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2 **Innate Immune-gene expression during experimental Amyloidinosis in**
3 **European seabass (*Dicentrarchus labrax*)**
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5 Omkar Byadgi^{1,*}, Michela Massimo¹, Ron P Dirks², Alberto Pallavicini^{3,4}, James E Bron⁵,
6 Jacquie H Ireland⁵, Donatella Volpatti¹, Marco Galeotti¹, Paola Beraldo¹
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8

9 ¹*Section of Animal and Veterinary Sciences, Department of Agricultural, Food, Environmental*
10 *and Animal Sciences (DI4A), University of Udine, 33100 Udine, Italy.*
11

12 ²*Future Genomics Technologies B.V., Leiden, The Netherlands.*
13

14
15 ³*Laboratory of Genetics, Department of Life Sciences, University of Trieste, Via Licio Giorgeri*
16 *5, 34126 Trieste, Italy.*
17

18
19 ⁴*National Institute of Oceanography and Applied Geophysics, via Piccard 54, 34151, Trieste,*
20 *Italy.*
21

22
23 ⁵*Institute of Aquaculture, University of Stirling, Stirling, Scotland, UK.*
24
25
26
27

28 ** Corresponding author. Omkar Byadgi, Section of Animal and Veterinary Sciences, Department*
29 *of Agricultural, Food, Environmental and Animal Sciences (DI4A), University of Udine, 33100*
30 *Udine, Italy. Tel: +393917580339, E-mail: omkar.byadgi@uniud.it*
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35 Abstract

36 The ectoparasite protozoan *Amyloodinium ocellatum* (AO) is the causative agent of
37 amyloodiniosis in European seabass (ESB, *Dicentrarchus labrax*). There is a lack of information
38 about basic molecular immune response mechanisms of ESB during AO infestation. Therefore, to
39 compare gene expression between experimental AO-infested ESB tissues and uninfested ESB
40 tissues (gills and head kidney) RNA-seq was adopted. The RNA-seq revealed multiple
41 differentially expressed genes (DEG), namely 679 upregulated genes and 360 downregulated
42 genes in the gills, and 206 upregulated genes and 170 downregulated genes in head kidney. In
43 gills, genes related to the immune system (perforin, CC1) and protein binding were upregulated.
44 Several genes involved in IFN related pathways were upregulated in the head kidney.
45 Subsequently, to validate the DEG from amyloodiniosis, 26 ESB (mean weight 14g) per tank in
46 triplicate were bath challenged for 2h with AO (3.5×10^6 /tank; 70 dinospores/ml) under controlled
47 conditions (26-28°C and 34‰ salinity). As a control group (non-infested), 26 ESB per tank in
48 triplicate were also used. Changes in the expression of innate immune genes in gills and head
49 kidney at 2, 3, 5, 7 and 23 dpi were analysed using real-time PCR. The results indicated that the
50 expression of cytokines (CC1, IL-8) and antimicrobial peptide (Hep) were strongly stimulated and
51 reached a peak at 5 dpi in the early infestation stage, followed by a gradual reduction in the
52 recovery stage (23 dpi). Noticeably, the immunoglobulin (IgM) expression was higher at 23 dpi
53 compared to 7 dpi. Furthermore, *in-situ* hybridization showed positive signals of CC1 mRNA in
54 AO infested gills compared to the control group. Altogether, chemokines were involved in the
55 immune process under AO infestation and this evidence allows a better understanding of the
56 immune response in European seabass during amyloodiniosis.

57
58 **Keywords:** *Amyloodinium ocellatum*, Dinoflagellates, Ectoparasite, Innate immunity, Illumina
59 RNA-seq

60
61 **1. Introduction**

62 The ectoparasite dinoflagellate *Amyloodinium ocellatum* (AO) is one of the most problematic
63 parasites causing disease among brackish and marine water fish, known as marine velvet disease
64 (Brown EM., 1934). AO causes a parasitic branchitis associated with high mortality and significant
65 economic losses in farming conditions worldwide (Cruz-Lacierda et al., 2004; Fioravanti et al.,
66 2006; Benetti et al., 2008; Saraiva et al., 2011; Soares et al., 2011; Dequito et al., 2015; Gomez et
67 al., 2018; Byadgi et al., 2019). This parasite mainly infests the gills, skin, and entire oropharyngeal
68 cavity of almost all species of brackish and marine water fish, including European seabass
69 (*Dicentrarchus labrax*) (Benetti et al., 2008; Alvarez-Pellitero, P et al., 1993; Byadgi et al., 2019).

70 Fish surviving the infestation may develop protective immunity, which suggests that the
71 immunoprophylactic control of this disease through vaccination could be feasible (Smith et al.,
72 1994; Cobb et al., 1998; Cecchini et al., 2001). However, the information regarding host responses
73 to *A. ocellatum* infestation is limited (Byadgi et al., 2019).

74 Transcriptomics has been used extensively to explore the host response towards fish parasite
75 infestations (Sudhagar et al., 2018). Results from the large yellow croaker (*Larimichthys polyactis*)
76 after *Cryptocaryon irritans* infestation indicated enrichment of the Toll-like receptor pathway
77 (TLR), chemokine signalling, complement system and coagulation cascades (Wang et al., 2016).
78 Low, non-lethal infestation by *C. irritans* enhanced a significant local immune response in large
79 yellow croaker (*Larimichthys crocea*) and induced immunosuppression (Yin et al., 2016).
80 Similarly, in skin of orange spotted grouper (*Epinephelus coioides*) affected by *C. irritans*, a local
81 immune response with intense leukocytes recruitment was observed (Hu et al., 2017).
82 Interestingly, three-spined stickleback (*Gasterosteus aculeatus*) infested by three different
83 genotypes of the trematode parasite, *Diplostomum pseudospathaceum*, revealed differential
84 mechanisms by which the host immune system reacts to the immunological threat (Haase et al.,
85 2016 & 2017). Moreover, in large yellow croaker upon infestation with the intestinal myxozoan
86 parasite *Enteromyxum scophthalmi* an inadequate adaptive immune activation was observed
87 (Robledo et al., 2014). However, during early phase of infestation in turbot (*Scophthalmus*
88 *maximus*) by *E. scophthalmi* an IFN-mediated immune response was recorded (Ronza et al., 2016).
89 During mild natural infestation of *Sparicotyle chrysophrii* in Gilthead sea bream (*Sparus aurata*)
90 a strong enrichment of differentially expressed genes in gills, related to apoptosis, inflammation
91 and cell proliferation was observed, whereas inhibition of DEG related to apoptosis, autophagy,
92 platelet activation, signalling and aggregation in the spleen was observed (Piazzon et al., 2019).
93 *Ichthyophthirius multifiliis* infestation in rainbow trout (*Oncorhynchus mykiss*) gills triggered an
94 innate immune response by enhancing the Chemokine signalling pathway, platelet activation, Toll-
95 like receptor signalling (TLR) pathway, NOD-like receptor signalling pathway, and Leukocyte
96 transendothelial migration (Syahputra et al., 2019). RNA-Seq-based transcriptome analyses were
97 also employed to study the parasites themselves such as *C. irritans* (Yin et al., 2016; Mo et al.,
98 2016) and salmon louse *Caligus rogercresseyi* (Allardo-Escárate et al., 2014), in order to
99 understand the host-parasite antigens interactions and to identify potential vaccine candidates.

100 Previous studies have indicated that Interleukin-1 (IL-1) and Tumor Necrosis Factor α (*Tnf- α*)
101 were activated in infested ESB reared in an aquaponics system (Nozzi et al., 2016). Experimental
102 infestation of AO in yellowtail (*Seriola lalandi*) enhanced the TLR22 expression and involved in
103 response to AO infestation (Reyes-Becerril, M et al., 2015). Moreover, natural outbreaks of AO
104 in ESB resulted in pronounced and sustained inflammation (*il-8*, *cc1*, and *cox-2*) involving many

105 novel molecules (Hepcidin) at the site of parasite attachment. Moreover, some of the genes related
106 to pro-inflammation such as TNF- α and IL1 β were down regulated, and this may be a result of a
107 transient process. Therefore, this recent work highlighted the immediate local immune responses
108 of ESB to natural AO infestation (Byadgi et al., 2019). However, further studies are needed to
109 understand the time course expression of these upregulated immune genes under laboratory
110 experimental infestation, in order to describe the physiological status of ESB during AO infestation
111 and the subsequent recovery processes.

112 Therefore, the objectives of this study were to evaluate immune gene expression in gills and head
113 kidney after AO infestation using RNA-seq, to evaluate the most differentially expressed genes in
114 AO infested ESB and to investigate the chemokine *cc1* mRNA using *in-situ* hybridization (mRNA
115 FISH) in order to survey the involvement of *cc1* against AO in the gills of ESB. Altogether, this
116 study will provide a more comprehensive understanding of the roles of ESB immune genes during
117 AO infestation and recovery.

118

119 **2. Materials and methods**

120 **2.1 Ethics statement**

121 All the experiments included in the present study have been carried out in the facilities (fish
122 stabularium ID 5E7A0) of Department of Agricultural, Food, Environmental and Animal Sciences
123 (University of Udine), as authorized by the Italian Ministry of Health (decree n 14/2018-UT,
124 12/11/2018). The animal care and protocols adopted adhere to the Directive 2010/63/EU of the
125 European Parliament, implemented at a national level by the D.L. n. 26 of 4 March 2014.

126 **2.2 Fish and parasite origin for experimental infestations**

127 AO-naïve ESB (mean weight 14g) juveniles, sourced from an Italian Northeast hatchery, were
128 acclimatized for two weeks in two separate recirculation systems, one for infection trial consisting
129 of three 120 L fiberglass tanks (temperature 22.5 \pm 2 $^{\circ}$ C, salinity 30 \pm 2‰ with natural photoperiod)
130 and one structurally equal for control and with the same abiotic parameters. Fish was fed daily
131 with commercial pellets and submitted to periodical veterinary control in order to assess their
132 health status.

133 Trophonts obtained from natural infestations were collected and purified following the lab protocol
134 (Beraldo et al., 2020; Byadgi et al., 2019) and subsequently early tomites (before first division)
135 were maintained *in vitro* at 16 \pm 0.5 $^{\circ}$ C (hibernation status) until experimental infestation.
136 Approximately 2 days before the experimental infestation the developmental process was
137 reactivated by bringing them to 24 $^{\circ}$ C to obtain viable dinospores.

138 **2.3 Small scale infestation and RNA-sequencing**

139 A dedicated small scale experimental infestation for RNA-seq was carried out in two 300L tanks
140 (25‰ salinity and 24±2°C) at the University of Udine facilities, in one of which 5 ESB were
141 infested by adding 4 dinospores/ml, whereas the other was left as control (5 uninfested ESB). After
142 one week, infested and control ESB were euthanized (MS-222, 400 mg/L; E10521, Sigma-
143 Aldrich) to collect gills and head kidney as study target organs. Total RNA from gills and anterior
144 kidney was extracted using RNeasy Mini Kit (Qiagen, TX, USA).

145 **2.4 Library construction and sequencing**

146 Total RNA purity and degradation were checked on a 1% agarose gel. RNA integrity was assessed
147 using the RNA Nano 6000 Assay Kit on the Bioanalyzer 2100 system (Agilent Technologies, CA,
148 USA). Complementary DNA (cDNA) libraries were constructed using the TruSeq stranded mRNA
149 Library Prep kit (Illumina®, USA). Libraries were sequenced (2×150bp) using the Illumina®
150 platform NovaSeq 6000. Bbmap ver38.32 was used to remove remaining Illumina adapters from
151 the sequencing reads. Bowtie2 (Langmead et al., 2012) (very sensitive settings) was used to align
152 the cleaned reads against the *Dicentrarchus labrax* Transcriptome CDS (diclab1_cds.fasta;
153 downloaded from NCBI) and unaligned reads were recovered in FASTQ format.

154 **2.4.1 RNA seq data analysis**

155 Raw data was analyzed using CLC Genomics Workbench v.12 (Qiagen Bioinformatics). Briefly,
156 clean reads were obtained by removing low quality reads through trimming. High quality reads
157 were aligned to the *D. labrax genome* downloaded from European seabass Genome Browser
158 Gateway database (<http://seabass.mpipz.mpg.de>) with related annotations Mapping parameters:
159 length fraction: 0.80, similarity fraction: 0.97, mismatch cost: 2, indels cost: 3.

160 **2.4.2 Differentially expressed gene**

161 Gene expression values were reported as TPM (Transcript per Million mapped reads). Genes that
162 were identified as being differentially expressed met the following criteria: absolute fold change
163 (FC) of >4, FDR *p-value* of <0.01 and Max group means of >10. Functional annotation of ESB
164 genes were refreshed using Blast2Go (BioBam Bioinformatics S.L.).

165 **2.5 AO experimental infestation and tissue collection for validation**

166 Twenty-six fish/tank (mean weight 14 g) were stocked in three different tanks for infection and
167 other three tanks for control in two independent recirculation systems (one for infested group, the
168 other for the control group respectively) were used for the trial. Three days before infestation the
169 water temperature was increased daily until 26±2°C and maintained for all experimental trial.
170 Seventy Dinospores/ml were added to the designated “infested” tanks at a concentration of
171 3.5×10⁶/tank. The time-course of infestation was monitored by the observation of clinical signs
172 and by fresh gill microscopical examination. Fish became infested after 2 hours and at 2 days post-

173 infestation (dpi) the AO trophont burden in the gills was evident, with slight clinical symptoms.
174 During the infestation, the maximum AO burden was observed at 10-12 dpi, thereafter fish started
175 to recover even if positive for amyloodiniosis. Throughout this period, the total mortality was 18%.
176 In the control group, fish were healthy, and no mortality was registered.
177 Differences in the expression levels of gill and head kidney innate immune genes at 2, 3, 5, 7 and
178 23 dpi were analysed using real-time PCR. Gills and head kidney were sampled (n=3/time point)
179 as reported in paragraph 2.3 and preserved in RNA later until required.

180 **2.6 Total RNA isolation and cDNA synthesis**

181 Total RNA was isolated using TRIzol® reagent (Invitrogen Corp., Carlsbad, CA, USA,
182 <https://www.thermofisher.com/>) according to manufacturer's instructions and the quantity and
183 quality determined spectrophotometrically. The quality was also checked by running each sample
184 on a 2% agarose gel and the RNA samples then stored at -80°C until required. For qPCR, 2 µg of
185 total RNA was reverse-transcribed in a 20 µL reaction according to the manufacturer's protocol
186 (iScript™ cDNA synthesis kit, Bio-Rad, <http://www.bio-rad.com/>).

187 **2.7 Real-time PCR assays**

188 The target and reference gene primers used in this study are detailed in Table 1. Amplifications
189 were performed according to Byadgi et al., 2019 in a final volume of 10 µL. Each reaction
190 contained 5 µL of IQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 µL
191 of each primer set (10 µM), 1 µL of template cDNA and 3 µL of DEPC-water. Real time PCR
192 determinations were performed in triplicate in 96-well PCR plates and carried out in an CFX96
193 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with an
194 initial denaturation cycle of 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for
195 10 sec. Amplification was followed by a standard melting curve from 55°C to 95°C, in increments
196 of 0.5°C for 5 sec at each step, to confirm that only one product was amplified and detected.
197 Samples were run in parallel with three reference genes, beta-actin, hsp90 and L13a, for cDNA
198 normalization (Mitter et al., 2009; Buonocore et al., 2017). Relative mRNA expression was
199 calculated using the $2^{-\Delta\Delta C_t}$ method (Livak et al., 2001), normalizing with geometric average of
200 three reference genes (β -actin, hsp90 and L13a) and relative to each control group.

