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Acquired protective immune response in a fish-myxozoan model encompasses specific antibodies and inflammation resolution

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ABSTRACT

The myxozoan parasite *Enteromyxum leei* causes chronic enteritis in gilthead sea bream (GSB, *Sparus aurata*) leading to intestinal dysfunction. Two trials were performed in which GSB that had survived a previous infection with *E. leei* (SUR), and naïve GSB (NAI), were exposed to water effluent containing parasite stages. Humoral factors (total IgM and IgT, specific anti-*E. leei* IgM, total serum peroxidases), histopathology and gene expression were analysed. Results showed that SUR maintained high levels of specific anti-*E. leei* IgM (up to 16 months), expressed high levels of immunoglobulins at the intestinal mucosa, particularly the soluble forms, and were resistant to re-infection. Their acquired-type response was complemented by other immune effectors locally and systemically, like cell cytotoxicity (high *granzyme A* expression), complement activity (high *c3* and *fucolectin* expression), and serum peroxidases. In contrast to NAI, SUR displayed a post-inflammatory phenotype in the intestine and head kidney, characteristic of inflammation resolution (low *il1β*, high *il10* and low *hsp90α* expression).

1. Introduction

Aquaculture production is constantly increasing, reaching 110 million tons globally, with a value of over 210 billion Euros in 2016 [1]. However, infectious diseases threaten its future and sustainability. The most common etiological agents in aquaculture are bacteria and viruses, followed by parasites. Parasite infections are favoured by fish farming conditions, often in over-crowded cages, and with stressful handling procedures, which also contribute to the propagation of opportunistic pathogens, provoking important economic losses [2].

The myxozoan clade, which belongs to the phylum Cnidaria, comprises more than 2180 species spread worldwide, most of which are fish endoparasites, infecting any tissue and host organ [3]. They have a large impact on freshwater and marine fish production [4] since there are no available effective control measures, such as vaccines or commercial treatments. *Enteromyxum leei* is a serious risk to gilthead sea bream (GSB, *Sparus aurata*) aquaculture due to its high rate of horizontal transmission and its high pathogenic potential [5]. This myxozoan is a histozoic parasite that dwells between gut epithelial cells, causing different degrees of anorexia, delayed growth with weight loss, cachexia, reduced marketability and increased mortality [6]. Its typical cell-in-cell division leads to the formation of mature proliferative stages that are capable of releasing new daughter cells, which invade the host intestine following a posterior-anterior pattern [7]. In advanced GSB infections with *E. leei,* the intestine displays hypertrophy of the lamina propria-submucosa and loss of the epithelial palisade structure, together with an intense local inflammatory response [6–9]. These intestinal changes heavily impair nutrient absorption reducing farming performances. Nowadays, *E. leei* constitutes a threat for many other cultured sparids, such as sharpsnout sea bream, *Diplodus puntazzo* [10]; black spot seabream, *Pagellus bogaraveo* [11]; common sea bream, *Pagrus pagrus* [12]; and red sea bream, *Pagrus major* [13].

Fish possess a complete immune system in which innate (natural) and adaptive (acquired) immunity are integrated, and mechanisms of immunologic specificity and memory are developed. The importance of mucosal interfaces in the triggering of antigen responses has been acknowledged in this primitive vertebrate clade [14,15]. In the last decade, studies on fish immunity are shedding some light on the

Abbreviations: AI, anterior intestine; dpe, days post-exposure; GSB, gilthead sea bream; HK, head kidney; NAI, naïve fish never exposed before to *E. leei*; PI, posterior intestine; PO, peroxidases; SUR, fish that survived and cleared an established *E. leei* infection

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immune effectors involved in the response to some myxozoan infections [16–19], but a long road lies ahead before therapeutics and vaccines will be available.

Immune response to myxozoans is highly species- and tissue-specific. The host-parasite encounter usually takes place at the mucosal surfaces, and thus, the first line of interactions and responses against myxozoa originate at the mucosal epithelia orchestrated locally by the mucosa-associated lymphoid tissue (MALT). Some myxozoan species reside only transitorily in mucosal epithelia, their route of entry into the host before they spread and reach their target sites. This is the case of some *Sphaerospora* spp. [20], *Tetracapsuloides bryosalmonae* [21,22], and *Myxobolus cerebralis* [23]. An inflammatory reaction is common at the target tissues of histozoic myxozoans [17], and by contrast, infection does not elicit any immune response while the parasite infects or moves along some immune-privileged sites, as the nerves in the case of *M. cerebralis* and the testes in the case of *S. testicularis* [24].

In GSB enteromyxosis, serum immunoglobulins, lysozyme, peroxidases and complement are involved in the humoral response [25–28], and B cells, T cells, mast cells and melanomacrophages act locally and systemically during the inflammatory response [29,30]. The mucin-constituted mucus barrier overlying the epithelia is the attachment site for the parasite before it penetrates the tissue and proliferates or spreads. Mucin composition and expression, and mucus-secreting goblet cell distribution are also modulated during enteromyxosis [8,31–34]. Chronic exposure to the parasite stimulated expression of genes related to interferon signalling and antigen processing and presentation in the intestine of exposed non-parasitized fish, which may point to the local triggering of immune resistance mechanisms against the infection [35]. An increase in the splenic melanomacrophage centres during GSB enteromyxosis may also indicate the onset of an acquired immune response [8,29]. Interleukin (IL) expression during GSB enteromyxosis was modulated from a pro-inflammatory to an anti-inflammatory profile, and this shift was apparently driven by the up-regulation of *il10* [36]. More recently, the involvement of a cytotoxic response in the clearance of the parasite was suggested from the higher expression of *cd8* receptors and cytotoxic molecules in exposed non-parasitized fish intestines [30].

Resistant fish strains or acquired protection was reported for some myxozoan infections, as in the case of *Kudoa thyrsites* [37], *T. bryosalmonae* [38] and *Ceratonova shasta* [39] infecting salmonids, or in *E. leei* affecting some sparid species [40,41]. Fish recovered from some myxozoan infections are less susceptible to reinfection, however, acquired immune mechanisms involved in resistance upon re-exposure to myxozoans have been studied in few cases. Specific antibodies conferred resistance against *E. scophthalmi* in turbot [42]. The severity of infection with *K. thyrsites* was decreased in re-exposed Atlantic salmon, which exhibited an acquired protective immune response mounted on antigen-presenting monocyte/macrophage and dendritic cells, along with B and T cells in the muscle tissue [43]. Rainbow trout re-exposed to *T. bryosalmonae* developed immune protection through the increase of circulating IgM^+ B cells and up-regulated mRNA levels of secretory IgM in the posterior kidney [44]. However, there are no previous studies on immune response upon re-challenge with *E. leei*.

The aim of this study was to determine the bases of the protective mechanisms involved in GSB surviving a first exposure to *E. leei* and re-exposed to the parasite. We focused on innate and adaptive immune parameters in order to elucidate the differential response of these re-exposed animals, compared to animals challenged for the first time with the parasite. Special attention was given to the role played by immunoglobulins and by the inflammatory response at the mucosal interface.

2. Materials and methods

2.1. Fish and experimental infections

Specific-pathogen-free (SPF) and clinically healthy gilthead sea bream (GSB) juvenile specimens from a commercial fish farm were kept in 5 µm-filtered and UV irradiated sea water (salinity 37.5 g/L) at temperatures between 18 °C and 27 °C, with natural photoperiod at our latitude (40°5′N; 0°10′E). The SPF status was confirmed by qPCR according to the protocol described in section 2.2. Fish were fed *ad libitum* a commercial diet (BioMar, Palencia, Spain). Animals were kept according to the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. All efforts were made to minimize suffering of the fish used for the experiments in accordance with national (Royal Decree RD1201/2005, for the protection of animals used in scientific experiments) and the current European Union legislation (86/609/EU) and the CSIC National Committee on Bioethics under approval number 151–2014. Two challenge trials with *E. leei* were performed at the facilities of IATS-CSIC (Castellón, Spain): T1, started in February and lasted 175 days (water temperature range 18°C-26.1°C) and T2 started in June, lasted 86 days (water temperature range 22.9°C-27°C). The reason for having two trials was mainly due to the low prevalence of infection found in T1. T2 was performed at higher water temperatures to ensure higher infection rates. Differences in sampling dates between trials were due to the faster progression of infection in T2, probably as a result of the different starting temperature. For clarity, a summary of the timings and experimental groups can be found in Table 1. In both trials, fish were challenged with *E. leei* by exposure to infected effluent, mimicking the natural route of infection. Briefly, it consisted of tanks holding recipient fish (RCPT) receiving water effluent from a donor tank holding *E. leei*-infected GSB, as previously described [45]. Heavily infected donor fish were selected using

Table 1
Experimental groups and sampling details of the two *Enteromyxum leei* transmission trails (T1 and T2) to *Sparus aurata*.

	T1				T2		
Initial water temperature	18 °C				22.8 °C		
Mean water temperature	19.6 °C				24.3 °C		
Sampling time (dpe)	0	66	105	175	0	61	86
Samples taken	NL-PCR, blood	NL-PCR, blood	NL-PCR, blood	HIS (AI, MI, PI), blood	NL-PCR, blood	NL-PCR, blood	HIS (AI, MI, PI), GE (HK, AI, PI), blood
Fish group	NAI		SUR		NAI		SUR
Fish number	20		20		20		24
Initial weight (g)	95		607		246		175

NAI (naïve), SUR (surviving), dpe (days post exposure), NL-PCR (non-lethal rectal mucosa probe for PCR diagnosis), HIS (tissue sample for histology), GE (tissue sample for gene expression), HK (head kidney), AI (anterior intestine), MI (middle intestine), PI (posterior intestine).

a non-lethal diagnostic technique described in section 2.2. The renewal of the water in the effluent system was kept to a minimum to ensure longer exposure to the parasite but without affecting water quality. The effluent exposure was not interrupted until the final sampling.

