Fish and Shellfish Immunology 84 (2019) 62–72

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Expression of infection-related immune response in European sea bass (*Dicentrarchus labrax*) during a natural outbreak from a unique dinoflagellate *Amyloodinium ocellatum*

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ARTICLE INFO

Keywords:
Natural infection
Amyloodinium ocellatum
European sea bass
Immune response
Immune related genes

ABSTRACT

In the Mediterranean area, amyloodiniosis represents a major hindrance for marine aquaculture, causing high mortalities in lagoon-type based rearing sites during warm seasons. *Amyloodinium ocellatum* (AO) is the most common and important dinoflagellate parasitizing fish, and is one of the few fish parasites that can infest several fish species living within its ecological range. In the present study, *A. ocellatum* was recorded and collected from infected European sea bass (*Dicentrarchus labrax*) during a summer 2017 outbreak in north east Italy. Histological observation of infected ESB gill samples emphasized the presence of round or pear-shaped trophonts anchored to the oro-pharingeal cavity. Molecular analysis for small subunit (SSU) rDNA of *A. ocellatum* from gill genomic DNA amplified consistently and yielded 248 bp specific amplicon of *A. ocellatum*, that was also confirmed using sequencing and NCBI Blast analysis. Histological sections of ESB gill samples were addressed to immunohistochemical procedure for the labelling of ESB *igm*, *inos*, *tlr2*, *tlr4*, *pcna and* cytokeratin. Infected gills resulted positive for *igm*, *inos, pcna and* cytokeratin but negative to *tlr-2* and *tlr-4*. Furthermore, ESB immune related gene response (innate immunity, adaptive immunity, and stress) in the course of *A. ocellatum* infection using quantitative polymerase chain reaction (*qpcr*) for infected gills and head kidney was analysed. Among the twenty three immune related gene molecules tested, *cc1*, *il-8*, *il-10*, *hep*, *cox-2*, *cla*, *cat, casp9*, and *igt* were significantly expressed in diseased fish. Altogether, these data on parasite identification and expression of host immune-related genes will allow for a better understanding of immune response in European sea bass against *A. ocellatum* and could promote the development of effective control measures.

1. Introduction

Amyloodinium ocellatum (AO) is an ectoparasite protozoan belonging to the phylum Dinoflagellata and is the unique species belonging to *Amyloodinium* genus (class Blastodiniphyceae, order Blastodiniales, family Oodiniaceae) [1,2]. AO is worldwide distributed and affects brackish and sea water fish in tropical and temperate regions. Furthermore, AO is the particular dinoflagellate capable to infect elasmobranchs other than teleosts [3]. In fact, cumulative evidence shows that it has a very low host species-specificity, being isolated from four aquatic organisms Phyla: Chordata, Arthropoda [4], Mollusca [5] and Platyhelminthes [6]. The parasite represents a serious problem for both farmed and aquarium fish [7], since amyloodiniosis can lead the host to death in less than 12 h [3] with acute morbidity and mortality around 100%. However, these two parameters considerably vary on the basis of farming condition, parasite burden, fish species and season consideration [8–13]. AO biological life cycle is direct but triphasic and it can be completed in less than a week dependent on the favourable environmental factors [14]. The trophont is the parasitic stage and it is sessile and strictly anchored to host epithelia (gill or skin) through rhizoids. The parasite feeds on the host through the stomopode [14] and, after feeding (2–6 days), trophonts detach from host, becoming tomonts (cystic reproductive stage). In 2–4 days dinospores (infective stage) hatch from tomonts and actively search a new host using flagella. After the adhesion to the host, dinospores transform into trophonts within few minutes. Even if sessile, trophonts constantly turn and twist slowly, facilitating the severance of host cell fragmentation [14]. AO inflicts moderate-to-intense tissue damage associated with serious gill hyperplasia with the subsequent lamellar fusion, inflammation, hemorrhage and necrosis [8,9,11,12,15–19]. In heavy infections, death can occur in

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https://doi.org/10.1016/j.fsi.2018.09.069
Received 4 July 2018; Received in revised form 6 September 2018; Accepted 24 September 2018
Available online 25 September 2018
1050-4648/

less than 12 h, however, mortalities have been documented also in subclinical or mild infestations as a probable consequence of osmoregulatory impairment and secondary microbial infections in damaged epithelial cells [20].

European sea bass (ESB) (*Dicentrarchus labrax*) is one of the most extensively aquacultured fish species in Mediterranean and is susceptible to *Amyloodinium ocellatum* infection [21,22]. Previous studies have indicated that after AO infection in ESB, Interleukin-1 (IL-1) and Tumor Necrosis Factor α (*tnf-α*) were activated in intestine [19]. Furthermore, peroxisome proliferator-activated receptor α (PPAR α), codifying for a protein involved in lipid metabolism, was upregulated in liver of the infected fish reared in an aquaponics system due to the need to produce energy in order to maintain homeostasis [19]. Noticeably, plasma proteome changes in gilthead head seabream (*S. aurata*) were observed as physiological response to AO and results indicated that several proteins belong to acute-phase response, inflammation, lipid transport, homoeostasis, osmoregulation, wound healing, neoplasia and iron transport were affected [23]. Moreover, the AO parasitic burden have enhanced the physiological stress response in gilthead seabream (*S. aurata*) [24]. To our knowledge, there are no further studies especially on ESB emphasizing immune gene response to AO infection through the screening of large panels of host immune related genes. Therefore, in order to improve the knowledge on the mechanisms at the basis of the host-parasite interactions with a particular attention to host immune response and tissue damages provoked by AO, histological, immunohistochemical and biomolecular analysis have been performed in the present study. Based on the current availability and functional characterization of European sea bass immune genes, we targeted genes that encompass innate/inflammatory/adaptive immune ligands, pathogen recognition receptors, antimicrobial peptides, and stress related factors. Thus, our study provides a balance between potential innate and adaptive immune mechanisms shaping amyloodiniosis pathogenesis and host response.

2. Material and methods

2.1. Fish sampling

Tissues samples from the infected fish and non-infected fish (gills and head kidney) were collected from ESB juveniles (mean weight 45 g) in a farm (lagoon-type rearing site) located in the delta area of the Po River (Porto Viro-RO, Italy), during a severe AO outbreak at the end of July 2017. The disease episode was characterized by high morbidity and mortality (100% within 7 days after the outbreak identification) and the etiology was microscopically confirmed by trophonts identification in gill biopsies. Clinical symptoms were dyspnea, superficial swimming, flushing, anorexia, and lethargy (in the advanced stage of the disease). ESB were reared in a 400 m^3 raceway supplied with brackish water (7‰ salinity and 28–30 °C). As a control, organs from ESB not infected by AO (asymptomatic and negative to the gill biopsy observation), reared in a different raceway of the same farm, were sampled for this study.

Before immersion in RNA later® Solution (Ambion®, https://www.thermofisher.com), gills and head kidney from infected and non infected fish (n = 5/group) were hygienically dissected and cutted to ≤0.5 cm in any single dimension. Tissue samples were placed into 1 ml of RNA-later (Ambion®, Life technologies), kept at 4 °C for 24 h and stored at −80 °C prior to RNA extraction and PCR analysis.