201 **2.8 *In situ* hybridization**

202 The experimental infestation for the *in-situ* hybridization (ISH) was carried out in a 300L tank
203 (25‰ and 24±2°C) at the University of Udine facilities, by adding dinospores (Dehority BA.,
204 2003) to a final concentration of 4/ml. After one week, infested and control ESB juveniles were
205 euthanized (MS-222, 400 mg/L; E10521, Sigma-Aldrich) to collect gills. Gills from AO-infested
206 and control ESB juveniles were fixed in 4% paraformaldehyde in PBS (pH 7.4) (16005 & P5368,
207 Sigma-Aldrich) overnight at 4°C, transferred to 70% ethanol and stored at -20°C. AO-infested and

208 control gills were also preserved in RNA later® (AM7021, Thermo Fisher Scientific) according
209 to manufacturer's instructions and stored at -20°C.

210 The paraffin embedding of samples was performed at the Institute of Aquaculture, University of
211 Stirling (Stirling, UK), and paraffin blocks stored at -20°C. Five µm sections were cut from the
212 4% paraformaldehyde-fixed, wax-embedded tissues, mounted onto Plus+ Frost positively charged
213 microscope slides (MSS51012WH, Solmedia) and stored at -20°C.

214 **2.8.1 Probes production.** Total RNA was extracted from RNA later® preserved gill tissues using
215 TRI Reagent (T9424, Sigma-Aldrich) as per manufacturer's instructions. The RNA was quantified
216 using the Nanodrop (ND2000c, ThermoFisher Scientific) and the quality was assessed on a 1%
217 agarose gel run with a 1Kb size marker (SM3014, Thermo Fisher Scientific). The gel was prepared
218 with and run in 0.5× TAE buffer and contained ethidium bromide (EtBr) to a final concentration
219 of 0.05µg/ml (E1510, Sigma-Aldrich). A 50µl, one-step reverse transcriptase PCR was performed
220 with MyTaq One-Step RT-PCR kit (BIO-65408, BioLine) as per manufacturer's instructions,
221 using 500ng total RNA and a final concentration of 400nM of each primer. The primers used were
222 Chemo2FW and Chemo2RV (table 1b) which had been designed to amplify the immune-related
223 transcript Chemokine CC1. The cycling profile was as follows: 45°C for 20 min, 95°C for 1 min,
224 40 cycles of 95°C for 10s, 60°C for 10s, 72°C for 30s, 72°C for 2 min and 10°C for 30 s. One
225 point five µl of each PCR product was visualized on 1% agarose gel containing EtBr (0.05µg/ml)
226 under UV light. The PCR products were purified using the QIAquick PCR Purification Kit (28104,
227 QIAgen), as per manufacturer's instructions with minor modifications. Purified PCR products
228 were then sent to GATC Biotech ([www.eurofinsgenomics.eu/en/custom-dna-sequencing/gatc-](http://www.eurofinsgenomics.eu/en/custom-dna-sequencing/gatc-services)
229 [services](http://www.eurofinsgenomics.eu/en/custom-dna-sequencing/gatc-services)) for sequencing using their LightRun service. For the *in situ* hybridization step a second
230 set of primers were produced to generate sense and anti-sense RNA probes by adding the T7
231 promotor sequence to the nucleotide sequences (Table 1b). The cycling protocol used was the
232 same as that reported above. Digoxigenin (DIG)-labelling was performed using the DIG-RNA
233 Labeling Kit (11 175 041 910, Sigma-Aldrich) following manufacturer's instructions. DIG-
234 labelled probes were aliquoted (1 µl) and stored at -70°C until required. To determine the yield of
235 the DIG-labelled riboprobes a dot-blot analysis was carried out according to Sigma Aldrich
236 protocol (DIG Application Manual for Nonradioactive In Situ Hybridisation, p59-64).

237 **2.8.2 Fluorescent mRNA In-situ Hybridization (FISH)**

238 Gill sections were dewaxed with xylene, rehydrated through a graded ethanol series, and then
239 incubated in 2× Saline Sodium Citrate (SSC) (BP1325-1, ThermoFisher Scientific) for 1 min. Ten
240 µg/ml Proteinase K (P2308, Sigma-Aldrich) was pipetted onto the tissue and digestion conducted,
241 in a humidified box, at 37°C for 5 min. The reaction was stopped by immersing the slides in ice-

242 cold, 4% paraformaldehyde (in PBS) for 5 min, followed by two washes in PBS for 2 min each at
243 RT. The slides were then dried as much as possible before a GeneFrame (AB-0578, Thermo Fisher
244 Scientific) was placed over the sections to localise the reagents to the tissue. Slides were incubated
245 with a Pre-hybridization solution (50% formamide, 20% 20× SSC and 30% nuclease-free ddH₂O)
246 at 37°C for 10 min in a humidified box. The riboprobes (300-800ng/ml final concentration) were
247 resuspended in the following hybridisation buffer: 50% (deionised) formamide (F9037, Sigma-
248 Aldrich), 5× SSC, 10% dextran sulphate (D8906, Sigma-Aldrich), 5× Denhardt's solution (D2532,
249 Sigma-Aldrich), 250 µg/ml yeast tRNA (15401-011, Invitrogen), 500 µg/µl herring sperm DNA
250 (D1811, Promega) and 1% blocking solution (11585762001, Sigma-Aldrich). The hybridisation
251 mix + riboprobes were heated at 80°C for 5 min and then cooled on ice before approximately 150
252 µl was pipetted onto the appropriate slide and covered with a GeneFrame coverslip. Hybridisation
253 was performed overnight at 60°C in a humidified box. The following day, the coverslips and
254 Geneframes were removed by rinsing in 2× SSC, slides were transferred into separate, individual
255 50 ml centrifuge tubes and washed twice in 2× SSC (30 min each) at RT on a rocking platform
256 (Stuart Scientific). A high-stringency wash step was performed at 65°C for 30 min in 50% 2× SSC
257 plus 50% deionised formamide, without agitation. This was followed by two washes in 2× SSC at
258 37°C for 10 min each on the rocking platform.

259 After post-hybridisation washes, transcripts were identified using the DIG nucleic acid detection
260 kit (11175041910, Sigma-Aldrich) and DIG Wash and Block Buffer set (11585762001, Sigma-
261 Aldrich), prepared as per the kit protocol. Sections were transferred to 1× Wash Buffer at RT for
262 5 min on rocking platform, followed by an incubation in 1× Blocking solution buffer at RT for 30
263 min with agitation. Sections were incubated for 2 h at RT with Anti-Digoxigenin-AP conjugate
264 antibody (11175041910, Sigma-Aldrich) diluted 1:5,000 in 1× Blocking solution and then washed
265 twice with 1× Wash buffer for 15 min at RT on rocking platform. Sections were dried as much as
266 possible and equilibrated in 1× Detection buffer for 5 min at RT. Bound antibody was localized
267 using Fast Red tablets dissolved in TRIS buffer (F4648, Sigma-Aldrich) as per manufacturer's
268 instructions. 150 µl of Fast Red solution was added to each slide and incubated in the dark at RT
269 in the humidified box (without agitation). Slides were monitored under a light microscope to
270 prevent over-development and high background. As soon as a signal was detected or after a
271 maximum of 30 min, the reaction was stopped by gently washing the slides with nuclease-free
272 ddH₂O. Finally, the slides were dried and mounted with a coverslip using Vectamount AQ
273 Aqueous Mounting Medium (H-5501, Vector Laboratories). Coverslip edges were sealed with
274 clear nail varnish and the sections were incubated overnight to dry. Light and fluorescence
275 microscopy images were captured using ArcturusXT™ Laser Capture Microdissection System
276 (Nikon) microscope with an attached digital camera. Negative control slides, incubated with

277 hybridization mix only, were included (i.e. no riboprobes); a positive control slide consisted of a
278 salmon louse (*Lepeophtheirus salmonis*) intestine section labelled with a trypsin antisense
279 riboprobe.

280 **2.9 Statistical analysis**

281 Results were analysed using the SPSS16 (SPSS Inc., Chicago, IL, USA) statistical software. Data
282 was tested for homogeneity of variance before ANOVA evaluation. Data distribution was
283 determined using descriptive statistics. Data were given as mean \pm standard deviation (SD).
284 Statistical significances obtained from qRT-PCR analysis were subjected to one-way analysis of
285 variance (one-way ANOVA) and compared using Duncan's multiple range test. Differences of
286 means among the groups were considered statistically significant when $p < 0.05$. Principal
287 component analysis (PCA) and heat map was conducted using CLC Genomics Workbench v.12
288 (Qiagen Bioinformatics) on normalized RNA-seq data transcripts per million (TPM) of *D. labrax*
289 gills and head kidney in response to infestation with *A. ocellatum*.

290 **3. Results**

291 **3.1 Assembly and sequence description**

292 Reads from control (n=3) and infested (n=5) tissues (gills and head kidney) were analyzed via
293 Illumina sequencing in a paired-end 2x 150-nt run. This generated, on average, 23,321,464 raw
294 reads per sample with a GC content of 52% (supplementary table 1). On average, 92.82% of the
295 raw reads (21,646,982 reads) mapped to the European seabass (*D. labrax*) reference genome
296 (supplementary table 2). The raw FastQ file has been deposited in the National Center for
297 Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession
298 number PRJNA588185.

299 **3.2 PCA and heat map**

300 PCA of the expression of all differentially expressed (DE) transcripts showed a clear clustering by
301 tissue type and infestation group. This was as expected due to the specific expression pattern per
302 tissue. Infestation with the parasite induced a shift in infested gills and the head kidney
303 (supplementary Fig.1 & 2). Cluster analysis not only separated the different tissues from one
304 another but also the gills from control-uninfested animals, suggesting that the most significant
305 effects were taking place in the target tissue (supplementary Fig. 3). Cluster analysis also clearly
306 separated the head kidney data from the control and infested groups (supplementary Fig 4).

307 **3.3 Differentially expressed genes after *Amyloodinium ocellatum* infestation**

308 Comparison of gene expression levels between AO infested and control groups revealed 1039 and
309 376 differentially expressed genes (DEG) in gills and head kidney respectively ($p < 0.01$). These
310 included 679 upregulated genes and 360 downregulated genes in gills and 206 upregulated genes
311 and 170 downregulated genes in head kidney (Fig. 1). The differentially expressed genes from

312 gills and head kidney were mainly annotated into biological process and molecular function. In
313 gills, the highest number of DEG for the biological process (Fig 2a) were annotated to proteolysis
314 (38), Oxidation-reduction process (30), immune response (18), proton transmembrane transport
315 (11) and chemotaxis (9). The highest molecular processes (Fig 2b) in gills were annotated to
316 protein binding (131), zinc ion binding (65), DNA binding transcription factor activity (25),
317 calcium ion binding (25) and DNA binding (19). In head kidney (Fig 2c) the highest number of
318 DEG for the biological processes were annotated to proteolysis (28), regulation of transcription
319 DNA template (11), immune response (8), oxidation-reduction process (8), transmembrane
320 transport (6) and protein phosphorylation (6). The highest molecular processes (Fig 2d) in head
321 kidney were annotated to protein binding (39), calcium ion binding (19), ATP binding (18), zinc
322 ion binding (13) and DNA binding (9).

323 In gills, among the immune response genes, the highest upregulation fold change (table 2) was
324 recorded for c-c motif chemokine 21-like (581-fold), cc chemokine 1 (46-fold) and interleukin-12
325 subunit alpha-like (39 fold). On the contrary the major downregulated genes were tumor necrosis
326 factor ligand superfamily member 12-like (-14-fold), somatomedin-b and thrombospondin type-1
327 (-1.70 fold), tumor necrosis factor ligand superfamily member 13b (-1.50 fold). Similarly, the top
328 3 upregulated and downregulated genes for proteolysis and protein binding are mentioned in (table
329 2). In head kidney among the immune response genes, the highest upregulated fold change (table
330 3) was recorded for cc chemokine 1 (22-fold), interleukin 10 precursor (22-fold) and c-x-c motif
331 chemokine 10 precursor (13-fold). On the contrary the main downregulated genes were
332 somatomedin-b and thrombospondin type-1 (-6-fold), tumor necrosis factor ligand superfamily
333 member 10-like (-4-fold) and tumor necrosis factor alpha (-2.5-fold). Similarly, the top 3
334 upregulated and downregulated genes in head kidney for proteolysis and protein binding are
335 mentioned in table 5.

336 Based on the DEG with highest fold change, the lists of the top 20 genes that were up-or down
337 regulated in the gills (supplementary table 3) and head kidney (supplementary table 4) were
338 observed in non-infested gills and head kidney, respectively. In gills, among the top 20, three
339 highly up-regulated immune genes were perforin 1 like (1806.91-fold), gtpase (800.18-fold), and
340 receptor transporting protein 3 (675.40-fold). Similarly, the top 3 downregulated genes were
341 protein-glutamine gamma-glutamyl transferase 5 isoform 1 (-2151.91-fold), Caspase 1 (-169.66-
342 fold), and gastrin cholecystokinin-like peptide-like (-161.77-fold). In head kidney (table 7), three
343 of the most highly up-regulated immune genes were interferon inducible Mx protein (4869.73-
344 fold), tubulin alpha chain (872.52-fold) and receptor transporting protein 3 (687.97-fold).
345 Similarly, the top 3 downregulated immune genes were arylamine n- pineal gland isozyme nat-10-

346 like (-6427.56-fold), dual specificity tyrosine-phosphorylation-regulated kinase 1a-like (-787.65-
347 fold) and carboxypeptidase b (-272.84-fold).

348 **3.4 Validation of RNA-seq by qPCR and *in situ* hybridization**

349 Throughout the time course infestation trial, the total mortality was 18%. In the control group, fish
350 were not infested, and no mortality was registered. Expression changes of innate immune genes in
351 gills and head kidney were analyzed at 2, 3, 5, 7 and 23 dpi. In gills (Fig 3), among the pro-
352 inflammatory molecules, CC1 (2-fold) and IL-8 (2-fold) started to peak at 5 dpi and were
353 significantly higher at 7 dpi while COX-2 was unaffected during the course of infestation.
354 Hepcidin (4.2-fold) and CLA (2-fold) were significantly higher at 2 and 3 dpi and declined at 5
355 and 7 dpi, while CASP9 was not affected post-infestation. Immunoglobulins IgM and IgT were
356 unaffected in gills during the course of infestation until 7dpi (Fig 5 a & b).

357 In head kidney (Fig 4), CC1 (2-fold) was significantly higher at 2 dpi and 3 dpi but decreased
358 thereafter. However, IL-8 and COX-2 were not affected by the infestation. Hepcidin (40-fold)
359 peaked at 7 dpi while CASP9 (2-fold) peaked at 5 dpi. CLA was not affected by infestation. IgM
360 (6.2-fold) peaked at 3 dpi and declined thereafter but IgT did not show significant changes in
361 expression until 7dpi (Fig 5 c & d).

362 During the recovery phase (23 dpi) the expression of CC1 (18-fold), hepcidin (20-fold) and IgM
363 (9-fold) in the gills (Fig 6a), was significantly increased compared to other genes while in head
364 kidney (Fig 6b), IgM (10-fold) and IgT (6 fold) were higher compared to control.