In both trials, two groups of RCPT fish were used. One group consisted of surviving fish (SUR), recovered from two previous independent experimental *E. leei*-infections. For T1, 20 SUR adult fish (initial mean body weight: 607 g) were used. In T2 the number of SUR fish was 24 (175 g). These animals were routinely tested by the non-lethal molecular diagnosis described in section 2.2. All SUR fish had yielded a 100% prevalence of infection (9 weeks post-exposure) and later on, tested negative for the presence of the parasite at 16 (T1) and 9 (T2) months post-exposure. Thus, all SUR animals had recovered from an established infection. The second group consisted of naïve GSB (NAI), never exposed to the parasite. Twenty NAI fish were used in, both, T1 and T2 (initial mean body weight 95 and 246 g, respectively). For both trials, SUR and NAI fish were individually tagged with passive integrated transponders (PIT-tags) and equally distributed into two replicated-500 L RCPT-tanks. All NAI and SUR fish were obtained from the same fish farm, and were held in the same conditions until the trials were carried out. In T2 NAI and SUR fish belonged to same stock and the only difference was the previous exposure to the parasite, which was reflected in the difference in weight. Thus, gene expression analyses were only performed in T2 to avoid differences due to age or genetic background.

2.2. Samplings and parasite diagnosis

In all samplings, fish were starved for two days before sampling and blood samples (1 ml) from SUR and NAI were taken from the caudal vessels by puncture with heparinized sterile needles. After overnight clotting at 4 °C and centrifugation at 3000 × *g* for 30 min, sera were recovered and maintained at −80 °C until further use. In the intermediate non-lethal (NL) samplings, fish were additionally sampled for parasite diagnosis by a non-lethal probe of the rectal mucosa with a cotton swab and PCR was carried out with specific primers for *E. leei* 18S rRNA gene, as previously described [6,46]. This NL-method was also used to determine the historical infection status of the SUR fish described above. At the final lethal samplings, all SUR and NAI fish were killed by overexposure to anaesthesia (MS-222, 0.1 g/L; Sigma). Head kidney, anterior and posterior intestine samples from all fish were taken in RNAlater (Qiagen) and kept at −20 °C for gene expression studies (final sampling of T2). Anterior, middle and posterior intestine samples from all fish were taken in 10% buffered formalin for standard histological procedures at the final sampling of both trials. Infection intensity was semiquantitatively evaluated on Giemsa stained histological sections of anterior intestine (AI), middle intestine (MI) and posterior intestine (PI) following the ranges: low = 1–10; medium = 11–50; high = 51 to > 100 parasite stages per microscope field observation at 120 × . A fish was considered positive for infection, when the parasite was found at least in one intestinal segment. Number of fish and type of samples taken at each time point are listed in Table 1. Fish that died outside the scheduled sampling points were checked post-mortem for the presence of the parasite and were no longer included in the results.

2.3. Circulating antibody detection

Total IgM in SUR and NAI serum samples was measured by ELISA. Briefly, 96- flat-bottomed well microplates (Maxisorp, Nunc) were coated with 50 µl of the GSB sera diluted 1:3000 in coating buffer (carbonate-bicarbonate buffer, pH 9.6) overnight at 4 °C. Between incubation steps, three successive washings with TTBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.2 (TBS) with 0.05% Tween 20) and one with TBS were performed. A blocking incubation step was carried out with 5% skimmed milk in TBS for 2 h at 37 °C, before incubating with a custom-made rabbit polyclonal antibody (Ab) against GSB IgM (1:80,000) [47] for two further hours at 37 °C. Thereafter, goat anti-rabbit IgG horseradish peroxidase conjugate (Sigma) diluted 1:2000 in TBS was added, incubated for 1 h at 37 °C and the last washing performed. TMB peroxidase substrate (3,3′,5,5′-tetramethylbenzidine solution + H_2O_2; Bio-Rad) was applied, incubated at room temperature in the dark with gentle shaking for 30 min, and finally the reaction was stopped by addition of 1 N H_2SO_4. The plates were then read at 450 nm with an automatic plate reader (Tecan Group Ldt.).

Total IgT in GSB serum samples was analysed by ELISA as previously described [26] using a custom-made anti-GSB IgT Ab (1:1000).

The presence of specific IgM against *E. leei* in serum samples was immunohistochemically detected using paraffin embedded sections of *E. leei*-infected GSB intestines obtained from previous and independent infection trials, following a protocol that can be described as an immunohistochemical sandwich ELISA. This method was chosen due to the unavailability of an optimized ELISA for this parasite. In addition, it has the advantage of providing information on the different parasite structures labelled by each serum. Briefly, tissue sections were deparaffinised and hydrated, endogenous peroxidase activity was quenched by incubation for 30 min in 3% H_2O_2 and slides were blocked with 1.5% normal goat serum (VECTOR Laboratories) for another 30 min. The standard washing procedure consisted of 5 min successive immersions in TTBS and TBS between incubation steps, which were performed in a humid chamber at room temperature. Tissue sections were then incubated for 1 h with each individual SUR or NAI serum (1:25 in TBS 1% bovine serum albumin; Sigma) and thereafter for one further hour with the rabbit polyclonal Ab against GSB IgM (1:17,000) [47]. Finally, tissues were incubated with a biotinilated goat anti-rabbit Ab (VECTOR Labs.) for 1 h and the avidin-biotin-peroxidase complex (ABC; VECTOR Labs.) was applied for one further hour. Bound peroxidase was visualized by addition of 3,3′-diaminobezidine tetrahydrochloride chromogen (Sigma) and the reaction stopped after 2 min with deionized water. Tissue sections were subsequently counterstained with Gill's haematoxylin, dehydrated and mounted in di-*N*-butyl-phthalate in xylene. Each serum sample was tested in duplicate. Negative controls were carried out omitting the fish serum, the primary and secondary Abs or the ABC. Intensity of immunoreactivity of each fish serum against the parasite was evaluated by microscopic examination of the immunolabelled tissue sections according to a semiquantitative scale ranging from 0 to 6 (scaling: 0 = no immunoreactivity with the parasite; 1 = very slight reactivity; 2 = slight reactivity; 3 = medium reactivity; 4 = medium-intense reactivity; 5 = intense reactivity; 6 = very intense reactivity).

2.4. Total serum peroxidases

Total serum peroxidases (PO) in SUR and NAI serum samples were measured in a plate assay following the procedure as previously described [48]. Briefly, individual sera diluted 1:20 in Hanks' balanced salt solution were incubated in 96-well microplates (Nunc) with 50 µl of TMB peroxidase substrate (Bio-Rad) (final volume 200 µl/well). After 2 min incubation at room temperature in the dark with gentle shaking, the reaction was stopped by addition of 1 N H_2SO_4. Then, plates were read at 450 nm with an automatic plate reader (Tecan Group Ldt.).

2.5. Gene expression analysis

2.5.1. RNA isolation

Total RNA from 100 mg of AI, PI and head kidney (HK), of 8 individuals of each T2 group (SUR and NAI) obtained at the final sampling (86 dpe) was isolated by means of the MagMax-96 for Microarray Total RNA Isolation kit (Ambion, Applied Biosystems), following the manufacturer's instructions. Tissue homogenization was performed in Lysing Matrix D tubes (MP Biomedicals) with 1 ml of TRI reagent using a FastPrep-24 homogenizer (MP Biomedicals, 2 cycles of 30 s at 6 m/s).

Table 2
Prevalence of infection with *Enteromyxum leei* by PCR and histology (for the last samplings, 175 and 86 days post-exposure (dpe) in T1 and T2, respectively), and fish number of naïve (NAI) and surviving (SUR) gilthead sea bream along trials 1 and 2 (T1 and T2).

		T1				T2		
	dpe	0	66	105	175	0	61	86
	Group							
Sampled fish	NAI	10	10	10	10	20	20	12
	SUR	20	20	20	16	24	23	18
Cumulative mortality (%)	NAI	0	0	15	40	0	0	40
	SUR	0	0	0	20*	0	4.2*	25*
Prevalence of infection (%)	NAI	0	0	40	20	0	75	83.3
	SUR	5	15	0	0	0	4.4	0

* Cumulative mortality in SUR fish was due to technical issues. Dead SUR fish were diagnosed post-mortem and found negative for *E. leei*, whereas dead NAI fish were always positive for the parasite and presented acute disease signs.

RNA quantity and purity was determined by Nanodrop (Thermo Scientific). The 8 individuals selected for the NAI group were the ones with highest infection intensity at 86 dpe. The 8 SUR fish chosen were the ones showing highest serum immunoreactivity against *E. leei* at 0 dpe. The criteria for the targeted selection was our prediction based on previous results, that pre-exposure to the parasite confers resistance to re-infection based, at least in part, on the presence of specific antibodies.

