Atlantic salmon post-smolts adapted for a longer time to seawater develop an effective humoral and cellular immune response against Salmonid alphavirus

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ABSTRACT

Salmonid alphavirus (SAV) causes pancreas disease (PD) in Atlantic salmon (*Salmo salar* L.) and disease outbreaks are mainly detected after seawater transfer. The influence of the smoltification process on the immune responses, specifically the adaptive response of Atlantic salmon after SAV infection, is not fully understood. In this study, Atlantic salmon post-smolts were infected by either bath immersion (BI) or intramuscular injection (IM) with SAV subtype 3, 2 weeks (Phase A) or 9 weeks (Phase B) after seawater transfer. The transcript levels of genes related to cellular, humoral and inflammatory responses were evaluated on head kidney samples collected at 3, 7, 14, 21, and 28 days post-infection (dpi). Corresponding negative control groups (CT) were established accordingly. Significant differences were found between both phases and between the IM and BI groups. The anti-inflammatory cytokine IL-10 was up-regulated in Phase A at a higher level than in Phase B. High mRNA levels of the genes RIG-1, SOCS1 and STAT1 were observed in all groups except the BI-B group (BI-Phase B). Moreover, the IM-B group showed a higher regulation of genes related to cellular responses, such as CD40, MHCII, and IL-15, that indicated the activation of a strong cell-mediated immune response. CD40 mRNA levels were elevated one week earlier in the BI-B group than in the BI-A group (BI-Phase A). A significant up-regulation of IgM and IgT genes was seen in both IM groups, but the presence of neutralizing antibodies to SAV was detected only in Phase B fish at 21 and 28 dpi. In addition, we found differences in the basal levels of some of the analysed genes between non-infected control groups of both phases. Findings suggest that Atlantic salmon post-smolts adapted for a longer time to seawater before they come into contact with SAV, developed a stronger humoral and cell-mediated immune response during a SAV infection.

Keywords: *Salmo salar*, bath immersion, adaptive response, smoltification,, Immunoglobulin, B cells, RIG-1, SOCS1, CD40, Interleukin.

1. Introduction

Pancreas disease (PD) is a systemic disease characterized by inflammation and cellular necrosis in exocrine pancreas and subsequent severe cardiac and skeletal myopathies, which results in high morbidity and mortality of farmed Atlantic salmon (*Salmo salar*) [1]. Salmonid alphavirus (SAV), a single-stranded, positive-sense RNA virus of the family *Togaviridae*, is the aetiological agent of PD. Six geographically distributed subtypes of SAV have been described on the basis of partial sequences of the E2 and nsP3 genes [2]. PD has been described in Atlantic salmon farms in Scotland, Norway, Ireland, France, Spain, and North America [3]. PD outbreaks in south-western Norway are mainly associated with Salmonid alphavirus subtype 3 (SAV3), which can be spread horizontally between neighboring salmon farms [4,5].

Atlantic salmon aquaculture is normally carried out in two phases: firstly, in land-based hatcheries where smolts are produced in freshwater tanks using flow-through and re-circulation systems; and later in an on-growing phase in seawater cages until harvest [6]. It is during the seawater phase that the farmed Atlantic salmon are most vulnerable to infection from diseases and parasites partly due to less possibility of controlling the culture conditions. For instance, natural outbreaks of PD in Atlantic salmon have only been reported in the seawater phase [7] and therefore PD outbreaks and higher losses are reported in the months after seawater transfer (SWT). It is known that smoltification and the initial period following seawater transfer is a time of high energy requirement due to the necessary physiological changes to adapt to seawater. The smoltification process (also known as Parr-Smolt transformation) is a series of physiological, morphological and behavioral changes [8,9]. Moreover, immune suppression characterized by a decrease of total white blood cells and circulating lymphocytes, a decrease

of plasma lysozyme activity, total serum immunoglobulin (Ig) M and total serum protein, and a down-regulation of some immune genes has been described during smoltification [9-12]. These data suggest that Atlantic salmon in the process of smoltification and in the post-smolt period may be especially vulnerable to infectious diseases. A link between smoltification and susceptibility to Infectious Salmon Anemia Virus (ISAV) was proposed by Glover et al., 2006 [13]. Despite the use of vaccines, the PD challenges in salmon farms persist, but few studies have evaluated immune responses of Atlantic salmon to PD a few weeks after seawater transfer. Previous studies employing injection or cohabitation challenge models have demonstrated a high level of expression of IFN-γ, two IFN-γ responsive genes, and TCR-β genes, suggesting a sustained acquired immune response in the skeletal muscle [14]. Moreover, a rapid activation of MHC I and II responses during the early stages of SAV subtype 1 infection have been reported [15]. Enhanced expression of T cell-related genes such as, T cell co-receptors CD4-1 and CD8α, and TCR-β, as well as MHCII, normally expressed only on antigen-presenting cells, have been detected during the later stages of SAV3 infection [16,17,14].