365 Through *in situ* hybridization it was possible to observe that in the uninfested control fish the gene
366 signal CC1 was evident in the gill associated lymphoid tissue (GIALT) (Fig. 7 a,b), in the lumen
367 of the central venous sinus and in the capillaries of the apical portion of the primary lamellae (data
368 not shown). In the infested fish, a higher abundance of CC1 positive leukocytes was observed in
369 the hyperplastic regions of the secondary lamellae (Fig. 8 a-d) and in the vessel wall (diapedesis)
370 of the central venous sinus of primary lamellae (Fig. 8 a-d). In general, the signal intensity was
371 lower in uninfested control fish, whereas in the infested subjects the signal was visibly higher. In
372 the gills of infested fish, no positive signal was detected near the trophont adhesion sites (Fig. 12
373 c & d). In the negative controls (no riboprobes) and in the samples hybridised with Chemokine
374 CC1 sense probe, no positive signal was detected in the examined gill tissues (Fig. 7 c-f; Fig. 9 a-
375 d). Sea louse (*L. salmonis*) intestine labelled with Trypsin antisense probe (Fig. 9e & f) represented
376 a reference positive control.

377

378 **4. Discussion**

379 The purpose of the present investigation is to provide general insights on how host and parasite
380 interact. In our previous study dealing with ESB response after natural AO infection (Byadgi et

381 al., 2019) we observed that none out of four genes codifying for molecules related to adaptive
382 immunity (mhc i, mhc ii and igm) show upregulation in gills and head kidney. This phenomenon
383 could be due to the nature (characteristics) of the host species and to the infection dynamics
384 (intended as mode of response in relation to the days post infection). Consequently, to the host
385 interaction with the parasite there was a pronounced and sustained inflammation (il-8, chemokine
386 cc1, cox-2) that brought many novel molecules (Hepcidin) to the site of parasite adhesion.
387 Therefore, from the present study and from the previous study as well it can be confirmed that
388 local innate immunity plays a major role during AO infection in ESB. During experimental
389 infection, RNA-seq was analysed at one time point during initial stage of infection and most of the
390 DEG recorded are therefore related to innate immunity which could be consequent to AO infection.
391 Based on the information we obtained from these two studies we can speculate that innate immune
392 responses with CC1, antimicrobial peptides, IL-8, COX-2 dominate the response in ESB after AO
393 infection, although we cannot exclude the involvement of specific antibodies and lymphocytes at
394 the systemic level.

395 We conducted the present study under “controlled infestation conditions” in order to understand
396 the time course expression of upregulated immune genes, the physiological status of the host, and
397 to determine their potential as functional markers in the ESB infested by AO. This is the first time
398 that this interaction has been studied for ESB and the present study was performed to extend our
399 understanding on this matter. In our previous study, we used Real-time quantitative PCR to
400 investigate immune gene expression and demonstrated their importance when fish responded to
401 AO exposure (Byadgi et al., 2019). However, the genes previously investigated are likely to
402 account for only a small proportion of the immune processes occurring in such tissues. The RNA-
403 seq analysis presented here aims to fill the gaps in our understanding of these pivotal immune
404 processes post-AO infestation in ESB.

405 We found more differentially expressed genes in gills (1039) than in head kidney (376) after AO
406 infestation. This is not that surprising since the gills are the main target tissue of AO infestation
407 and the parasite attachment in ESB is mostly restricted to this organ. Additionally, the distinctive
408 clinical sign of the disease, i.e. anaemia, is attributable to the alteration of the gill physiology
409 induced by the parasite. Furthermore, kidney analysis revealed 170 down-regulated genes, an
410 interesting result that can be attributed to the common lympho-haematopoietic functions and to
411 the cellular depletion as similarly observed in the spleen and kidney of turbot (*S. maximus*) infested
412 by *Enteromyxum scophthalmi* (Bermúdez et al., 2010; Haase et al., 2014).

413 Based on the highest fold change observed, our findings from RNA-seq in gills suggest that
414 perforin might play significant roles in the immune system and in the ESB immune defense against
415 AO. Meantime, the differential expression dynamics seem to imply possible different cellular

416 locations or functional differences. Perforin was first characterized as a lytic pore-forming protein
417 isolated from cytotoxic T lymphocytes (CTLs) (Podack et al., 1985). In humans and mice perforin
418 is a single-copy gene and its immunological function has been well studied. However, unlike
419 humans and mice, there is more than one perforin gene in fish genomes (Toda et al., 2011; Varela
420 et al., 2016), although only one isoform has been reported, in teleosts, to date (Hwang et al., 2004;
421 Athanasopoulou et al., 2009; Jung et al., 2014; Taylor et al., 2016).

422 In head kidney several genes related to the interferon-mediated immune response and the
423 promyelocytic leukemia protein (PML) gene were upregulated. The PML gene positively regulates
424 the type I interferon response by promoting transcription of IFN-stimulated genes (ISGs) (Kim et
425 al., 2014). Also, during AO infestation in head kidney, we observed genes related to IFN
426 signalling, with an increased expression of IFN-gamma showed the highest number of DE genes.
427 Overall, these results point towards a response mediated by both type II IFNs, as observed in early
428 stages of several protozoan infestations occurring in mammals (Beiting et al., 2014). In teleosts
429 parasitized by amoebae and myxozoan parasite, the IFN-mediated immune response was shown
430 to play a major role, with implications for fish resistance or susceptibility to the disease (Young et
431 al., 2008; Davey et al., 2011; Bjork et al., 2014). It was observed that in turbot facing advanced
432 stages of enteromyxosis, IFN-related genes were markedly downregulated, suggesting a
433 differential immune response during the different phases of infestation to enteromyxosis in turbot
434 (Robledo et al., 2014). Having observed the upregulation of several interferon genes during AO
435 infestation, we could speculate that IFN expression might depend on the stage of infestation or on
436 the localization of the parasite during the infestation.

437 In the RNA-seq data, we found four chemokines (*cc21*, *cc1*, *cc10*, and *cc19*) significantly
438 upregulated in infested gills and head kidney compared with control. These results suggest that
439 more immune cells were recruited in the infested sites. A previous study demonstrated that IL-8
440 produced in human intestinal mucosa during infestation was capable of recruiting blood monocytes
441 and maintaining the macrophage population in the mucosa (Smythies et al., 2006). In rainbow
442 trout, IL-8 had the analogous capability of attracting monocyte-macrophage cells during
443 infestation (Montero et al., 2008). IL8 and CXCR1 correspond to chemokine (CK) and chemokine
444 receptors (CR) in mammals, while other CKs and CRs have no ligand-receptor correlation. In this
445 study, the fold-change of the up-regulation of *cc21* was extremely high (581-fold). Also, CCL19
446 in this study was 11-fold up regulated in the head kidney. CCL19 and CCL21 are homeostatic
447 chemokines, which play an important role in T and B cell trafficking and migration to peripheral
448 lymphoid tissues in mammals (Choi et al., 2003), whereas in turbot CCL19 was reported to attract
449 head kidney leukocytes and augment host immune defense (Chen et al., 2013). CXCL9, CXCL10
450 and CXCL11 are known in mammals as a group of interferon inducible chemo attractants

451 recruiting activated CD4 β Th1 cells, CD8 β T cells and NK cells (Liu et al., 2007; Cheng et al.,
452 2011). Therefore, the immune response induced by all upregulated chemokines probably leads to
453 the enhanced resistance against AO pathogen in ESB. Hence, further studies should be directed
454 towards understanding the biological activity and functionality of ESB chemokines during
455 amyloodiniosis.

456 In gills and head kidney among the proteolysis category, the most common upregulated gene was
457 matrix metalloproteinase, after AO infestation. Matrix metalloproteinases (MMPs) have important
458 functions in extracellular matrix (ECM) degradation and tissue repair (Page-McCaw et al., 2007).
459 Mmp-9 has been found to contribute to leukocyte migration, thus participating in mammalian
460 inflammation and immunity (Van den Steen et al., 2007; Greenlee et al., 2007). Mmp-9 in
461 zebrafish (*Danio rerio*) is expressed notably in the head-kidney and in peritoneal and peripheral
462 blood leukocytes upon infestation, indicating its role in immune responses (Chadzinska et al.,
463 2008). In *Mycobacterium marinum* infested zebrafish, Mmp-9 was found to enhance recruitment
464 of macrophages and to contribute to granuloma formation and bacterial growth (Volkman et al.,
465 2010). Therefore, our findings suggest that Mmp is a protective molecule against AO in ESB via
466 a proteolytic role.

467 In gills and head kidney of ESB after AO infestation, the RNA-seq data revealed that the TNF- α
468 and TNF superfamily genes were downregulated. In our previous study, we similarly observed
469 that there was a lack of *il-1 β* and *tnf- α* expression in gills (Byadgi et al., 2019). Toxins or enzymes
470 released by the parasites might have damaged the leucopoietic system resulting in reduction in the
471 expression of most of the immune-related genes, including *tnf- α* (Kar et al., 2016). However,
472 functionally assessed to have pro-inflammatory activity in fish, *il-1 β* and *tnf- α* are often co-
473 expressed with other macrophage-derived inflammatory mediators such as *il-8*, *cox-2*, and *inos* in
474 parasitic and bacterial infestations (Harun et al., 2011; Bruijn et al., 2012; Oladiran et al., 2011;
475 Alvarez-Pellitero et al., 2008; Covello et al., 2009). Some cytokine genes were mainly down
476 regulated including TNF- α , an important proinflammatory cytokine in fish (Sigh et al., 2004).
477 Although this cytokine may play a role in the initiation of the early immune response and has
478 multiple effects on gene expression during inflammation (Dinarello et al., 2009), IL-1 β expression
479 was likely suppressed in rainbow trout in late stages of the infestation. Depression of pro-
480 inflammatory cytokine production in rainbow trout macrophages infested with *Renibacterium*
481 *salmoninarum* was previously demonstrated (Grayson et al., 2002). In addition, TNF- α , considered
482 as an important component in the inflammatory response in fish (Secombes et al., 2001) and
483 activated in rainbow trout after i.p. injection of live theronts of *I. multifiliis* (Jørgensen et al., 2008),
484 was not triggered or up regulated by infestation. In *Gymnocypris przewalskii*, *I. multifiliis*
485 infestation enhanced TNF synthesis because of the up regulation of TLR genes (Tian et al., 2017).

486 Therefore, in AO infested ESB the lack of evidence for TNF- α and IL-1 β expression at the time
487 point under study could be due to the transient fluctuation of expression of these genes throughout
488 the infestation.

489 In ESB after AO infestation, we observed the downregulation of complement *c3* and *c9* genes,
490 which may be associated with susceptibility to AO and mortality in ESB. The complement system
491 (Wood P., 2011) is an essential part of the innate immunity (Holland et al., 2002) in alerting the
492 host to the presence of potential pathogens (Boshra et al., 2006) and plays a crucial role in the
493 response or resistance against Ich (Buchmann et al., 1999; Buchmann et al., 2001; Heidarieh et al.,
494 2015). In rainbow trout, complement factor C9 played a role in the skin and gills during parasite-
495 host interaction (Sigh et al., 2004; Jørgensen et al., 2008; Olsen et al., 2011) and against the
496 bacterial pathogen *Yersinia ruckeri* (Chettri et al., 2012). Although other studies indicated high
497 expression of *c3* in the liver, head kidney, skin, gill and spleen of infested individuals, our results
498 indicated its down-regulation (Heidarieh et al., 2015; Sigh et al., 2004; Jørgensen et al., 2008;
499 Olsen et al., 2011). Altogether, it is evident that the complement system plays a much more
500 important role in infested gills sites where it comes into direct contact with AO. It is still not known
501 whether and how complement components affect the AO infestation outcome in ESB but it can be
502 hypothesized that AO also evolved with a strategy to evade or counteract the complement system
503 of ESB.

504 Therefore, from the present study and from the previous study as well (Byadgi et al., 2019), it can
505 be confirmed that local innate immunity plays a major role during AO infestation in ESB. During
506 experimental infestation, RNA-seq was analysed at onetime point during initial stage of infestation
507 and most of the DEG recorded are therefore related to innate immunity which could be consequent
508 to AO infestation. Based on the information we have from the two studies we can speculate that
509 innate immune responses with CC1, antimicrobial peptides, IL-8 dominate the response in ESB
510 after AO infestation at the early phase, although we cannot exclude the involvement of specific
511 antibodies and lymphocytes which requires further studies. The fact that the “post infestation
512 course” in AO episodes (either natural or experimentally induced) is usually rather rapid, therefore
513 we can reasonably speculate that the genes of innate immunity are more expressed than those of
514 specific immunity.

515 **RNA-seq data validation**

516 In our previous study, we observed that *cc1* expression was upregulated in AO infested gills,
517 indicating that a pro-inflammatory stimulus was activated by the host response *versus* the parasite
518 (Byadgi et al., 2019). In our present investigation, gills and head kidney showed a high fold change
519 of *cc1* post-AO infestation. Interestingly, chemokine expression increases when there is tissue
520 damage and most chemokines are recognized as pro-inflammatory factors; they have been shown

521 to exert regulatory functions in a wide range of pathological and physiological contexts, such as
522 hypersensitivity reactions, infestation, angiogenesis, inflammation, tumor growth and
523 haematopoietic tissues development (Suresh et al., 2006; Nibbs et al., 2013; Stone et al., 2017).
524 Given their critical role in inflammation, many chemokines and chemokine receptors have been
525 identified as potential therapeutic targets in a wide range of inflammatory diseases (Proudfoot et
526 al., 2002). Chemokines (CKs) known as chemotactic cytokines recruit immune cells into the sites
527 of injury or infestation in acute inflammation. They act via binding to specific G protein-coupled
528 receptors on target cells, which orchestrate immune cell migration and positioning at the
529 organismic level in the host. The expression of *cc1* and *il-8* in infested gill tissues on day 5
530 indicates that the genes were expressed during the inflammation process. In rainbow trout
531 (*Oncorhynchus mykiss*), CC chemokines were up regulated in liver after challenge with
532 haemorrhagic septicaemia virus (VHSV) and infectious pancreatic necrosis virus (IPNV)
533 (Montero et al., 2008). Up-regulation of CC chemokines was detected in spleen (15-fold) and liver
534 (29-fold) of miiuy croaker (*Miichthys miiuy*) infected by *Vibrio anguillarum* (Cheng et al., 2011).
535 Significant up-regulation of *cc1* in all tissues of *Lates calcarifer* infested by *C. irritans* was also
536 reported (Mohd-Shaharuddin et al., 2013). Although the regulation of *cc1* genes in affected host
537 organs has previously been reported (Byadgi et al., 2019), the present study demonstrated that gills
538 (harbouring a relevant parasite load) responded significantly stronger compared to head kidney.
539 Also, the time dependent high up-regulation of *cc1* and *il-8* in ESB post AO infestation may
540 indicate that *cc1* and *il-8* have a prominent role in the response against AO.