For histology and immunohistochemistry 2–3 gill arches deriving from 10 infected and from 5 healthy ESB were fixed in 4% buffered formaldehyde and Bouin's solution (Bio-Optica, Milano, www.bio-optica.it), embedded in paraffin, sectioned, and stained with Haematoxylin-Eosin, PAS-Alcian blue and Masson's trichrome, or submitted to immunohistochemical protocol.

2.2. Amyloodinium ocellatum isolation

Amyloodinium ocellatum was collected from infected ESB based on [25] with some modifications. AO trophonts were detached by placing moribund ESB in a clean 1 L plastic jar containing 0.5 L of freshwater for 2–3 min. Then, fish were removed from the jar, the water salinity was adjusted to 20 ppt by addition of 0.5 L of 40 ppt salt water and the jar content poured into a glass jar through a plastic funnel lined with two layers of 100 µm nylon filter mesh, to remove large debris. The filtrate was set aside for 15–20 min to allow sedimentation and transformation of trophonts into tomonts; the saltwater overlay was removed and tomonts transferred in sterile 50 ml tubes. Tomonts were washed twice by centrifugation for 10 min at 150 × *g*, saltwater overlay removal and re-suspension in sterile saltwater (20 ppt). Successively, 2 ml of the tomonts suspension in sterile saltwater were gently layered onto 2 ml of Percoll® (Sigma-Aldrich, https://www.sigmaaldrich.com/) in 15 ml tubes and tomonts purified by centrifugation for 10 min at 180 × *g*. The supernatant was removed and the pelleted tomonts were washed three times with 15 ml of sterile saltwater, then stored at −20 °C until further use. The thawed pellet was disrupted with a disposable pestle. The DNA was extracted from the homogenate using the DNeasy Tissue Kit (Qiagen, https://www.qiagen.com) according to the manufacturer's instructions.

2.3. Polymerase chain reaction (PCR) and sequencing

Amyloodinium ocellatum – specific primers AO18SF (5′-gaccttgccc-gagaggg-3′) and AO18SR (5′-ggtgttaagattcaccacactttcc-3′) were used for PCR amplification of a 248 bp segment of the 3′ end of the SSU rDNA gene [26]. PCR amplification of the AO DNA using the AO18SF/R primer set was carried out in a final volume of 50 µL according to [26]. PCR reaction was performed using a Bio-Rad thermocycler (Bio-Rad Laboratories Inc., CA, USA, http://www.bio-rad.com/) with a reaction mixture containing 100 ng DNA, 100 ng of each primer, and 1.25 Units HotStart Taq (Invitrogen, USA, https://www.thermofisher.com/) under the following conditions: initial denaturation at 94 °C for 15 min followed by 35 cycles of initial denaturation (94 °C for 1 min), annealing (58 °C for 1 min), extension (72 °C for 1 min), and a final elongation (72 °C for 5 min). Subsequently, 5 µL of the PCR product were analysed by 2% agarose gel electrophoresis stained with ethidium bromide and visualiszed with an UV transilluminator. PCR product molecular weight was determined using a 100 bp DNA ladder (Thermo Fisher Scientific, Pittsburgh, PA, USA). To identify the type of AO isolate used in the present study, PCR products were then purified using a QIAquick Purification kit (Qiagen, https://www.qiagen.com/it/) and directly sequenced using the AO18SF/R primer set (https://www.eurofinsgenomics.eu/). Using a BLAST search, the sequences obtained were compared with those published in NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The multiple sequence alignment was performed using the CLUSTALW (www.ebi.ac.uk/clustalw2).

2.4. Immunohistochemistry

Immunohistochemistry was performed on gills samples in order to label some inflammation related antigens during ESB response to amyloodiniosis. For this purpose, 4 µm histological sections of ESB gills fixed in Bouin's were addressed to immunohistochemical procedure using a HRP-based anti-rabbit or anti-mouse kit (EnVisionTM FLEX, K8009, K8021 and K8023 - Dako, Agilent). Briefly the slides were routinely dewaxed and rehydrated and all incubations were performed at room temperature (RT) in a humid chamber. Tissue endogenous peroxidase was inactivated by slides immersion in H_2O_2 for 30 min at RT, antibodies aspecific binding was blocked with 1:20 normal goat serum (30 min), then the procedure included an antigen retrieval treatment (10 min at 90 °C) (High pH, K8004; or Low pH, K8005 - Dako, Agilent) and a 2 h incubation with primary antibodies specific for the

following antigens: ESB IgM (rabbit polyclonal, Trieste University, Italy, 1:24.000); Inducible Nitric Oxide Synthase (*inos*) (RB-1605, Thermo Scientific, 1:200); Toll-like Receptor 2 (*tlr2*) (ab1655, Abcam, 1:50); Toll-like Receptor 4 (*tlr4*) (76B357, Imgenex, 1:50); PCNA (SC-56, Santa Cruz Biotechnologies, 1:50); cytokeratin (ab9377, Abcam, 1:50). After the use of the EnVision linkers and the EnVision HRP reagent, Diaminobenzidine (Sigma Aldrich) was used as chromogen (7 min) and haematoxylin as counterstain. Negative control sections were included by replacement of the primary antibodies with dilution buffer.

2.5. Total RNA extraction from ESB tissues

Total RNA was isolated using TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA, https://www.thermofisher.com/) according to the manufacturer's instructions. Total RNA purity and degradation was checked on a 2% agarose gel. The quality of total RNA was analysed by a spectrophotometer using 260/280 nm UV. From individual fish, total RNA from tissues was dissolved equally in RNase-free water and stored at −80 °C until further use. For q-PCR, 2 μg of total RNA was reverse-transcribed in a 20 μL reaction system according to the manufacturer's protocol (iScript™ cDNA synthesis kit, Bio-Rad, http://www.bio-rad.com/). Total RNA with no reverse transciptase enzyme in the reaction was also included as negative RT control.

2.6. Real-time PCR assays

One microliter of each cDNA synthesis reaction was employed as the template in the qPCR reactions to analyze each gene transcription. Primers to detect expression of genes are mentioned in Table 1 [27]. Primer efficiency was determined and multiple reference was used for normalizing the target gene. Amplifications were performed in a final volume of 10 μL. Reaction mixture contained 5 μL of IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.2 μL of each primer set (10 mM), 1 μL of template cDNA and 3.6 μL of DEPC-water. Real time PCR determinations were performed in triplicate in 96-well PCR plates and carried out in an CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with an initial denaturation cycle of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. Amplification was followed by a standard melting curve from 55 °C to 95 °C, in increments of 0.5 °C for 5 s at each step, to confirm that only one product amplified and detected. Samples were run in parallel with three reference genes, *β-actin*, *hsp90* and *l13a*, for cDNA normalization [28]. Relative mRNA expression was calculated using the $2^{-\Delta\Delta CT}$ method [29], normalizing with geometric average of three reference genes (*β-actin*, *hsp90* and *l13a*) and relative to control group.