2.5.2. cDNA synthesis and amplification

Prior to reverse transcription, 500 ng of RNA were treated with DNaseI amplification grade (Invitrogen) following the manufacturer's instructions. Five hundred ng of input RNA were reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) following the manufacturer's protocol (10 min at 25 °C, 2 h at 37 °C, and 5 min at 85 °C).

2.5.3. PCR array

Real-time (RT) quantitative PCR was carried out using the CFX96 Connect™ Real-Time PCR Detection System (Bio-Rad) in a 96-well layout designed for simultaneously profiling the genes of interest for each tissue and individual. The primers used in this study (Supplementary Table 1) were selected to detect target genes characteristic of the specific responses to be studied. The primers were designed and/or checked for specificity by data mining of the GSB transcriptomic database (http://nutrigroup-iats.org/seabreamdb/). All the primers were checked to have similar efficiency above 90% ($R^2 > 0.98$). Each RT reaction of 20 µl contained 3.3 ng of total input RNA sample, 5 × PyroTaq EvaGreen qPCR Mix Plus (Cultek Molecular Bioline) and specific primers at a final concentration of 0.45 µM. The PCR reaction conditions consisted of an initial step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. The specificity of the reactions was verified by visual analysis of melting curves for each reaction performed. Fluorescence data acquired during the PCR extension phase were normalized by the delta-delta Ct method [49] using *β-actin* as a reference gene. This particular reference gene was chosen among several other candidates as the most stable among conditions in each tissue with the GeNorm software. To allow comparisons, all individual *β-actin* Ct values were verified to have differences lower than 1 Ct value.

2.6. Cluster analysis

Partitioning cluster analysis was performed to evaluate the similarity of all individual fish used in the gene expression analysis. All the information available for each of the SUR (n = 8) and NAI (n = 8) fish used for the PCR array from T2 was used to perform K-means clustering. This includes individual biometric parameters (weight, length and condition factor), serum peroxidases, total serum IgM, total serum IgT and specific serum IgM levels for all three sampling points; individual infection status determined by NL-diagnostic in the intermediate sampling and by histological scoring in the final sampling; and the expression level of the 21 genes studied in the three tissues (head kidney, anterior and posterior intestine) in the final sampling. The raw data used for the cluster analysis are shown in Supplementary Table 2. Clustering was performed in R (v3.4.3) using the *kmeans* function with scaled data, $k = 3$ and *nstart* = 50 to obtain stable results [50]. Visualizations were performed using the factoextra R package [51].

2.7. Statistics

Data on specific serum IgM, total serum IgM, total serum IgT, serum PO and gene expression were analysed for statistically significant differences between SUR and NAI among sampling points by one-way analyses of variance (ANOVA-I) followed by Student–Newman–Keuls test. Data which failed the normality or equal variance test were analysed with Kruskal–Wallis ANOVA-I on ranks followed by Dunn's method. Differences were considered significant at a $p < 0.05$. A chi-square test was run to assess whether having specific IgM against *E. leei* and being parasitized was dependent on the NAI/SUR condition.

3. Results

3.1. Parasite infections

In T1 SUR fish, the parasite was only detectable at 66 dpe (15% prevalence) and no infection was detectable at 105 and 175 dpe, whereas T1 NAI fish reached their maximum prevalence of infection (40%) at the third sampling (105 dpe). At the last sampling point (175 dpe), prevalence of infection of NAI dropped to 20% (Table 2). Giemsa stained sections obtained at the last sampling allowed to depict the intensity and extension of the infection along the intestinal tract. NAI fish showed medium infection intensities at the PI, consisting mainly of trophozoites with secondary (S) and tertiary cells (T), whereas AI and MI segments had low infection intensity, with mostly single cell trophozoites (primary (P) cells) (Fig. 1). Inflammatory infiltrates and hypertrophy of the intestinal submucosa were often observed in NAI fish. By contrast, no parasite stages were detected in SUR fish. Scattered macrophage-like cells with engulfed cell debris in the epithelium, infiltrated lymphocyte-like cells close to the basal membrane, and eosinophilic granular cell aggregates in the lamina propria-submucosa were often seen in SUR intestines (Fig. 1).

Since prevalence of infection of T1 NAI fish turned out to be lower than in average infections in our laboratory, a second infection trial (T2) was performed, with additional focus on immune gene expression in tissues, besides humoral immunity. At the second sampling point (61 dpe), prevalence of infection in NAI fish was 75%, whereas only one SUR fish was infected (4.4%). At the last sampling point (86 dpe), prevalence of infection of NAI increased to 83.3%, whereas it dropped to 0% in SUR fish (Table 2). The histological examination of T2 intestinal sections of NAI fish at 86 dpe also showed medium infection intensity at the PI (trophozoites with S and T cells), a low infection intensity at the AI (P cells) and no infection at MI. As in T1, no parasite stages were observed in SUR intestines, and scattered cell debris were found in the epithelium (Fig. 1).

Mortalities in T1 rose to 40% in NAI fish and 20% in SUR at the last sampling point. Similarly, in T2, mortality was 40% and 25% in NAI and SUR fish, respectively. No parasites were found in dead SUR fish, and all NAI dead fish were positive for the parasite.

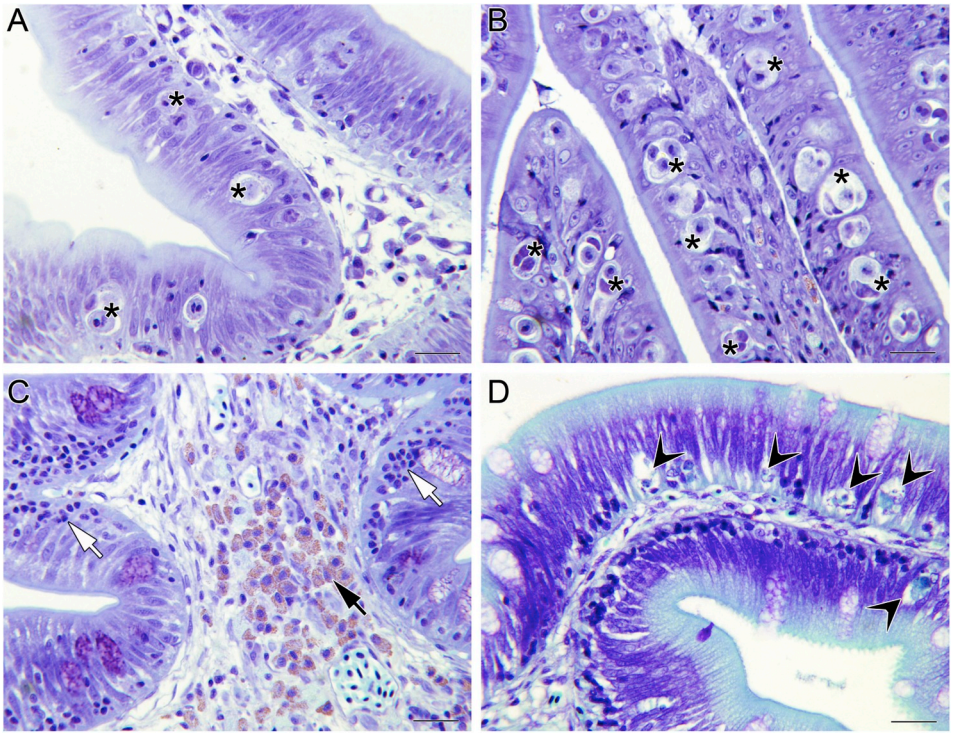


Fig. 1. Representative images for T1 and T2 Giemsa stained intestinal sections of naive (A, B) and surviving (C, D) gilthead sea bream. (A) Anterior intestine segment with low infection intensity: *E. leei* stages (*) consist mainly of primary and secondary cells. (B) Posterior intestine (PI) with high infection intensity: parasite stages (*) are trophozoites with secondary and tertiary cells. (C, D) Non-infected PI with evident cellular immune response. Aggregates of eosinophilic granular cells in the lamina propria-submucosa (black arrow) and infiltration of lymphocyte-like cells in the epithelium (white arrows) (C) and macrophages with cell debris in the epithelium (arrow heads) (D). Scale bars = 20 μm.

3.2. Total serum IgM and IgT

In T1, SUR IgM was significantly higher than NAI IgM before exposure (0 dpe). Serum IgM of NAI fish was significantly higher at 66 dpe and 105 dpe than before exposure to *E. leei*, whereas SUR sera IgM increased progressively and became significantly higher than at the beginning of the experiment from 105 dpe onwards. (Fig. 2A). In T2, no significant differences were found between circulating IgM of NAI and SUR groups at any sampling point. NAI IgM was significantly higher at 86 dpe when compared to 0 dpe. In SUR fish, IgM was significantly higher at 86 dpe than in the previous time points (Fig. 2B).

No significant differences in total serum IgT were found between NAI and SUR fish at any time point, in any trial. When analysing the IgT serum values within each fish group, only a significant change was detected in T1 in SUR fish, whose serum IgT dropped at 105 dpe significantly. In T2, no significant changes of circulating IgT levels were detected in any group (Fig. 3).