541 Another interesting result observed after AO infestation in ESB, was the time related change in
542 Heparin expression in both gills and head kidney. In gills, it was significantly increased at 2 dpi
543 but in head kidney, it was highest at 7dpi. Heparin transcription was described as increased in
544 anaemic fish due to *Photobacterium damsela* infection (Rodrigues et al., 2006). Elevation of
545 heparin (42-fold) in the liver of rainbow trout (*Oncorhynchus mykiss*) infected by *Y. ruckeri* has
546 also been reported (Raida et al., 2009). Up-regulated expression of the heparin gene at days 3 and
547 10 post *C. irritans* infestation was found in *Lates calcarifer* liver, kidney, gill and spleen tissues
548 (Mohd-Shaharuddin et al., 2013). Time-dependent changes in heparin expression were recorded
549 in *Labeo rohita* at 3 dpi with *Argulus siamensis*. These were significantly high in liver and kidney
550 tissues but negligible in skin (Kar et al., 2015). In healthy organisms, iron concentration is
551 maintained at a stable level in plasma, and this element is stored in hepatocytes and splenic/hepatic
552 macrophages at constant levels, despite unstable absorption of iron from the diet (Hermenean et
553 al., 2017). It has been reported that heparin-mediated low serum iron level functions as a host
554 defense mechanism that evolved to restrict iron availability for pathogen growth and development

555 (Drakesmith et al., 2012; Ganz et al., 2015). This validates the results obtained from our previous
556 study on natural infestation of ESB with AO and suggests that hepcidin could possibly be
557 considered a relevant immune marker in ESB, providing a protective effect in the fish when highly
558 expressed.

559 In our previous study, the lower level or no expression of *igm* in infected samples indicated that
560 the parasite toxins might negatively influence the systemic specific immune response (like
561 production of *igm*) (Covello et al., 2009) or alternatively that the disease reached its onset very
562 rapidly and the individuals did not have enough time to activate a specific humoral response.
563 Therefore, in the present study we evaluated time course *Igm* and *Igt* expression and similar results
564 were observed until 7dpi but at 23 dpi indicated that in gills and head kidney there was an
565 upregulation of *igm* and *igt*. There are few reports on *igm/igt* expression in gills and skin after
566 parasite infestation (Zhang et al., 2010; Olsen et al., 2011; Xu et al., 2013). Further research should
567 be directed to evaluate the immune response in skin to complete the evidence that mucosal immune
568 response plays an important role during AO infestation in ESB.

569 In this study, a fluorescent mRNA *in situ* hybridization (FISH) protocol was developed to
570 investigate the host-parasite interactions by determining the presence or absence of Chemokine
571 CC1 mRNA sequences in affected tissues. The fluorescent signal was localized within specific
572 cells allowing a semi-quantitative estimation of the level of occurrence/expression. Based on our
573 knowledge, there is no documented information about previous FISH based approaches in ESB
574 gills infested by *A. ocellatum*. In uninfested fish, a faint hybridization signal was detected in the
575 GIALT, in the lumen of the central venous sinus and in the capillaries of the apical portion of the
576 primary lamellae. On the other hand, Chemokine CC1 signal was more evident in infested gill
577 tissue, with positive cells detected in the hyperplastic areas of the secondary lamellae and in the
578 vessel wall of the central venous sinus of the primary lamellae. Interestingly, a positive signal was
579 also detected in the cytoplasm of some trophonts, potentially suggesting the “phagotrophic” nature
580 of the dinoflagellate as previously speculated (Lom et al., 2002) and also observed in our previous
581 immunohistochemical survey (Byadgi et al., 2019). On the other hand, no positive signal was
582 detected in the tissue areas close to anchored parasites. A possible explanation for the absence of
583 positive cells in those areas may be due to evasive mechanisms adopted by the protozoan to avoid
584 detection and defense by the host immune system, as documented for other fish parasites
585 (Buchmann et al., 2002; Sitjà-Bobadilla 2008; Kumar et al., 2013). The results reported here also
586 support the observed differential expression of Chemokine CC1 transcript between uninfested and
587 infested European sea bass and provide a better understanding of the pattern of localisation of

588 leukocyte populations in ESB gills. These results suggest that *cc1* plays an important role against
589 AO attachment and pathogenicity.

590 **5. Conclusions**

591 In this study, the immune mechanisms in ESB gills and head kidney after infestation by AO,
592 indicated partial overview of the mucosal response. Gills showed a higher number of DEG
593 compared to head kidney, indicating the importance of the mucosal immune response at the site
594 of AO attachment. Several immune genes were altered after AO infestation, such as chemokine
595 *cc1*; multiple genes of the interferon-mediated immune response were upregulated. This points to
596 a recruitment of immune cells towards the site of AO attachment in ESB gills. Moreover, the
597 downregulation of tumor necrosis factors and complement factors in ESB indicated the potential
598 temporary nature of TNF- α expression and of the invasion mechanisms triggered by AO to
599 counteract the ESB. The upregulation of chemokines was also validated by qPCR and *in situ*
600 hybridization, specific for chemokine CC1, evidencing that chemokines play an important role in
601 the local immune response during AO infestation in ESB. Therefore, the molecular modifications
602 at the base of host and pathogen interaction identified here provide a basis to better understand
603 processes that may influence ESB immune performance during AO infestation.

604 **Conflict of Interests**

605 The authors declare that there is no conflict of interest.

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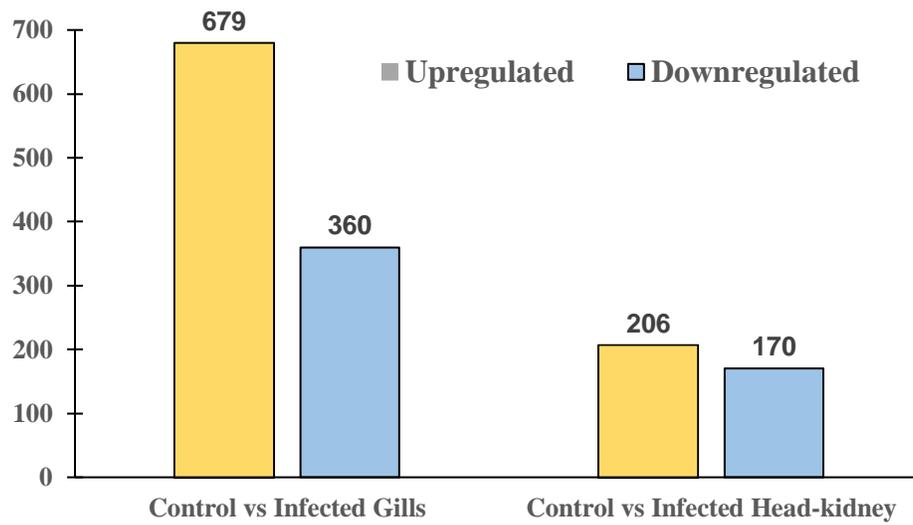


Figure 1 - Upregulation and down regulation of differentially expressed genes in gills and head kidney after challenge with *A. ocellatum* in *D. labrax*.

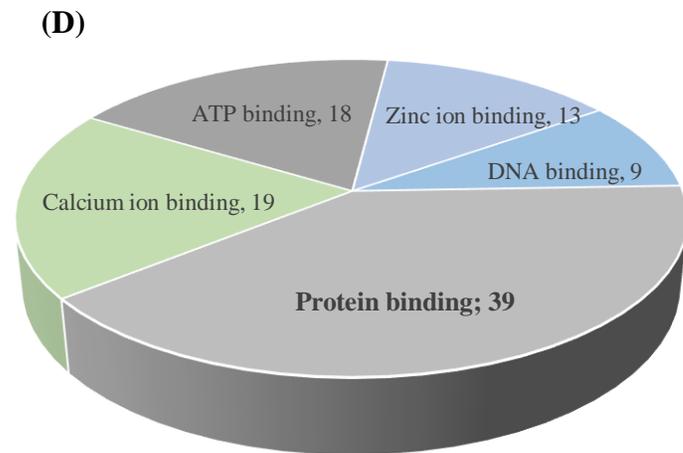
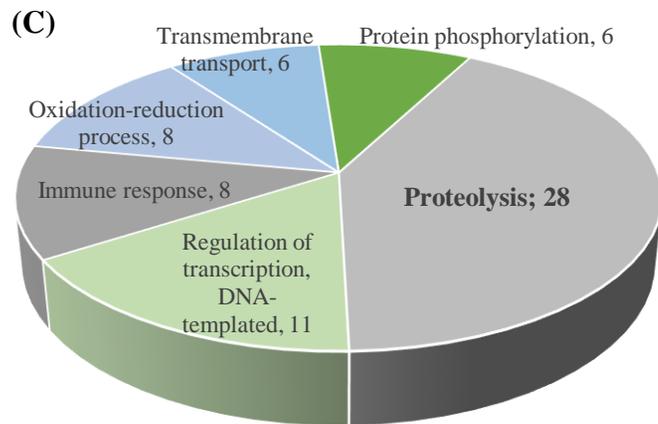
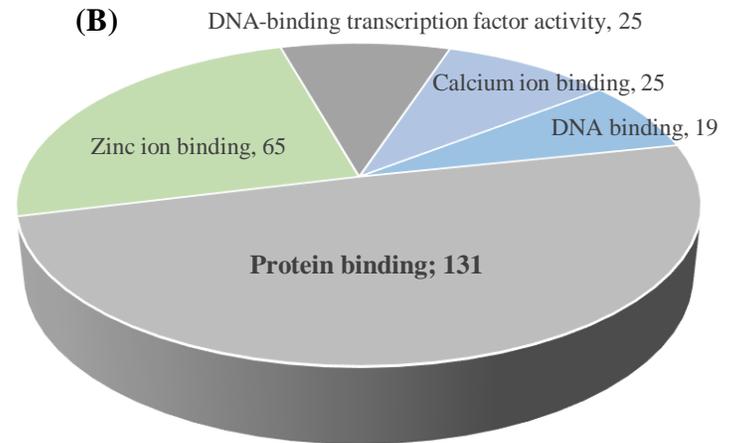
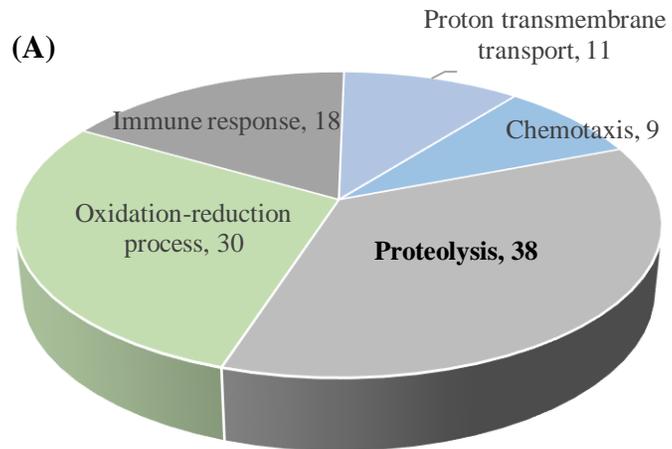


Figure 2. Gene ontology (GO) enrichment analysis of differentially expressed genes in gills (a & b) and head kidney (c & d). The results of GO enrichment analysis of differentially expressed genes were classified into two categories: biological process (a and c), and molecular function (b & d). The functional classification of GO with the corresponding number of genes.

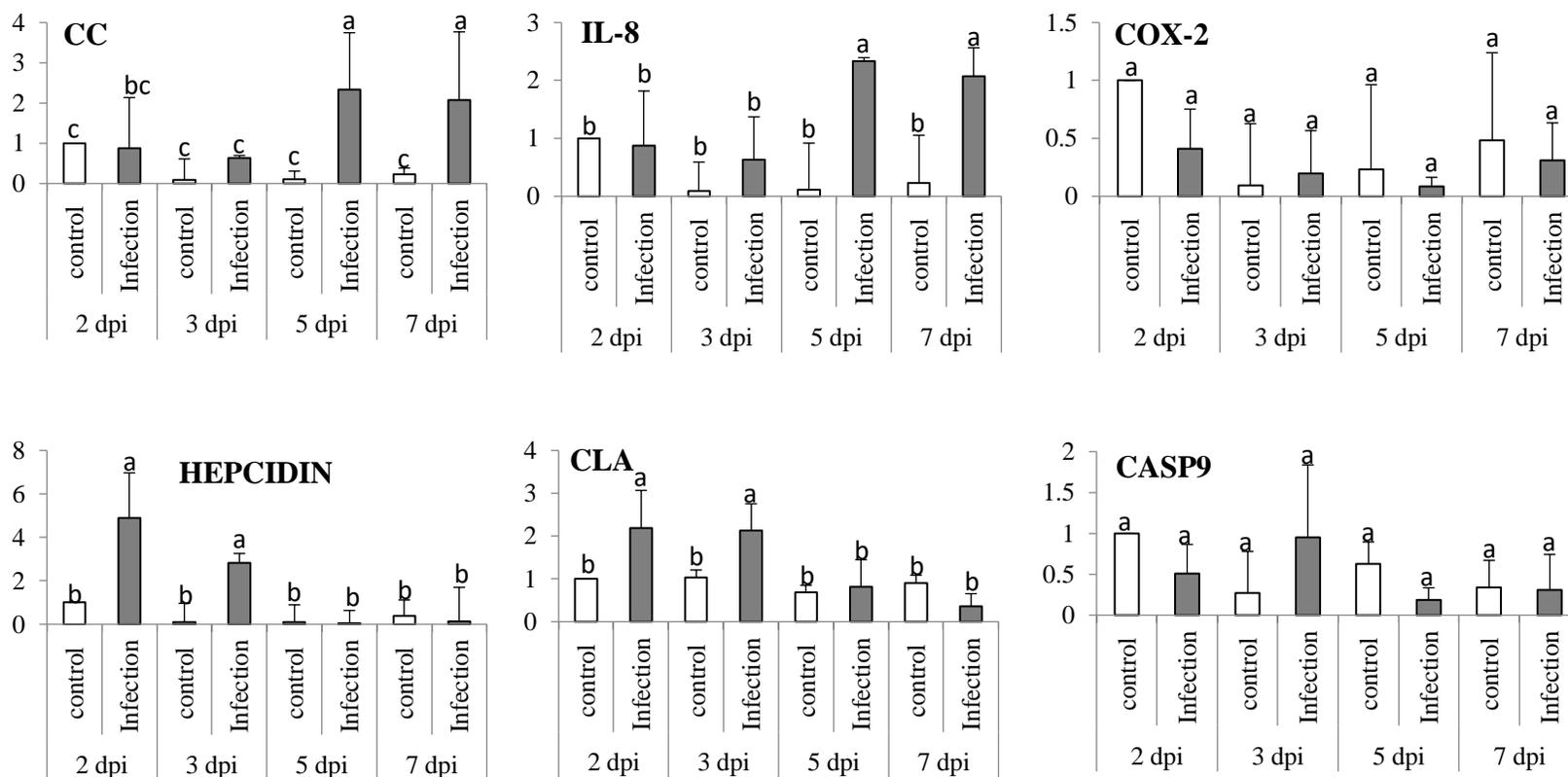


Figure 3- Relative mRNA expression of innate, immune-related genes chemokine CC1, interleukin-8, cyclooxygenase-2, hepcidin, c-type lectin A and Caspase-9 at 2, 3, 5, 7 days post infection **in gills of ESB** infested with AO as measured by quantitative real-time PCR. Data are presented as mean \pm SD and multiple reference genes were used to normalize with the target gene (n=3). Different alphabetical letters indicate the significant difference with $p < 0.05$.