2.7. Statistical analysis

Data were analysed using the SPSS16 (SPSS Inc., Chicago, IL, USA) statistical software. Data were tested for normality of the variables using Shapiro-Wilk test and homogeneity of variance before ANOVA evaluation. Descriptive statistics which includes mean, standard deviation, 95% confidence interval for mean, was used to determine the data distribution. Later, analysis of variance (ANOVA) was used to determine the differences in mean among different group, and compared with post hoc multiple comparison using Duncan's multiple range test. Differences of means among the groups were considered statistically significant when $p < 0.05$.

3. Results

3.1. Evaluation of AO infection

This natural AO outbreak in ESB juveniles was a very severe amyloodiniosis case characterized by a massive parasite burden. Clinical symptoms were dyspnea, superficial swimming, flushing, anorexia, and lethargy (in the advanced stage of the disease). Infected ESB showed marked diffuse gill anaemia and in some cases hemorrhages. Histologically, several trophonts at different growth stages (size 27–130 × 23–60 μm) were attached to the oro-pharingeal cavity epithelium causing a severe and diffuse hydropic degeneration of epithelial and chloride cells, oedema, necrosis and hyperplasia of the gill epithelium especially in the distal third of the primary lamellae (Fig. 1a). Along the gill filaments lymphocytes, macrophages, mast cells

Table 1
Functional group, primer name and sequence of genes of interest and three reference genes. Primers were either designed using sequences from GenBank (see accession number) or taken from literature (see reference).

Functional group	Sl.No	*Primer Name*	Forward Sequence (5′–3′)	*Primer Name*	Reverse Sequence (5′–3′)	GenBank Accession number
Innate Immunity	1	*cc1-F*	tgggttcgccgcaaggttgtt	*cc1-R*	agacagtagacgaggggaccacaga	AM490065.1
	2	*ifn-F*	gtacagacaggcgtccaaagcatca	*ifn-R*	caaacagggcagccgtctcatcaa	AM765847.2
	3	*il-1β-F*	caggactccggtttgaacat	*il-1β-R*	ttgtccccttttgaatggac	AJ311925.1
	4	*il8-F*	gtctgagaagcctgggagtg	*il8-R*	gcaatgggagttagcaggaa	AM490063.1
	5	*il6-F*	acttccaaaacatgccctga	*il6-R*	ctccttagactgaccagcgg	Cordero et al., 2016
	6	*il*-10-F	cagtgctgtcgttttgtggagggtttc	*il*-10-R	tctctgtgaagtctgctctgagttgcctta	Azeredo et al., 2015
	7	*fer-F*	atgcacaagctctgctctga	*fer-R*	tttgcccagggtgtgtttat	Sarropoulou et al., 2009
	8	*hep-F*	aagagctggaggagccaatgagca	*hep-R*	gactgctgtgacgcttgtgtctgt	DQ131605.1
	9	*tlr1-F*	gcctctgcctcaatacctgatccca	*tlr1-R*	aacaacctgtgcttggccctgtc	KX399287
	10	*tlr9-F*	tcttggtttgccgacttcttgcgt	*tlr9-R*	tactgttgccctgttgggactctgg	KX399289
	11	*tnfα-F*	agccacaggatctggagcta	*tnfα-R*	ggacagctacagaagcggac	DQ070246.1
	12	*cox2-F*	agcacttcacccaccagttc	*cox2-R*	aagcttgccatccttgaaga	Cordero et al., 2016
Adaptive immunity	13	*mhc class ia-F*	tgtacggctgtgagtgggatgatgag	*mhc class ia-R*	agcctgtggtcttggagcgatgaa	JX171695.1
	14	*mhc class iia-F*	agtccgatgatctaccccagagacaac	*mhc class iia-R*	acaggagcaggatagaaaccagtcaca	FN667955.1
	16	*Ighm-F*	aggacaggactgctgctgtt	*ighm-R*	acaacagcagacagcaggtg	FN908858
	17	*Ight-F*	tcacttggcaaattgatgga	*ight-R*	agaacagcgcactttgttga	FM010886
Complement system	18	*cla-F*	gatggcagcaagctccggtattca	*cla-R*	tctgacctatgaccccagccaaca	EU660935.1
	19	*gal-F*	tgcaactcttaccagggaggcaact	*gal-R*	gtcacgaggaactctgtaggggtga	EU660937.1
	20	*casp3-F*	ctgatttggatccaggcatt	*casp3-R*	cggtcgtagtgttcctccat	DQ345773.1
	21	*casp9-F*	ggcaggactcgacgagatag	*casp9-R*	ctcgctctgaggagcaaact	DQ345776.1
Stress	22	*cat-F*	tgatggctaccgccacatgaacg	*cat-R*	ttgcagtagaaacgctcaccatcgg	FJ860003.1
	23	*hsp70*-F	acaaagcagacccagaccttcacca	*hsp70*-R	tggtcatagcacgttcgccctca	AY423555.2
Reference	24	*actb-F*	tgaaccccaaagccaacagggaga	*actb-R*	gtacgaccagaggcatacagggaca	AJ537421.1
	25	*l13a-F*	tctggaggactgtcaggggcatgc	*l13a-R*	agacgcacaatcttgagagcag	Mitter et al., 2009
	26	*hsp90*-F	gctgacaagaacgacaaggctgtga	*hsp90*-R	agatgcggttggagtgggtctgt	AY395632.1

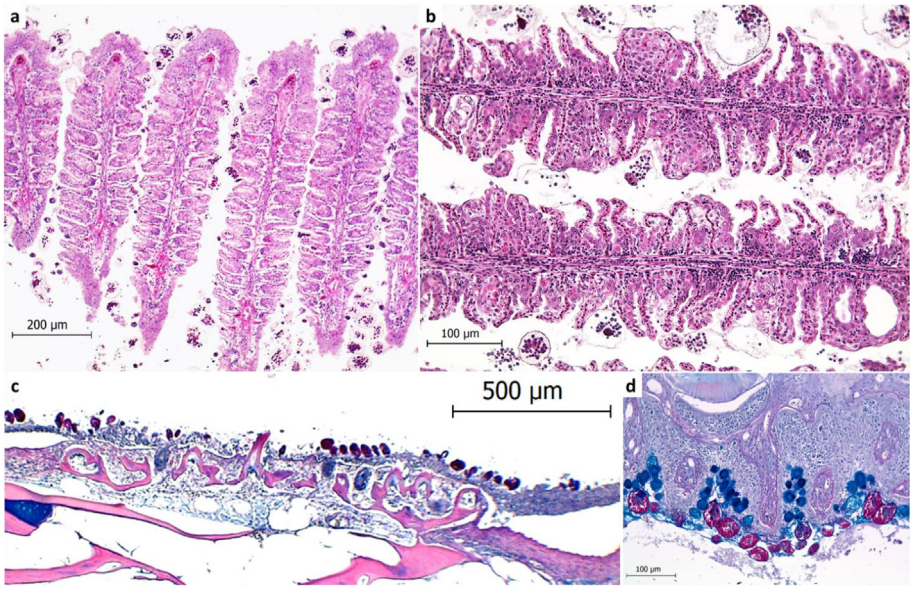

Fig. 1. Heavy *Amyloodinium ocellatum* infection in ESB. A) attached trophonts inducing severe and diffuse epithelial degeneration and hyperplasia of the distal third of lamellae, H-E; b) epithelial damage, hyperplasia with lacunae formation between lamellae, and cellular infiltrate, H-E; c) floor of buccal cavity showing attached trophonts and epithelial damage with hyperplasia, Pas-Alcian blue; d) starch granules in AO trophonts stained in red and very abundant mucous cells in the epithelium of pharyngeal tract, Pas-Alcian blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

were discernible; the same cell infiltrate pattern was evident even in the buccal cavity and pharynx, where mucous cells were very abundant in the epithelium (Fig. 1b). AO trophonts anchoring and feeding on epithelial cells induced also vascular system damages (rupture of pillar cells, aneurysms and microhaemorrhages).

