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Effects of temperature on amoebic gill disease development: does it play a role?

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Abstract

A relationship between increasing water temperature and amoebic gill disease (AGD) prevalence in Atlantic salmon (*Salmo salar*) has been noted at fish farms in numerous countries. In Scotland (UK) temperatures above 12°C are considered to be an important risk factor for AGD outbreaks. Thus, the purpose of this study was to test for the presence of an association between temperature and variation in the severity of AGD in Atlantic salmon at 10°C and 15°C. The results showed an association between temperature and variation in AGD severity in salmon from analysis of histopathology and *Paramoeba perurans* load, reflecting an earlier and stronger infection post amoebae exposure at the higher temperature. Whilst no significant difference between the two temperature treatment groups was found in plasma cortisol levels, both glucose and lactate levels increased when gill pathology was evident at both temperatures. Expression analysis of immune and stress related genes showed more modulation in gills than in head kidney, revealing an organ-specific response and an interplay between temperature and infection. In conclusion, temperature may not only affect the host response, but perhaps also favours higher attachment/growth capacity of the amoebae as seen with the earlier and stronger *P. perurans* infection at 15°C.

Keywords: temperature, *Paramoeba perurans*, amoebic gill disease.

1. INTRODUCTION

The causative agent of amoebic gill disease (AGD) in farmed Atlantic salmon is *Paramoeba perurans* (synonym *Neoparamoeba perurans* (Feehan et al., 2013)), an amphizoic amoeba (15-40 µm diameter) that has successfully fulfilled Koch's postulates (Crosbie et al., 2012). Relatively little is known about the biology of *P. perurans* and its potential environmental risk factors and reservoirs in relation to AGD outbreaks. A relationship between increasing water temperature and AGD prevalence has been noted in numerous studies that report outbreaks in Atlantic salmon farms in Tasmania (Australia), Scotland (UK), Norway, Chile, and South Africa (Adams & Nowak, 2003; Bustos et al., 2011; Clark & Nowak, 1999; G. M. Douglas-Helders et al., 2003; M. Douglas-Helders et al., 2001; M. Douglas-Helders et al., 2005; Mouton et al., 2013; Steinum et al., 2008). For example, in Scotland (UK) temperatures above 12°C are considered to be an important risk factor for AGD outbreaks (Marine Harvest, personal communication).

Temperatures of 15°C or above have also been associated with a metabolic depression and non-optimal rates of growth of Atlantic salmon in terms of thermal growth coefficient (TGC), when compared to fish at 13°C, indicative of a chronic stress response (Olsvik et al., 2013). However, in lower latitude production areas, such as Ireland and Tasmania, Atlantic salmon can be cultured at temperatures of ca. 15°C and other studies showed a larger range of survival/growth temperatures for Atlantic salmon (Johansson et al., 2009; Oppedal et al., 2011; Stehfest et al., 2017) as, e.g., up to maximum 22°C (Elliott & Elliott, 2010). Atlantic salmon thermal tolerance seems to be correlated with a previous acclimation to temperature and differences among studies depend on the methods used. For this study, Atlantic salmon acclimated to temperatures found in Scottish waters, and previously held at 10°C were used.

In intensive aquaculture, many different factors may cause stress in fish, impacting negatively on immunity and resulting in increased disease susceptibility. Stress impacts can be found in farmed fish subjected to non-optimal environmental variables, such as temperature, dissolved oxygen, nitrogen compounds, salinity, pH, presence of chemicals, contaminants, and the presence of pathogens (Tort, 2011). Among these, temperature is also *per se* an important factor for poikilothermic animals, such as fish, which has an effect on immune function (Bowden, 2008; Mikkelsen et al., 2006; Nikoskelainen et al., 2004; Pettersen et al., 2005; Raida & Buchmann, 2007). For instance, higher water temperatures can also lead to the up regulation of cytokine genes (*il-1β*, *il-10* and *ifn-γ*) and increases in secreted *IgM* in fish, with higher expression at 25°C compared to 15°C and 5°C, in rainbow trout vaccinated with

94 *Yersinia ruckeri* serotype O1 (Raida & Buchmann, 2007); the lytic activity of both total and
95 alternative complement pathways was higher in rainbow trout acclimated at 20°C compared
96 to 5°C and 10°C (Nikoskelainen et al., 2004); and the number of leucocytes in blood of
97 Atlantic salmon post-smolts showed higher proportions of neutrophils and lower proportions
98 of Ig^+ cells at 18°C compared to fish at lower temperatures (Pettersen et al., 2005).
99 In teleost fish, the head kidney itself is both an immune and endocrine organ: the fish putative
100 hematopoietic tissue is located adjacent to the endocrine tissue, the chromaffin cells produce
101 catecholamines, and the interrenal cells produce cortisol (Bernier et al., 2009). Specific
102 cytokine receptors and cytokines are produced close to endocrine cells to allow the
103 neuroendocrine system to receive signals from the immune system and vice versa (Bernier et
104 al., 2009). In the context of a stress response in fish, glucocorticosteroids influence the
105 secretion of pro- and anti-inflammatory cytokines, while cortisol was shown to affect
106 apoptosis and proliferation of immune cells for effective deactivation and activation of the
107 teleostean immune response (Bernier et al., 2009), leading, in the case of deactivation, to
108 increased fish susceptibility to infections (Gadan et al., 2012; Tort, 2011).
109 Thus, the primary purpose of this study was to test for the presence of an association between
110 temperature, potentially acting as a stressor, and variation in the severity of AGD in Atlantic
111 salmon, at two different temperatures relevant to Scottish salmon aquaculture, 10°C and
112 15°C, the latter important in summer. A secondary aim was to explore a causal explanation
113 for this association by investigating hormonal and molecular responses affected by
114 temperature focusing on primary and secondary stress responses (plasma cortisol, glucose
115 and lactate levels) and on immune and stress related gene expression.
116

2. MATERIALS AND METHODS

2.1 Experimental set-up and fish challenge

Before the experiment, samples from five Atlantic salmon (approx. 150 g) were screened for the presence of viral pathogens (infectious salmon anaemia, infectious haematopoietic necrosis, viral haemorrhagic septicaemia, infectious pancreatic necrosis) by signs of cytopathic effects on different fish cell lines and by real time RT-PCR, for *P. perurans* by real time RT-PCR, for bacterial pathogens using culture techniques (head kidney swabs in tryptic soy agar plates with 2% sodium chloride, and gill swabs in *Flexibacter maritimus* medium plates). Gills were also examined histologically for AGD and signs of other gill diseases/damage. Fish were acclimatised to 10 ± 1°C for two weeks prior to the commencement of the experiment and fed 1% body weight/day using a commercial Skretting Atlantic salmon smolt diet throughout the experimental period. The experiment had two treatment groups challenged with 500 cells/l of the B8 clonal culture of *P. perurans* (Collins et al., 2017) for which previous data were used to inform the experimental design. The B8 clonal culture was chosen for use because previous experimental studies showed that a challenge dose of 500 cells/l resulted in a median gill score (~2, based on histological analysis for AGD) towards the end of the experiment for fish held at 10 ± 1°C (Collins et al., 2017). Therefore, the same challenge dose of this clonal culture was used anticipating that, in stress related experiments, there was scope to see if the fish become more susceptible to infection (Gadan et al., 2012).

The experiment was designed to establish an AGD challenge assuming a power analysis of 80%. Briefly, the gill scores for individual fish within treatment groups were modelled from previously observed median values (Collins et al., 2017) assuming a binomial distribution, and a proportional odds model was used to evaluate the capability of different group sizes to detect a difference in scores between treatment groups with a type I error of 5%. For the experiment the first treatment group was held at 10 ± 1°C and the second treatment group at 15 ± 1°C, with the increase in temperature (1°C/day for 5 days) starting from 10 days prior to amoeba challenge. The experiment also had two negative control groups, which were exposed to filtered (before the challenge) medium from amoeba cultures to account for any effects of the co-occurring bacteria. Negative and control groups are described in Table 1. The amoeba cultures were acclimatised at the two different temperatures (10°C and 15°C) for three months before starting the experiment, and four passages of the cultures have been were

performed over this time to maintain their survival until the starting of the experiment as described by Benedicenti et al. (2018). Amoebae were cultured as described previously (Benedicenti et al., 2015) and aliquots of amoebae were numerically equal and randomly distributed to experimental tanks. Three technical replicates (three tanks each containing 21 fish and 350 l of 34-35 ppt sea water, with a flow-through of 180 l/h and a 12 h light/dark regime) were used for the treatment groups and one tank for each negative control group. Four sampling points were chosen for the experiment, the first three days before *P. perurans* exposure (dbe), and the others at 2, 10 and 21 days post *P. perurans* exposure (dpe). Fish were anaesthetised with a lethal dose of 12.5 mg/l of metomidate hydrochloride (DL-1-(1-phenylethyl)-5-(metoxycarbonyl) imidazole hydrochloride) (Aquacalm, Syndel, Canada) resulting in death within 2.5 min to reduce the cortisol release into the blood due to handling (Gadan et al., 2012; Gamperl et al., 1994; Iversen et al., 2003; Olsen et al., 1995). To reduce blood contamination of gill samples, fish were bled by caudal venous puncture and the heart was removed. Samples from the dorsal part of the third gill arch (left side) targeted to include the interbranchial lymphoid tissue as described in Benedicenti et al. (2015), and irrespective of presence or absence of visible gill lesions, and head kidney were collected for gene expression and *P. perurans* load analyses and stored in RNAlater (RNAlater® Stabilization Solution, Ambion®) at -80°C. Blood samples were also collected and placed on ice in heparin tubes (BD Vacutainer®), then centrifuged at 4,000 x g for 15 min at 4°C to separate the plasma and blood cells. The plasma was collected, stored at -80°C and subsequently used for cortisol, glucose and lactate analyses.

2.2 Histopathology

For histological analysis and assessment of the pathology associated with AGD, samples from the entire second gill arch (left side) were fixed in 10% buffered neutral formalin solution for a minimum of 24 h, washed in 100% EtOH, and then stored in 70% EtOH until processing. Samples were then washed three times in 100% EtOH, in xylene (3 dips) and embedded in paraffin wax. Sections (3 µm) were stained with haematoxylin and eosin (H&E stain) and scored (category 0 - 5) (Table 2). The final score was based on a median of all the histopathology features shown in Table 2, which was a system developed in previous work relating to the exposure of *P. perurans*-infected fish to H_2O_2 treatment (McCarthy et al., 2015). Representative pictures showing the different scored categories are presented in supplementary figures. Histopathology statistical analysis was performed in R (R software,

software 3.0.1) using the `polr` function, a proportional odds logistic regression which fits a logistic or probit regression model to an ordered factor response (Agresti, 2010). For the statistics, the different treatments/controls were grouped together based on the most similar parameter estimates in a stepwise *a posteriori* procedure used to combine non-significant factor levels until the models' comparison was significant after models' comparison with the anova (`aov`) function ($p \leq 0.05$). Diagnostic plots of the final model were always performed to validate that the model assumptions were met.

2.3 Assessment of P. perurans infection

P. perurans load (18S rRNA) was assessed on gill cDNA samples using a TaqMan assay (Fringuelli et al., 2012). Relationship between the *P. perurans* load (18S rRNA) Cp values between treatments (fish exposed to *P. perurans* at 10°C and 15°C) was tested with a generalised liner mixed-effects model (Bates et al., 2015) using the `lmer` function in the `lme4` package in R; while single comparisons per each sampling day were performed by a liner mixed-effects model (Pinheiro & Bates, 2000) using the `lme` function in the `nlme` package in R. A generalized linear mixed-effects model in R was used to describe the relationship between fixed response variables (Cp values between treatments) and a random categorical covariate (tank effect), which influences the variance of the response variable (`lmer` function in the `lme4` package in R). The `AOV` function was used to compare mixed-effects models and diagnostics plots were used to validate the final model showing that the response variable was a reasonably linear function of the fitted values, residuals vs fitted values were symmetric around a zero line and errors were normally distributed.