We have established a bath challenge model based on the viral shedding capacity of Atlantic salmon experimentally injected with SAV. When used in a bath challenge of naïve fish this SAV3 containing water mimics the natural exposure to water-borne virus. Compared to cohabitation challenge, a bath challenge model has the advantage of limiting the infection time to a small window which is useful when studying the time frame of the subsequent immune responses. The susceptibility of Atlantic salmon post-smolts at 2 and 9 weeks post seawater transfer (wpt) to SAV3 by comparing fish infected via the bath challenge (BI) model and by intramuscular (*i.m.*) injection (IM) has been reported [18]. In addition, we have studied the immune-related gene regulation of some antiviral immune genes and genes involved in the pathways leading to interferon (IFN) regulation and the production of ISGs. Smolts fully acclimatized to seawater showed an increased innate immune response to SAV infection and a

higher basal abundance of several innate immune genes relative to the smolts that were infected shortly after seawater transfer [19].

In the present work, we have focused on analysing transcript levels of genes directly or indirectly involved in adaptive responses, including genes that express cell surface receptors/markers, in Atlantic salmon post-smolts transferred to seawater seven weeks apart and then infected with SAV3 by BI or IM. The obtained data will give a more comprehensive understanding of the factors determining survival of salmon suffering from pancreas disease after seawater transfer and the associated immunological events.

2. Materials and Methods

2.1 Experimental design, tissue collection, and RNA extraction

Atlantic salmon post-smolts from the same batch of fish (Aquagen strain) were challenged with SAV3 either 2 (Phase A, average weight of 41g) or 9 (Phase B, average weight of 89 g) weeks after seawater transfer (oxygen saturation of >85 %; 12 °C; salinity 34.5‰). Na^+, K^+-ATPase (NKA) enzyme activity was evaluated in the gills of fish from Phase A and acceptable NKA activity was observed, and thus this was deemed to have no influence on SAV infection [18]. NKA levels were not evaluated in fish from Phase B because both groups belonged to the same production batch and after 9 weeks, the fish appeared well adapted to seawater.

The experimental setup consisted of three groups: negative control (CT); intramuscular injection (IM); or bath immersion (BI) and each of these groups were in triplicate tanks with 65 fish per tank (Fig. 1A). Fish in the IM group were infected with 10^4 $TCID_{50}$ SAV3 propagated in Chum salmon heart-1 (CHH-1) cells [20] and quantified by end-point dilution assay (TCID50) [21]. Fish in the BI group were bath immersed for 6 h in seawater containing SAV3, which was shed by shedder fish injected with 10^4 TCID50 SAV3/fish one week before the experiment started. The bath immersion dose was estimated by a one-step RT-qPCR assay

of filtered/concentrated shedder tank water, which resulted in an average Ct value of 28 and 34 in Phases A and B, respectively [18]. Infected groups from Phase A were named IM-A and BI-A, and infected groups from Phase B were named IM-B and BI-B. Fish belonging to the control group from Phase A (CT-A) and from Phase B (CT-B) were injected with supernatant of non-infected cultures of CHH-1 cells. The SAV3 isolate prepared for use in this experiment was later found to be contaminated with low levels of infectious pancreatic necrosis virus (IPNV). However, compared with the SAV3, the level of IPNV was so low that it is unlikely to have caused any discernable effect on the interpretation on the immune gene regulation evaluated post-infection. In fact, anterior kidney samples from the BI-A group were IPNV negative, and only 6 fish in the IM-A group and 4 fish in the IM-B group were IPNV positive, with very low virus loads (Cts all >36) [22]. Before handling, the fish were bath anaesthetized with a mixture of Metomidate (10 mg L^{-1}) and Benzocaine (60 mg L^{-1}), and before sampling, fish were killed employing Metomidate and a higher dose of Benzocaine (160 mg L^{-1}). The animal care and experimental setup were performed in accordance with the established guidelines and approved by the Norwegian Animal Research Authority (ID: 5651). Further information on experimental procedures, fish, and virus have been published previously [18].