3.2. PCR detection of A. ocellatum

The parasite was previously identified as *A. ocellatum* through morphological characterization. However, based on genomic DNA isolation and molecular analysis using the specific primers of *A. ocellatum* AO18SF and AO18SR have amplified consistently and yielded 248 bp of 18S rDNA, a specific amplicon of *A. ocellatum* was confirmed using sequencing (Fig. 2). The sequence was edited and submitted to NCBI GenBank with the accession number KY474336.1. Further, multiple sequence alignment was performed using the CLUSTALw and indicated that sequenced AO was conserved with the isolates from Mediterranean sea (DQ490256.1), Red sea (DQ490257.1), Fujian, China (KU761581.1 and KR057921.1), Southern Mississippi, USA (JX905204.1) (Fig. 3).

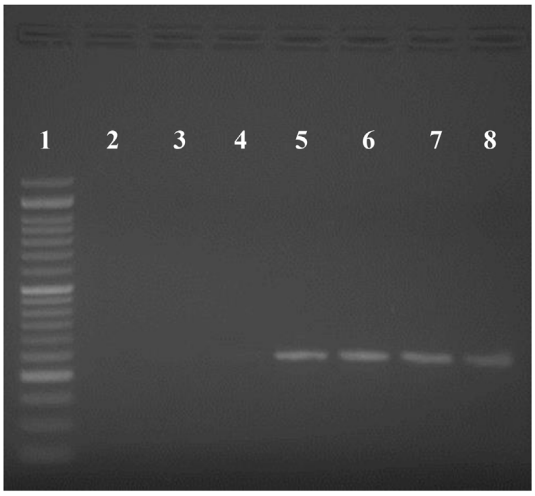

Fig. 2. Detection of *A. ocellatum* using Polymerase chain reaction. Lane 1: 50 bp DNA ladder, lane 2: negative PCR without DNA as template, lane 3–4: genomic DNA of gill from non-infected ESB as template, lane 5–6: genomic DNA of gill as template from *A. ocellatum* infected ESB, lane 7–8: purified parasitic DNA as template from infected ESB.

3.3. Immunohistochemistry

The markers were selected based on their relevance as indicators of tissue inflammatory and proliferative response and on their previously assessed reactivity on ESB tissues. Hyperplastic gill lamellae revealed the presence of cell populations positive for *igm*, *inos*, *pcna* and cytokeratin. The antibody specific for *igm* marked cells whose morphology was ascribable to plasma cells and, in some cases, to macrophages, diffusely distributed in the epithelium of lamellae (Fig. 4b). The antibody for *inos* highlighted cell populations whose morphology is similar to macrophages (Fig. 4d). They were mainly localized in the gill areas were *lamellae* were hyperplastic and fused. The application of the same markers on gill tissue samples from healthy fish allowed to detect very few positive cells whose number was negligible if compared to the one observed in the pathological samples (Fig. 4c). It is noteworthy to underline that the antibodies for *inos* reacted also with antigens expressed by AO. The antibodies for *tlr2*, *tlr4* did not label cell populations in the gills samples under evaluation (data not shown). A relevant part of the epithelial cells resulted positive also for PCNA (Fig. 4f). The cytokeratin antibody marked very clearly the gill epithelial cells, underlying the condition of hyperplasia in the diseased individuals (Fig. 4h).

3.4. Immune gene expression

The gene expression analysis of infected and uninfected ESB including twenty two (Table 1) immune related genes revealed the expression of seven genes in gills and nine in head kidney, specifically encoding Chemokine *cc1*, *il-8*, *il-10*, *cox-2*, *hepcidin*, *C type lectin*, *casp9*, *catalase*, and *igt*. In gills seven genes [*cc1*, *il-8*, *Hep*, *cox-2*, (Fig. 5a), *igt* (Fig. 6a), *cla* and *casp9* (Fig. 6b)] were differentially expressed ($P < 0.05$) in ESB infected by *A. ocellatum*. In head kidney nine genes were expressed in infected ESB [*cc1*, *il-10*, *hep*, *tlr9*, *cox-2*, (Fig. 5b), *igt* (Fig. 7a), *casp3*, *casp9* and *cat* (Fig. 7b]. Among the seven innate immune genes differentially expressed in gills, *hepcidin* (25 folds) and *cc1* (12 folds) (Fig. 5a) showed the highest expression. Whereas in head kidney, among the eight innate immune genes, *cox-2* (15 folds) and *hep* (10 folds) (Fig. 5b) showed the highest fold change.

Among the adaptive related genes analysed, *igt* showed a significantly different expression in both gills (Fig. 6a) and head kidney (Fig. 7a) from infected fish. The expression of the reference genes (*β-actin*, *il-3a* and *hsp-90*) remained constant and no significant change was detected.

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DQ490257.1      CTTGACCTTGCCCGAGAGGGTTGGGTAATCTTCTCAAAGTGCATCGTGATGGGGATAGAT
DQ490256.1      CTTGACCTTGCCCGAGAGGGTTGGGTAATCTTCTCAAAGTGCATCGTGATGGGGATAGAT
JX905204.1      ---GACCTTGCCCGAGAGGGTTGGGTAATCTTCTCAAAGTGCATCGTGATGGGGATAGAT
KU761581.1      CTTGACCTTGCCCGAGAGGGTTGGGTAATCTTCTCAAAGTGCATCGTGATGGGGATAGAT
KY474336.1      ----------------------------------------ATCGTGATGGGGATAGAT
KR057921.1      CTTGACCTTGCCCGAGAGGGTTGGGTAATCTTCTCAAAGTGCATCGTGATGGGGATAGAT
                                                        ******************

DQ490257.1      TATTGCAATTATTAATCTTGAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGC
DQ490256.1      TATTGCAATTATTAATCTTGAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGC
JX905204.1      TATTGCAATTATTAATCTTGAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGC
KU761581.1      TATTGCAATTATTAATCTTGAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGC
KY474336.1      TATTGCAATTATTAATCTTGAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGC
KR057921.1      TATTGCAATTATTAATCTTGAACGAGGAATTCCTAGTAAGCGCGAGTCATCAGCTCGTGC
                ************************************************************

DQ490257.1      TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTTCCGG
DQ490256.1      TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTTCCGG
JX905204.1      TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTTCCGG
KU761581.1      TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTTCCGG
KY474336.1      TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTTCCGG
KR057921.1      TGATTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGGGTGTTCCGG
                ************************************************************