3.3. Specific IgM levels against E. leei

In T1, the percentage of SUR sera with specific IgM remained high throughout all sampling points (65–75%, Table 3) and there were no significant differences in the mean intensity of specific immunodetection among SUR sera along the trial (Fig. 4A). Among all the probed SUR sera that had IgM against *E. leei* (72.37%), 69.74% were non-infected fish and only 2.63% were infected (Table 3, pie chart). All immunoreactive SUR sera labelled P cells and most of them also S cells and spore valves throughout the trial (Fig. 4C). The intensity of parasite immunodetection of SUR sera was significantly higher than that of NAI except at 175 dpe, when NAI intensity reached SUR values (Fig. 4A). No specific IgM against the parasite was detected in NAI fish until 105 dpe. At this time point, only 20% of NAI sera immunoreacted against *E. leei* with low intensity (Table 3 and Fig. 4A). A significant increase of the mean intensity of specific immunodetection was found in 60% of the NAI sera at 175 dpe. Only 20% of all analysed NAI sera throughout the trial had specific IgM against the parasite, of which 12.5% were non-infected fish and 7.5% were infected (Table 3, pie chart). Immunoreactive sera of 105 dpe NAI fish consistently labelled P and S cells of the parasite stages and spore valves only inconsistently, whereas some 175 dpe NAI sera labelled P cells, S cells, spore valves and spore accompanying cells consistently (Fig. 4A, C).

In T2, specific parasite immunodetection by SUR sera was statistically higher than that of NAI sera only at 0 dpe, with 83.3% of SUR having specific IgM against the parasite. A significantly higher intensity of specific immunodetection was detected with 61 dpe and 86 dpe SUR sera, when compared to 0 dpe serum. The percentage of SUR sera with specific IgM also remained high in T2, being 91.3% at 61 dpe, and 83.3% at 86 dpe (Table 3 and Fig. 4B). All immunoreactive SUR sera labelled P cells and most of them also S cells and spore valves throughout the trial. Only one SUR fish, which had specific anti-*E. leei* IgM, was PCR positive for *E. leei* at 61 dpe (Tables 2 and 3). A significant increase in the intensity of specific immunodetection was found in NAI sera at 61 dpe, compared to 0 dpe NAI sera. All NAI sera at 61 and 86 dpe had specific IgM against the parasite, though, 75% and 83,3% were infected fish, respectively (Table 3). At these time points, NAI immunoreactive sera labelled consistently P and S cells and spore valves only inconsistently (Fig. 4B and C). Interestingly, 15% of the NAI sera immunoreacted against the parasite, though with low intensity, before exposure (0 dpe).

In both trials, contingency analysis demonstrated that there was a significant relationship between previous exposure to the parasite (SUR/NAI variable) and production of specific IgM (anti-*E.leei*$^{+}$/anti-*E.leei*$^{-}$ variable) ($p < 0.001$). There was also a significant relationship between the production of specific IgM (anti-*E.leei*$^{+}$/anti-*E.leei*$^{-}$ variable) and infection status (parasitized/non-parasitized variable) ($p < 0.001$).

3.4. Total serum peroxidases (PO)

Total serum PO at T1 were significantly higher in NAI fish than in SUR fish at the first three samplings and dropped at the last sampling to SUR values, which remained lower and constant (Fig. 5A).

At T2, no significant differences were found between NAI and SUR groups at any sampling point. SUR values showed a progressive and significant decrease in each sampling point, though NAI PO had no significant variations along the trial (Fig. 5B).

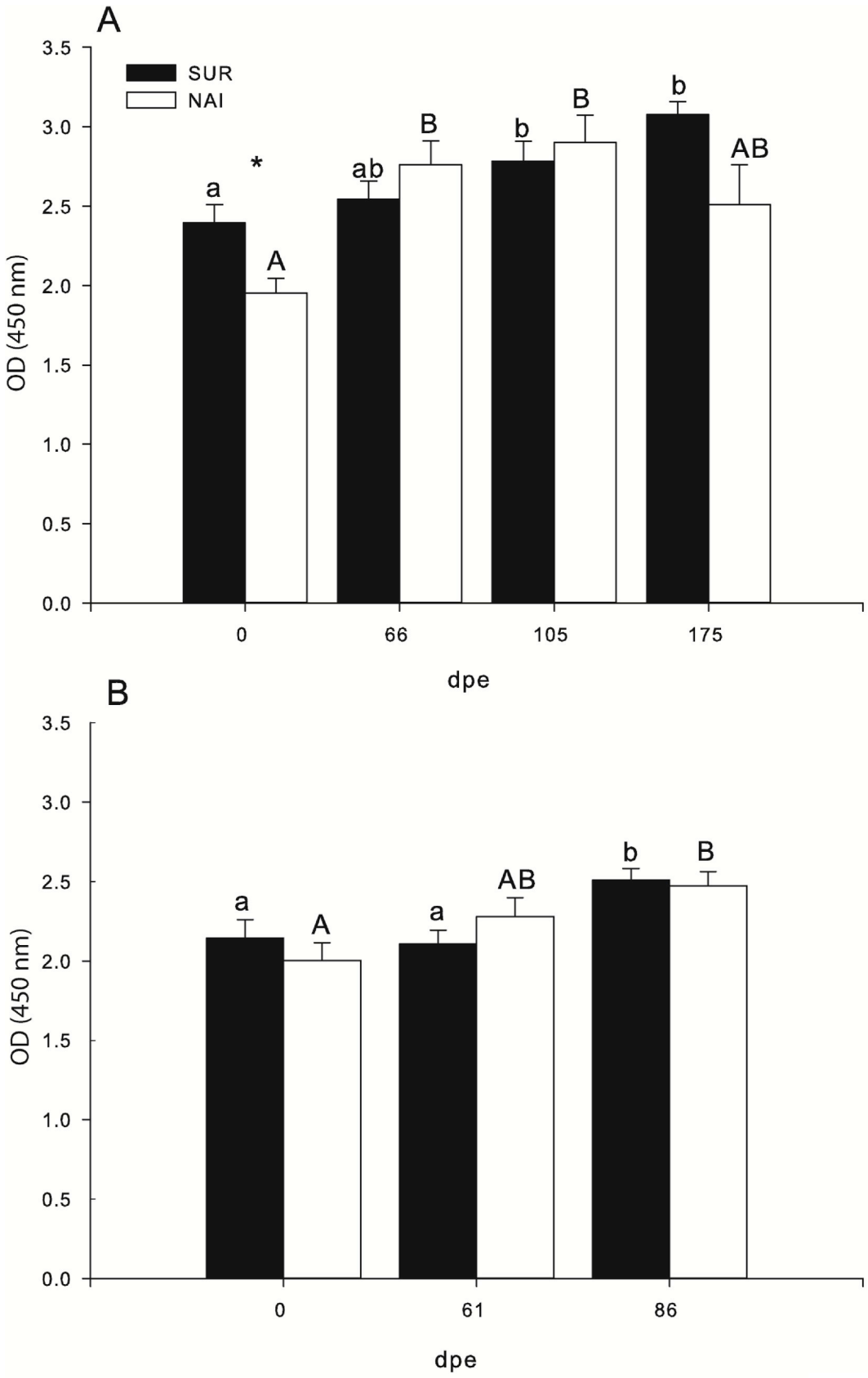


Fig. 2. Total serum IgM (mean +SEM) of naïve (NAI) and surviving (SUR) gilthead sea bream after exposure to *E. leei* in T1 (A) and T2 (B) by ELISA. Different lower case (SUR) or upper case (NAI) letters indicate statistically significant differences ($p < 0.05$) within each experimental group along samplings. Asterisks indicate statistically significant differences ($p < 0.05$) between experimental groups at the same time point. dpe = days post exposure.

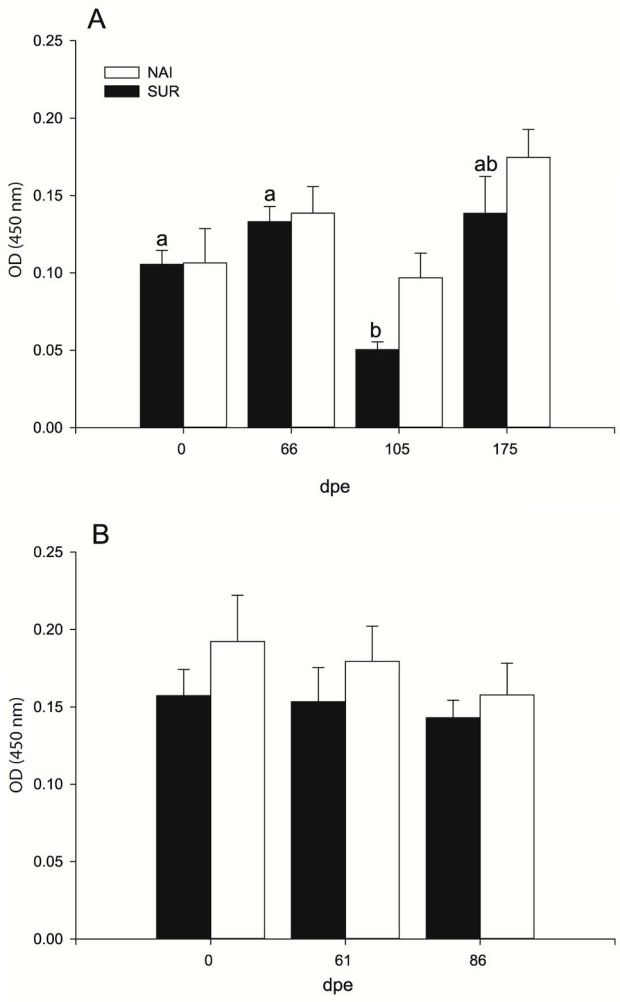


Fig. 3. Total serum IgT (mean +SEM) in naïve (NAI) and surviving (SUR) gilthead sea bream after exposure to *E. leei* for T1 (A) and T2 (B) by ELISA. Different lower case letters (A) indicate statistically significant differences ($p < 0.05$) within the SUR group along samplings.