2.3 Cortisol assay

Cortisol concentrations [ng/ml] were determined by radioimmunoassay (RIA) as described by (Pottinger & Carrick, 2001). Briefly, plasma samples (200 µl) were extracted by vortex mixing with 1 ml of ethyl acetate (AnalaR®, VWR, UK) (1:5 of plasma: ethyl acetate), and after centrifugation aliquots of the resulting supernatant were transferred to 3.5 ml polypropylene assay tubes (Sarstedt, Germany). For the determination of a standard curve, tubes with aliquots of 100 µl of ethyl acetate containing between 0 and 800 pg (0, 6.25, 12.5, 25, 50, 100, 200, 400, 800 pg) of inert cortisol (Sigma-Aldrich®, UK), in duplicate, were used. Blanks consisted of tubes with only 100 µl ethyl acetate (AnalaR®, VWR, UK). All the tubes, including the unknown assay tubes, received a 25 µl aliquot of ethyl acetate containing

20,000 disintegration per minute (dpm) of [1,2,6,7- ^{3}H]cortisol (GE Healthcare Life Sciences, UK, 60 Ci/mmol) and the solvent was evaporated under a vacuum. 200 µl of phosphate buffered saline (PBS, Sigma-Aldrich®, UK) containing anti-cortisol antibody (IgG-F-2; IgG Corp.; 1:600, Abcam, UK) and 0.1% of bovine serum albumin (suitable for RIA, pH 5.2, ≥96%, Sigma-Aldrich®, UK) was then added to each tube and the tubes incubated overnight at 4°C. After incubation, the assay tubes were placed on ice and unbound cortisol was retrieved by adding to each tube 100 µl of chilled, stirred, dextran-coated charcoal suspension (1.0% activated charcoal; 0.2% dextran in PBS). Tubes were next vortexed, incubated on ice for 5 min and centrifuged at 3,000 x g for 10 min at 4°C. 200 µl of the supernatant was transferred to 4.5 ml scintillation fluid (Ecoscint A; National Diagnostics, US) in a scintillation vial (VWR, UK), mixed by inversion, and counted under standard [^{3}H] conditions for at least 5 min. The concentration of cortisol in the plasma samples was calculated from the equation of a 3-parameter hyperbolic function fitted to a plot of the percentage of [^{3}H]cortisol bound against pg of inert cortisol (SigmaPlot® Regression Wizard; SPSS Science). A generalised liner mixed-effects model (Bates et al., 2015) using the `lmer` function in the `lme4` package and a liner mixed-effects model (Pinheiro & Bates, 2000) using the `lme` function in the `nlme` package in R for single comparisons at each sampling day were applied for statistical analysis as described before. Diagnostics plots were used to validate the final model and they showed that the response variable was not a reasonably linear function of the fitted values, residuals vs fitted values were not symmetric around a zero line and errors were not normally distributed. Therefore, data were transformed to decrease the variability among biological replicates in the same treatment. Firstly, data were transformed with the squared roots as some values were not detectable (cortisol concentration below the detection limit had an assigned value of zero), however, also in this case, the diagnostics was not satisfactory. Thus, 0.005 ng/ml was applied as the lowest concentration in the undetectable samples because it was less than the minimum value of 0.010 ng/ml detected by the RIA assay, and this allowed the model to analyse Log transformed data.

2.4 Glucose assay

Plasma glucose levels were measured by the glucose oxidase method (GAGO-20, Sigma-Aldrich®, UK). 50 µl of diluted plasma samples in dH_2O (3 µl of the sample + 47 µl dH_2O) was incubated for 30 min at 37°C with 100 µl of assay reagent (o-dianisidine reagent mixed with glucose/ peroxidase reagent as described in the technical bulletin) in a 96 well

microplate (Greiner Bio-One, VWR, UK). The reaction was stopped by the addition of 100 µl of 12N H_2SO_4 (ACS reagent, 95.0-98.0%, Sigma-Aldrich®, UK) and the absorbance was measured for each sample (triplicate reactions) against the reagent blank (dH_2O processed as for the samples) at 540 nm in a spectrophotometer (SpectraMax® Plus 384 Microplate Reader, Molecular Devices, US). The glucose concentration was calculated using a linear standard curve produced at the same time using different dilutions (0, 20, 40, 60, 80 µg glucose/ml) of the glucose standard solution (1.0 mg/ml in 0.1% benzoic acid). Statistical analysis was performed as described above without data transformation (section 2.3).

2.5 Lactate assay

Plasma lactate was measured using the D-lactate colorimetric assay (MAK058, Sigma-Aldrich®, UK), where D-lactate is oxidised by D-lactate hydrogenase and generates a colorimetric product measured at 450 nm. 30 µl of plasma samples were mixed with D-lactate buffer to bring the volume to 50 µl and then 50 µl of reaction mix (D-lactate assay buffer and enzyme mix, as described in the technical bulletin) added into 96 well microplates (Greiner Bio-One, VWR, UK). The mix was incubated for 30 min at room temperature before the absorbance was measured for each sample (triplicate reactions) against the reagent blank (dH_2O processed as for the samples) at 450 nm on a spectrophotometer (SpectraMax® Plus 384 Microplate Reader, Molecular Devices, US). The lactate concentration was calculated using a linear standard curve produced at the same time using different dilutions of the standard solution (0, 2, 4, 6, 8, 10 µl of a 1mM standard solution which corresponds to 0, 2, 4, 6, 8, 10 nmole, respectively). Statistical analysis was performed as described above without data transformation (section 2.3).

2.6 Gene expression analysis

Total RNA was isolated from the gill samples using TRIzol, following the manufacturer´s instructions (TRIzol® Reagent, Ambion®). Total RNA was dissolved in 50 – 60 µl diethylpyrocarbonate (DEPC)-treated water and concentration [ng/µl] determined on a NanoDrop ND-1000 Spectrophotometer (PEQLAB GmbH, Germany). To assess the sample quality, the A260/A280 and A260/A230 ratios were checked to ensure that the RNA had an A260/A280 ratio of ~2.0 and that the A260/A230 ratio was in the range of 1.8 – 2.2.

To guarantee constant and comparable amounts of RNA in the analyses, the concentration of RNA was set to approximately 1000 ng of total RNA per reaction for the reverse

transcription (RT). The RNA was treated with gDNA Wipeout Buffer (QuantiTect Reverse Transcription Kit, Qiagen) to remove genomic DNA (gDNA) contamination and incubated for 2 min at 42 °C. Each RT was performed in a mix containing: 14 µl RNA previously treated to eliminate gDNA (approximately 50 ng/µl of input total RNA), 1 µl of reverse-transcription master mix (reverse transcriptase and RNase inhibitor), 4 µl of Quantiscript RT Buffer, and 1 µl of RT Primer Mix optimized blend of oligo-dT and random primers dissolved in water (QuantiTect Reverse Transcription Kit, Qiagen). The mixture was incubated at 42 °C for 30 min and afterwards the enzyme was inactivated at 95 °C for 3 min. A negative cDNA control sample with DEPC-treated water (Invitrogen™, Carlsbad, USA) instead of reverse transcriptase was included to check for genomic contamination. The generated cDNA template was diluted 1:10 with DEPC-treated water and then stored at - 20 °C until real time RT-PCR analysis. Real time RT-PCR was carried out using a LightCycler® 480 (Roche Applied Science) in a 20 µl reaction using SYBR® Green I Nucleic Acid Gel Stain (Invitrogen™, Carlsbad, USA) and IMMOLASE™ DNA Polymerase (Bioline, UK). 4 µl cDNA were used in each reaction to maintain data integrity for gene expression comparisons. The real time analysis program consisted of 1 cycle of denaturation (95 °C for 10 min), 40 cycles of amplification (95 °C for 30 s, 63 °C for 30 s, 72 °C for 20 s, 84 °C for 5 s), followed by 95 °C for 5 s and 75 °C for 1 min. Melting curve analysis was carried out to check that primers were giving a specific PCR product. Real time RT-PCR primers are given in Table 3. A negative control was included in the reverse transcription (cDNA synthesis), containing all the reagents, except the reverse transcriptase, to confirm absence of contaminating DNA since not all primer pairs crossed exon-intron boundaries. Primer efficiency was tested using 4 fold serial dilutions of cDNA from pooled RNA samples and calculated by the 'LightCycler® 480 software version 1.5.1.62' (Roche Applied Science) as $E = 10^{(-1/s)}$, where s is the slope generated from the Log dilution of cDNA plotted against Cp (cycle number of crossing point). The relative expression level of the candidate genes was expressed as arbitrary units which were calculated from the serial dilutions of references run in the same 384-well plates and then normalised against the expression level of the house-keeping gene *ef1α*. Statistical analysis was performed as described above (section 2.3). Gene expression analysis was performed in gill and head kidney samples, as gills are directly affected during *P. perurans* infection and the head kidney is an important immune and endocrine organ. In higher vertebrates, it has been shown that glucocorticoid-treated monocytes/macrophages produce significantly less Il-12, leading to a decreased capacity of

these cells to induce Ifn-γ production in $CD4^+$ T cells and, therefore, affecting the T_H1/T_H2 balance (Blotta et al., 1997; DeKruyff et al., 1998; Elenkov et al., 1996; Elenkov & Chrousos, 1999). Thus, for this study, it was decided to investigate the following immune related genes: T_H2 putative markers (*il-4/13* isoforms), markers of macrophage activation and polarization in mammals (*arg2a*, *arg2b*, *inos*), and also cellular markers of antigen presenting cells, B cells and T cells (*mhcI (UBA)*, *mhcII (DAB)*, *cd4*, *cd8a*, *cd8b*, *IgM*, *IgT*, and *IgD*).

Stress related genes include heat shock proteins (Hsp), which are highly conserved molecular chaperones, ubiquitously expressed, classified into families based on their approximate molecular mass in kilodaltons (kDa), and with a functional relationship between their expression and the HPI axis in higher vertebrates (Ackerman et al., 2000; Celi et al., 2012). Two Hsp90 cytosolic isoforms have been reported, Hsp90α and Hsp90β (Celi et al., 2012). Hsp90α is inducible and associated with stress-induced cytoprotection (Celi et al., 2012) and four different isoforms are present in Atlantic salmon, Hsp90α1a, Hsp90α1b, Hsp90α2a, and Hsp90α2b (de la Serrana & Johnston, 2013). In contrast, Hsp90β is constitutively expressed, mainly associated with early embryonic development and several cellular pathways, and two isoforms are present in Atlantic salmon, Hsp90β1 and Hsp90β2 (Celi et al., 2012; de la Serrana & Johnston, 2013).

2.7 Ethics statement

All handling of fish was conducted in accordance with the Animals (Scientific Procedures) Act 1986 and all proposed experiments were first subject to detailed statistical review to ensure that a minimum number of fish was used, which would allow statistically meaningful results to be obtained.

3. RESULTS

3.1. Histopathology and P. perurans load

The histopathology features were assessed following Table 2 and the final gill score was calculated as a median among all fish and tanks for each treatment and control. During the pre-challenge and first (2 dpe) sampling points, the treatments and control fish showed a median gill score of 1. The second sampling, at 10 dpe, showed a median gill score of 2 in the infected fish (A and B groups) and a median of 1 for the controls (C and D groups), while the third sampling at 21 dpe showed an increased median gill score of 3 only for infected fish at 15°C (B) (Fig. 1). At 21 dpe, both control tanks and group A remained at scores of 1 and 2 respectively. Statistical analysis (using the `polr` function in R) was performed for the second and third sampling points separately, as these treatments had a gill score ≥ 1. The final model for both the second and the third sampling points showed that all the negative controls grouped together, while the infected fish at 10°C and 15°C could not be grouped with the other treatments showing a statistical difference ($p \leq 0.05$) and therefore a temperature effect in the infected groups.

P. perurans load (18S rRNA) assessment was performed on cDNA samples from gill used also for gene expression analysis. Fish exposed to *P. perurans* at 10°C (A) showed higher Cp values (lower expression) for amoeba 18S rRNA relative to fish exposed to *P. perurans* at 15°C (B) (Fig. 2). The negative controls (C and D) and the fish health screening before the amoeba challenge showed Cp values of 0 or values greater than 35, regarded as the upper Cp threshold for reliable detection (Collins et al., 2017), therefore the statistical analysis was performed only between the infected groups A and B. The generalized linear mixed-effects model showed that there is an interaction between the two treatments (A and B groups), among sampling days and a covariance of the random tank effect: `(model<-lmer(Amoeba_load ~ Treatment + Sampling_Day + Treatment : Sampling_Day + (1 | taskA$Tankf)))` showing an effect of the temperature in the infected groups over time. Single analysis performed at each sampling day, using a linear mixed-effects model, showed a significant difference between the two treatments ($p \leq 0.05$, $n = 30$), at all sampling points after *P. perurans* exposure, with lower Cp values for group B indicating higher amoebae numbers.