DQ490257.1      TGAATAACTCGGACTGCTGCAGTCTTCTGCTTCTGAAAACTGTGGCGGAAAGTGTGGTGA
DQ490256.1      TGAATAACTCGGACTGCTGCAGTCTTCTGCTTCTGAAAACTGTGGCGGAAAGTGTGGTGA
JX905204.1      TGAATAACTCGGACTGCTGCAGTCTTCTGCTTCTGAAAACTGTGGCGGAAAGTGTGGTGA
KU761581.1      TGAATAACTCGGACTGCTGCAGTCTTCTGCTTCTGAAAACTGTGGCGGAAAGTGTGGTGA
KY474336.1      TGAATAACTCGGACTGCTGCAGTCTTCTGCTTCTGAAAACTGTGGCGGAAAGTGTGGTGA
KR057921.1      TGAATAACTCGGACTGCTGCAGTCTTCTGCTTCTGAAAACTGTGGCGGAAAGTGTGGTGA
                ************************************************************

DQ490257.1      ATCTTAACACCTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA
DQ490256.1      ATCTTAACACCTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA
JX905204.1      -TCTTAACACC----------------------------------------------------
KU761581.1      ATCTTAACACCTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA
KY474336.1      ATCTTAACACC----------------------------------------------------
KR057921.1      ATCTTAACACCTAGAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAA
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Fig. 3. Nucleotide sequence of small subunit ribosomal RNA gene alignment of *Amyloodinium ocellatum* isolate with other isolates from red sea (DQ490257.1); Mediterranean Sea (DQ490256.1); Gulf of Mexico (Mississippi, JX905204); Fujian, China (KU761581.1); Mediterranean Sea from this study (KY474336.1); Fujian, China (KR057921.1);. —— Sequence gaps, *identical residues,: conserved substitution,. Semi-conserved substitution.

4. Discussion

Amyloodiniosis represents a serious problem for several farmed or ornamental fish species in many different aquaculture rearing systems (tanks, ponds or aquaria) supplied with brackish and marine water in tropical and temperate regions. Above all, amyloodiniosis is a major threat for land-based rearing sites in Southern Europe, where, without an early treatment, it can cause high mortality (reaching 100% regardless of size) during warmer months. The outbreak under study represented exactly this situation, being a severe amyloodiniosis in ESB juveniles, in which the anatomopathological features are serious and consistent with what has already been described in ESB and in many other fish species [16–23]. Furthermore, polymerase chain reaction (PCR) performed on the genomic DNA of infected ESB gills amplified the specific product of small sub unit rDNA of AO and confirmed by sequencing the disease etiology. It is noteworthy that the intraspecific variation between different geographical isolates of *A. ocellatum* has been found to be very low [26]. Similarly, our multiple sequence alignment indicated that sequenced AO from natural infected ESB was conserved when compared with all the Mediterranean isolates from the database available in NCBI GenBank. Based on the histological and molecular observations, it was evident that the infection in ESB was due to AO. In order to identify the host immune gene response specific for this infection, we broadly evaluated innate immune-related, adaptive and stress genes during the initial phase of parasite infection and consequently insights on the pathogenesis of AO infection in ESB were discussed. Such knowledge highlights the immune molecules and pathways that could be targeted for immunization intervention against the parasite. However, there is also a difficulty with respect to determining specific immune gene response to parasite in natural infection mainly because of the individual genetic variability. Since the ESB during stocking were derived from the same hatchery, we can exclude the presence of genetic variability among the infected ESB as well as the controls. Our data could be reliable for further studies on experimental infection which can benefit the ESB farming. The results demonstrated that natural infection by AO in ESB elicits significant changes in immune-related gene expression. These findings will facilitate the identification of specific molecules which are being regulated during AO infection and this could help us to understand the pathogenicity of AO towards the host, as well as the pathogenesis of the disease. Subsequently, using these upregulated molecules (for example IgT) as markers, further studies could be directed towards mucosal related immune response during vaccine development, aiming at mitigating AO outbreaks in aquaculture.

For the immunohistochemical labeling, a panel of antibodies (markers) was selected based on their relevance as indicators of tissue reactivity in terms of inflammatory/proliferative response and on their previously assessed reactivity on ESB tissues by the authors. Based on our knowledge, there are no documented data in which IHC was performed on ESB gills infected by *A. ocellatum*. Some of the markers selected for the present study have already been employed on fish species for anatomo-pathological and immunological surveys. Immunoglobulin (*igm*) bearing cells have been marked in ESB juveniles head kidney/spleen to evaluate the outcomes of a vaccination protocol against vibriosis [30]. The *inos* molecule has been detected by immunohistochemistry in tissues of *Scophthalmus maximus* in response to natural as well as experimental infection with *A. salmonicida* [31,32] and upon vaccination against the same pathogen [33]. *inos* was also marked by IHC in wild *Abramis brama* during a natural infestation by *Ergasilus sieboldi* [34], in wild *Hypostomus francisci* gills to investigate the impact of environmental pollutants [35] and in farmed turbot (*Psetta maxima)* submitted to *Enteromyxum scophthalmi* infection [36,37]. Similarly *tnf-α* positive cells have been labelled in tissues of *Scophthalmus maximus* infected by *A. salmonicida* [32,37] and in the gills of *Oncorynchus mykiss* infected by *Loma salmonae* [38]. On the contrary, the antibody markers for *tlr2* and *tlr4* have never been used by