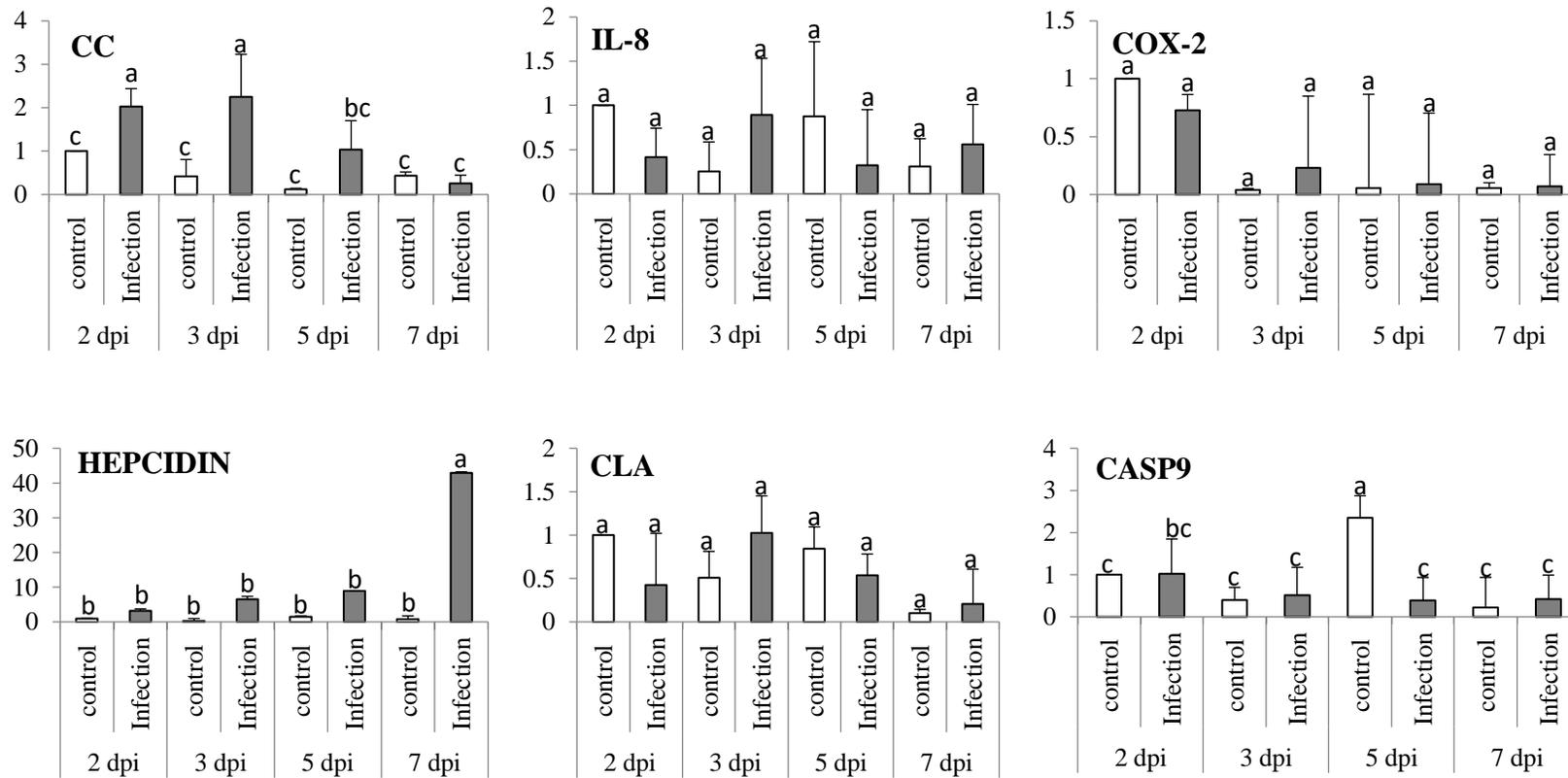


Figure 4- Relative mRNA expression of innate, immune related genes chemokine CC1, interleukin-8, cyclooxygenase-2, hepcidin, c-type lectin A and Caspase-9 at 2, 3, 5, 7 days post infection **in head kidney** of ESB infested with AO as measured by quantitative real-time PCR. Data are presented as mean \pm SD and multiple reference genes were used to normalize with the target gene (n=3). Different alphabetical letters indicate the significant difference with $p < 0.05$.

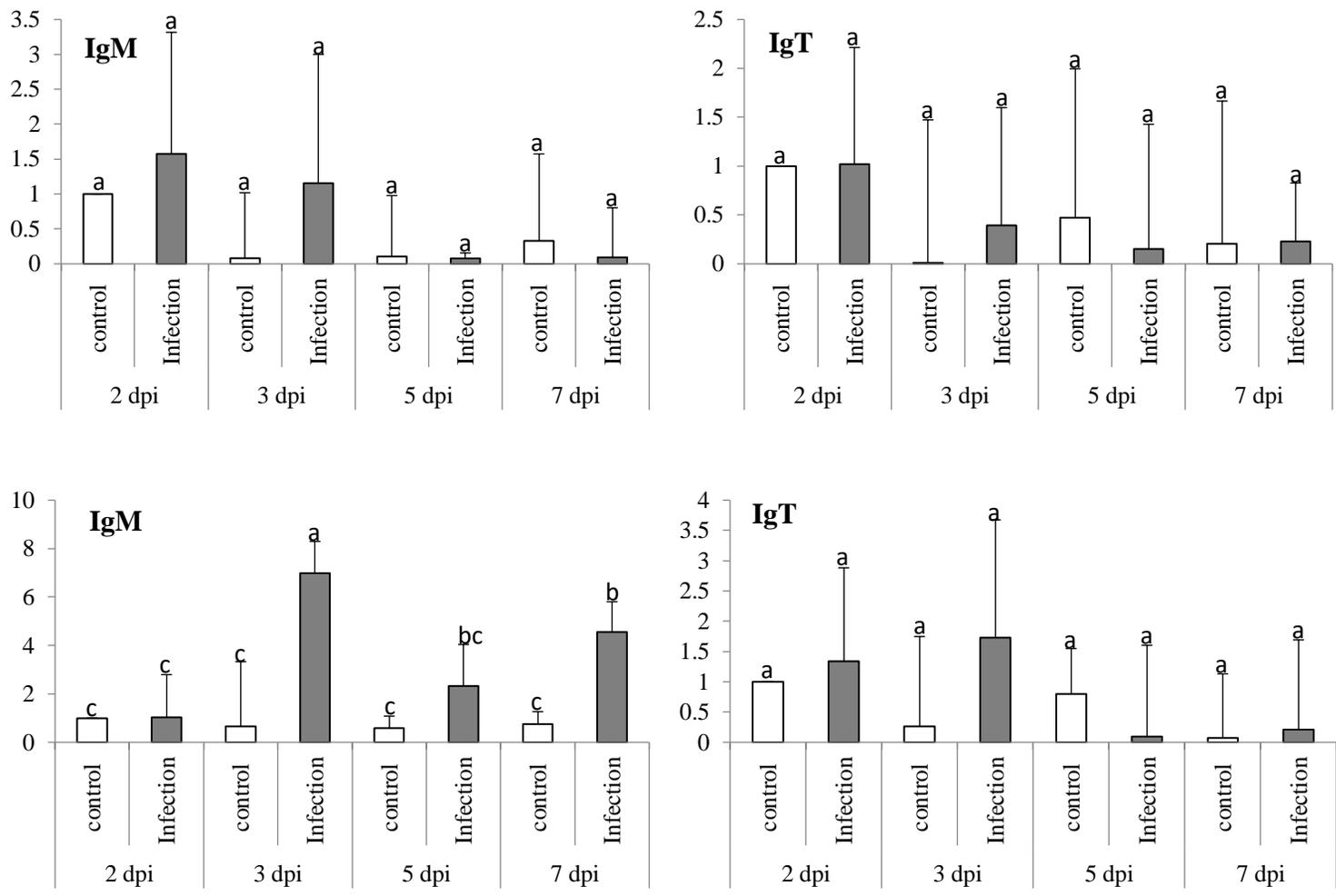


Figure 5- Relative mRNA expression of immunoglobulin M and immunoglobulin T at 2, 3, 5, 7 days post infested **in gills (A and B) head kidney (C and D) of ESB** infected with AO as measured by quantitative real-time PCR. Data are presented as mean \pm SD and multiple reference genes were used to normalize with the target gene (n=3). Different alphabetical letters indicate the significant difference with $p < 0.05$.

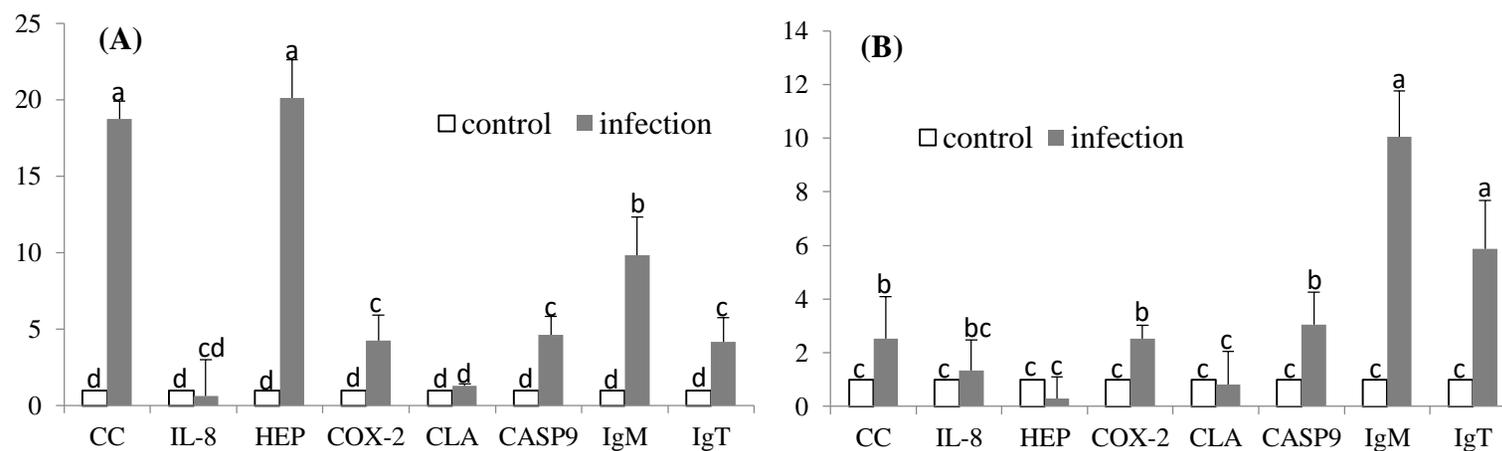


Figure 6- Relative mRNA expression of immune genes at 23 days post infection **in gills (A)** and **head kidney (B)** of ESB infested with AO as measured by quantitative real-time PCR. Data are presented as mean \pm SD and multiple reference genes were used to normalize with the target gene (n=3). Different alphabetical letters indicate the significant difference with $p < 0.05$.

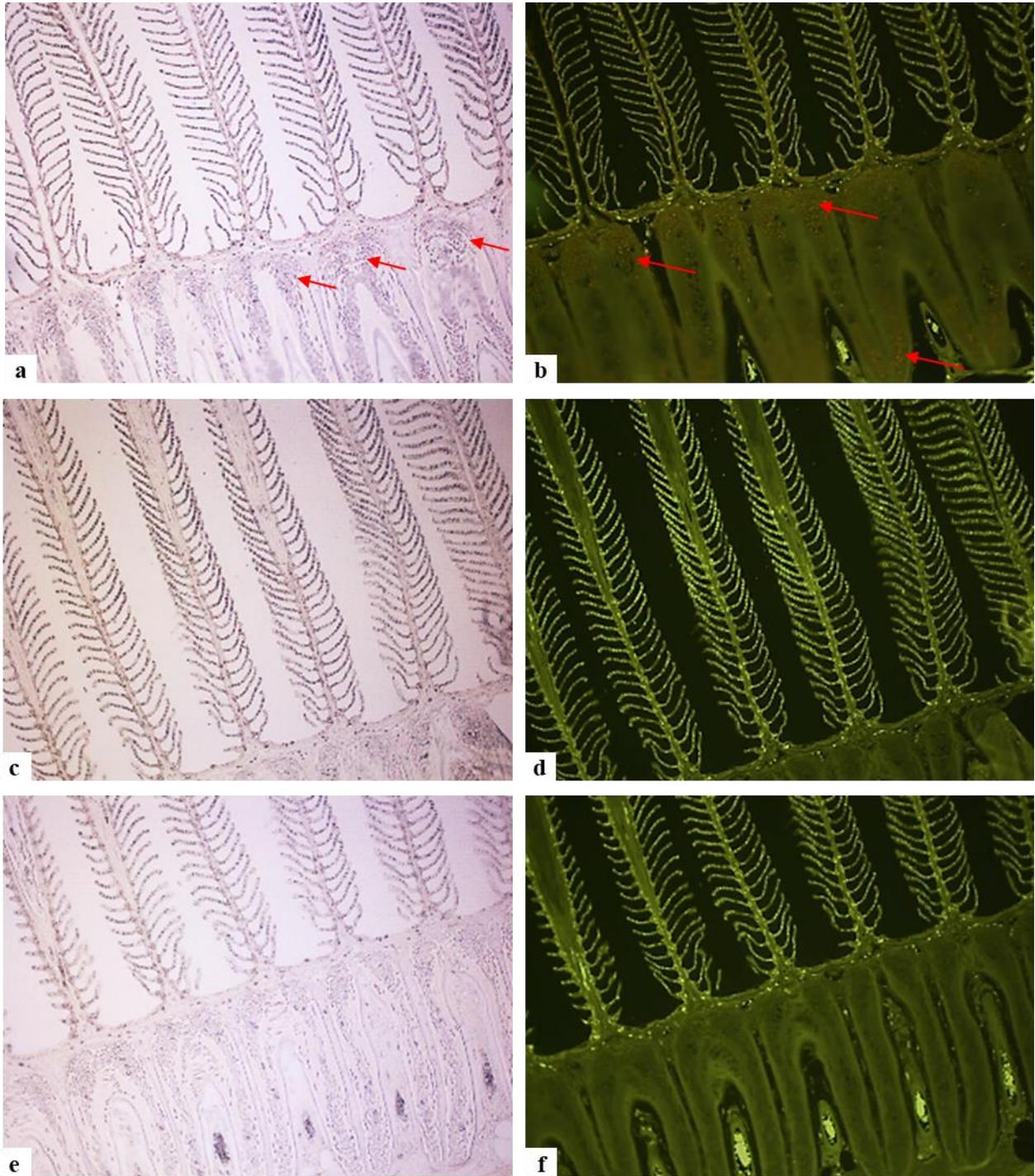


Figure 7. ESB gill tissue from uninfested control fish. a) and b) Leukocytes expressing Chemokine CC1 in the GIALT (Gill Associated Lymphoid Tissue) (arrows). c) and d) sections incubated with Chemokine CC1 sense probe. e) and f) Negative control (no riboprobes). Images captured in brightfield are on the left, images captured using fluorescence microscopy are on the right. All at 10X magnification.