3.5. PCR array: T2

The expression of 21 immune-related genes was analysed in HK, AI and PI upon infection with *E. leei* in NAI and SUR fish from T2. Markers of immune response expected to be affected by *E. leei* infection, such as cytokines, complement, lectins, growth factors, stress markers, antiproteases, cytotoxic enzymes, antigen presentation and mucins were included in this study. The PCR-array also included some markers of adaptive immune response, such as immunoglobulins and a *pan* T cell maker. The individual expression of 21 analysed genes relative to *β-actin* expression is shown in Supplementary Table 2, and the ratio SUR/NAI of their mean expression in Fig. 6.

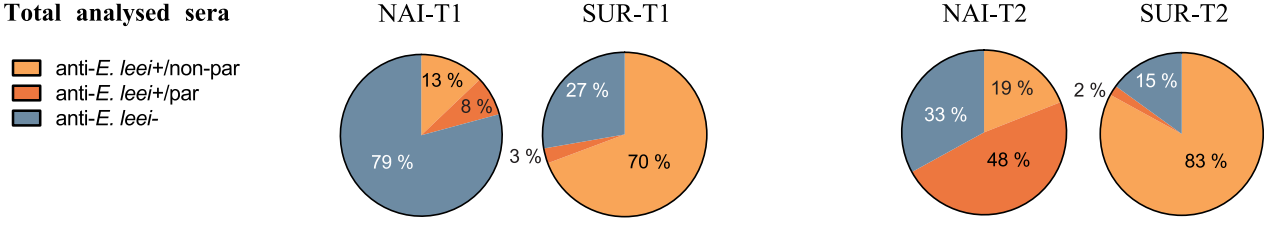
E. leei re-exposure induced a significantly higher expression of soluble *IgM*, soluble *IgT* and membrane *IgT* in AI of SUR fish when compared to NAI animals. However, at the HK and PI of SUR, *IgT* was less expressed. SUR fish also showed higher expression of *il10* and lower of *il1β*, at the AI and a higher expression of the cytotoxic enzyme (*gzma*) in the AI and PI. Both, complement related genes, *c3* and *fcl*, were higher in HK and PI of SUR fish, and *zap70* (marker of T lymphocytes) was higher in HK. Antigen presentation related genes showed no significant differences between groups. The stress marker *hsp90α* was lower in the PI of SUR. The expression of the two included antiproteases had opposite patterns, appearing *lcp1* down-regulated and *α2m* up-regulated in SUR animals. The expression of *imuc* was lower at the AI of SUR fish (Fig. 6).

3.6. Cluster analysis

To have an overview of all the results, K-means clustering was performed gathering all available data of T2 samples (Fig. 7A). All individual data used in this analysis can be found in Supplementary Table 2. Due to the high variability and the heterogeneous nature of the different data sets, dimension reduction was not very efficient, with the first two principal components (PC) explaining only 28.9% of the total variability (PC1 17.42% and PC2 11.7%). However, cluster analysis clearly separated SUR from NAI fish. One NAI fish (NAI_2) clustered separated from the other two groups, creating a third cluster. Interestingly, this particular individual was the one showing the highest level of infection in both, the non-lethal sampling at 61 dpe and in the histological scoring of the PI at 86 dpe. The variables that had more weight driving the separation of the clusters were the expression of *il10*

Table 3
Naïve (NAI) and surviving (SUR) gilthead sea bream exposed to *Enteromyxum leei* in trial 1 and 2 (T1 and T2). For each sampling, percentage of fish with specific IgM against the parasite (anti-*E. leei*$^+$) are given. Within anti-*E. leei*$^+$ fish, percentages of non-parasitized (anti-*E. leei*$^+$/non-par) and parasitized (anti-*E. leei*$^+$/par) fish are indicated. Pie charts show the percentages of total analysed sera in each trial for three fish categories: anti-*E. leei*$^+$/non-par (light orange); IgM anti-*E. leei*$^+$/par (dark orange); the remaining, are fish without specific IgM against the parasite (anti-*E. leei*$^-$) (blue). dpe = days post exposure.

		T1				T2		
	dpe	0	66	105	175	0	61	86
	Group							
anti-*E. leei*$^+$ (%)	NAI	0	0	20	60	15	100	100
	SUR	65	75	75	75	83.3	91.3	83.3
anti-*E. leei*$^+$/non-par (%)	NAI	0	0	10	40	15	25	16.7
	SUR	65	65	75	75	83.3	87	83.3
anti-*E. leei*$^+$/par (%)	NAI	0	0	10	20	0	75	83.3
	SUR	0	10	0	0	0	4.3	0



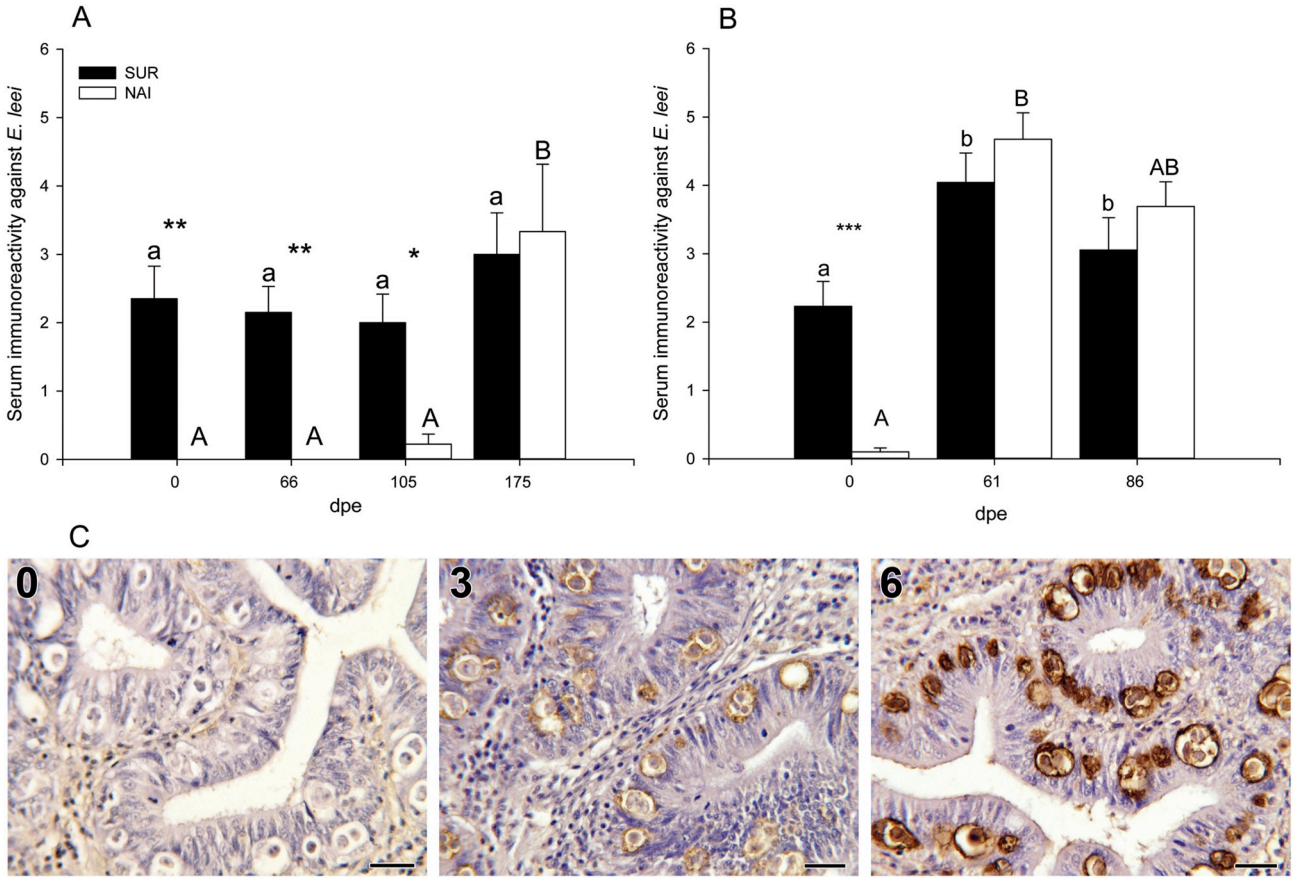


Fig. 4. Specific serum immunoreactivity (mean +SEM) against *E. leei* of naïve (NAI) and surviving (SUR) gilthead sea bream in T1 (A) and T2 (B). Intensity of immunoreactivity to the parasite was observed by immunohistochemistry (scaling example is shown in C for none = 0, medium = 3 and maximum = 6). Different lower case (SUR) or upper case (NAI) letters indicate statistically significant differences ($p < 0.05$) within each experimental group along samplings. Asterisks indicate statistically significant differences between experimental groups at the same time point (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$). dpe = days post exposure. Scale bars = 20 μm.

in AI and the presence of specific antibodies at 0 dpe in SUR sera (Fig. 7B). The high expression of *ifn*γ, *sIgM* and *mIgM* in PI; *sIgT* in HK; and the high intensity of infection, drove NAI_2 to cluster separately. This overall data analysis demonstrated the differential profile of NAI and SUR fish upon parasite infection and highlighted immunoglobulin expression, both at systemic and local levels, as important markers of the response of the fish to the disease.