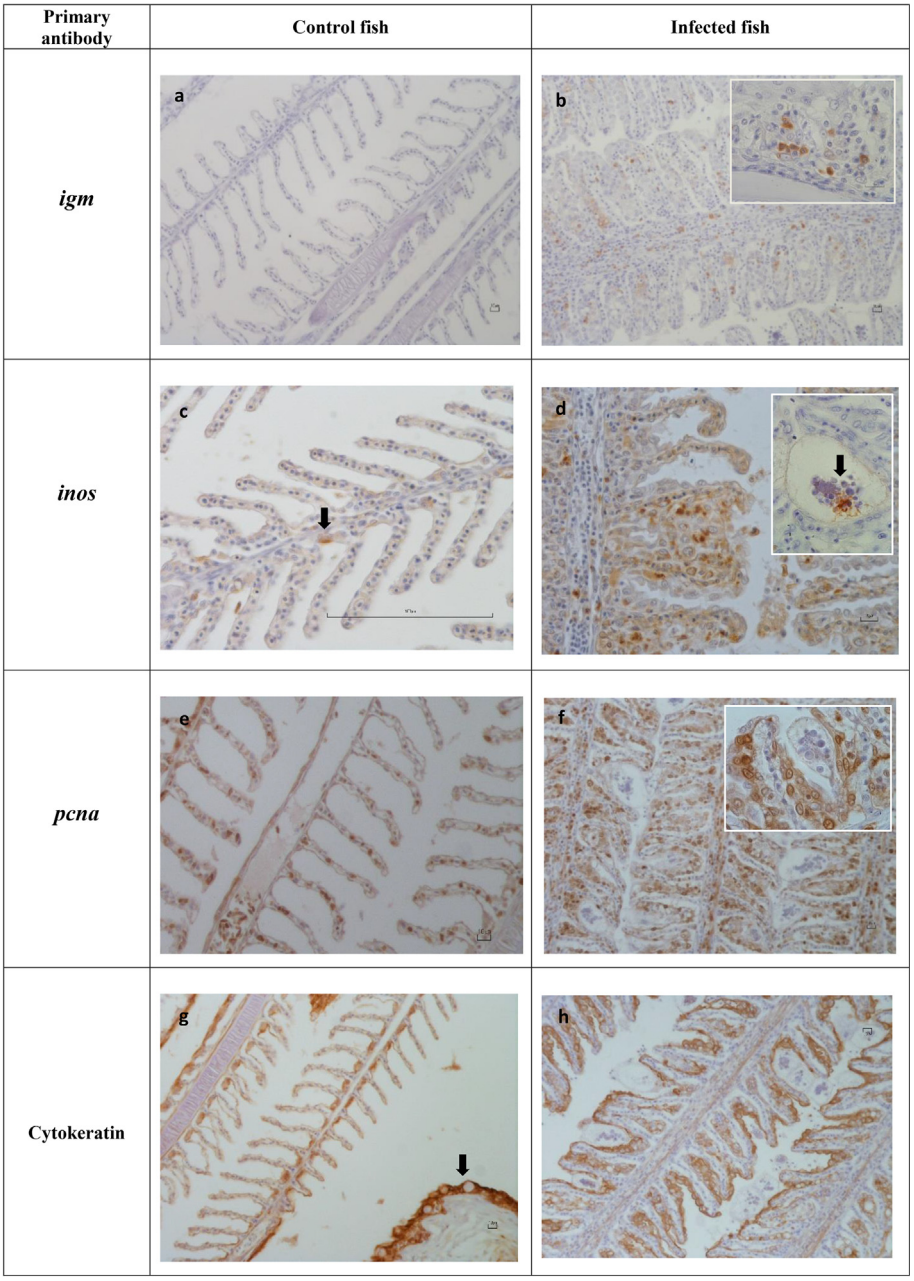
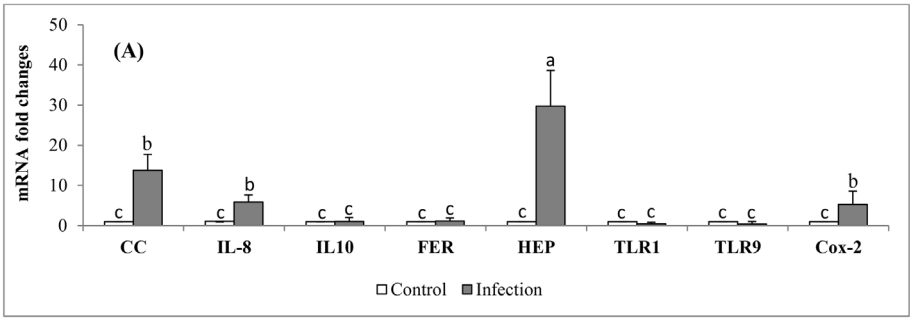


Fig. 4. ESB (*D. labrax*) branchial tissue infected by AO labelled with antibody markers. 4a-Immunolabelling of gills from healthy ESB with anti IgM antibody indicating absence of positive cells. 4b-Immunolabelling of gills from infected ESB with anti *igm* antibody, evidence of positive cells within the gills epithelium, possibly ascribable to plasma cells and IgM-bearing macrophages. 4c- Immunolabelling of gills from healthy ESB with anti *inos* antibody. Slight positivity of few intra-epithelial cells (arrow). 4d-Immunolabelling of gills from infected ESB with anti *inos* antibody, highlighting cell populations whose morphology is similar to macrophages, mainly localized in the gill areas were *lamellae* are hyperplastic and fused. An internal area of attached trophonts results positive. 4e-Immunolabelling of gills from healthy ESB with anti PCNA antibody and the number of epithelial cell under turnover is limited. 4f-Immunolabelling of gills from infected ESB with anti PCNA antibody and the number of epithelial cell under turnover is relevant, the parasites are negative. 4g- Immunolabelling of gills from healthy ESB with anti-cytokeratin antibody indicating slight positivity of the gill mucosa and intense positivity of the mucosa (arrow) in the gill cavity. 4h- Immunolabelling of gills from infected ESB with anti-cytokeratin antibody. It marked very clearly the gill epithelial cells, underlying the condition of hyperplasia.

other researchers for immunohistochemical studies on fish, prior to this experience.

As reported in the results section *inos* antibodies marked cells with a morphology potentially ascribable to macrophages; while the *igm* antibody labelled cell populations referred as plasma cells or Ig bearing macrophages. These results suggest that the mucosal immune response adopted by ESB against AO includes the activation of GALT (gill associated lymphoid tissue) components such as phagocytic cells and antibody producing cells. As other protozoa, AO can stimulate the host immune response promoting the release of pro-inflammatory cytokines and enzymes such as *inos*. Further investigations will be necessary to determine the number of positive cells to the different markers and to correlate them with the lesion severity and parasite burden. Other than host tissue antigens, also adherent trophonts showed positivity for *inos* markers. This observation allows to speculate on the possible ability of the parasite itself to synthesize molecules that are ancestrally expressed also by very simple organisms. For example the nitric oxide production pathway has been described in many organisms ranging from single cell moulds to humans, being a defence mechanism early developed in the evolution [39]. AO could possibly use these molecules to interact with the host gills to which it adheres [40]. Moreover we can also hypothesize that the iNOS molecule we labelled internally to the trophonts derives from material that the parasite incorporates from the gills by the stomopode. In fact some dinoflagellates, among them *A. ocellatum*, are known as organisms able to perform “phagotrophy” in order to get nutrients from the host cell cytoplasm, as observed ultrastructurally by Ref. [41] Lom and Lawler (1973). Several studies confirmed that there is a good cross-reactivity between mammalian and fish cytokeratins as well as markers associated with cell proliferation [42–45]. The positive staining obtained in the present study with the anti-cytokeratin and the anti-PCNA antibodies respectively allowed for a better morphological description of the mucosal cell proliferation and consequent hyperplasia, which often characterises the amylodiniosis post-infection pattern in the gills.



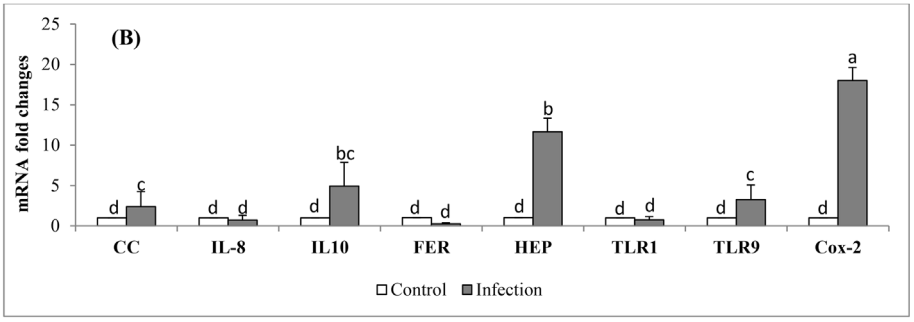
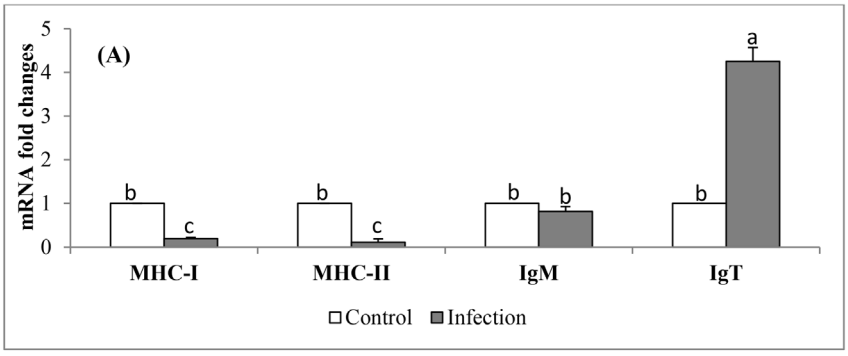


Fig. 5. Relative mRNA expression of innate immune related gene in gills (A) and head kidney (B) in ESB naturally infected with AO as measured by quantitative real-time PCR. Data are presented as mean ± SD and multiple reference genes were used to normalize with the target gene (n = 5). Different alphabetical letters indicate the significant difference with $p < 0.05$.