Total RNA from head kidney tissue samples described in this study were previously used to study innate immune gene responses [23,19]. Briefly, head kidney tissue samples were collected from fish in each treatment group and frozen in liquid nitrogen at 3, 7, 14, 21, and 28 days post-infection (dpi). Total RNA was extracted using an iPrep™ PureLink® Total RNA Kit (Invitrogen, USA.) with TRIzol® reagent (Ambion) and following the manufacturer's instructions. RNA concentration was measured using a Nanodrop ND-1000. Five percent of the RNA samples from tissues were randomly chosen and checked for integrity on a Bioanalyser (Agilent Instruments), resulting in RINs of ≥ 9 for all samples tested.

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148 **2.2 cDNA synthesis and quantitative PCR (RT-qPCR)**

149 qScript™ SuperMix (Quanta Biosciences) was used to transcribe cDNA from 1 µg head kidney
150 total RNA in a 20 µl reaction including priming with both random hexamers and Oligo-dT as
151 described in the manufacturer's instructions. The cDNA was diluted 1:10 with AMRESCO's
152 sterile, Nuclease-free water (VWR) as this was previously demonstrated to be an optimal
153 dilution for the analysis performed in this study. Assays for CD40, IgM and IgT, SOCS1, CD4-
154 1, CD8α, Retinoic-acid-inducible protein 1 (RIG-1), Nuclear factor kappa-light-chain-
155 enhancer of activated B cells (NFκB), four Interleukins (IL), and the IL-2 Receptor β chain
156 protein (IL2Rβ) were designed for use in this study. Target-specific RT-qPCR primers were
157 designed to either span exon-exon boundaries, or to have at least one primer spanning an exon
158 boundary of the gene of interest. All assay products were visualized on a 3% MetaPhor®
159 Agarose gel (Lonza) and sequenced to verify the specificity of the assay. Assays for Signal
160 transducer and activator of transcription 1 (STAT1), MHCIIβ, and Elongation factor 1A
161 (EF1A) were adapted from previously published studies [24-26]. Efficiency was calculated for
162 each primer set using triplicates of a five-point, 4 x dilution series of pooled cDNA. EF1A was
163 used for normalization and is considered the best option of several endogenous reference genes
164 evaluated for use with Atlantic salmon during SAV infection [27]. All primers and efficiency
165 data for the assays used in this study are listed in Table 1.
166 RT-qPCR was run in 384 well plates using Brilliant III Ultra-Fast SYBR® Green master mix
167 (Agilent) and Applied Biosystems 7900HT Fast Real-Time PCR system in a 7 µL reaction
168 volume containing 2 µL diluted cDNA and 400 nM of each primer. The running conditions
169 were as recommended by the manufacturer and a melting curve analysis was included for each
170 run. The Ct values were normalized using the Ct values from the EF1A assay run on the same
171 plate for each individual (ΔCt). Fold change ($2^{-\Delta\Delta Ct}$) of transcript level for each individual was

calculated by subtracting the relevant mean ΔCt values obtained from 3 calibrator fish, sampled before the start of the experiment (Day 0). Outliers were not removed from any of the data sets for either analysis or presentation in the figures, as they represent the real biological diversity within these groups.

2.3 Neutralizing antibody titres to SAV

Blood was collected by caudal vein puncture and plasma was obtained by centrifuging the blood at 16000 g for 15 min at 4 °C the day of collection. Serum was allowed to clot overnight and similarly separated the following day. All sera and plasma samples were stored at -80°C until analysis. Eighteen plasma samples were randomly chosen from all groups in both Phase A and B and eighteen serum samples were randomly chosen from all groups in Phase B at 21 and 28 dpi (with the exception that all individuals positive for SAV in the BI-B group were included). Serum samples were only taken from Phase B fish. Neutralizing antibody titres to SAV were measured by Agri-Food and Biosciences Institute (AFBI) in Belfast (UK) [28].