4. Discussion

We have demonstrated that GSB that survived a previous infection with the myxozoan parasite *E. leei* are resistant to enteromyxosis upon re-challenge in two different trials, with different infective pressure. Re-exposed fish (SUR) exhibited higher levels of circulating IgM (total and specific against *E. leei*) and differential immune gene expression, when compared to naïve fish (NAI) exposed for the first time to the parasite. At the beginning of both trials, a higher percentage of SUR fish possessed specific anti-*E. leei* IgM, and these antibodies immunoreacted with stronger intensity against the parasite, which apparently helped them to cope with the parasite during the re-exposure. Furthermore, SUR fish had a higher expression of Igs in AI, higher local cytotoxic activity, and higher local and systemic activation of molecules related to the lectin pathway of complement. The higher expression of the regulatory cytokine *il10* and the lower expression of the acute pro-inflammatory cytokine *il1β* in AI, together with the histopathological observations, point towards an anti-inflammatory or resolution phenotype in the intestines of SUR fish.

Parasite transmission and establishment in recipient fish was slower in T1 than in T2, evidenced by the longer duration of the experiment (175 *vs* 86 days), and the lower prevalence of infection in T1. This difference was probably due to differences in the infective pressure on recipient fish, mainly due to the different water temperature. T1 started in winter when water was kept at 18 °C, whereas T2 started in summer, with an initial natural water temperature of 22.8 °C. Also, mean water temperature was lower in T1, being 19.6 °C *vs* 24.3 °C in T2 (Table 1). As previously shown, water temperature is critical for *E. leei* infections [52].

Regardless of these differences, in both trials, total circulating IgM increased over time in both experimental groups. The high level of total IgM of SUR fish in T1 before challenge (0 dpe) might be related to the greater age and size of this fish, but the higher levels of specific IgM of SUR fish in both trials (65% in T1, 83.3% in T2) point to a primed basal status after a first infection. In fact, global cluster analysis highlighted the importance of the initial high anti-*E. leei* specific IgM in SUR fish for their differential response when compared to NAI. In addition, immunoreactivity of the anti-*E. leei* specific IgM in all SUR sera was significantly stronger than in NAI sera, pointing to a greater affinity of these Igs for the parasite's antigens, at least initially, in both trials. The higher percentage of fish with specific anti-*E. leei* IgM and the higher affinity of these antibodies likely contributed to limit parasite spreading in SUR fish.

An early immune response after re-exposure is indicative of an acquired immune response (anamnestic response), in which a specific antigen activates its counter-specific B cell into differentiation and clonal expansion for early specific Ig production [53]. In the current study, anti-*E. leei* specific IgM in the SUR group remained high up to 16

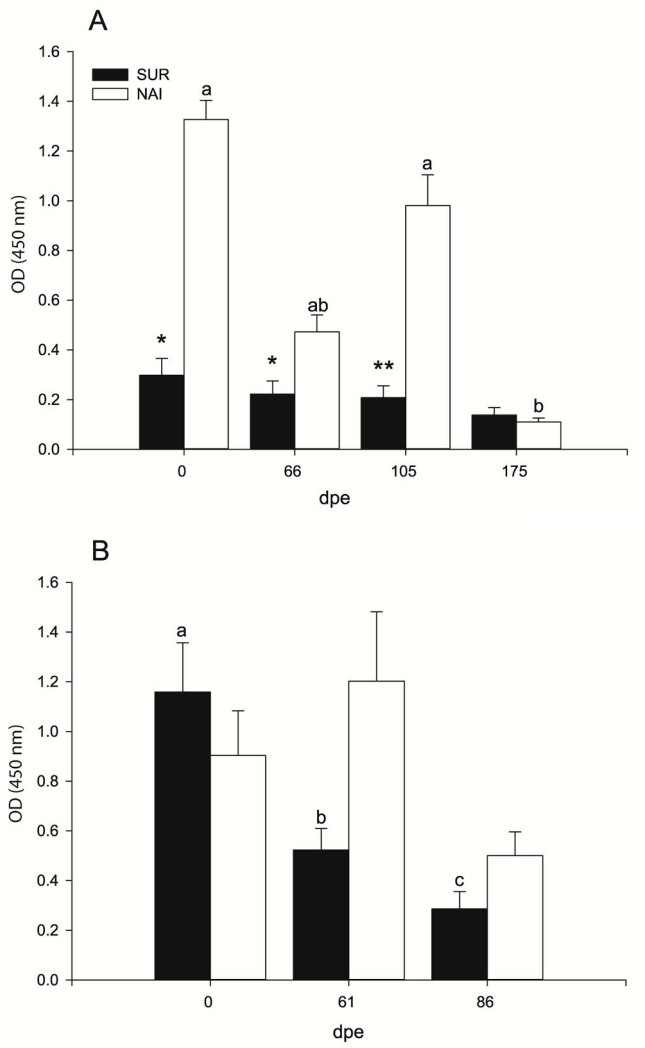


Fig. 5. Total serum peroxidases (mean +SEM) of naïve (NAI) and surviving (SUR) gilthead sea bream after exposure to *E. leei* in T1 (A) and T2 (B). Different letters indicate statistically significant differences within each group along samplings. Asterisks indicate statistically significant differences (*$p < 0.05$; **$p < 0.001$) between experimental groups. dpe = days post exposure.

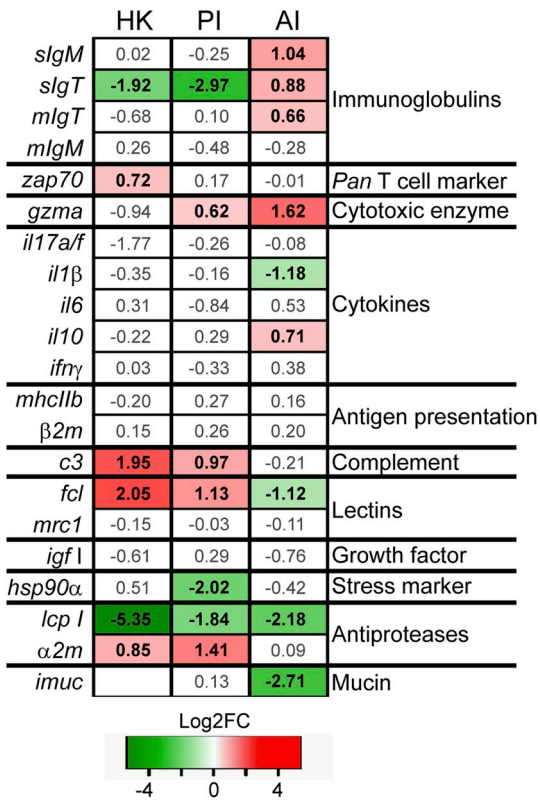


Fig. 6. Heatmap depicting the differential expression of 21-immune related genes in trial 2, categorized by their functional categories, in head kidney (HK), posterior (PI) and anterior (AI) intestine, at 86 days post-exposure (final sampling point). The log 2 fold change (Log2FC) of the ratio between the mean relative expression of the selected surviving (SUR, n = 8) and naïve (NAI, n = 8) gilthead sea bream is shown for each gene. The colour scale was only applied to the significantly differentially expressed genes (ANOVA, $p < 0.05$). Bold numbers in red indicate significantly higher expressed genes in SUR animals. Bold numbers in green indicate significantly lower expressed genes in SUR animals. For clarity, non-differentially expressed genes appear in white. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

months after these fish were first infected by the parasite, though we do not know exactly when parasite clearance occurred. Long-term protection against *E. leei* by B cells could be feasible, as such long-term B cell responses have been suggested for some teleost species. Specific antibodies against the pathogenic flagellates *Amyloodinium ocellatum*, *Cryptobia salmositica*, *Trypanoplasma borreli* and *Trypanosoma carassii* are found after recovery from disease outbreaks and experimental exposure and immunization trials in several teleost species [54]. Rainbow trout re-exposed after 1 year to *T. bryosalmonae* responded with significantly increased circulating IgM^+ B cells and up-regulated secretory IgM expression in the posterior kidney, compared to unexposed and naïve-exposed fish already at 10 dpe [44]. Similarly, channel catfish re-challenged 9 weeks after first infection with the ciliate parasite *Ichthyophthirius multifiliis* had higher specific antibody titres from 5 to 22 weeks after re-exposure [55]. No data on the starting IgM/antibody status of the re-exposed fish at 0 dpe is available for the latter examples, but those fish maintained specific antibody secreting cells and detectable specific antibody titres in serum for approximately 1 year. Goldfish that recovered from *T. carassii* infection produced protective specific IgM with high *in vitro* lytic activity even before re-challenge [56]. Concerning IgM affinity, 10-fold increases were detected in rainbow trout after stimulation with diverse immunogens [57–59]. In resistant turbot to *E. scophthalmi*, low levels of specific IgM were detected long after re-exposure (three years later) [42,45]. In the current study, GSB showed long-lasting and protective anti-*E. leei* seroprevalence, which would imply the existence of persistent antigen-specific antibody-secreting plasma cells besides memory B cells.