4.1. Innate immunity post AO infection in ESB

The cytokines, *il-1β* and *tnf-α*, are crucial mediators of pro-inflammatory responses and in the activation of B and T cells [46]. In our study, there was a lack of *il-1β* and *tnf-α* expression in infected gills, this may be due to the variation in the host susceptibility towards AO parasites, and toxins or enzymes released by them might have damaged the leucopoietic system resulting in reduction in the expression of most of the immune-related genes including *tnf-α* [47]. Therefore, this host susceptibility is due to the species and parasite infection [47]. However, functionally assessed to have pro-inflammatory activity in fish, *il-1β* and *tnf-α* are often co-expressed with other macrophage-derived inflammatory mediators such as *il-8*, *cox-2*, and *inos* in parasitic and bacterial infections [48–52]. *il-8*, *cox-2* and *cc1* expression was upregulated in AO infected gills, which indicates that a proinflammatory stimulus was activated by the host response *versus* the parasite. Based



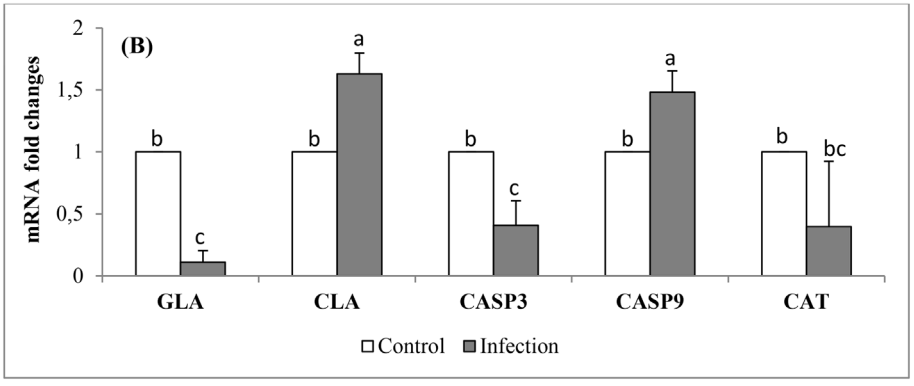
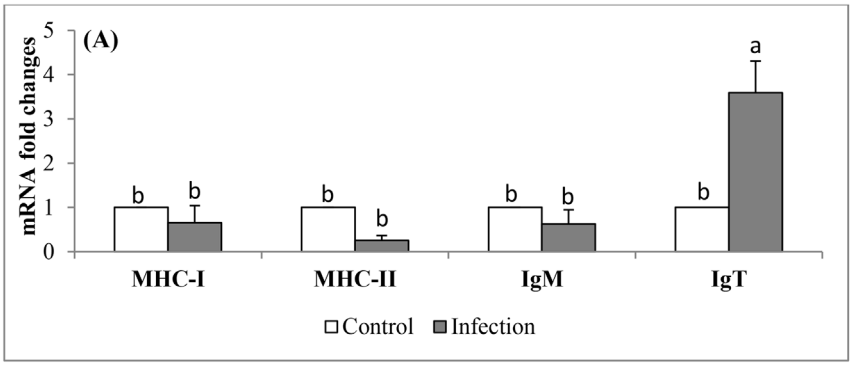


Fig. 6. Relative mRNA expression of adaptive (a), complement and stress response (b) related gene in gills in ESB naturally infected with AO as measured by quantitative real-time PCR. Data are presented as mean ± SD and multiple reference genes were used to normalize with the target gene (n = 5). Different alphabetical letters indicate the significant difference with $p < 0.05$.



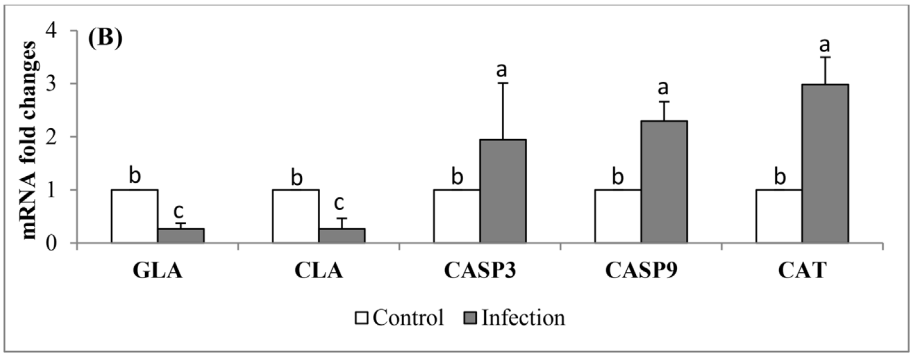


Fig. 7. Relative mRNA expression of adaptive (a), complement and stress response (b) related gene in head kidney in ESB naturally infected with AO as measured by quantitative real-time PCR. Data are presented as mean ± SD and multiple reference genes were used to normalize with the target gene (n = 5). Different alphabetical letters indicate the significant difference with $p < 0.05$.

on previous studies, *il-8*, *cox-2* and *cc1* inflammatory response in the hosts was found to be positively correlated with the resistance of the hosts to ectoparasites [53,54]. In particular CC chemokine plays a significant role in the wound healing due to the parasite invasion [55]. The upregulation of *il*-8 might indicate a chemotactic stimulus for neutrophils, aimed at inducing their recruitments towards the site of infection. This phenomenon was previously observed in carp stimulated with zymosan, in which *il-8* played a crucial role by involving neutrophilic granulocytes during the early phase of inflammation [56].

This study highlighted the important results of increased expression in hepcidin, which is an iron regulator that reduces the availability of iron for pathogen growth. It is generally up-regulated by bacterial and parasitic infection in salmonids [49,57,58]. In the case of European sea bass it was recorded that hepcidin was upregulated in all the tissues after infection with *V. anguillarum* [59], but no upregulation was detectable in ESB infected tissues with Nodavirus, hence, hepcidin considered as a marker for bacterial infection in ESB [59]. In healthy organisms, iron concentration is maintained at a stable level in plasma, and it is stored in hepatocytes and splenic/hepatic macrophages at constant levels, despite unstable absorption of iron from the diet [44]. It has been reported that hepcidin-mediated low serum iron level functions as a host defense mechanism that evolved to restrict iron availability for pathogen growth and development [60,61]. Recombinant hepcidin in grass carp protected from *F. columnare* via regulating iron availability and immune gene expression [62]. These results demonstrate that hepcidin in ESB post AO infection played a major role as host defense and could be considered as AO infection related marker in ESB but further studies are required to evaluate the protective role of hepcidin in this fish species.