361 *5.3.2 Cortisol assay*

362 Group A showed mean plasma cortisol concentrations of 5.96 ± 1.11 ng/ml (mean ± SEM, n
363 = 15) at the pre-challenge sampling, 1.00 ± 0.22 ng/ml (mean ± SEM, n = 15) at 2 dpe, 0.87 ±
364 0.54 ng/ml (mean ± SEM, n = 15) at 10 dpe, and 6.43 ± 2.06 ng/ml (mean ± SEM, n = 15) at
365 21 dpe. Group B showed mean plasma cortisol concentrations of 9.20 ± 3.18 ng/ml (mean ±
366 SEM, n = 15) at the pre-challenge sampling, 0.60 ± 0.14 ng/ml (mean ± SEM, n = 15) at 2
367 dpe, 1.23 ± 0.30 ng/ml (mean ± SEM, n = 15) at 10 dpe, and 9.78 ± 1.78 ng/ml (mean ±
368 SEM, n = 15) at 21 dpe. Groups C and D showed, respectively, mean plasma cortisol levels
369 of 0.55 ± 0.38 ng/ml and 1.76 ± 0.30 ng/ml (mean ± SEM, n = 5) at the pre-challenge
370 sampling point, 0.12 ± 0.06 ng/ml and 0.23 ± 0.06 ng/ml (mean ± SEM, n = 5) at 2 dpe, 0.97
371 ± 0.11 ng/ml and 1.43 ± 0.72 ng/ml (mean ± SEM, n = 5) at 10 dpe, and 5.12 ± 1.87 ng/ml
372 and 2.18 ± 0.70 (mean ± SEM, n = 5) at 21 dpe. The generalized linear mixed-effects model
373 in R was used to analyse the relationship between cortisol concentration (the fixed response
374 variable) and tank effect (the random categorical covariate), which influences the variance of
375 the response variable. The Log-likelihood function showed that there was evidence of a
376 random (tank) effect among replicates, showing a variability of values among tanks. The `AOV`
377 function was used to compare mixed-effects models and the final model showed that there
378 was no difference among all groups (A, B, C and D) while the sampling day had an effect
379 (`model<-lmer(Cortisol ~ Sampling_Day + (1 | taskA$Tankf) + (1 |`
380 `(taskA$Tankf:Sampling_Day)), REML=TRUE)`). After a stepwise *a posteriori*
381 procedure (to combine non-significant factor levels until the models' comparison was
382 significant and with the models' comparison of the `aov` function) it was shown that the A
383 and B treatment groups could be grouped together, as could the C and D control groups.
384 Diagnostics plots validated the final model: `model<-lmer(taskA$logCortisolA ~`
385 `treat + Sampling_Day + treat : Sampling_Day + (1 | taskA$Tankf),`
386 `REML=TRUE)`. Analysis of each sampling day, performed using a linear mixed-effects model,
387 showed no significant difference between the infected groups (A and B) at each sampling day
388 ($p > 0.05$, n = 40) but significant differences were seen for 1) the pre-challenge sampling
389 point between C and A+B ($p \leq 0.05$, n = 40) and 2) the first sampling point between A and
390 C+D ($p \leq 0.05$, n = 40), with higher levels in the infected groups in both cases (Fig. 3a).

391 *5.3.3 Glucose assay*

392 A mixed-effects model in R was used to analyse the relationship between glucose
393 concentration (the fixed response variable) and tank effect (the random categorical covariate),
394 which influences the variance of the response variable. The Log-likelihood function showed
395 that no random (tank) effect was detectable among technical replicates. The `AOV` function was
396 used to compare mixed-effects models and showed that there was a difference among
397 treatments and the sampling day (including the interaction between treatments and sampling
398 days). After a stepwise *a posteriori* procedure (to combine non-significant factor levels until
399 the models' comparison was significant and with the models' comparison of the `aov`
400 function) it was shown that A+C and B+D can be grouped together (Fig. 3b), showing similar
401 estimates in the general model between the two temperatures for plasma glucose levels
402 irrespective of infectious status. Diagnostics plots validated the final model: `model<-`
403 `lmer(Glucose ~ treat + Sampling_Day + treat : Sampling_Day + (1 |`
404 `Tankf) , REML=TRUE)`. Single analysis performed for each sampling day, using a linear
405 mixed-effects model, showed no significant difference between the treatment groups at the
406 pre-challenge and first sampling points ($p > 0.05$, $n = 40$) but significant differences were
407 found at 1) the second sampling point between A+B+D and C with lower values in C ($p \leq$
408 0.01, $n = 40$), and at 2) the third sampling point between A and B+D with lower values in B
409 and D groups ($p \leq 0.01$ and $p \leq 0.05$, respectively, $n = 40$).

410 *5.3.4 Lactate assay*

411 A mixed-effects model in R was used to analyse the data and the Log-likelihood function
412 showed that no random (tank) effect was detectable among technical replicates. The `AOV`
413 function was used to compare mixed-effects models and the final model showed that there
414 was a difference among groups, and that the sampling day (including the interaction between
415 treatments and sampling days) had an effect. After a stepwise *a posteriori* procedure it was
416 shown that A+B treatments and C+D controls could be grouped together (Fig. 3c), having
417 similar estimates in the general model between the two infection groups and the two control
418 groups in terms of plasma lactate concentration. Diagnostics plots validated the final model:
419 `model<-lmer(Lactate ~ treat + Sampling_Day + treat : Sampling_Day +`
420 `(1 | Tankf) , REML=TRUE)`. Single analysis performed for each sampling day (using a
421 linear mixed-effects model), showed no significant difference among all groups at the first
422 and third sampling points post exposure ($p > 0.05$, $n = 40$), but significant differences were

found at 1) the pre-challenge sampling point between B and D, with higher values in the control group D ($p \leq 0.05$, $n = 40$), and at 2) the second sampling point between A+B+D and C with lower values in C ($p \leq 0.05$, $n = 40$).

5.3.5 Gene expression analysis

Gene expression analysis was performed in gills and head kidney samples for immune and stress related genes and the statistical analysis was performed in R using mixed-effects models. The Log-likelihood function showed no random (tank) effect was detectable among technical replicates for all the genes and a single analysis was performed for each sampling day using a linear mixed-effects model. Detailed results are presented in supplementary tables.

5.3.5.1 Markers of macrophage activation

Analysis of immune genes related to macrophage activation/polarization showed that **1)** *arg2a* (Benedicenti et al., 2017) had significantly lower expression in the treatment group A compared to B and C ($p \leq 0.05$, $n = 39$) during the first sampling point in gills (Fig. 4a), while no significant difference in expression was detected in head kidney (Fig. 4a); **2)** *arg2b* (Benedicenti et al., 2017) showed a significant difference between the treatment groups (A and B) and controls (C and D) in gills at the pre-challenge sampling point ($p \leq 0.05$, $n = 40$) and between the two temperature treatments A and B at the first sampling point ($p \leq 0.05$, $n = 39$), with higher expression in the higher temperature group B (Fig. 4b), while in head kidney samples significant differences between the two temperature treatment groups were detected at the pre-challenge sampling ($p \leq 0.05$, $n = 40$) and at the third sampling ($p \leq 0.01$, $n = 38$) points, with higher expression in the lower temperature group in this organ (Fig. 4b); **3)** *inos* expression was only detectable in gills with significant differences seen between groups B and C and between the control groups (C and D) during the pre-challenge sampling ($p \leq 0.05$, $n = 40$), and between the two temperatures during the first (A and B, A and D, B and C, $p \leq 0.01$, $n = 39$) and the second sampling (A and B, A and D, $p \leq 0.01$, $n = 38$), with higher expression in the higher temperature groups (Fig. 4c).

5.3.5.2 Cellular markers of antigen presenting cells

Major histocompatibility class (MHC) I and II molecules present antigen and interact respectively with CD8 molecules on the surface of cytotoxic T cells or with CD4 molecules

on the surface of helper T cells. In gills, *mhcI (UBA*) (Jørgensen et al., 2006) showed a significantly lower expression in treatment group A in comparison to the other groups during the first sampling point (Fig. 5a), and a significantly lower expression in treatment group B compared to groups A and C during the second and the third sampling points after *P. perurans* exposure ($p \leq 0.05$, n = 38). These results are similar to the mRNA expression of *cd8a* (Fig. 5b) and *cd8b* (Fig. 5c) during the second and the third sampling points after challenge in gills, where group B was lower in comparison to the other treatments at the second sampling point and the two treatment groups (A and B) were significantly different at the third sampling point, with a lower expression seen in group B. In gills, *mhcII (DAB)* (Fig. 5d) (Belmonte et al., 2014) and *cd4* (Fig. 5e) showed a significantly higher expression in treatment group B compared to group A at the first sampling after challenge ($p \leq 0.05$, n = 39), while the *cd4* mRNA level was decreased at the second sampling after challenge in treatment group B, which was significantly different compared to group C ($p \leq 0.05$, n = 38). In head kidney, *mhcI (UBA)* (Fig. 5a) only showed a significant difference at the second sampling, with lower expression in treatment group B compared to group C ($p \leq 0.05$, n = 38), while *mhcII (DAB)*, *cd4*, *cd8a*, and *cd8b* showed no significant effects (Fig. 5b-e).

5.3.5.3 T_H2 markers

In gills, *il-4/13a* (Fig. 6a) and *il-4/13b1* (Fig. 6b) showed significant differences only between treatment groups and control groups (A with C + D, and B with D for *il-4/13a*; A with C+ D and B with C + D for *il-4/13b1*) with higher expression levels in the treatment groups during the second and the third sampling points. In contrast, *il-4/13b2* showed a significant difference between the two treatment groups during the third sampling point after challenge ($p \leq 0.05$, n = 39), with a higher expression level seen in group B (Fig. 6c). In head kidney, *il-4/13a* expression level showed a significant difference between the two treatment groups pre-challenge, and the first and second sampling points after challenge ($p \leq 0.05$, n = 39), with a higher expression level at the lower temperature (Fig. 6a). No effects were seen on *il-4/13b1* (Fig. 6b).

5.3.5.4 Immunoglobulins

The three immunoglobulins produced in salmon, IgM, IgT and IgD were screened with primers amplifying both secreted and membrane forms (m/s) (Tadiso et al., 2011). In gills (Fig. 7a-c), *IgM (m/s)* showed no significant differences, *IgD (m/s)* showed a significant

484 difference between groups A and B ($p \leq 0.05$, n = 40) during the pre-challenge sampling
485 point, while *IgT (m/s)* was significantly different between groups A and B during the three
486 sampling points after challenge, with a higher expression seen in group B only at the first
487 sampling after challenge ($p \leq 0.01$, n = 39). In head kidney, *IgM (m/s)* showed a significant
488 difference between groups B and C ($p \leq 0.05$, n = 40) in the pre-challenge samples, with a
489 lower expression seen in group B, while *IgD (m/s)* and *IgT (m/s)* showed no significant
490 differences (Fig. 7a-c).

491 *5.3.5.5 Stress related genes*

492 Stress related genes studied included heat-shock proteins, which are classified into families,
493 based on their approximate molecular mass (*hsp90* isoforms, *hsp70* and *hsp30*). *hsp90α1a* (de
494 la Serrana & Johnston, 2013) was only detected in gills, with a significant difference found at
495 the third sampling point between the treatment and control groups at 15°C (B and D), with
496 lower expression in the presence of AGD (Fig. 8a). *hsp90α2b* was significantly different both
497 in gills and head kidney: in gills at the first sampling after challenge between groups B and C
498 ($p \leq 0.05$, n = 39), with a higher expression detected at the higher temperature; in head kidney
499 at the first and the second sampling points, with differences found between the two treatment
500 temperatures (i.e., A + C vs B + D) with higher expression at the higher temperature (Fig.
501 8b). At the first sampling after challenge, *hsp90β1* was differentially expressed only in head
502 kidney (Fig. 8c), between groups B and C ($p \leq 0.05$, n = 39), while *hsp90β2* (Fig. 8d) was
503 modulated significantly only in gills (A and B, $p \leq 0.01$; A and D $p \leq 0.05$, n = 39). *hsp30*
504 showed no changes in expression in both gills and head kidney (Fig. 8e), while *hsp70* (Fig.
505 8e) was affected only in gills pre-challenge (C lower than A and B, $p \leq 0.05$, n = 40) and at
506 the first sampling after challenge (A having reduced expression in comparison to the other
507 treatments).

508

4. DISCUSSION

The relationship between increasing water temperature and AGD prevalence has been mentioned in numerous studies which recorded outbreaks in Atlantic salmon farms in Tasmania (Australia), Scotland (UK), Norway, Chile, and South Africa (Adams & Nowak, 2003; Bustos et al., 2011; Clark & Nowak, 1999; G. M. Douglas-Helders et al., 2003; M. Douglas-Helders et al., 2001; M. Douglas-Helders et al., 2005; Mouton et al., 2013; Steinum et al., 2008). Indeed, in Scotland temperatures above 12°C are considered to be an important risk factor for AGD outbreaks (Marine Harvest, personal communication), therefore, the main purpose of this study was to investigate the effect of temperature (10ºC vs 15ºC) on variation in severity of AGD in Atlantic salmon, with the higher temperature potentially acting as a stressor for fish previously acclimated to 10°C. A secondary goal was to gain a better understanding of this effect by investigating hormonal and molecular responses affected by temperature, focusing on primary and secondary stress responses, reflected in plasma cortisol, glucose, and lactate levels, and on immune and stress related gene expression analysis.