2.4 Data Analysis

The data from RT-qPCR were transformed (+1, $\log_{10}$) and the normal distribution was proved by Levene's test employing the Statistica software package. Statistical analysis for immunological parameters was performed by Graph Pad Prism 6.0 statistical software package. The statistical significance between groups in Phase A and Phase B and between both phases was determined using One-way ANOVA on log transformed data followed by Tukey's multiple comparisons test. Results showing $p < 0.05$ are considered significantly different. Although these methods use averages in their calculations, medians were used for discussion and visual representation of the data, because of the asymmetric distribution of the data.

Average transcript levels of the genes analysed in positive and negative fish in both infected groups and in the control group are also included (Supplementary figures S1 and S2). The statistical significance between control fish and fish either positive or negative for SAV3 RNA was estimated for each tested gene and also between all sampling time-points and also for both phases when there was more than one positive or negative fish per sampling time-point (data not shown).

3. Results

3.1 SAV3 infection

Two groups of Atlantic salmon post-smolts from the same batch, which were transferred to seawater 7 weeks apart, were infected with SAV3 by IM or BI. The percentage prevalence at each time point was determined by analysing the SAV3 RNA in heart tissue [18] (Fig. 1B). Briefly, at the earliest sampling time-points the fish in the IM-A group had a higher prevalence of SAV infection and higher viral loads than fish in the BI-A group. By 14 dpi the prevalence in the IM-A and BI-A groups was 95.8% and 100%, respectively, but viral loads were still lower in the BI-A group. At later time-points, all the BI-A fish were positive (100% prevalence) and significantly higher amounts of SAV3 were detected. A maximum of 2 fish were negative in the IM-A group at the last two sampling time-points.

In Phase B, 95% of the fish in the IM-B group were SAV3 positive at 7 dpi and they remained almost 100% positive at all later time-points. The viral load in the IM-B group increased up to 14 dpi after which it decreased, falling to levels similar to those seen at 7 dpi levels by 28 dpi. The BI-B group had only a few positive fish at 14 dpi and this remained the same until the end of the experiment. At 21 dpi the SAV positive fish in the BI-B group also had relatively low viral loads.

3.2 Transcript Profiles of Immune Response Genes

We have performed RT-qPCR on head kidney samples collected at 3, 7, 14, 21, and 28 dpi in order to study the effect of the extra time in seawater on transcript levels of immune genes related to cellular, humoral, and inflammatory responses. The reported changes in transcript levels are from median values calculated for the whole group that comprised 8 fish sampled from triplicate tanks (altogether 24 individuals per treatment at each sampling time-point). Many of the changes are heavily influenced by the infection status of the fish since not all fish exposed to SAV were SAV3- positive at all time-points. Specifically, infected fish in the BI-B group from Phase B were only found at 14, 21, and 28 dpi. Where these differences between positive and negative fish are significant, they are mentioned.

Inflammatory response

Transcript levels of IL-18, IL-10, RIG-1, SOCS1 and STAT1 genes, were also evaluated in this study to supplement our previous studies describing transcript abundance of other genes involved in the inflammatory response [23,19]. The mRNA level of pro-inflammatory fish cytokine IL-18 was down-regulated in all groups in Phase A compared to the calibrator fish taken before the experiment started. Conversely, in Phase B, IL-18 mRNA levels remained stable in most individuals throughout the experiment (Fig. 2). Interestingly, a significantly higher transcript abundance was detected at 7 dpi in SAV3-positive fish in the IM-B group. At 14 dpi some of the individuals from the IM-B group presented a 5- and 17-fold increase when the median value of the group was 1-fold.

IL-10, an anti-inflammatory cytokine and member of the class II cytokine family, was highly regulated at 7 and 14 dpi in the IM-A fish, reaching a peak at 7 dpi with 20-fold increases. IL-10 displayed a 5-fold up-regulation in the BI-A group at 14 dpi and it remained significantly elevated at 21 and 28 dpi. In contrast, IL-10 mRNA levels in fish from the IM-B group only

showed a 4-fold increase at 14 dpi in Phase B and was almost unchanged in the BI-B group at all time-points (Fig. 2).