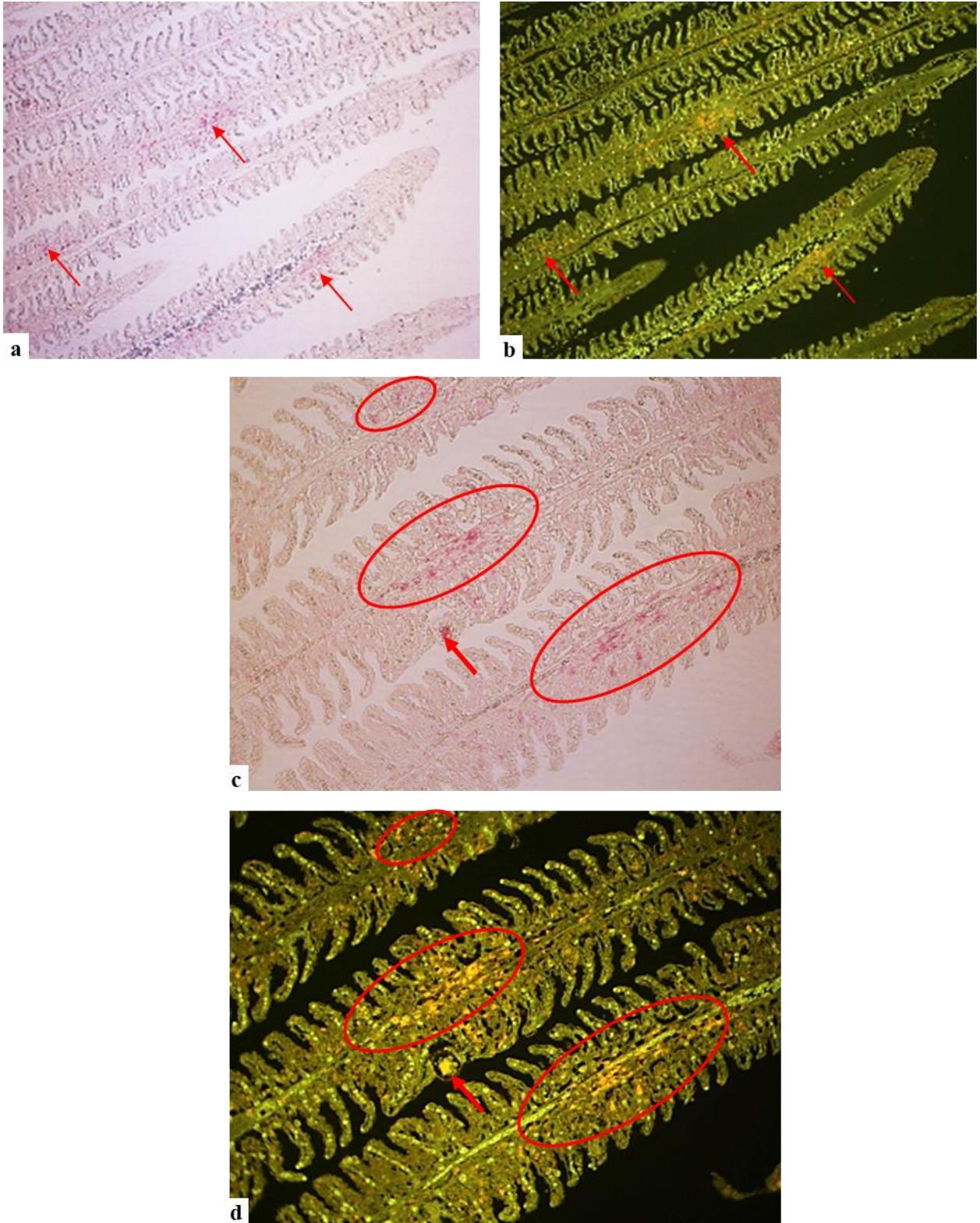


Figure 8. ESB gill tissue infested by *A. ocellatum*. a) to d) CC1 positive leukocytes in the hyperplastic regions of the secondary lamellae and in the vessel wall (diapedesis) of the central

venous sinus of the primary lamellae. c) and d) A positive signal for CC1 in the hyperplastic areas of secondary lamellae and within cytoplasm of *A. ocellatum* trophonts (arrows). a) and b) 10X magnification; c) and d) 20X magnification. Images 8a and 8c were captured in brightfield, figures 8b and 8d using fluorescence.

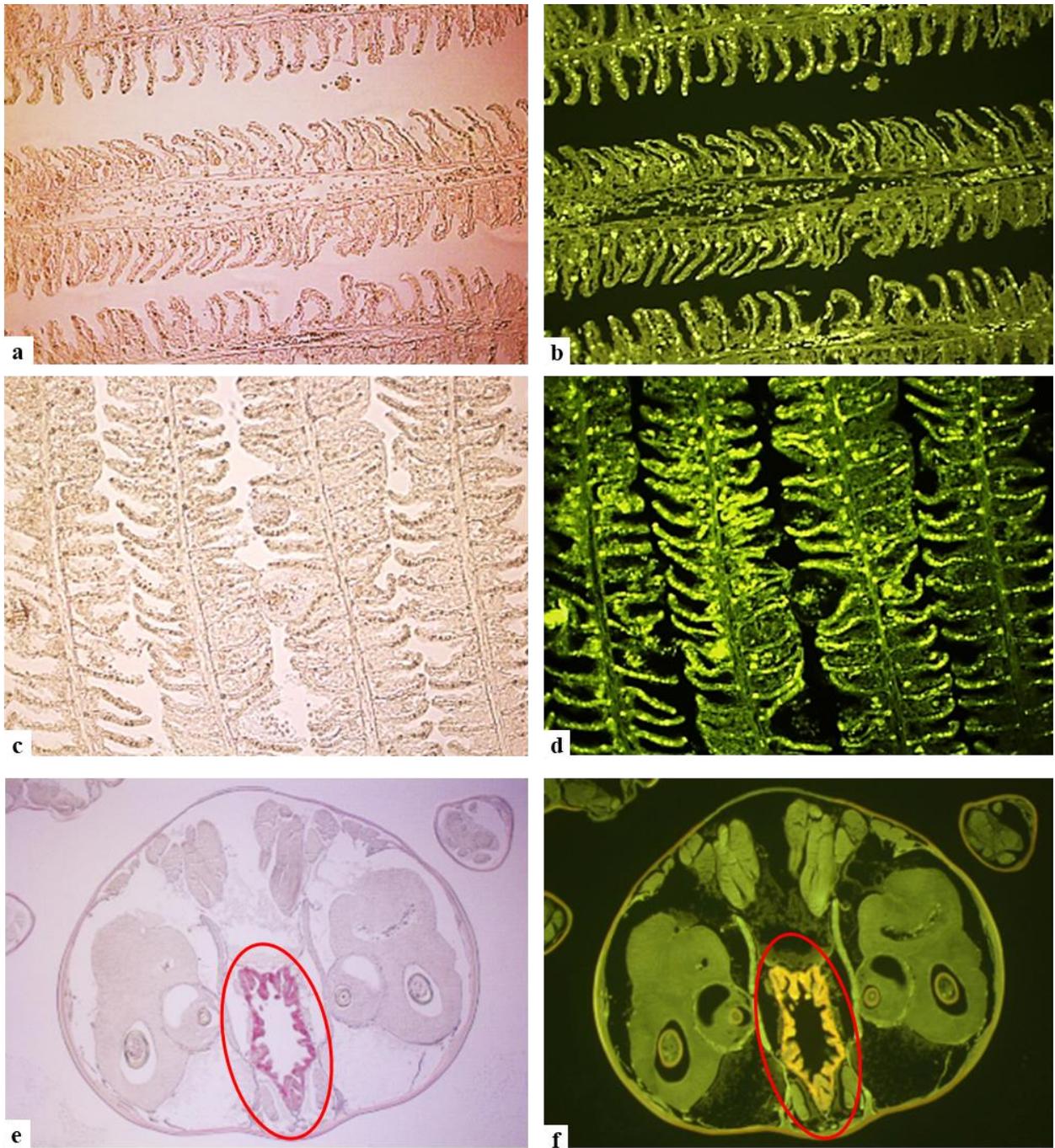


Figure 9. ESB gill tissue infested by *A. ocellatum*. a) and b) Chemokine CC1 sense probe, 10X magnification. c) and d) Negative control (no riboprobes), 20X magnification. e) and f) Reference positive control represented by sea louse (*L. salmonis*) intestine labelled with Trypsin antisense probe, 10X magnification. Images on the left captured with brightfield and on the right using fluorescence.

Table 1a. Functional group, 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 | Name | Forward Sequence | Name | Reverse Sequence | GenBank Accession number |
|----|--------------------------|----------------|---------------------------|----------------|---------------------------|------------------------------|
| 1 | <i>Innate Immunity</i> | <i>cc1-F</i> | tgggttcgccgaaggttgtt | <i>cc1-R</i> | agacagtagacgaggggaccacaga | AM490065.1 |
| 2 | | <i>il8-F</i> | gtctgagaagcctgggagtg | <i>il8-R</i> | gcaatgggagttagcaggaa | AM490063.1 |
| 3 | | <i>hep-F</i> | aagagctggaggagccaatgagca | <i>hep-R</i> | gactgctgtgacgcttgtgtctgt | DQ131605.1 |
| 4 | | <i>cox2-F</i> | agcacttcaccaccagttc | <i>cox2-R</i> | aagcttgccatccttgaaga | Cordero <i>et al.</i> , 2016 |
| 5 | <i>Adaptive immunity</i> | <i>Ighm-F</i> | aggacaggactgctgctgtt | <i>ighm-R</i> | acaacagcagacagcaggtg | AM493677 |
| 6 | | <i>Ight-F</i> | cggacttcattcagttaccctg | <i>Ight-R</i> | caactgtacacatcagggcc | KM410929.1 |
| 7 | <i>Complement system</i> | <i>cla-F</i> | gatggcagcaagctccggtattca | <i>cla-R</i> | tctgacctatgaccccagccaaca | EU660935.1 |
| 8 | | <i>casp9-F</i> | ggcaggactcgacgagatag | <i>casp9-R</i> | ctcgtctgaggagcaact | DQ345776.1 |
| 9 | <i>Reference</i> | <i>actb-F</i> | tgaaccccaagccaacagggaga | <i>actb-R</i> | gtacgaccagaggcatacagggaca | AJ537421.1 |
| 10 | | <i>l13a-F</i> | tctggaggactgtcaggggatgc | <i>l13a-R</i> | agacgcacaatcttgagagcag | Mitter <i>et al.</i> , 2009 |
| 11 | | <i>hsp90-F</i> | gctgacaagaacgacaaggctgtga | <i>hsp90-R</i> | agatgcggttgagtggtctgt | AY395632.1 |

Table 1b. Primers plus T7 region used for designing the riboprobes for mRNA FISH.

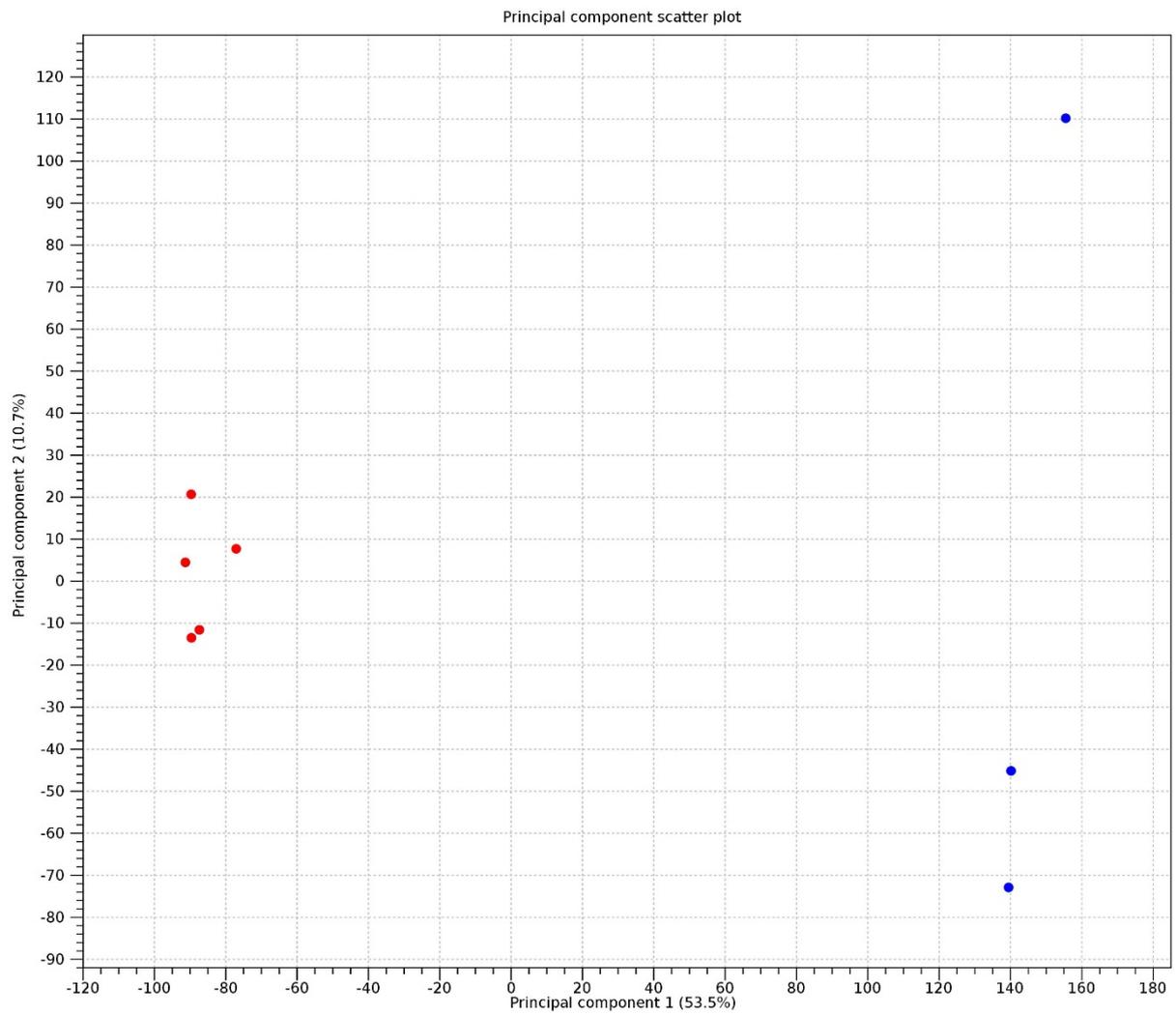
| Probes | Oligo name | Sequence (5' → 3') |
|-------------------------------|--------------|---|
| Chemokine CC1 sense probe | Chemo2_T7_FW | <u>taatac</u> gactcactataggggtctctggagaggaacggaga |
| | Chemo2RV | ggtgttttcattggccggag |
| Chemokine CC1 antisense probe | Chemo2FW | tctctggagaggaacggaga |
| | Chemo2_T7_RV | <u>taatac</u> gactcactataggggggttttcattggccggag |

Table 2. Differentially expressed genes (DEGs) regulated after AO infestation in gills of ESB.