Histopathology confirmed *P. perurans* infection at 10 dpe to 500 cells/l of the B8 clonal culture of *P. perurans*, with a median gill score of 2 for the two infected groups, while a median gill score of 1 was applied to the control groups relating to background gill condition of the aquarium animals, and not associated with *P. perurans* gill lesions. However, at 21 dpe infected fish held at 15°C (group B) showed a higher median gill score of 3, while the gill score of infected fish held at 10°C (group A) remained at 2. A stepwise *a posteriori* procedure used for statistical analysis indicated that a stronger AGD pathology was associated with the higher temperature (15°C) treatment. *P. perurans* load (18S rRNA) on gills also showed a significant difference between infected fish held at 10°C and 15°C during the first, second and third samplings after *P. perurans* exposure with an earlier detection and higher numbers (or possibly higher expression activity) of *P. perurans* associated with the higher temperature (15°C). A recent study using the same *P. perurans* clone as in this study (B8) showed *in vitro* a significantly higher increase in attached amoebae over time at 15°C than at 10°C (while amoebae in suspension increased to a greater extent at 10 °C) and this phenomenon perhaps contributes to the findings here (Benedicenti et al., 2018).

Cortisol is the principal corticosteroid secreted by interrenal cells of the head kidney in teleost fish and it has been classified as part of the primary response after a stress event (Barton, 2002; Barton & Iwama, 1991). The mixed-effect model used for statistical analysis

showed an influence of the biological technical replicates, i.e. tank effect, reflecting a variability in cortisol concentration among tanks. The sampling of blood was performed in a way to minimize the release of cortisol due to handling procedure and, therefore, fish were anesthetised and killed within 2.5 min as previously described (Gadan et al., 2012; Gamperl et al., 1994; Iversen et al., 2003; Olsen et al., 1995). However, variability among individuals might have influenced the results, with no significant changes between the treatment groups. Therefore, reliable inferences regarding stress induction, based on differences in cortisol levels seen between different groups, are difficult to make. The statistical results showed that the concentration of cortisol was not different between infected groups at 10°C and 15°C for all sampling points, including the pre-challenge group, while a significant difference was detected between infected and control groups in the pre-challenge samples and at the first sampling point post exposure (group C different to A and B; group A different to C and D, respectively). The results may reflect an experimental artefact, with some tanks inadvertently disturbed, since it was not expected that group C would be different to group A at the pre-challenge stage, both groups being uninfected and held at 10°C.

Secondary stress responses include changes in plasma (e.g., glucose and lactate levels) and gene expression, which are related to physiological adjustments such as energy metabolism, respiration, immune function and cellular responses (Barton, 2002; Barton & Iwama, 1991). The mixed-effect model showed no influence of tank effect for all these analyses. The neuro-endocrine stress response affects energy metabolism in stressed organisms, causing levels of circulating glucose to increase (Ackerman et al., 2000). No significant changes in the level of glucose in plasma were shown at the pre-challenge and first sampling points among treatment and control groups, while a significantly lower concentration was found in control group C compared to infected group A, and in group C compared to group D at the second sampling point. This may indicate an increased level of glucose, potentially indicative of stress due to prolonged higher temperature (groups C and D) and/or the presence of AGD pathology (groups A and B). At the third sampling point, a significant difference in glucose levels is no longer seen due to infection, i.e. between infected groups and their relevant controls (A and C, B and D), nor due to temperature i.e. between controls at the different temperatures (C and D). However, glucose levels in both the infected and control groups at 10°C (A and C), were greater than in corresponding groups at 15°C (B and D). This could be explained by a higher oxidative catabolism of glucose at the higher temperature.

More variability among fish was shown by the lactate analysis probably reflecting a possible effect of the metomidate anaesthesia on blood lactate levels in fish, as has been described previously (Olsen et al., 1995). However, at the second sampling, a significant difference in lactate levels was observed between control groups C and D, indicating an effect of temperature, with higher levels at 15°C. A significant difference was also observed between infected and control groups at 10°C, indicating an increase in lactate levels due to AGD, but not between infected and control groups at 15 °C (B and D). The cause of the differences between infected groups and their controls may be that increase in lactate due to temperature alone at 15 °C masks any effect of AGD. No differences were seen among all groups at the third sampling point post exposure, perhaps explained again by habituation/exhaustion of the response. Similar results for cortisol and glucose have been described previously in Atlantic salmon subjected to a daily handling stress (15 s out of the water) for 4 weeks, where no significant differences were found for plasma cortisol levels, while glucose increased after 1 week (Fast et al., 2008).

In this study, different expression levels of most of the Hsp genes analysed were found between gills and head kidney, showing an organ-specific response as previously described by Ackerman et al. (2000). *Hsp90α1a* was only detected in gills, with a significant difference between the infected and control groups at 15°C (B and D) at the third sampling point after challenge, with higher expression in the control. Differences in expression in gill were not observed between infected and control groups at 10°C, nor between controls at 10°C and 15°C, indicating that infection and temperature alone are not responsible for differences. One possible explanation in the context of infection is that increased temperature can increase *hsp90α1a* expression to some extent, but that parasite infection/AGD pathology suppresses it and the higher parasite load/pathology at the third sampling point is sufficient to generate a significant difference in gene transcripts between groups B and D. Again, as for *hsp90α1a*, neither infection groups (A vs C, B vs D), nor temperature (C vs D) gave rise to significant differences in *hsp90β2* expression in gills, but the combination of both may have generated the significant differences seen between A and B at sampling point 2 dpe. Based on control values (C and D), the difference was due to possible suppression of *hsp90β2* by *P. perurans* infection at 10°C in group A. *hsp70* expression levels also appear suppressed in gills by infection at 10°C at 2 dpe, with infected group A having significantly lower levels of gene transcripts compared to control group C. No significant differences in expression were observed between infected and control groups at 15°C nor between controls groups at 10°C

and 15°C. However, significant differences were observed in gills between groups A and C at the pre-challenge stage, indicating that the findings for groups A vs C at 2 dpe may not be reliable. Marcos-López et al. (2017) reported up regulation of *hsp70* in gills from fish infected with *P. perurans* at 21 dpe at 10.5°C to 11.5°C, and an average gross gill score of 3.3, in contrast to findings in this study. Similarly, *hsp70* expression was found elevated in fish following infection with the parasite *Enteromyxum leei* (Sitjà-Bobadilla, 2008), and in different viral and bacterial infections (Ackerman et al., 2000; Song et al., 2016). Elevated *hsp70* has been suggested previously to be involved indirectly in cell proliferation (Marcos-López et al., 2017) and, therefore, it could be speculated that apparent down regulation of the *hsp70* gene in *P. perurans* infected salmon at 10°C may have resulted in less severe pathology compared to infection at 15°C.

Only *hsp90α2b* showed significant differences in expression between treatment groups in both gills and head kidney, with a lower expression detected at 10°C in both infected and control groups, showing an effect of both the pathology and the temperature. In contrast to results in gills for *hsp90β2* expression, which showed significant differences between treatment groups A and B, no significant differences were found within or between infected and control groups in head kidney. However, the significant difference in the isoform *hsp90β1* expression in head kidney between groups B and C may indicate interplay between temperature and infection, with greatest differences seen between higher temperature and infection loads (suppressed expression) of group B, and lower temperature/uninfected control group (C), with no differences seen between infected groups and their corresponding controls. *hsp70* was not modulated in head kidney, while *hsp30* was not modulated in gills and head kidney. Overall, in relation to *hsp* gene expression in gill from Atlantic salmon with AGD, there appeared to be a down regulation of, or no effect on, these genes compared to non-infected salmon. This is in contrast to findings elsewhere (Marcos-López et al., 2017).

In gills, *il-4/13a* and *il-4/13b1* (markers of a putative T_H2 response in fish) showed significant differences between infected and control groups at 10 and 21 dpe due to AGD pathology, with higher mRNA levels in the infected groups A and B, but no significant differences relating to temperature within the infected and control groups. This indicates induction/cell migration-proliferation in response to infection (pathology and/or parasite) but no significant modulation due to temperature. Expression of *il-4/13b2* also showed significantly higher levels in infected treatment groups A and B compared to their respective control groups C and D at the third sampling after exposure (21 dpe), indicating induction

due to AGD. However, significant differences were also found between the infected groups A and B and between the control groups C and D, indicating that temperature significantly modulated expression/cell numbers, with higher expression/cell numbers expressing *il-4/13b2* at the higher temperature. This trend of higher mRNA levels with higher temperature was reflected across the other sampling points, but not consistently so. The higher *il-4/13b2* mRNA levels in infected group B may also reflect the higher pathology/amoebae numbers found in this group during the third sampling. Moreover, a recent study showed the up regulation of *il-4/13a* and *b1* isoforms in gills after *P. perurans* infection at 12°C, with higher expression/cell migration-proliferation linked to higher AGD pathology with*a* and *b1* up regulated similarly for different levels of pathology, while *b2* expression was more correlated with infection level (Benedicenti et al., 2015; Benedicenti et al., 2017). Therefore, these results suggest a putative different expression among the different *il4/13* isoforms with *il4/13a* and *il4/13b1* providing a high basal expression but is less responsive to pathogen-associated molecular patterns (PAMPs) and pathogen challenge whilst *il4/13b2*, when activated, provides an enhanced type-2 immunity, which may have an important role in specific cell-mediated immunity (Wang et al., 2016). In head kidney, there was a significant difference in *il-4/13a* mRNA levels between groups A and B and between control groups C and D at the pre-challenge indicating an effect of temperature, with higher levels at 10°C. No significant differences were seen between infected groups and their respective controls post challenge indicating no effect of infection alone on expression/cell numbers in head kidney. Neither was a difference seen between the control groups C and D at sampling points post *P. perurans* exposure, indicating that any effect of temperature on expression/cell migration-proliferation had disappeared. However, there was a significant difference between infected groups A and B at 2 and 10 dpe with a higher mRNA level at the lower temperature, while *il-4/13b1* did not show any significant differences and *il-4/13b2* mRNA was not detectable by real time RT-PCR.

Two main types of macrophage populations are known that differ in terms of activation triggers and effector function: 1) the classically activated M1 macrophages induced by T_H1 cytokines that convert L-arginine to L-citrulline, producing NO and reactive nitrogen species, and 2) the alternatively activated M2 macrophages that express arginase after activation with T_H2 cytokines. In this study, gill results for *arg2a* indicate an interplay between infection and temperature in relation to its modulation. Infection at 10°C appears to suppress baseline mRNA levels compared to controls and compared to infection at 15°C at early infection

673 stages. No significant differences were observed at later infection stages. mRNA levels for
674 *arg2b* again indicate an interplay between temperature and infection, giving a significant
675 difference in levels between infected groups A and B at early infection stages, with infection
676 seeming to suppress baseline levels at 10°C but induce levels at 15°C. Temperature
677 differences alone, nor infection alone induced significant differences, such that neither
678 infected group had significantly different *arg2b* levels compared to their respective controls,
679 and control groups C and D also did not differ significantly. However, unexplained
680 significant differences in *arg2b* levels were observed between groups A and C, and B and D
681 pre-challenge which makes interpretation uncertain. No significant differences in mRNA
682 levels were detected for *arg2a* in head kidney. However, *arg2b* levels showed significant
683 differences in head kidney between infected groups A and B at pre-challenge and 21 dpe, and
684 between A and its control C at 21 dpe, with higher expression seen at the lower temperature
685 and in association with infection at the lower temperature respectively. This induction in
686 *arg2b* in group A in head kidney at 21 dpe contrasts with the *arg2b* suppression seen in
687 infected gills at 2 dpe. A similar pattern to that observed for *arg2a* is also observed with
688 respect to *inos* mRNA levels, with neither infection nor temperature differences alone giving
689 rise to significant differences, but the two combined resulting in significantly lower *inos*
690 mRNA levels in group A compared to group B at 2 and 10 dpe. There was a general trend for
691 higher levels at higher temperatures, in both infected and control groups. In head kidney, *inos*
692 expression levels were not sufficiently high to be detected by real time RT-PCR.
693 MHC I and II molecules interact respectively with CD8 on the surface of cytotoxic T cells or
694 with CD4 on the surface of helper T cells. In gills, *mhcI (UBA)*, *cd8a* and *cd8b* mRNA levels
695 showed a significant difference between the two infected groups mainly at 10 and 21 dpe,
696 with lower levels at 15°C. However, at 10 dpe, *cd8a* and *cd8b* mRNA levels in group B were
697 also significantly down regulated with respect to its control, indicating suppression of *cd8a*
698 and *cd8b* markers/cell types due to higher temperature/higher amoebae load at this stage. At 2
699 dpe, mRNA of *mhcI* was significantly suppressed compared to its control group C. At 21 dpe
700 infected groups A and B differed significantly in expression. However, the infected groups
701 did not differ in relation to their respective controls, indicating that differences were not due
702 to infection alone. Similarly, the two control groups C and D did not differ significantly in
703 mRNA levels indicating that temperature alone was not responsible for the difference in
704 groups A and B. Therefore, an interplay between infection and temperature may have driven
705 the difference between the infected groups, with infection and higher temperatures

suppressing *mhcI* expression in group B. *mhcII (DAB)* and *cd4* showed significantly higher mRNA levels in treatment group B (higher temperature and amoebae load) compared to group A at 2 dpe, perhaps again due to an interplay of temperature and infection, with no significant differences observed between infected fish and their control groups, nor between different temperature control groups. In head kidney, *mhcI (UBA)*, *mhcII (DAB)*, *cd4*, *cd8a*, and *cd8b* showed no significant differences between infected and respective control groups nor between temperature control groups at all sampling points. This might be explained as a local pathology acting at only the mucosal level and not the systemic level.