The viral RNA sensor, RIG-1, was highly up-regulated in Phase A. In the IM-A group, the RIG-1 mRNA levels peaked with 17- and 14-fold increases at 7 and 14 dpi, respectively, followed by a drop at 21 dpi. Fish in the BI-A group also showed significant up-regulation of RIG-1 transcript levels already at 7 dpi, which peaked with significant 20- and 15-fold increases at 14 and 21 dpi, respectively. The transcription pattern of RIG-1 was different in Phase B, where the maximum regulation was seen at 14 dpi in both the IM-B and BI-B groups, with moderate fold increases of 7.5 and 3, respectively. Furthermore, mRNA levels of RIG-1 had decreased by 28 dpi (Fig. 3).

Similar to RIG-1, the mRNA level of SOCS1 was higher in Phase A than in Phase B, with an increase for both infected groups at 7, 14 and 21 dpi in Phase A (Fig. 3). In the IM-A group, SOCS1 transcript levels peaked at 7 dpi but quickly decreased, while the BI-A group peaked at 14 dpi to a level higher than the IM-A group at 21 dpi. Both returned to control levels at 28 dpi. Fish positive for SAV3 RNA in the IM-A and BI-A groups exhibited higher SOCS1 transcript levels than the negative fish at 7 dpi (S1). Similarly, in Phase B, positive fish showed much higher mRNA levels of SOCS1 at 7 and 14 dpi in IM-B and at 14 and 21 dpi in BI-B relative to the negative fish at the same time-points (S2).

STAT1 had a similar pattern of modulation to SOCS1 with both the IM-A and BI-A groups showing increases, while in Phase B, only the IM-B group had significant fold increases (Fig. 3). However, the same positive individuals exhibiting elevated fold increases of SOCS1 also had high fold increases of STAT1 (S1 and S2).

Cellular response

The regulation of CD40, CD8α, CD4-1, MHCIIβ, NFκB, IL-2Rβ, IL-2 and IL-15 genes was measured in an effort to evaluate cellular immune responses during the experiment. The level of the costimulatory molecule CD40 mRNA was up-regulated in both the IM and BI groups in Phase A and B. The highest fold change in the IM-A and BI-A groups, corresponding to a 2-fold up-regulation compared to the control group, and was observed at 21 dpi. Interestingly, at 21 dpi, two negative individuals in the IM-A group showed more than a 7-fold increase when the median value in the IM-A group was 2-fold. Approximately 2-fold increases were apparent in the IM-B and BI-B groups at 14 dpi, one week earlier than groups in Phase A (Fig. 4). CD40 mRNA levels in the IM-A, BI-A and IM-B groups reached a peak at 21 dpi and the BI-B group showed only a moderate increase during the course of the experiment. SAV-positive fish had a higher average value of CD40 mRNA abundance than SAV-negative fish in the IM-A and IM-B groups at 7 and 3 dpi, respectively.

The T cell co-receptors, CD4-1 and CD8α, were relatively unchanged showing a slight down-regulation in all groups (Fig. 4). CD4-1 transcript abundance in Phase A was slightly downregulated at 7 dpi, but by 28 dpi it had increased 2-3-fold in all groups. A different CD4-1 transcription pattern was observed in infected groups in Phase B where the basal mRNA level was lower than in Phase A and unchanged throughout the sampling period. (Fig. 4). In the IM-B group at 3 dpi, three SAV-positive individuals showed more than a 4-fold increase, while in the BI-B group two negative individuals showed 2- and 4-fold increases, when the median value in both groups was 1-fold (S2 and Fig 4).

Differences in median values of the CD8α transcript levels between infected groups and control groups in Phase A and B were small, but there was a slight downward regulation in Phase A. Whereas in Phase B transcripts were stable throughout the sampling period (Fig. 4). Interestingly, some of the individuals from the IM-A and BI-A groups, which had high transcript levels of CD8α at 21 and 28 dpi, also showed elevated levels of CD40 and CD4-1.

The MHCIIβ gene regulation pattern was different in Phase A and B for both infected groups compared to their respective controls (Fig. 5). MHCIIβ was relatively unchanged in Phase A. The IM-B group showed a significant increase in MHCIIβ mRNA levels at 14 and 21 dpi, corresponding to a doubling compared to the control group, followed by a decrease at 28 dpi. Moreover, the basal mRNA levels of the MHCIIβ was higher in the CT-B group than in the CT-A group significantly so at 14 dpi (S3).