In this study we have evaluated *tlr1* and *tlr9* as pathogen recognition receptors (PRRs). Results indicated that there was no significant expression of *tlr1* in gills and head kidney but *tlr9* was significantly higher in head kidney. Although gills and skin are the target organs for *A. ocellatum*, it is interesting to note the expression of relevant immune receptor genes also in internal organs in ESB. This may indicate that the parasitic DNA as PAMP are being recognized by *tlr9* in head kidney but this should be elucidated in the future studies. However, the immunohistochemical labelling on gills indicated the absence of *tlr4* and *tlr2*. Moreover, the role of *tlr* as anti-parasite in fish is still largely unknown but few studies in *Labeo rohita* infected by Argulus [63] and in *Seriola lalandi* experimental infected with *A. ocellatum* [64] indicated that the role of *tlr* is important for host innate immune response. Therefore, it can be inferred that the AO infection in ESB and the response of the host towards AO are specific in terms of type of *tlr* expression as our results indicated only the expression of *tlr9* in head kidney. Interestingly, we recorded that, C-type lectin was upregulated in infected gills but not in head kidney. Noticeably, *clr* are certainly essential to the recognition of different carbohydrates present on the surface or in the excretory/secretory products of different parasites. This recognition can promote the uptake, internalization and processing of parasite antigens that can consequently trigger the specific immune response. Therefore, it is evident that the complement system, although activated, does not constitute an effective response to AO infection. Eventhough, complement system was upregulated in this study, AO was able to avoid the host response and therefore induced a severe outbreak.

In the present study, there was an upregulation of *casp9* in gills and *casp3*, *casp9* were upregulated in head kidney. Several caspases have been characterized in teleosts, including pro-inflammatory caspases, such as caspase-1 [65] and caspases related to apoptosis, such as caspase-9 [66] or caspase-3 [67]. Our data on apoptotic related caspase 3 and caspase 9 indicate that there was a maintenance of homeostatis order in ESB effector mechanisms of innate and adaptive response to AO infection and that the apoptosis is important in maintaining the homeostasis between innate and adaptive mechanisms for effective host response [68]. It is noteworthy that, the infected cells with caspase and inflammatory expression should limit the activation of pathogen including parasite through the recruitment of phagocytes. However, AO could still survive in the host and continue its cycle determining the amplification of the infection in ESB. A similar evidence was reported from a variety of mammalian pathogens which are capable of surviving

inside host cells by interfering with host cell apoptotic processes [69–71]. Therefore, our results indicate that eventhough apoptotic caspase 3 and 9 are being activated by ESB, the host response could not limit the attach and development of AO in ESB.

4.2. Adaptive immunity post AO infection in ESB

Noticeably, innate immunity is the initial defence against parasite invading like AO, and host innate immunity is intrinsically linked to the initiation of the adaptive immune response. In the present study, three out of four gene codifing for molecules related to adaptive immunity (*mhc i*, *mhc ii* and *igm*) did not show upregulation in gills and head kidney. This phenomenon could be due to the nature (individual characteristics) of the host species and to the infection dynamics (intended as mode of response in relation to the days post infection). The post infection sampling for the expression analysis in fact has been carried at a defined time point, and further time course studies are required to determine an eventual onset of adaptive response *versus* AO infection in ESB. It is interesting to speculate that the downregulation of *il-1β*, *tnf-α* and *mhc* genes may have prevented the activation and development of acquired immunity and this could possibly explain the high susceptibility of ESB to *A. ocellatum* recorded during the present outbreak. Eventhough, *igm* also has an important role in adaptive immune response, the lower level or no expression of *igm* in infected samples indicated that the parasitic toxins might negatively influence the systemic specific immune response (like production of *igm*) [52] or alternatively that the disease reached its onset very rapidly and the individuals did not have enough time to activate a specific humoral response. Interestingly, we recorded a significant high expression of *igt* in AO infected gills and head kidney in ESB. There are only few studies which have demonstrated the increased expression of *igt* after parasite infection. For example, *igt* was demonstrated to be specifically involved in gut response against the parasite *Ceratomyxa shasta* [72] and in gills and skin response against the parasitic ciliate *Ichthyophthirius multifiliis* [73,74] in rainbow trout and in head kidney response against the ectoparasite *Argulus siamensis* in rohu (*Labeo rohita*) [52]. Also, the upregulation of heavy and light chain of *igt* was detected in head kidney of gilthead sea bream post *E. leei* challenge [75]. This is the first evidence of *igt* expression post AO infection in ESB and hence further studies should be directed towards understanding the local *igt* expressing cells in the gills and head kidney with the availability of anti-IgT antibody in ESB and correlate with the mRNA expression post AO infection in ESB.

5. Conclusion

These preliminary results depict that inflammatory and local immunity played a significant role in determining the susceptibility of host to the parasite. ESB was naturally infected by *A. ocellatum,* as confirmed by histological and specific PCR analysis. Immunohistochemical labelling resulted positive for ESB *igm*, *inos*, *pcna* and cytokeratin but negative to *tlr2*, *tlr4*. Besides, the pronounced and sustained inflammation (*il-8*, *chemokine cc1*, *cox-2*) that brought many novel molecules (Hepcidin) to the site of parasite adhesion. This work signifies the immediate local immune responses of ESB to AO parasite infection. However, further studies should be directed to understand the time course expression of these upregulated immune genes, the physiological status of the host, and to determine their potential as functional markers in the ESB infected by AO.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgements

The present study was performed under the European Union support as the Horizon 2020 project Parafishcontrol (grant agreement No. 634429). This output reflects only the authors view, and the European Union cannot be held responsible for any use that may be made of the information contained herein (http://www.parafishcontrol.eu/). The authors wish to thanks technical Pierluigi Bagatella and Carla Calligaro for the technical assistance (https://sav.uniud.it/en/) and Prof. Emilio Tibaldi for his help and discussion during the study (Department of Agricultural, Food, Environmental and Animal Science, University of Udine).

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Abbreviations

AO: Amyloodinium ocellatum
ESB: European sea bass
SSU: small sub unit

NCBI: National Center for Biotechnology Information
PPAR α: Peroxisome proliferator activated receptor α
PCR: Polymerase chain reaction
q-PCR: quantitative polymerase chain reaction
IHC: immunohistochemistry
PRR: pathogen recognition receptor
PAMP: pathogen associated molecular pattern
PCNA: Proliferating cell nuclear antigen
igm: immunoglobulin M
inos: inducible nitric oxide synthase
tlr2: toll like receptor 2
tlr4: toll like receptor 4
tlr9: toll like receptor 9
tnf-α: tumor necrosis factor alpha
cc1: chemokine CC1
il-8: interleukin-8
hep: hepcidin
cox-2: cyclooxygenase-2
cla: c type lectin
cat: catalase
casp9: caspase 9
igt: immunoglobulin T