The three immunoglobulins known in teleosts, IgM, IgT and IgD were screened with primers amplifying both secreted and membrane forms (m/s) (Tadiso et al., 2011). In gills and kidney, *IgM (m/s)* was not significantly different between temperatures, nor between infected groups, nor between infected and respective control groups. *IgD (m/s)* showed a significant difference in gills between the infected groups A and B at the pre-challenge sampling, but no other differences were observed. *IgT (m/s)* was significantly reduced in gills of infected group A at 2 dpe compared to its control group C. No differences in mRNA levels were observed between infected and control groups at 15°C (B and D), nor between control groups C and D, indicating a suppression of IgT expression/associated cell type due to infection at 10°C, at early stages of the pathology. At 10 and 21 dpe, expression in infected group A was significantly higher than in infected group B, though neither A nor B were significantly different to their controls, indicating an interplay between infection and temperature resulting in lower IgT expression due to infection at higher temperature/higher amoebae load. However, a previous study on cellular markers of cell-mediated immunity (T cell receptor (*tcr*)-*α* chain, *cd4*, *cd8*, *mhcI*, *mhcIIα*), and antibody-mediated immunity (*IgM*, *IgT*) showed a classical inflammatory response in the gills of *P. perurans*-infected Atlantic salmon, with all the genes significantly up regulated in AGD-affected fish in comparison to control fish at 10 days post exposure to 2,000 amoebae/l at 16°C (Pennacchi et al., 2014). Moreover, a positive correlation between the *tcr-α* chain and *cd8* was shown, and it was hypothesized that the T-cells within the AGD affected gills were mainly constituted of $CD8^+$ cells and not $CD4^+$ T-cells. (Pennacchi et al., 2014). However, no transcriptional changes of *IgM*, *IgT*, *tcr*, and *cd8* mRNA levels were found in another study at a later stage of infection (31 days post exposure to 150 amoebae/l, and re-exposed to the parasite at the same density 5, 8 and 14 weeks later, to emulate a recurrent infection) at 16°C (Valdenegro-Vega et al., 2015), suggesting a down regulation during advanced stages of AGD. Although not significantly up regulated or down

regulated in the current study, the trend in *IgT* expression, with increased expression at earlier stages and decreased expression at later stages/higher amoebae load (15°C group B), resembles the results of the latter study. It is important to note however that differences exist between the studies (current and previous) including differences in pathogen exposure, temperature, and potentially in the pathology/infection status of the specific tissue sample taken, e.g. a second gill arch was sampled by Pennacchi et al. (2014) and a re-exposure to the parasite was performed by Valdenegro-Vega et al. (2015), potentially leading to differences in the results among studies.

In conclusion, this study shows an association between temperature and variation in AGD severity in Atlantic salmon, reflecting an earlier and stronger AGD histopathology, and higher amoebae numbers at the higher temperature (15°C). No significant difference between the two infected groups (A and B) was seen in cortisol levels in plasma, however glucose and lactate had increased levels associated with temperature (groups B and D) and with the presence of AGD (infection groups A and B) at the second sampling point, when gill pathology was first evident. Thus higher temperature and AGD pathology combined elevated these potential stress markers. Immune and stress related gene expression analysis showed modulation in gills rather than in head kidney, mainly during the first sampling point after challenge, with different expression levels between the two organs revealing an organ-specific response. Therefore, higher temperature (at 15°C) while linked mainly to earlier and stronger *P. perurans* infection through supporting greater proliferation of *P. perurans* on gills, at least for the amoeba clone used here (Benedicenti et al., 2018), may also act as a potential stressor in terms of changes in hormone levels in the plasma during early stages of pathology.

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6. TABLES

Table 1. Description of treatment and control groups.

Groups	Description
A	Fish exposed at 10°C to 500 cells/l of B8[§] clonal culture of *P. perurans* cultured for 3 months at 10°C
B	Fish exposed at 15°C to 500 cells/l of B8 clonal culture of *P. perurans* cultured for 3 months at 15°C
C	Fish exposed at 10°C to filtered culture media.
D	Fish exposed at 15°C to filtered culture media.

§ Collins et al. (2017)

Table 2. Histopathology features (category 1 - 5) used for AGD scoring system, based on the work of (McCarthy et al., 2015).

1	2	3	4	5
Stratification *focal* †	**Stratification** *focal to multifocal*	**Stratification** *multifocal* §	**Stratification** *multifocal to diffuse*	**Stratification** *diffuse* ¶
Hyperplasia *focal* • partial interlamellar filling • total interlamellar filling (1-10 ILU)	**Hyperplasia** *focal to multifocal* • partial interlamellar filling • total interlamellar filling (1-10 ILU)	**Hyperplasia** *multifocal* • partial interlamellar filling • total interlamellar filling (1-10 ILU)	**Hyperplasia** *multifocal to diffuse* • partial interlamellar filling • total interlamellar filling (> 10 ILU)	**Hyperplasia** *diffuse* • partial interlamellar filling • total interlamellar filling (> 10 ILU)
Mucous cells lined up *focal*	**Mucous cells** lined up *focal to multifocal*	**Mucous cells** lined up *multifocal*	**Mucous cells** lined up *diffuse*	**Mucous cells** lined up *diffuse*
Fusion of lamellae *focal*	**Fusion of lamellae** *focal to multifocal*	**Fusion of lamellae** *multifocal*	**Fusion of lamellae** *multifocal to diffuse*	**Fusion of lamellae** *diffuse*
		Stratification of filaments *absence*	**Stratification of filaments** *focal*	**Stratification of filaments** *multifocal*
	Fusion of filaments *absence*	**Fusion of filaments** *focal*	**Fusion of filaments** *multifocal*	**Fusion of filaments** *diffuse*
Spongiosis *absence* **Vesicles** or lacunae *absence*	**Spongiosis** *focal* **Vesicles** or lacunae *focal*	**Spongiosis** *focal* **Vesicles** or lacunae *focal to multifocal*	**Spongiosis** *multifocal* **Vesicles** or lacunae *multifocal*	**Spongiosis** *multifocal* **Vesicles** or lacunae *multifocal*
Epithelial and general hypertrophy *focal*	**Epithelial and general hypertrophy** *focal to multifocal*	**Epithelial and general hypertrophy** *multifocal*	**Epithelial and general hypertrophy** *multifocal to diffuse*	**Epithelial and general hypertrophy** *diffuse*
	Epithelial lifting & desquamation *focal*	**Epithelial lifting & desquamation** *focal to multifocal*	**Epithelial lifting & desquamation** *multifocal*	**Epithelial lifting & desquamation** *diffuse*
Necrosis *absence*	**Necrosis** *focal – one single spot*	**Necrosis** *focal to multifocal*	**Necrosis** *multifocal*	**Necrosis** *diffuse*
Infiltration inflammatory cells *focal* **Circulatory disturbance (thrombosis, aneurysm)** *absence*	Mild inflammatory response **Circulatory disturbance (thrombosis, aneurysm)** *focal*	Mild inflammatory response **Circulatory disturbance (thrombosis, aneurysm)** *focal to multifocal*	Mild inflammatory response **Circulatory disturbance (thrombosis, aneurysm)** *multifocal*	**Thrombosis** *diffuse* **Circulatory disturbance (thrombosis, aneurysm)** *diffuse* **Loss of pillar structure in affected areas in the middle of filaments**
> 90% gill without impairment	**70 - 90% gill without impairment**	**50 - 70% gill without impairment**	**20 - 50% gill without impairment**	**< 20% gill without impairment**

† *Focal:* a single lesion located within the colony surface and completely surrounded by living tissue.
§ *Multifocal:* two or more separated lesions surrounded by live tissue.
¶ *Diffuse:* Irregular patterns of tissue loss without a distinct margin and/or lacking a distinct annular or linear band or focal/multifocal lesion.
ILU= interlamellar unit, a unit is the distance between two lamellae.

956 **Table 3.** Atlantic salmon primer sequences used for Atlantic salmon gene expression analysis and *P. perurans*
957 load (by real time RT-PCR).

Gene		Oligonucleotides (5' – 3')	Accession number
ef1α	Forward	CAAGGATATCCGTCGTGGCA	AF321836
	Reverse	ACAGCGAAACGACCAAGAGG	
il-4/13a	Forward	CCACCACAAAATGCAAGGAGTTCT	NM_001204895
	Reverse	CCTGGTTGTCTTGGCTCTTCAC	
il-4/13b1	Forward	GCATCATCTACTGAGGAGGATCATGAT	HG794524
	Reverse	GCAGTTGGAAGGGTGAAGCATATTGT	
il-4/13b2	Forward	CTCAATGGAGGTTTGGAGTTTCAGG	HG794525
	Reverse	TGCAGTTGGTTGGATGAAACTTATTGT	
mhcI (UBA)	Forward	CTGCATTGAGTGGCTGAAGA	38 alleles
	Reverse	GGTGATCTTGTCCGTCTTTC	(Jørgensen et al., 2006)
mhcII (DAB)	Forward	AGATTCAACAGCACTGTGGGGAA	42 alleles
	Reverse	GTCTGACATGGGGCTCAACTGTCT	(Belmonte et al., 2014)
cd4	Forward	CGGAAGCGAGGGATATAAATGGTG	EU585750
	Reverse	GGCATCATCACCCGCTGTCT	
cd8a	Forward	GACAACAACAACCACCACGACTACAC	AY693393
	Reverse	GCATCGTTTCGTTCTTATCCGGTT	
cd8b	Forward	GATCAAACCCCAAAAGGCTGTG	AY693392
	Reverse	GACACTTTTTGGGTAGTTGGCTGAA	
arg2a	Forward	GACCACCTCTTGTCAAGGAAGCA	XP_014045709
	Reverse	CTCACGGGTCTGTCCTAGGGC	
arg2b	Forward	GACCACCTCTTGTCAAGGAAGCA	XP_014067199
	Reverse	CCATGGAAGCGGTGCTCG	
inos	Forward	GCTACACGACATGAAACACCCAGAGTT	DW469313 (EST)
	Reverse	GGACATCCTGGACATAGACCTTTGG	
hsp90α1a	Forward	AAAAAAACAGGAGGAGCTGAATT	KC150880
	Reverse	ATGTTGGCTGTCCACCCGTAGTTG	(de la Serrana & Johnston, 2013)
hsp90α2b	Forward	GAGAAGAAGGATGGGGAAGGAGAG	KC150879
	Reverse	CTTGTCCCCACATGCGCCATCG	(de la Serrana & Johnston, 2013)
hsp90β1	Forward	TGGATGAGGACAAGACAAAGTTCG	KC150882
	Reverse	GCTGAAGCCAGAGGAGAGGAGA	(de la Serrana & Johnston, 2013)
hsp90β2	Forward	AGGAGGACAAGACGAGGTTTGA	KC150883
	Reverse	GCTGAAGCCCGAAGAGAGCAATG	(de la Serrana & Johnston, 2013)
hsp30	Forward	CCGTTCAGGCAGATCAAACT	NP_001134440
	Reverse	GAGGAGCTGTCTGTCAAGCA	modified from (de la Serrana & Johnston, 2013)
hsp70	Forward	CCTGGTGAAGATGAGGGAGA	B5X4Z3
	Reverse	GTTCCCTGGACATGCCTTTG	(de la Serrana & Johnston, 2013)
IgM (m/s)	Forward	TGAGGAGAACTGTGGGCTACACT	XP_014058600.
	Reverse	TCTTAATGACTACTGAATGTGCA	modified from (Tadiso et al., 2011)
IgT (m/s)	Forward	CAACACTGACTGGAACAACAAGGT	ACX50292.1
	Reverse	CGTCAGCGGTTCTGTTTTGGA	(Tadiso et al., 2011)
IgD (m/s)	Forward	CCAGGTCCGAGTGGGATCA	AAD43527.1
	Reverse	TGGAGCAGGGTTGCTGTTG	(Tadiso et al., 2011)
P. perurans	Forward	GTTCTTTCGGGAGCTGGGAG	EF216903 – EF216905
	Reverse	GAACTATCGCCGGCACAAAAG	(Fringuelli et al., 2012)
	Probe	6-FAM-CAATGCCATTCTTTTCGGA	

