a central anti-inflammatory cytokine capable of inhibiting the expression of many pro-inflammatory cytokines [67], and directly affecting T cell activation [68]. IL-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.

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References


Kim DH, Austin B. Cytokine expression in leucocytes and gut cells of rainbow trout, Oncorhynchus mykiss Walbaum, induced by probiotics. Vet Immunol Immunopathol 2006;114:297-304.


Forlenza M, de Carvalho Dias JD, Vesely T, Pokorova D, Savelkoul HF, Wiegertjes GF. Transcription of signal-3 cytokines, IL-12 and IFN, coincides with the timing of CD8 up-regulation during viral infection of common carp (Cyprinus carpio L.). Mol Immunol 2008;45:1531-47.


Estensoro I, Calduch-Giner JA, Kaushik S, Pérez-Sánchez J, Sitjà-Bobadilla A. Modulation of the IgM gene expression and the IgM immunoreactive cell distribution by the nutritional background in gilthead sea bream (Sparus aurata) challenged with Enteromyxum leei (Myxozoa). Fish Shellfish Immunol 2012;33:401-10.


Smith DE. The biological paths of IL-1 family members IL-18 and IL-33. J Leukocyte Biol 2011;89:383-92.


**Figure captions**

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

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

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

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

**Supplementary Material**

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

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IL-1 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, IL-8RA
IL-10 family and related receptors: IL-10, IL-10RA, IL-10RB, IL-22R
IL-12 family: IL-12β
Other cytokines: IL-34, TNF-α
Table 2. Characteristics of new assembled sequences of interleukin-related genes of gilthead sea bream (*Sparus aurata*) according to BLAST searches.

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<th>size (nt)</th>
<th>Annotation&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup>Number of reads composing the assembled sequences.

<sup>b</sup>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.

<sup>c</sup>Best BLAST-X protein sequence match.

<sup>d</sup>Expectation value.

<sup>e</sup>Codifying domain sequence.
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**Figure 1**

Histology t1

Histology t2

- 20.0 μm

Histology t1

Histology t2
### 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.

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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