In SUR fish, the secondary exposure to the parasite apparently triggered the clonal selection and expansion of B cells expressing protective IgM with specificity for *E. leei* antigens. Thus, in both trials most of the SUR fish remained uninfected and the few SUR fish diagnosed as positive for *E. leei* at intermediate time points, scored negative at the end of the experiment, evidencing their acquired protection. By contrast, NAI fish had a faster systemic IgM response, which however, did not confer initial protection against the parasite, due to the lack of specific anti-*E. leei* IgM in the sera of NAI fish until advanced time points, when the infection was already established. In regard to myxozoan infections, in rainbow trout re-exposed to *T. bryosalmonae,* the cellular adaptive response (increase in IgM^+ circulating B cells) resulted in lower prevalence and intensity of infection [44]. In Atlantic salmon re-exposed to *K. thyrsites* the cell-mediated immune response by means of macrophages/dendritic cells, cytotoxic T cells, and B cells, resulted in lower parasite prevalence and infection intensity [43]. Those leukocyte populations probably also have a pivotal role in the adaptive immune response of the current SUR fish, but further studies

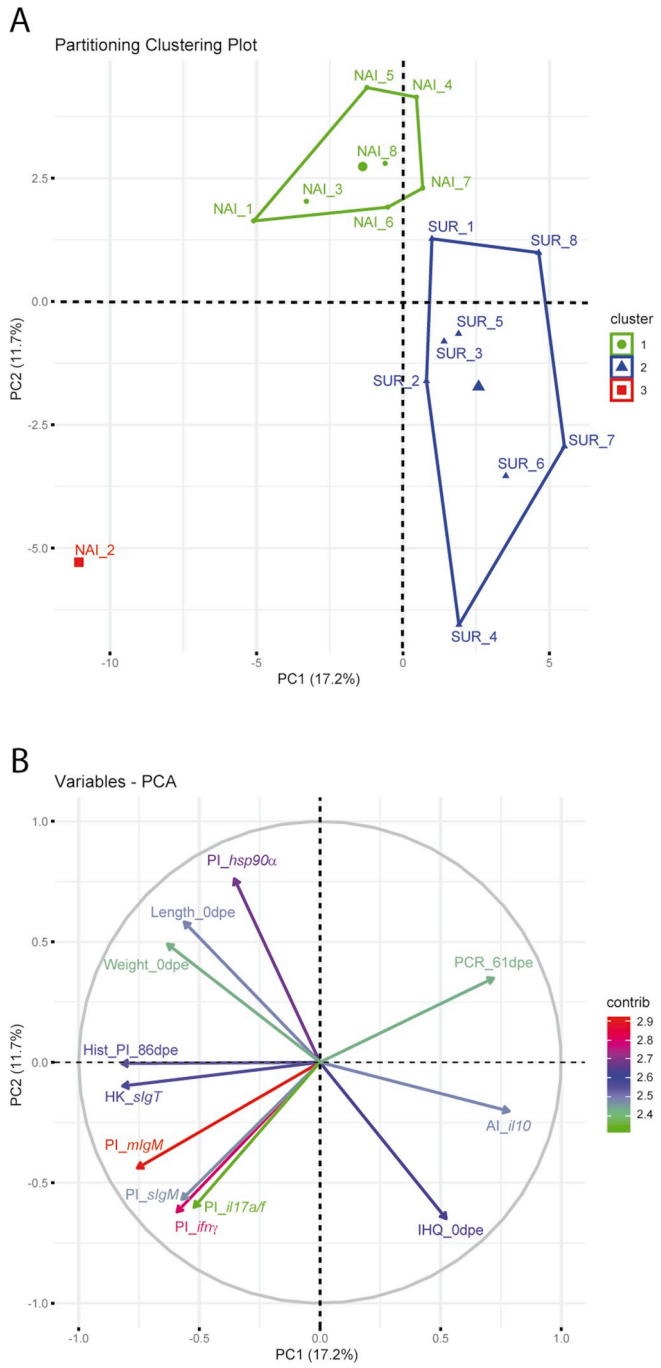


Fig. 7. K-means cluster analysis using all variables available in all the sampling points. Surviving (SUR, n = 8) and naïve (NAI, n = 8) fish, whose gene expression was analysed in T2, were used to construct the plots. (A) Cluster plot showing the first two principal components of the k-means analysis with k = 3. Cluster 1 (green) is entirely constituted by NAI fish, whereas all SUR fish group in cluster 2. Cluster 3 is constituted by a single individual from the NAI group that failed to group in any of the other two clusters. (B) Contribution plot for the 12 variables with more weight in the clustering. The colour scale represents the percentage of contribution for each variable. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

will be needed to decipher their implication.

Concerning NAI fish, total serum IgM increased initially faster than in SUR fish, and NAI presented significantly higher levels of total serum IgM already at 66 dpe (T1) and 86 dpe (T2), compared to 0 dpe. Focusing on naïve GSB, significantly higher IgM was previously reported at protein level in recipient fish sera [26] and at transcriptional level in the PI [27] after 133 days of effluent exposure to the parasite. One of these studies also detected up-regulation of soluble and membrane IgM only at the PI 64 days after anal intubation with the parasite, which provoke a faster progression of the infection [26]. With both experimental transmission methods a concomitant significant increase of IgM^+ B cells occurred in the PI and HK [27,32]. Thus, inflammation during enteromyxosis engaged local and systemic IgM response in GSB, supporting the current higher early levels of circulating IgM in NAI fish. Interestingly, during the inflammatory response elicited in Chinook salmon by *C. shasta*, a susceptible fish strain developed a premature response including early recruitment of Ig^+ cells to the intestine, unable to arrest parasite proliferation, while resistant salmon recruited Ig^+ cells to the site of infection later [60]. Such a premature or hyperactive Ig response in susceptible fish resembles the early response in the present NAI GSB.

Most SUR fish in both trials (69.7% in T1; 83.1% in T2) produced specific anti-*E. leei* IgM and were not infected upon re-exposure, probably because they were pre-exposed to the immunogen. By contrast, NAI fish with specific IgM were mostly infected. At 61 dpe, a specific/acquired IgM response seems to be already engaged during the intense exposure to the immunogen (T2), which only happened from 105 dpe onwards under the lower infective pressure of T1. However, specific anti-*E. leei* IgM was also present in non-infected NAI fish (12.5% in T1; 19.2% in T2), and even some NAI sera had *E. leei*-specific IgM immunoreactivity at 0 dpe in T2. Yet, the increase in NAI fish of IgM (total and *E. leei*-specific) over the course of the infections became evident, correlating with parasite load (T2 > T1). However, the Ig-response in NAI fish was unable to prevent parasite establishment, at least during the period of the trials. Timing and magnitude of the specific immune response against the parasite in NAI fish was probably responsible for its initial failure in parasite clearing during a first exposure, since selection and boost of the B cell clones expressing the protective Igs would occur later. Though detection of specific anti-*E. leei* IgM has not been reported before, previous studies also found increased local and systemic IgM and IgT, as well as IgM^+ B cells/plasma cells during enteromyxosis in naïve GSB [26,27,61]. These Ig-responses by the host were now shown to shift quite late during parasite invasion from non-specific to specific.

A high proportion of the non-specific, naturally occurring antibodies is constituted by IgM antibodies and is the basis of combinatorial/acquired immunity early since vertebrate evolution [62]. Our data suggest the presence and boost of natural IgM with specificity for certain antigens present in *E. leei*, at least for the 0 dpe immunoreactive IgM found in NAI fish (T2). However, the inability of NAI fish in both trials to generate substantial initial protection even after triggering an Ig-response, could be related to the lower affinity for the parasite of these antibodies. Correlation between natural antibody titres and ability to generate protective and specific primary and secondary antibody responses has been detected for some salmonids and cyprinids [63,64]. Whether such correlations influence the humoral immune response of naïve GSB was beyond the scope of the present study, but would deserve detailed investigation. Yet, vaccination trials performed on GSB against virus or bacteria have given contradictory results in regard to acquired protection associated to IgM titres [65–67].

The emergence of specific anti-*E. leei* IgM with significant immunoreactivity in NAI fish sera only happened after 175 dpe under the low infective pressure of T1, and earlier, at 61 dpe, with the higher infective pressure of T2. In both trials, sera of NAI fish apparently immunoreacted with the same parasite structures as SUR sera, namely P and S cells, and towards the end of the trials also consistently with spore valves and spore accompanying cells. Thus, P and S proliferative stages of *E. leei*, first to colonize the host tissues early during infection, were the most immunogenic parasite structures. Sporogonic stages, which chronologically develop later in the fish host, were less immunogenic, especially in NAI fish.

While the systemic IgM response seems to play a major part during the development of the acquired immune response against *E. leei*, serum IgT seemed to have a lesser role in this play. Serum IgT in T2 showed a decreasing trend over time, especially in NAI fish, which might reflect depletion after its consumption. In T1, IgT depletion seemed to occur at 105 dpe and recovered later at 175 dpe in both experimental groups. However, serum IgT might only reflect a transition state during IgT^+ B cell recruitment into its main action site, the mucosal compartment where infection is taking place. IgT has been described as an Ig isotype with an important mucosal role similar to the mammalian IgA [68] and plays an important function combating other myxozoans, such as *C. shasta* in the intestine [68], and *T. bryosalmonae* in the kidney [69]. It is tempting to suggest that the transient depletion of serum IgT at 105 dpe in T1 and the decreasing trend in T2 could reflect recruitment of circulating IgT^+ B cells towards the local site of infection for SUR and NAI fish, but the study of IgT dynamics in GSB during infection would deserve a thorough study. At transcriptional level, lower *sIgT* expression in HK together with a higher *sIgT* expression in AI in SUR, might indicate more intense recruitment of IgT^+ B cells/activated plasma cells into SUR fish intestines, than in NAI intestines. In PI, no increase of *sIgT* in SUR *vs* NAI was found, which can be explained by the high expression found in NAI during the first and more intense contact with the parasite at the target site of infection, according to a previous study [26]. Besides, a local accumulation of IgT^+ B cells at the AI from the previous exposure to *E. leei* might have occurred. Likewise, aggregates of IgT^+ cells were found in rainbow trout skin after 3 months of exposure with *I. multifiliis* [70], and in rainbow trout intestine an accumulation of IgT and IgT^+ B cells were found in the lamina propria three months after *C. shasta* infection [68]. Unfortunately, the available anti-GSB IgT antibody does not work in immunohistochemistry, so we were not able to study the levels of specific IgT in NAI and SUR fish.