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6. FIGURE LEGENDS

Fig. 1. Gill scores from 0 (no pathology) to 5 (greatest pathology) were used to assess the gill samples from the second gill arch (left-side). Treatments represent: **A)** fish exposed to 500 cells/l of *P. perurans* at 10°C; **B)** fish exposed to 500 cells/l of *P. perurans* at 15°C; **C)** fish exposed to filtered culture media at 10°C; **D)** fish exposed to filtered culture media at 15°C. Sampling points: 3 days before *P. perurans* exposure (dbe), and at 2, 10 and 21 days post exposure (dpe). Histopathology statistical analyses were performed in R (R software, software 3.0.1) using a proportional odds logistic regression which fits a logistic or probit regression model to an ordered factor response for the last two sampling points separately (Agresti, 2010). A stepwise *a posteriori* procedure was used to combine non-significant factor levels until the models' comparison was significant after models' comparison with the `anova` function ($p \leq 0.05$). Similar red letters indicate that different treatments/controls were grouped together based on the most similar parameter estimates.

Fig. 2. *P. perurans* load (18S rRNA) Cp values (mean ± 95% confidence interval) by treatment (fish exposed to 500 cells/l of B8 clonal culture of *P. perurans* at 10°C (A) or 15°C (B), and sampling points (2, 10 and 21 days post *P. perurans* exposure - dpe). The relationship between the *P. perurans* load (18S rRNA) Cp values among treatments and sampling points was tested with mixed-effects models in R (R software, software 3.0.1) and a statistical difference was found between the treatments at each sampling day ($p \leq 0.05$). Note: higher Cp values correspond to a lower expression of the 18S rRNA in the sample; lower Cp values correspond to a higher expression of the 18S rRNA in the sample.

Fig. 3. Cortisol (*a*), glucose (*b*) and lactate (*c*) concentrations by treatment and sampling point (mean ± 95% confidence interval). Treatments represent: fish exposed to 500 cells/l of B8 clonal culture of *P. perurans* at 10°C (A) or 15°C (B); fish exposed to filtered culture media at 10°C (C) or 15°C (D). Sampling points: 3 days before *P. perurans* exposure (dbe), and at 2, 10 and 21 days post exposure (dpe). Statistical analysis was performed in R (R software, software 3.0.1) with mixed-effects models. Statistical analysis of cortisol data was performed on Log transformed data. A stepwise *a posteriori* procedure was used to combine non-significant factor levels until the models' comparison was significant after models' comparison with the `aov` function ($p \leq 0.05$) for the generalised linear mixed-effects model. Values are expressed as mean ± 95% confidence interval and similar red letters indicate that different treatments/controls were not statistically different (linear mixed-effects model).

Fig. 4. Transcript expression level of genes related to markers of macrophage activation in gills and head kidney determined by real time RT-PCR and expressed as arbitrary units normalized against the expression level of ef1α (mean ± 95% confidence interval). Fish exposed to 500 cells/l of B8 clonal culture of *P. perurans* at 10°C (A) or 15°C (B); fish exposed to filtered culture media at 10°C (C) or 15°C (D). Sampling points: 3 days before *P. perurans* exposure (dbe), and at 2, 10 and 21 days post exposure (dpe). Statistical analysis was performed in R (R software, software 3.0.1) with a linear mixed-effects model. Similar letters indicate that different treatments were not statistically different ($p > 0.05$, $n = 40$).

Fig. 5. Transcript expression level of genes related to cellular markers of antigen presenting cells in gills and head kidney determined by real time RT-PCR and expressed as arbitrary units normalized against the expression level of ef1α (mean ± 95% confidence interval). Fish exposed to 500 cells/l of B8 clonal culture of *P. perurans* at 10°C (A) or 15°C (B); fish exposed to filtered culture media at 10°C (C) or 15°C (D). Sampling points: 3 days before *P. perurans* exposure (dbe), and at 2, 10 and 21 days post exposure (dpe). Statistical analysis was performed in R (R software, software 3.0.1) with a linear mixed-effects model. Similar letters indicate that different treatments were not statistically different ($p > 0.05$, $n = 40$).

Fig. 6. Transcript expression level of genes related to T_H2 markers in gills and head kidney determined by real time RT-PCR and expressed as arbitrary units normalized against the expression level of ef1α (mean ± 95% confidence interval). Fish exposed to 500 cells/l of B8 clonal culture of *P. perurans* at 10°C (A) or 15°C (B); fish exposed to filtered culture media at 10°C (C) or 15°C (D). Sampling points: 3 days before *P. perurans* exposure (dbe), and at 2, 10 and 21 days post exposure (dpe). Statistical analysis was performed in R (R software, software 3.0.1) with a linear mixed-effects model. Similar letters indicate that different treatments were not statistically different ($p > 0.05$, $n = 40$).

Fig. 7. Transcript expression level of genes related to immunoglobulins in gills and head kidney determined by real time RT-PCR and expressed as arbitrary units normalized against the expression level of ef1α (mean ± 95% confidence interval). Fish exposed to 500 cells/l of B8 clonal culture of *P. perurans* at 10°C (A) or 15°C (B); fish exposed to filtered culture media at 10°C (C) or 15°C (D). Sampling points: 3 days before *P. perurans* exposure (dbe), and at 2, 10 and 21 days post exposure (dpe). Statistical analysis was

performed in R (R software, software 3.0.1) with a linear mixed-effects model. Similar letters indicate that different treatments were not statistically different ($p > 0.05$, $n = 40$).

Fig. 8. Transcript expression level of genes related to stress in gills and head kidney determined by real time RT-PCR and expressed as arbitrary units normalized against the expression level of ef1α (mean ± 95% confidence interval). Fish exposed to 500 cells/l of B8 clonal culture of *P. perurans* at 10°C (A) or 15°C (B); fish exposed to filtered culture media at 10°C (C) or 15°C (D). Sampling points: 3 days before *P. perurans* exposure (dbe), and at 2, 10 and 21 days post exposure (dpe). Statistical analysis was performed in R (R software, software 3.0.1) with a linear mixed-effects model. Similar letters indicate that different treatments were not statistically different ($p > 0.05$, $n = 40$).

Fig. 1

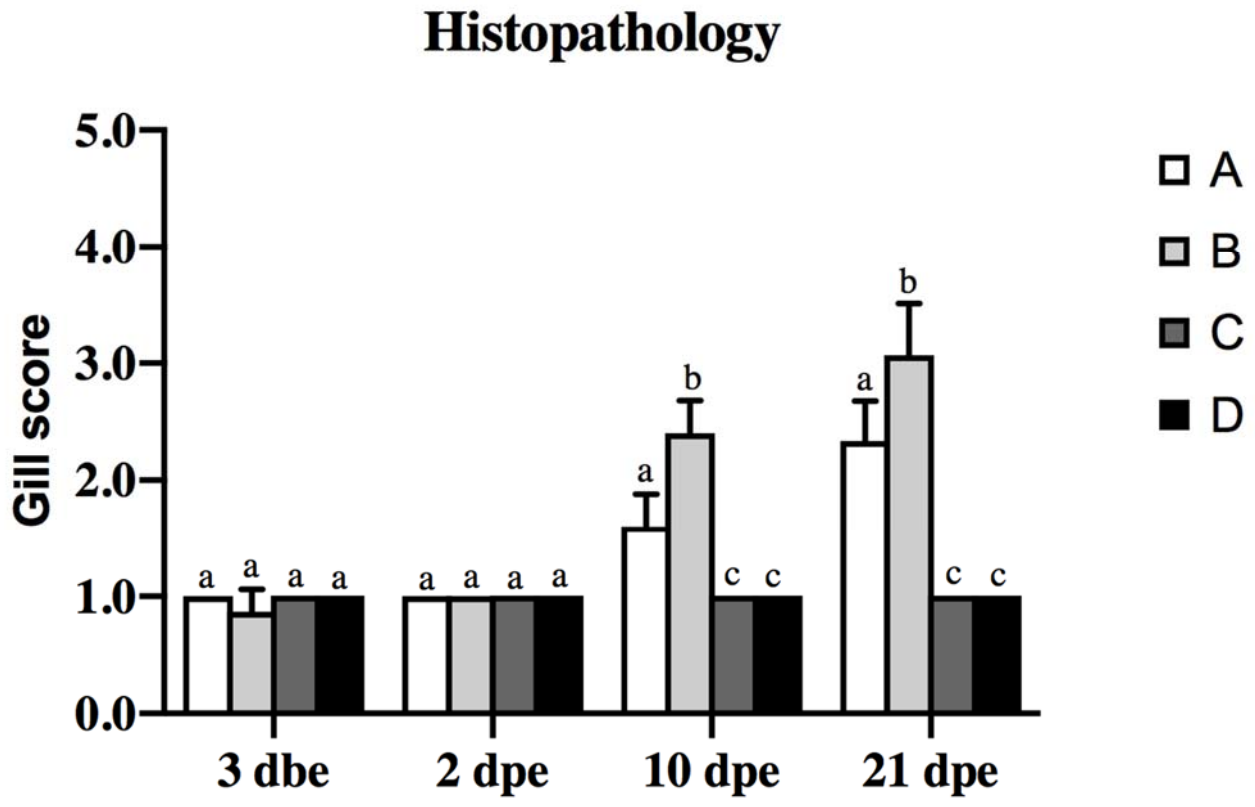

Histopathology
Gill score
5.0
4.0
3.0
2.0
1.0
0.0
a a a a
a a a a
a b
c c
a b
c c
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D