NFκB mRNA abundance was slightly up-regulated in both infected groups in Phase A whereas in Phase B transcript levels were stable. In Phase A, the median value of each group at all time-points was mostly higher than in Phase B, when the IM-A group was elevated over 2-fold at 21 and 28 dpi. In Phase B, both infected groups were highest at 14 dpi but decreased to control values at 28 dpi (Fig. 5).

The IL-2Rβ gene encodes a cytokine receptor protein that interacts specifically with IL-2. IL-2Rβ transcript levels were rapidly up-regulated after SAV3 infection, as indicated by the detection of elevated levels in both infected groups in both phases at 3 dpi (Fig. 5). These changes in transcript levels were accompanied by changes in the control groups resulting in significantly lower fold changes in the BI-A group at 28 dpi, when both the IM-A and CT-A groups showed over 2-fold increases.

The members of the IL-2 subfamily of cytokines, namely IL-2 and IL-15, was also analysed (Fig. 6). IL-2 mRNA level was regulated to only a small extent in both phases, while IL-15 mRNA abundance was unchanged in Phase A, except a 2-fold increase at 14 dpi in the IM-B group. Interestingly, the lower expression of IL-2 mRNA in the BI-A group at 28 dpi was reflected in the lower expression of its receptor, IL-2Rβ, at the same time-point.

Humoral response

Both IgM and IgT mRNA levels were up-regulated during the experiment, but a week later in Phase A compared to Phase B (Fig. 7). In Phase A, both infected groups exhibited the similar median value of IgM and IgT mRNA abundance and both were upregulated at 21 and 28 dpi. In the IM-B group, IgM and IgT transcript levels reached a peak at 14 and 21 dpi respectively, and a trend of down-regulation at 28 dpi could be seen. IgM mRNA was only up-regulated in SAV-positive fish in the BI-B group and 5 of 7 positive individuals showed ≥4-fold (4, 4, 4, 5, 9) increase at 28 dpi (S2). Moreover, IgT transcript levels in the BI-B group were also higher in SAV-positive fish relative to SAV-negative fish at 21 and 28 dpi, and such differences were statistically significant (S2).

3.3 Differences between control fish in both phases

We found differences in the basal transcript level in some of the analysed genes between the CT-A and CT-B groups (Table 2 and S3). CD40, CD4-1, CD8α, and SOCS1 were more abundantly regulated in the CT-A than in the CT-B, and the differences were significantly higher at 3 and 28 dpi (Table 2 and S3). The basal transcript level of NFκB, IL-15, and RIG-1 were also significantly higher in the CT-A group, but only at 28 dpi. In contrast, other genes showed a higher expression in the CT-B group compared to the CT-A group, as seen with the MHCIIβ at 14 dpi, IgM at 7 and 14 dpi, and the IL-18 with a peak at 7 dpi.

3.4 Neutralizing antibody titres

Plasma and serum samples collected at 21 and 28 dpi were assayed for the presence of SAV-neutralizing antibodies. In Phase A, the fish had no detectable neutralizing antibodies neither at 21 nor at 28 dpi.

Neutralizing antibodies were not detected in the plasma samples from the control fish nor in those from any of the infected groups at 21 dpi in Phase B. At 28 dpi 67% (12 of 18) of the

IM-B group and 11% (2 of 18) of the BI-B group plasma samples had neutralizing antibodies at detectable titres. All individuals showing an antibody response were positive for SAV RNA and in the BI-B group both SAV-positive fish with neutralizing antibodies had titres of ≥1:40 (Fig. 8). Similarly, in the serum samples, 17% (3 of 18) of the IM-B group at 21 dpi, 77% (14 of 18) of the IM-B group at 28 dpi and 22% (4 of 18) of the BI-B group at 28 dpi had neutralizing antibodies (Table 3).

4. Discussion

The salmon farming industry suffers huge economic losses due to PD outbreaks every year. Despite the use of vaccines against SAV by some of the farms, PD is still regularly observed during the grow-out phase in seawater. Although several studies have examined immune responses to SAV in Atlantic salmon, most of the mechanisms leading to protection still remain unclear. Therefore, the identification of measures that can potentially prevent or at least reduce SAV infection after seawater transfer is of particular interest. Thus, in this study, we have used our recently described BI challenge model to examine the relative regulation of genes involved in innate and adaptive responses to virus infection [23,19]. Taken together with previous studies from our laboratory, the results described here give a more detailed understanding of the differences in the anti-viral immune responses that develop in fish from the same production batch that are infected with SAV at either 2 or 9 weeks after seawater transfer.