Among the studied cytokines, only *il1β* and *il10* showed differential expression in AI of SUR animals when compared to NAI. The higher expression of *il10* and the lower expression of *il1β* in SUR animals could indicate a shift to an anti-inflammatory profile characteristic of inflammation resolution. The pleiotropic regulatory cytokine IL10 is considered one of the most important anti-inflammatory cytokines responsible for limiting and terminating inflammation, in order to prevent damage and help to the formation of long-lived memory cells against future threats. This cytokine has been extensively studied in mammals and fish with no apparent major functional differences [71,72]. One of its main activities is the inhibition of the expression of pro-inflammatory cytokines by phagocytes and T lymphocytes, among which, *il1β* is one of the main affected [73]. In fact, an anti-inflammatory profile 64 days after anal intubation with the parasite has been described in GSB intestines with significant up-regulation of *il10* [36]. IL10 also increases the cytotoxic activity of mammalian NK cells [74] and stimulates B cell proliferation, maturation and antibody secretion both in mammals and fish [73]. The up-regulation of *il10* during long-lasting parasitic infections of carp with *T. borreli* and *T. carassii* actually coincided with the appearance of higher concentrations of specific antibodies against the parasites and the concomitant down-regulation of pro-inflammatory genes [71,75]. In the current study, the higher expression of *il10* in AI of SUR fish is also parallel to lower expression of *il1β*, higher expression of immunoglobulins (particularly the soluble forms) and a higher expression of the cytotoxic enzyme *gzma*. Thus, these results point to a key role of IL10 in the resolution of *E. leei* infection and the accurate production of a protective memory pool.

In the current study, we did not detect significant differences in *zap70* expression in intestines of SUR and NAI fish. However, SUR fish show higher expression of this T cell marker in HK, probably pointing to a replenishment of T cells in this organ in those animals where the infection was successfully avoided or beaten. However, the significantly higher *zap70* SUR/NAI ratio could have also been induced by the previously reported HK depletion of T cells in naïve fish during intestinal recruitment or by T cell proliferation induced by the secondary exposure. Zap70 is a commonly used *pan* T cell marker, in both fish and mammals [73,76]. Previous studies revealed its up-regulation in intestines of *E. leei* infected GSB, parallel to a down-regulation in head kidney, due to T cell migration to the target tissue where they became activated [30].

In our study, the higher expression of *gzma* in SUR animals could indicate a higher activation or presence of cytotoxic cells during a secondary exposure helping to avoid parasite establishment. Gzma is one of the cytolytic effector molecules released by cytotoxic cells such as cytotoxic T lymphocytes (CTL) and non-specific cytotoxic cells (NCC) [77]. Both CTLs and NCCs express many receptors, transcription factors and effector molecules in common [78]. It is difficult to determine which cell population is involved in the current study, but previous studies seem to indicate that both NCCs [79] and CTLs [30] are involved in *E. leei* clearance and fish survival. Cytotoxic activity, mainly related to $CD8^+$ CTLs, was also linked to successful resolution of infection and protection upon re-exposure in Atlantic salmon infected with the myxozoan parasite *K. thyrsites* [43].

Concerning innate immune factors, total serum PO, involved in the oxidative response against pathogens, showed different kinetics in each trial, but all ended in PO depletion. SUR fish in T1 had significantly lower serum PO than NAI and remained stable, whereas in T2 SUR fish had remarkably higher levels than in T1, and showed a faster decrease than NAI. The differences found between SUR and NAI in T1 might be related to the different age or genetic background of these fish, one of the reasons for which T2 was conducted with animals from the same stock and age. The involvement of serum PO in the immune response against *E. leei* has previously been reported only in the context of primary responses in naïve fish [28,80,81]. Fish serum peroxidases, enclosed in the granules of phagocytic cells, participate in the respiratory burst cascade [82] and are capable of local and systemic regulation and intervention upon infections [83,84], dietary intervention [85–87] and exposure to waterborne toxics [87,88]. Peroxidase bearing granulocytes in GSB are neutrophils and acidophils [89]. The significant contribution of these cells to the resolution phase of inflammatory responses in teleosts [90] and specifically in GSB [91], has been pointed out, and their involvement in the immune response found in SUR fish might be related.

Other innate immune effectors were differentially expressed in SUR fish during the secondary immune response to the parasite. This was the case of *α2m*, whose expression was higher in SUR animals upon re-exposure to *E. leei.* This *pan*-protease inhibitor, involved in the clearance of virtually any host or foreign proteinase via endocytosis [92], was also up-regulated at gene and protein expression levels in other myxozoan infections in different fish species [6,25,80,93]. In all these cases, this up-regulation was measured during the peak of parasitic infections in naïve fish and the assumed role in these particular scenarios was the clearance of parasite proteinases to counteract tissue invasion. The biological importance of α2M during parasitic infections has been demonstrated from invertebrates to mammals, and its additional role in the maintenance of tissue homeostasis during the resolution of the inflammatory response has been described [94]. LCP1 is another protease inhibitor that belongs to the group of Stefins (members of the cystatin superfamily of cysteine proteinase inhibitors) [95]. The expression of *lcp1* in GSB was also up-regulated in previous *E. leei* infections and dietary intervention studies [35,96].

Molecules related to the complement system were also modulated in SUR fish. The genes *c3* and *fcl* had a higher expression in SUR fish during the secondary response to the parasite at local and systemic levels. In agreement with this, serum complement activity by the alternative pathway was also decreased in GSB during the primary acute response to *E. leei* [81,97] and the expression of complement components was down-regulated in PI and HK of *E. leei*-infected GSB [35], which was interpreted as a parasitosis-associated immunodepression. Regulation of the expression of *fcl* in naïve GSB intestines was also found in response to enteromyxosis (down-regulation) [96] and to

dietary intervention (up-regulation) [98]. The current re-exposed SUR fish showed no signs of such immunodepression by the parasite, and the expression of complement genes appeared to be re-established or even improved, compared to NAI fish. Complement, specifically C3 and its receptors, play an important role in antigen uptake, internalization, presentation, and generation of an antibody response in all vertebrates, thereby interconnecting innate and acquired immune responses [99]. F-type lectins, such as *fcl*, are pattern recognition receptors found in the serum of several fish species, including GSB, and play an active role in PAMP recognition, opsonisation, phagocytosis, and complement activation [100].

A lower expression of *i-muc* was found in the AI of SUR fish compared to NAI. Mucin expression in the gastrointestinal tract of GSB has tissue-specific distribution. The intestinal mucin (*i-muc*) is constitutively highly expressed at the PI and was down-regulated in this intestinal segment due to primary *E. leei* infection or to dietary intervention [34]. The modified mucin expression could imply changes in intestinal susceptibility to further biological or chemical offenders, as mucins are part of the mucosal surface of the gastrointestinal tract, that modulate many biological processes like cell adhesion, molecular trafficking and receptor activation, and are key players in host-pathogen interactions [100].

The expression of *hsp90α* was lower in the PI of SUR fish than in NAI. Molecular chaperones, including HSPs, are life-essential peptides that stabilize unfolded proteins and their expression is highly inducible under cellular stress in higher vertebrates [101] and fish [102]. In previous studies, several HSPs were consistently up-regulated in primary *E. leei*-infected GSB intestines [96]. The current results further highlight the different phenotypes of the two experimental groups. Namely, NAI fish develop an intense inflammatory response during their first exposure to the parasite, induced by the intestinal infection, and engage a late production of circulating specific antibodies and activation of the parasite-combatting machinery at the target site. SUR fish, by contrast, are characterised by the reestablishment of homeostasis and stress/inflammation resolution.

5. Conclusions

GSB re-exposed to *E. leei* mount an early and effective adaptive humoral immune response, involving higher circulating IgM, which is more specific against the parasite, than in GSB exposed to the parasite for the first time. Fish resistant to the parasite with high titres of specific IgM (SUR) do not exhibit the usual enteritis-associated signs, which naïve fish develop during enteromyxosis (pro-inflammatory gene expression profile, hypertrophy of intestinal submucosa), but instead have a phenotype distinctive of inflammation resolution. SUR Ig-response was supported by other involved immune mechanisms like cell cytotoxicity and complement. The current results shed some light on the limited knowledge of adaptive immune responses against myxozoan parasites in sparids. Future research efforts will have to be made in order elucidate which cell effectors are involved in the acquired resistance of GSB to *E. leei*.

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Conflicts of interest

The authors declare that they have no competing interests.

Author contributions

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Appendix A. Supplementary data

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