Fig. 2

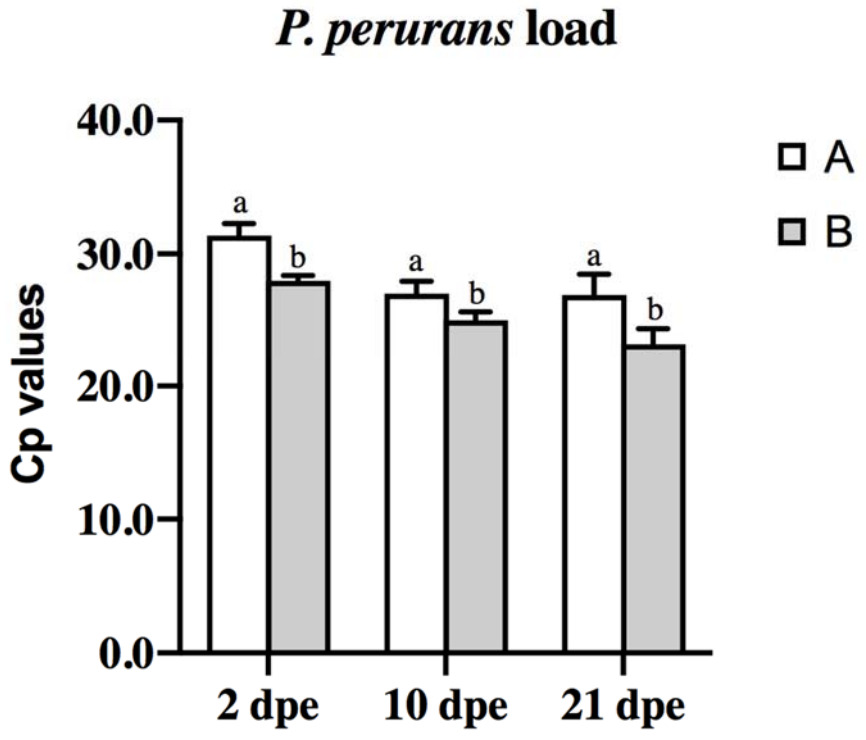

P. perurans load
Cp values
40.0
30.0
20.0
10.0
0.0
a b
a b
a b
2 dpe
10 dpe
21 dpe
A
B

Fig. 3

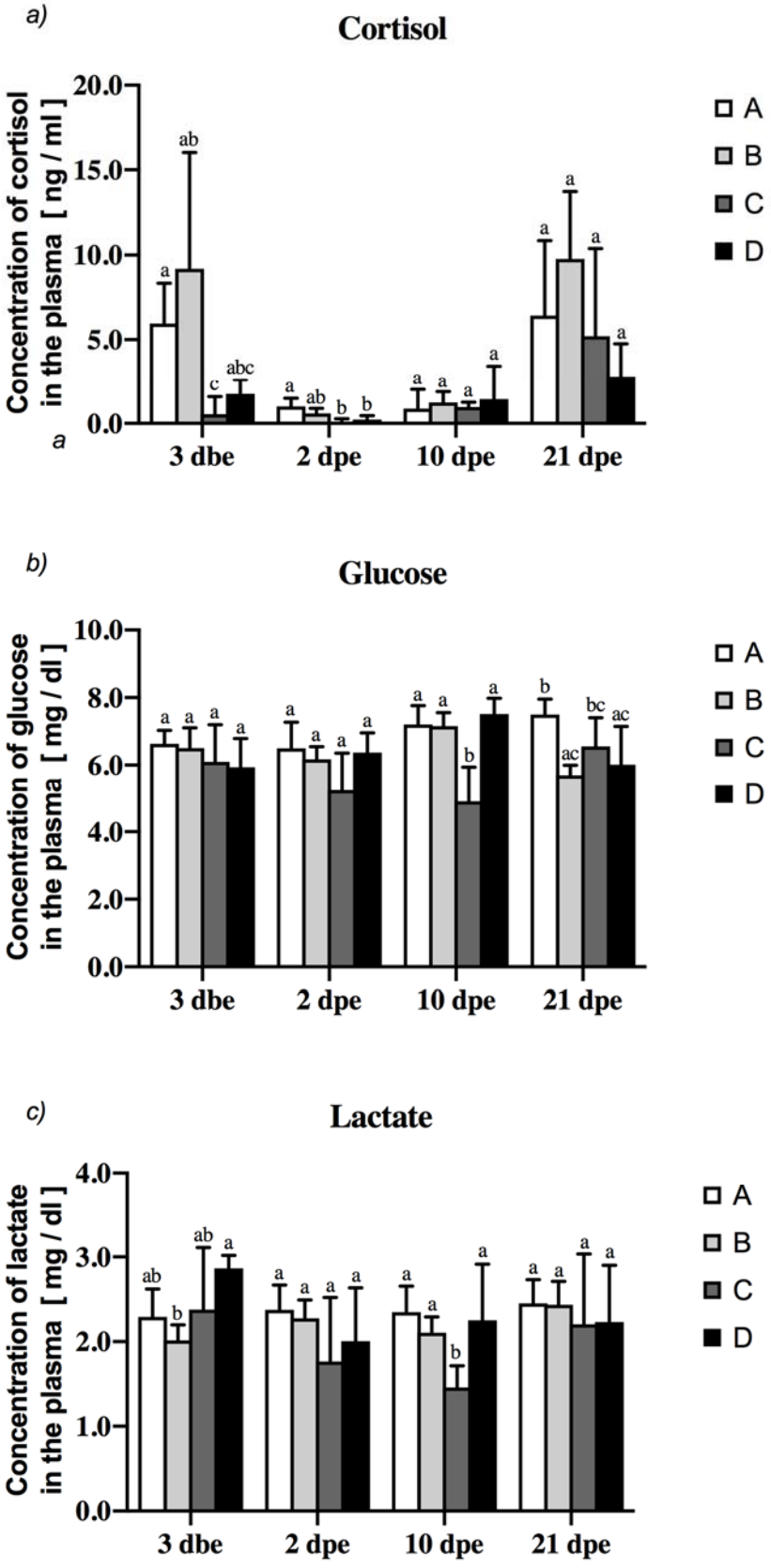

a)
Cortisol
Concentration of cortisol in the plasma [ng / ml]
20.0
15.0
10.0
5.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D
b)
Glucose
Concentration of glucose in the plasma [mg / dl]
10.0
8.0
6.0
4.0
2.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D
c)
Lactate
Concentration of lactate in the plasma [mg / dl]
4.0
3.0
2.0
1.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D

Fig. 4

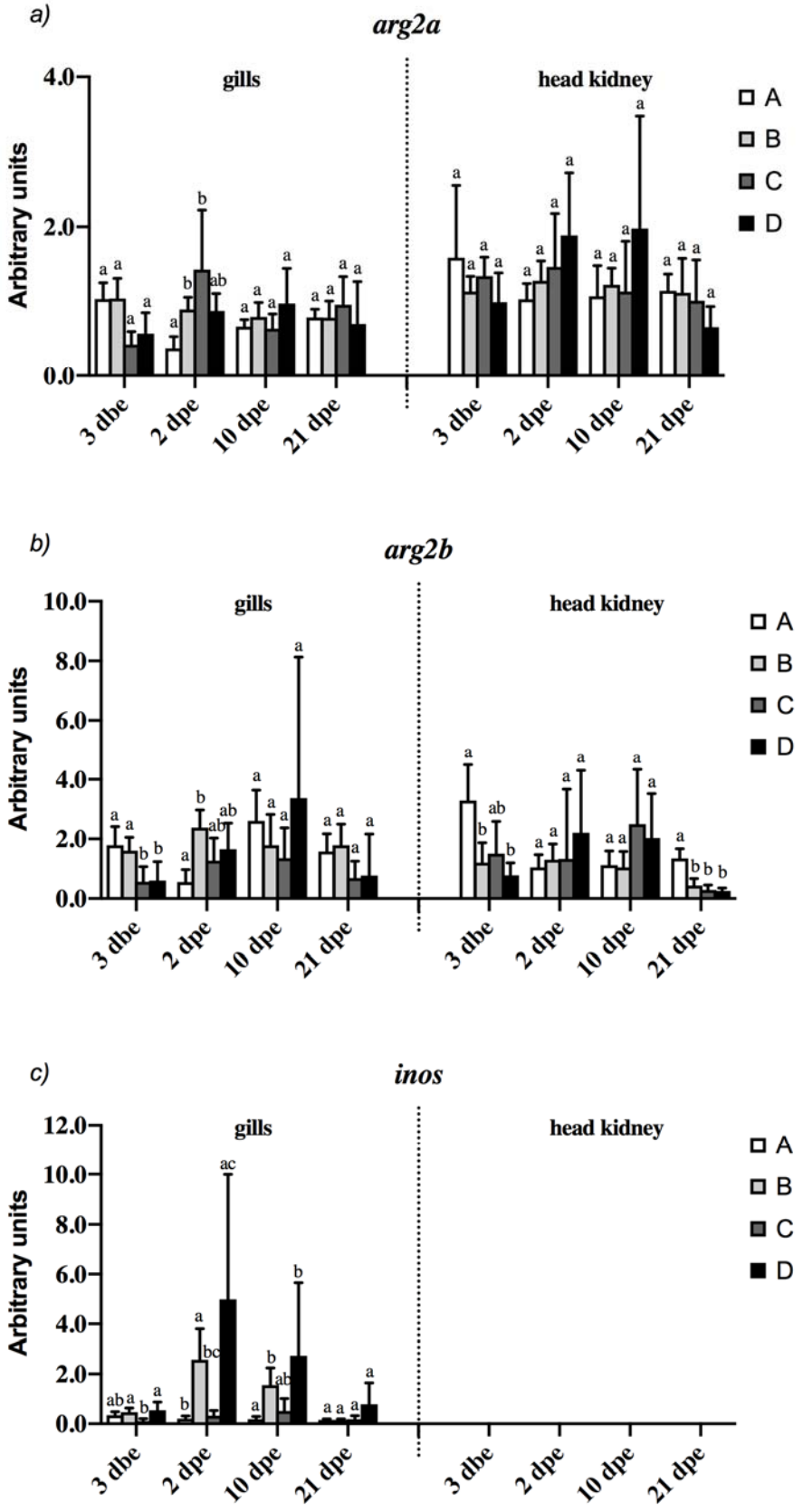

a)
arg2a
gills
head kidney
A
B
C
D
Arbitrary units
4.0
2.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
b)
arg2b
gills
head kidney
A
B
C
D
Arbitrary units
10.0
8.0
6.0
4.0
2.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
c)
inos
gills
head kidney
A
B
C
D
Arbitrary units
12.0
10.0
8.0
6.0
4.0
2.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe

Fig. 5

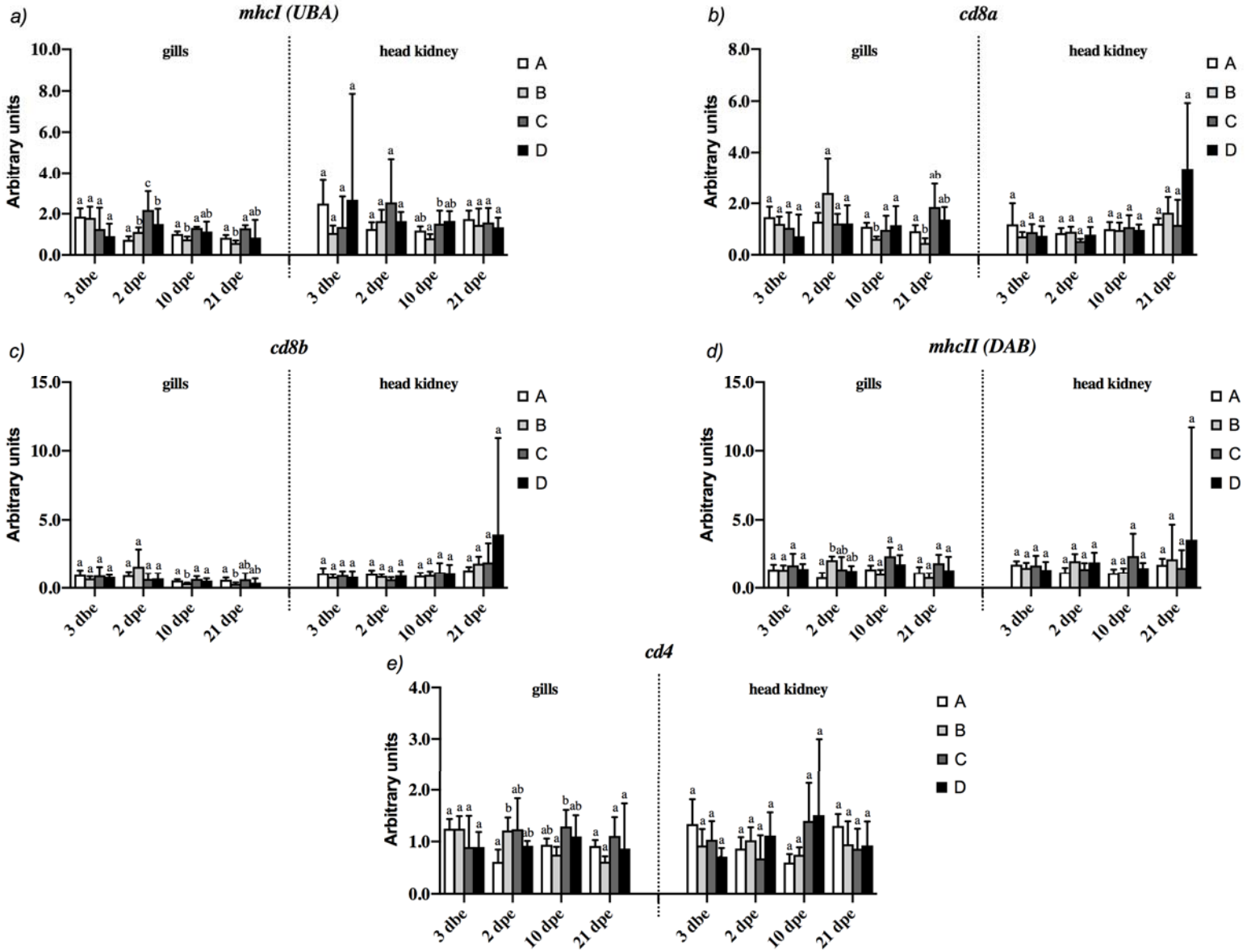

a)
mhcI (UBA)
b)
cd8a
c)
cd8b
d)
mhcII (DAB)
e)
cd4
gills
head kidney
Arbitrary units
A
B
C
D
3 dbe
2 dpe
10 dpe
21 dpe