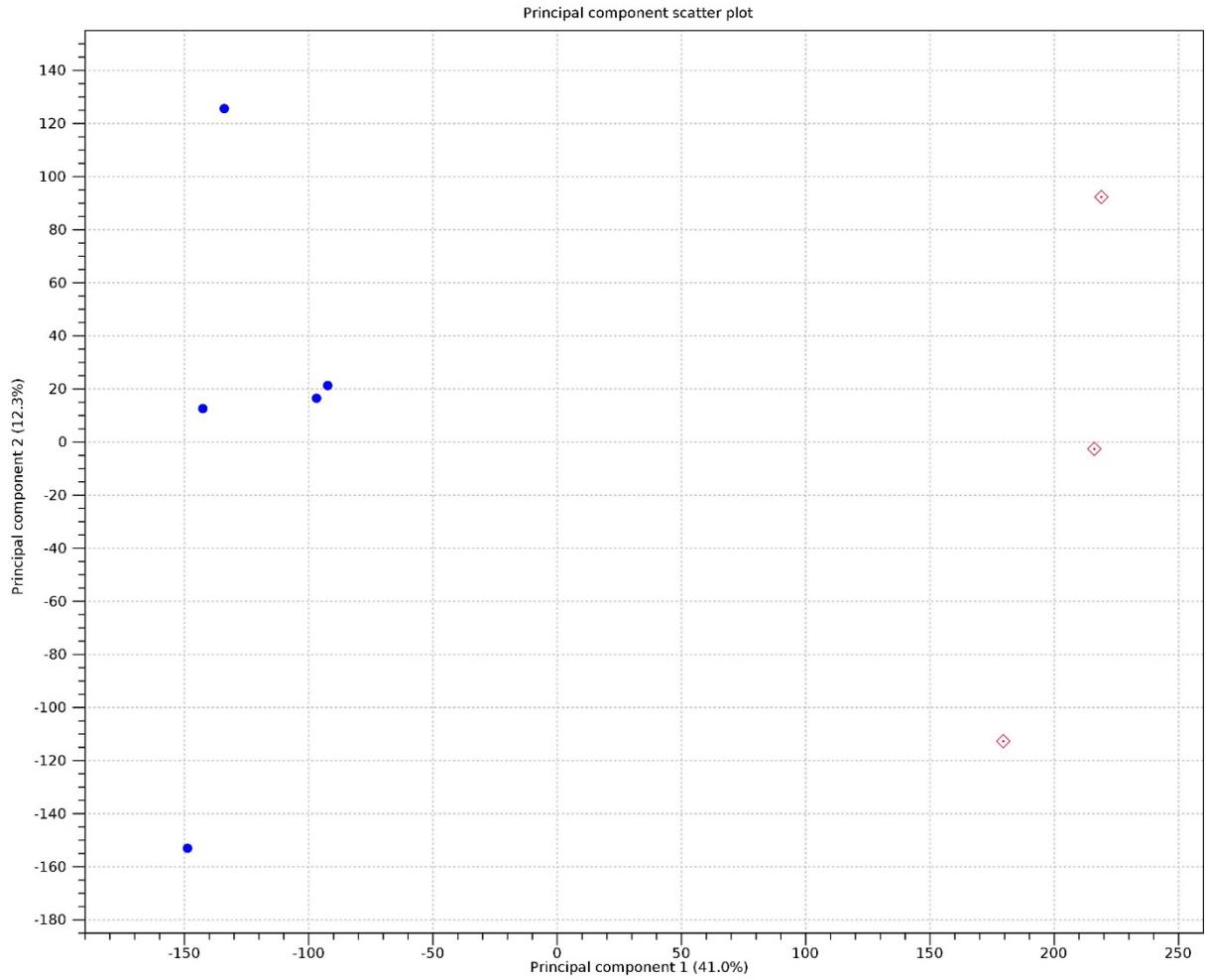
| Annotation | Gene abbreviation | Gene name | Fold change | Up / downregulated |
|------------------------|--------------------------|---|--------------------|---------------------------|
| Immune response | <i>ccl21</i> | c-c motif chemokine 21-like | 581,78 | ↑ |
| | <i>cc1</i> | cc chemokine 1 | 46,32 | ↑ |
| | <i>il12a</i> | interleukin-12 subunit alpha-like | 39,66 | ↑ |
| | <i>il10</i> | interleukin 10 | 5,52 | ↑ |
| | <i>gcsf</i> | granulocyte colony-stimulating factor-like | 5,18 | ↑ |
| | <i>tnfsf12</i> | tumor necrosis factor ligand superfamily member 12-like | -14,44 | ↓ |
| | <i>sbspon</i> | somatomedin-b and thrombospondin type-1 | -1,70 | ↓ |
| | <i>tnfs13b</i> | tumor necrosis factor ligand superfamily member 13b | -1,50 | ↓ |
| | <i>tnf-α</i> | tumor necrosis factor alpha | -1,40 | ↓ |
| | <i>tnfs10</i> | tumor necrosis factor ligand superfamily member 10-like | -1,31 | ↓ |
| Proteolysis | <i>mmp</i> | matrix metalloproteinase | 113,40 | ↑ |
| | <i>spe</i> | elastase-like serine protease | 41,32 | ↑ |
| | <i>pcsk6</i> | proprotein convertase subtilisin kexin type 6 | 39,86 | ↑ |
| | <i>casp3</i> | caspase 3b | 38,12 | ↑ |
| | <i>cela1</i> | pancreatic elastase | 35,86 | ↑ |
| | <i>mts16</i> | metalloproteinase with thrombospondin motifs 16 | -341,9 | ↓ |
| | <i>f11</i> | coagulation factor xi-like | -102,69 | ↓ |
| | <i>cpa6</i> | carboxypeptidase a6 | -25,94 | ↓ |
| | <i>klk8</i> | kallikrein-8 precursor | -13,78 | ↓ |
| | <i>capn15</i> | calpain-15 | -13,69 | ↓ |
| Protein binding | <i>ankrd1</i> | ankyrin repeat domain-containing protein 1 | 616,50 | ↑ |
| | <i>ctnnd2</i> | catenin delta-2 | 139,42 | ↑ |
| | <i>ifi17</i> | interferon-induced 17 kda protein precursor | 101,52 | ↑ |
| | <i>il28b</i> | interleukin-27 subunit beta-like | 93,41 | ↑ |
| | <i>nlrc3</i> | protein nlrc3-like | 88,88 | ↑ |
| | <i>ankdd1b</i> | ankyrin repeat and death domain-containing protein 1b | -8,20 | ↓ |
| | <i>ptprj</i> | receptor-type tyrosine-protein phosphatase eta-like | -7,79 | ↓ |
| | <i>muc5ac</i> | mucin-5ac- partial | -6,75 | ↓ |
| | <i>f11</i> | coagulation factor xi-like | -6,68 | ↓ |
| | <i>c3</i> | complement component c3 | -5,26 | ↓ |

Table 3. Differentially expressed genes (DEGs) regulated after AO infestation in head kidney of ESB.

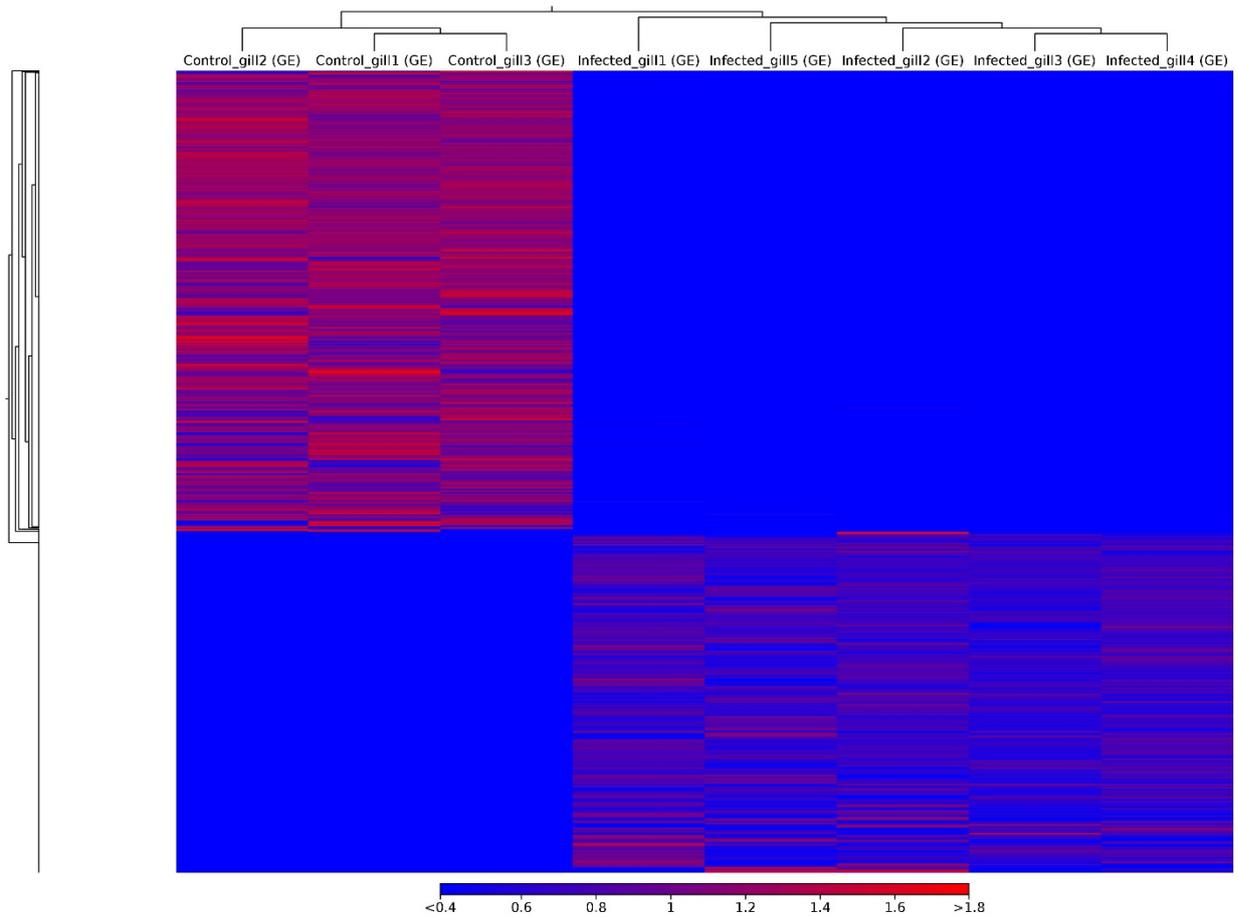
| Annotation | Gene abbreviation | Gene name | Fold change | Up / downregulated |
|------------------------|--------------------------|---|--------------------|---------------------------|
| Immune response | <i>cc1</i> | cc chemokine 1 | 22,97 | ↑ |
| | <i>il10</i> | interleukin 10 precursor | 22,40 | ↑ |
| | <i>cxcl0</i> | c-x-c motif chemokine 10 precursor | 13,66 | ↑ |
| | <i>cxcl9</i> | c-c motif chemokine 19 precursor | 11,45 | ↑ |
| | <i>cc21</i> | c-c motif chemokine 21-like | 7,30 | ↑ |
| | <i>sbspon</i> | somatomedin-b and thrombospondin type-1 | -6,19 | ↓ |
| | <i>tnfsf10</i> | tumor necrosis factor ligand superfamily member 10-like | -4,08 | ↓ |
| | <i>tnf-α</i> | tumor necrosis factor alpha | -2,50 | ↓ |
| | <i>c9</i> | complement component c9 | -1,47 | ↓ |
| | <i>eda</i> | ectodysplasin splice variant-8 exons | -1,17 | ↓ |
| Proteolysis | <i>ddn1</i> | duodenase-1 precursor | 50,99 | ↑ |
| | <i>bmp1</i> | bone morphogenetic protein 1 | 20,57 | ↑ |
| | <i>mmp</i> | matrix metalloproteinase | 19,63 | ↑ |
| | <i>mcpt1</i> | mast cell protease 1a-like | 18,34 | ↑ |
| | <i>gzma</i> | granzyme a-like | 14,22 | ↑ |
| | <i>cpb</i> | carboxypeptidase b | -272,84 | ↓ |
| | <i>ctrb</i> | chymotrypsin b-like | -252,15 | ↓ |
| | <i>prss1</i> | trypsin-1 precursor | -221,94 | ↓ |
| | <i>ctr2</i> | chymotrypsinogen 2 | -185,22 | ↓ |
| <i>trp</i> | trypsinogen 2 | -174,21 | ↓ | |
| Protein binding | <i>trim39</i> | e3 ubiquitin-protein ligase trim39-like | 129,68 | ↑ |
| | <i>nlrc3</i> | protein nlrc3-like | 79,16 | ↑ |
| | <i>ifi17</i> | interferon-induced 17 kda protein precursor | 77,45 | ↑ |
| | <i>setd8</i> | n-lysine methyltransferase setd8-like | 31,95 | ↑ |
| | <i>sdk2</i> | protein sidekick-2-like | -189,87 | ↓ |
| | <i>mybpc1</i> | myosin-binding protein slow-type | -124,58 | ↓ |
| | <i>mxra5</i> | matrix-remodeling-associated protein 5 | -112,65 | ↓ |
| | <i>cntn4</i> | contactin-4-like | -98,96 | ↓ |
| | <i>klhl34</i> | kelch-like protein 34 | -90,95 | ↓ |



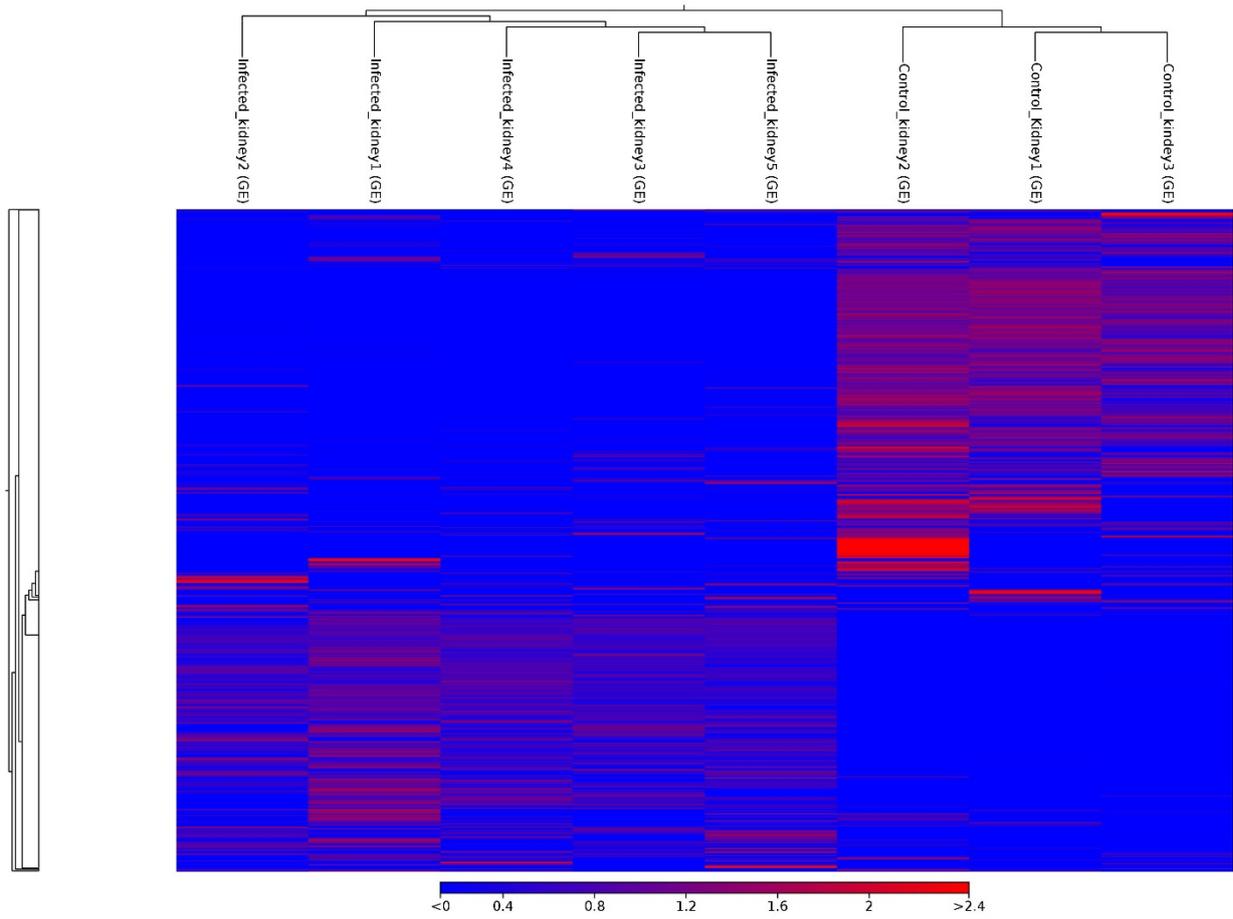
Supplementary Figure 1- Principal component analysis (PCA) of the normalized RNA-seq data transcripts per million (TPM) of *D. labrax* gills in response to infestation with *A. ocellatum*. Red dot represents infested gills and blue dot indicates non-infested gills.



Supplementary Figure 2- Principal component analysis (PCA) of the normalized RNA-seq data transcripts per million (TPM) of *D. labrax* head kidney in response to infestation with *A. ocellatum*. Blue dot represents infested head kidney and red dot indicates non-infested head kidney.



Supplementary Figure 3- A heat map based on gene expression levels of 1039 DEGs in gills of *D. labrax* infested with *A. ocellatum*. Hierarchical clustering heatmaps based on the DESeq-normalized gene expression levels. The genes with similar expression patterns are clustered together. The up-regulated genes are in red and the down-regulated genes are in blue.



Supplementary Figure 4 - A heat map based on gene expression levels of 376 DEGs in head kidney of *D. labrax* infested with *A. ocellatum*. Hierarchical clustering heat maps based on the DESeq-normalized gene expression levels. The genes with similar expression patterns are clustered together. The up-regulated genes are in red and the down-regulated genes are in blue.

Supplementary Table 1. Read count statistics

| Sample name | Read count | GC content % | Paired, mapped pairs % | Paired, broken pairs % | Paired, not mapped % |
|--------------------|-------------------|---------------------|-------------------------------|-------------------------------|-----------------------------|
| Control_gill1 | 23,160,288 | 54 | 65.20 | 17.24 | 17.57 |
| Control_gill2 | 22,780,654 | 52 | 72.37 | 9.01 | 18.62 |
| Control_gill3 | 19,241,532 | 54 | 62.81 | 18.24 | 18.95 |
| Control_Kidney1 | 21,220,732 | 52 | 55.56 | 26.64 | 17.81 |
| Control_Kidney2 | 21,777,880 | 54 | 62.76 | 19.77 | 17.47 |
| Control_Kidney3 | 23,005,570 | 52 | 64.53 | 18.76 | 16.71 |
| Infested_gill1 | 19,071,872 | 50 | 60.16 | 22.68 | 17.16 |
| Infested_gill2 | 28,364,912 | 52 | 61.55 | 20.70 | 17.76 |
| Infested_gill3 | 26,269,084 | 50 | 67.99 | 15.56 | 16.44 |
| Infested_gill4 | 24,975,596 | 52 | 70.73 | 13.86 | 15.42 |
| Infested_gill5 | 27,012,904 | 52 | 58.24 | 23.23 | 18.53 |
| Infested_Kidney1 | 23,440,752 | 50 | 60.88 | 23.14 | 15.99 |
| Infested_Kidney2 | 20,271,424 | 52 | 58.06 | 23.41 | 18.53 |
| Infested_Kidney3 | 25,841,066 | 52 | 62.37 | 22.07 | 15.55 |
| Infested_Kidney4 | 20,184,678 | 52 | 60.59 | 23.45 | 15.96 |
| Infested_Kidney5 | 26,524,480 | 52 | 62.86 | 21.88 | 15.25 |

Supplementary Table 2. Fragment count statistics

| Sample name | Mapped to genes % | Mapped to intergenic % |
|--------------------|--------------------------|-------------------------------|
| Control gill1 | 90.31 | 9.69 |
| Control gill2 | 87.66 | 12.34 |
| Control gill3 | 90.36 | 9.64 |
| Control Kidney1 | 93.92 | 6.08 |
| Control Kidney2 | 93.77 | 6.23 |
| Control Kindey3 | 93.83 | 6.17 |
| Infested gill1 | 91.65 | 8.35 |
| Infested gill2 | 92.36 | 7.64 |
| Infested gill3 | 92.14 | 7.86 |
| Infested gill4 | 92.11 | 7.89 |
| Infested gill5 | 92.02 | 7.98 |
| Infested Kidney1 | 94.63 | 5.37 |
| Infested Kidney2 | 94.85 | 5.15 |
| Infested Kidney3 | 94.88 | 5.12 |
| Infested Kidney4 | 95.37 | 4.63 |
| Infested Kidney5 | 95.27 | 4.73 |

Supplementary Table 3 - List of top 20 Upregulated and Downregulated genes in gills of *D. labrax* infested with *A. ocellatum* representing gene symbol, protein product, gene ontology class and fold change.

| UPREGULATED | | | | |
|---------------|------------------|---|--|-------------|
| Ranking | Gene symbol | Protein product | GO Class (Direct) | Fold change |
| 1 | <i>prf1</i> | perforin-1-like | immune system | 1,806.91 |
| 2 | <i>gimap4</i> | gtpase imap family member 4-like | cytosol | 800.18 |
| 3 | <i>rtf3</i> | receptor-transporting protein 3 | protein binding | 675.40 |
| 4 | <i>ankrd1</i> | ankyrin repeat domain-containing protein 1 | ankyrin repeat binding | 616.50 |
| 5 | <i>gig2</i> | gig2-like protein | cytoplasm | 541.34 |
| 6 | <i>ifit5</i> | interferon-induced protein with tetratricopeptide repeats 1-like | protein binding | 439.90 |
| 7 | <i>aloxe3</i> | epidermis-type lipoxygenase 3-like | epidermis development | 437.59 |
| 8 | <i>samd9</i> | sterile alpha motif domain-containing protein 9 | protein binding | 436.05 |
| 9 | <i>gad11</i> | glutamate decarboxylase-like protein 1 | protein binding | 252.53 |
| 10 | <i>gcnt1</i> | beta- -galactosyl-o-glycosyl-glycoprotein beta- -n-acetylglucosaminyltransferase-like | oxidation-reduction process | 251.50 |
| 11 | <i>asrg11</i> | isoaspartyl peptidase 1-asparaginase | beta-aspartyl-peptidase activity | 241.82 |
| 12 | <i>noxo1</i> | nadph oxidase organizer 1-like | NADPH oxidase complex | 172.73 |
| 13 | <i>tuba1a</i> | tubulin alpha-1a chain | structural constituent of cytoskeleton | 160.62 |
| 14 | <i>hsp70</i> | heat shock protein 70 | regulation of protein ubiquitination | 134.98 |
| 15 | <i>cc1</i> | cc chemokine 1 | chemokine activity | 117.50 |
| 16 | <i>nf-x-like</i> | melanophilin-like isoform x1 | integral component of membrane | 115.70 |
| 17 | <i>cxcr1</i> | c-x-c chemokine receptor type 1-like | chemokine binding | 115.07 |
| 18 | <i>arg1</i> | arginase-1 | cytoplasm | 109.71 |
| 19 | <i>viperin</i> | Virus inhibitory protein | suppression by virus of host transcription | 109.08 |
| 20 | <i>cnfn</i> | cornifelin homolog b-like | cytoplasm | 79.15 |
| DOWNREGULATED | | | | |
| Ranking | Gene symbol | Protein product | GO Class (Direct) | Fold change |
| 1 | <i>tgm1</i> | Protein-glutamine gamma-glutamyltransferase 5 isoform 1 | eukaryotic initiation factor 4E binding | -2,151.91 |
| 2 | <i>casp1</i> | caspase 1 isoform 2 | protein binding | -169.66 |
| 3 | <i>gast</i> | gastrin cholecystokinin-like peptide-like | signal transduction | -161.77 |
| 4 | <i>nefh</i> | neurofilament heavy polypeptide | neurofilament cytoskeleton organization | -108.02 |
| 5 | <i>muc5ac</i> | mucin-5ac- partial | cytoplasm | -107.85 |
| 6 | <i>f11</i> | coagulation factor xi-like | regulation of blood coagulation | -89.65 |

| | | | | |
|----|----------------|--|--|--------|
| 7 | <i>fut7</i> | alpha-()-fucosyltransferase-like | integral component of membrane | -80.29 |
| 8 | <i>mfap4</i> | microfibril-associated glycoprotein 4-like | protein binding | -73.63 |
| 9 | <i>krt13</i> | type i cytoskeletal 13-like | protein binding | -67.46 |
| 10 | <i>fel</i> | fish-egg lectin | cell | -53.68 |
| 11 | <i>sema5b</i> | semaphorin-5b isoform x6 | branching involved in blood vessel morphogenesis | -46.00 |
| 12 | <i>urea</i> | urea transporter | urea transmembrane transport | -45.37 |
| 13 | <i>c1ql4</i> | complement c1q-like protein 4 precursor | complement component C1q binding | -45.26 |
| 14 | <i>cxcl14</i> | c-x-c motif chemokine 14 precursor | chemokine activity | -43.81 |
| 15 | <i>cyp1</i> | cytochrome p450 1a | intracellular membrane-bounded organelle | -42.33 |
| 16 | <i>hsd17b3</i> | testosterone 17-beta-dehydrogenase 3 | intracellular membrane-bounded organelle | -35.82 |
| 17 | <i>rasd2</i> | gtp-binding protein rhes-like | negative regulation of protein ubiquitination | -34.72 |
| 18 | <i>igfbp-5</i> | insulin-like growth factor-binding protein 5-like | insulin-like growth factor binding | -29.01 |
| 19 | <i>sfrp5</i> | secreted frizzled-related protein 5 | negative regulation of canonical Wnt signaling pathway | -30.35 |
| 20 | <i>vwc2</i> | von willebrand factor c domain-containing protein 2-like | AMPA glutamate receptor complex | -26.80 |

Supplementary Table 4 – List of top 20 Upregulated and Downregulated genes in head kidney of *D. labrax* infested with *A. ocellatum* representing gene symbol, protein product, gene ontology class and fold change.

| UPREGULATED | | | | |
|---------------|--------------------|--|---|-------------|
| Ranking | Gene symbol | Protein product | GO Class (Direct) | Fold change |
| 1 | <i>mx</i> | interferon inducible mx protein | activation of innate immune response | 4,867.73 |
| 2 | <i>tuba1a</i> | tubulin alpha-1a chain | structural constituent of cytoskeleton | 872.52 |
| 3 | <i>rtp3</i> | receptor-transporting protein 3 | protein binding | 687.97 |
| 4 | <i>ifn-alpha-1</i> | type alpha 1 | B cell differentiation | 270.30 |
| 5 | <i>trim39</i> | e3 ubiquitin-protein ligase trim39-like | protein binding | 129.68 |
| 6 | <i>aste1</i> | protein asteroid homolog 1-like isoform x1 | nuclease activity | 98.21 |
| 7 | <i>rgs5</i> | regulator of g-protein signaling 5-like | G protein-coupled receptor signaling pathway | 70.16 |
| 8 | | inosine-uridine preferring nucleoside hydrolase-like | purine nucleoside catabolic process | 56.72 |
| 9 | <i>gad1</i> | glutamate decarboxylase-like protein 1 | protein binding | 55.83 |
| 10 | <i>mx</i> | mx protein | innate immune response | 48.64 |
| 11 | <i>gig2</i> | gig2-like protein | cytoplasm | 47.63 |
| 12 | <i>rnp3</i> | nucleolar complex protein 3 partial | nucleolar ribonuclease P complex | 43.45 |
| 13 | <i>herc4</i> | probable e3 ubiquitin-protein ligase herc4-like | ubiquitin-protein transferase activity | 43.34 |
| 14 | <i>ddit4</i> | dna damage-inducible transcript 4 | response to hypoxia | 39.55 |
| 15 | <i>cmpk2</i> | ump-cmp kinase mitochondrial | ATP binding | 36.97 |
| 16 | <i>ntf-2</i> | nuclear transport factor 2-like | positive regulation of antimicrobial peptide biosynthetic process | 34.99 |
| 17 | <i>irf3</i> | interferon regulatory factor 3 | interferon-alpha production | 34.75 |
| 18 | <i>sntx</i> | stonustoxin subunit alpha | toxin activity | 30.80 |
| 19 | <i>angptl4</i> | angiopoietin-related protein 4-like | protein binding | 28.68 |
| 20 | <i>gimap7</i> | gtpase imap family member 7-like | GTPase activity | 28.66 |
| DOWNREGULATED | | | | |
| Ranking | Gene symbol | Protein product | GO Class (Direct) | Fold change |
| 1 | <i>p13913</i> | arylamine n- pineal gland isozyme nat-10-like | arylamine N-acetyltransferase activity | -6,427.56 |
| 2 | <i>dyrk1a</i> | dual specificity tyrosine-phosphorylation-regulated kinase 1a-like | peptidyl-tyrosine autophosphorylation | -787.65 |

| | | | | |
|----|-----------------|--|--|---------|
| 3 | <i>cpdb</i> | carboxypeptidase b | proteolysis involved in cellular protein catabolic process | -272.84 |
| 4 | <i>prss1</i> | trypsin-1 precursor | proteolysis | -221.94 |
| 5 | <i>ctrb1</i> | chymotrypsinogen 2 | protein binding | -185.23 |
| 6 | <i>try</i> | trypsinogen 2 | proteolysis | -174.22 |
| 7 | <i>cela2a</i> | chymotrypsin-like elastase family member 2a-like | proteolysis | -157.37 |
| 8 | <i>hcea</i> | high choriolytic enzyme 1-like | metalloendopeptidase activity | -127.56 |
| 9 | <i>mybpc1</i> | myosin-binding protein slow-type | myosin binding | -124.58 |
| 10 | <i>actc1</i> | alpha cardiac-like isoform 1 | atpase activity | -108.93 |
| 11 | <i>ctn</i> | Cardiac troponin | troponin complex | -106.66 |
| 12 | <i>cela1</i> | elastase-1-like | proteolysis | -76.32 |
| 13 | <i>elsrp</i> | elastase-like serine protease | serine-type Endopeptidases inhibitor activity | -62.72 |
| 14 | <i>serpinb1</i> | pancreatic elastase | type B pancreatic cell proliferation | -48.85 |
| 15 | <i>tpm2</i> | tropomyosin beta chain-like isoform 1 | protein binding | -44.62 |
| 16 | <i>tcap</i> | telethonin | detection of muscle stretch | -43.25 |
| 17 | <i>astl</i> | astacin like metallo-protease | cytoplasm | -40.78 |
| 18 | <i>tnnc1</i> | troponin slow skeletal and cardiac muscles | troponin complex | -39.68 |
| 19 | <i>pvalb7</i> | parvalbumin-7-like isoform x1 | calcium ion binding | -35.21 |
| 20 | <i>e7</i> | type i keratin e7 | cytosol | -36.17 |