Fig. 6

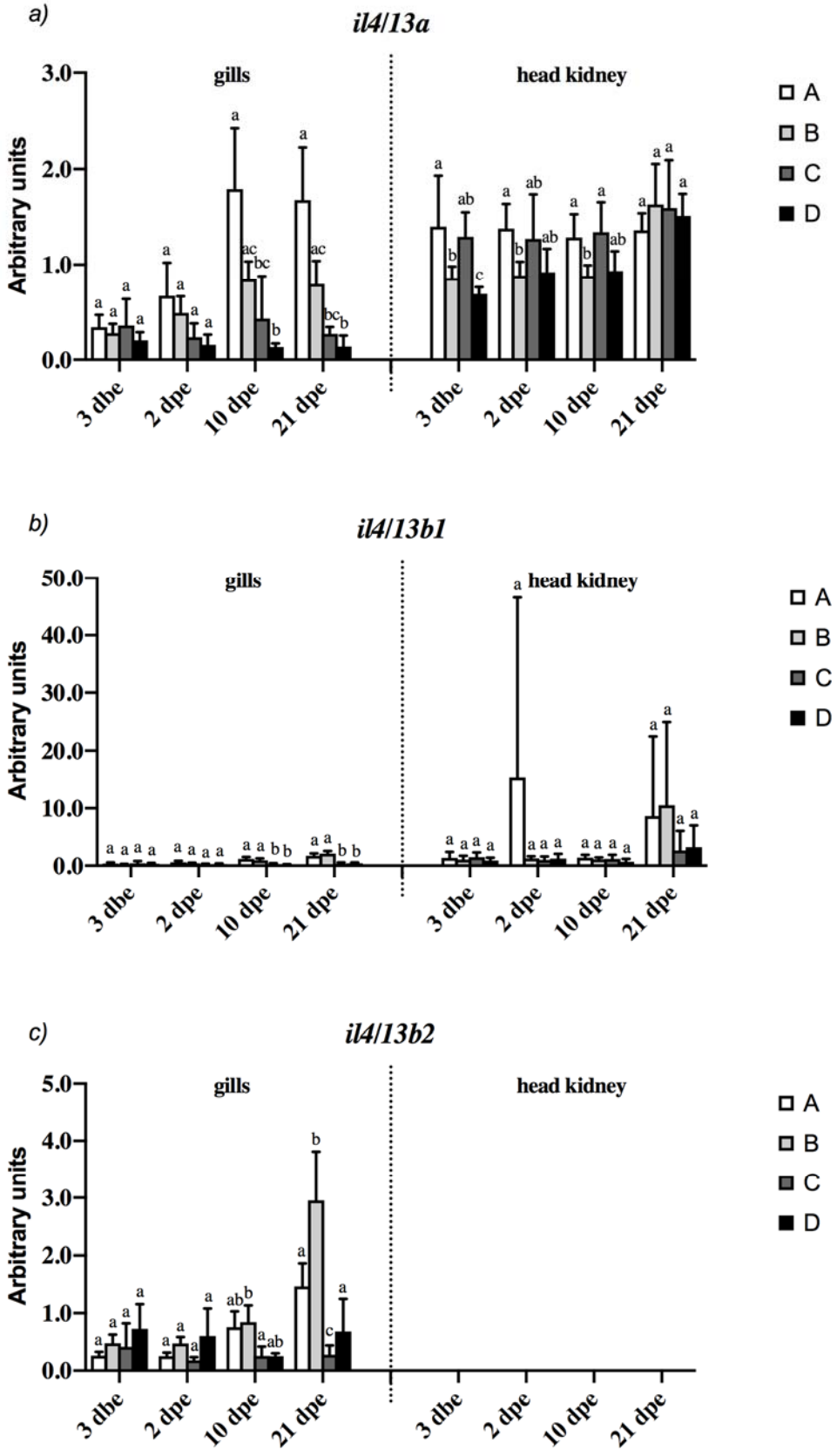

a)
il4/13a
gills
head kidney
Arbitrary units
3.0
2.0
1.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D
b)
il4/13b1
gills
head kidney
Arbitrary units
50.0
40.0
30.0
20.0
10.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D
c)
il4/13b2
gills
head kidney
Arbitrary units
5.0
4.0
3.0
2.0
1.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D

Fig. 7

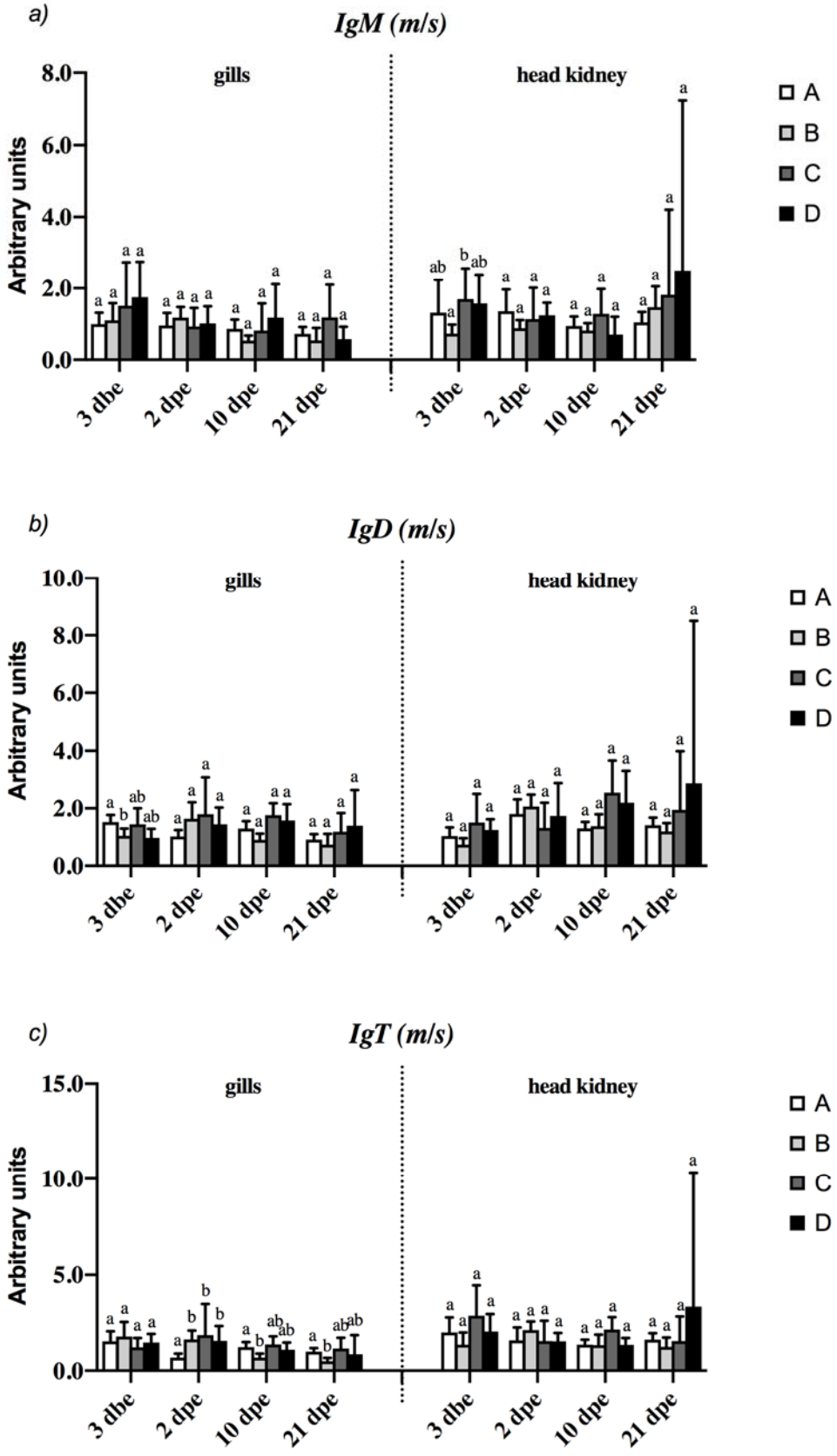
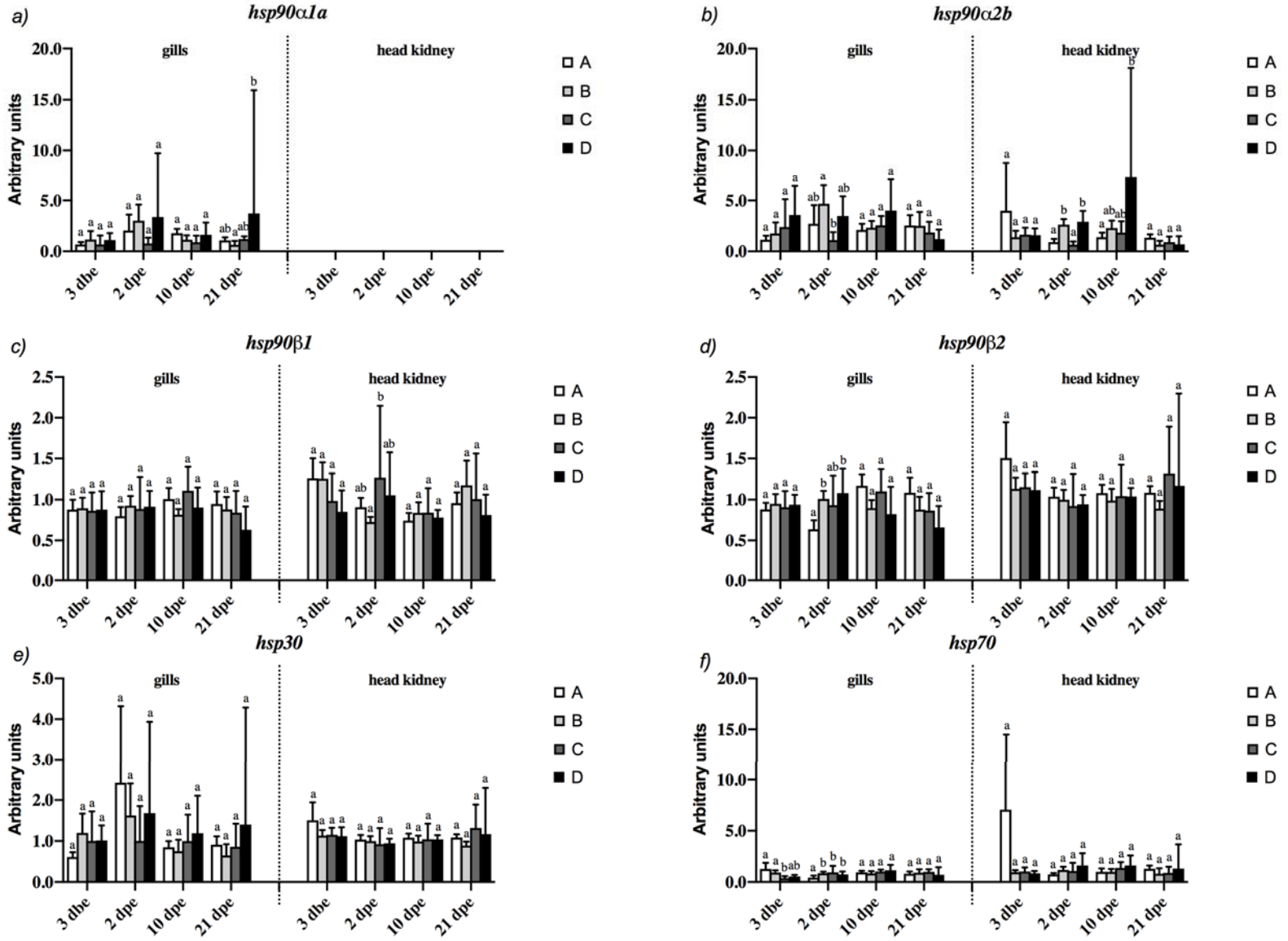

a)
IgM (m/s)
gills
head kidney
Arbitrary units
8.0
6.0
4.0
2.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D
b)
IgD (m/s)
gills
head kidney
Arbitrary units
10.0
8.0
6.0
4.0
2.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D
c)
IgT (m/s)
gills
head kidney
Arbitrary units
15.0
10.0
5.0
0.0
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D

Fig. 8


a)
hsp90α1a
b)
hsp90α2b
c)
hsp90β1
d)
hsp90β2
e)
hsp30
f)
hsp70
gills
head kidney
Arbitrary units
3 dbe
2 dpe
10 dpe
21 dpe
A
B
C
D