RIG-1 is a pattern recognition receptor (PRR) that interacts with dsRNA leading to the production of type I interferons (IFNs) and the expression of IFN-stimulated genes (ISGs) [29]. RIG-1 showed similar patterns of transcription as another PRR, LGP2a, in Phase A and B [19,23]. Both PRRs were highly up-regulated in both phases and the up-regulation in phase A was greater. Moreover, RIG-1 was one of the most highly constitutively expressed immune gene examined in this study.

IL-10 is a member of the class II cytokine family that inhibits the activity of Th1 cells, natural killer cells, and macrophages. It has been demonstrated that it plays an important role in both the earliest and later anti-inflammatory responses to SAV3 infection [16]. In this study, we analysed the regulation of IL-10 gene until 4 wpi and up-regulation at 7 dpi in the IM-A group and at 14 dpi in the BI-A, IM-B, and BI-B groups could be observed. Whereas, the pro-inflammatory fish cytokine, IL-18, which induces IFN-γ production and promotes Th1 immunity in vertebrates [30] was relatively unchanged in both phases during the whole experiment.

STAT1 and SOCS1 are an activator and an inhibitor of the JAK/STAT signaling pathway, respectively, furthermore, both are crucially involved in the control of inflammatory responses. Both genes showed a high level of mRNAs at 7 dpi in all infected groups and had similar profiles of expression in Phase A and B, suggesting that they are regulating each other. The upregulation in the BI-A group, although lower, was maintained for a longer time compared to the IM-A group. This pattern of expression was similar to that which was observed for a number of other innate immune genes in this group of fish after SAV infection [23]. The high transcript abundance of SOCS1 has been suggested to be a consequence of SAV infection and may be a survival strategy for the virus. Increasing SOCS1 mRNA expression may subsequently inhibit signal transduction via the JAK/STAT pathway and may contribute to viral replication [31,32]. In our previous study, we observed a reduction in ISG and Mx expression in the IM-B group [19] that partially may be due to up-regulation of the SOCS1 after SAV infection.

Regulation of IL-10, RIG-1, SOCS1, and STAT-1 genes in Phase A for both challenge models (represented by trend-lines on the figures) was consistent with our previous results on the innate immune genes associated with the IFN response or with inflammation [23]. Expression of these transcripts was higher in head kidney samples from Phase A relative to Phase B, which

suggested a greater inflammatory response in Phase A. Interestingly, an abundance of inflammatory cells was observed as early as 7 dpi in SAV3 target-organs (pancreas and heart) in Phase B post-smolts, indicating activation of a cellular immune response to SAV3, which was not observed in Phase A fish [18]. Furthermore, the regulation of the genes mentioned above, and also of IgT and CD40, was delayed by one week in the BI-A fish when compared to the IM-A fish. This suggests that, in the BI group, the immune response to SAV3 was delayed in comparison to the IM infection, probably due to the infection route and/or the reduced viral dose.

Most of the adaptive immune genes studied here did not change significantly during the experimental period. However, the accumulative effect of many small changes in the transcript level of several genes in the same signaling pathway may produce biological effects. This is illustrated by the slight up-regulation in both IM-A and CT-A at 28 dpi in several genes all involved in cellular immunity; CD40, CD4-1, CD8 α, IL-2, IL2Rβ and IL-15 when the BI-A group showed no change at this time-point. Furthermore, IgT and IgM mRNA transcript levels were seen to be higher in fish that also showed a high expression of CD40, CD4-1 and CD8α mRNA.

Also, of these genes the T-cell co-receptors genes, CD4-1 and CD8α, were not significantly up-regulated during this study at any time-point in any of the groups, suggesting that the T cell response was not induced in response to SAV3 infection, at least in the first 4 weeks after infection. This is consistent with previous results showing that up-regulation of CD4-1 and CD8α was not detected at the earlier time points in co-habitation trials. However, both genes were found to be significantly expressed at 6 and 8 weeks post-infection [33,16,17]. On the other hand, we detected high mRNA levels of MHCIIβ, CD40, and IL-15 in head kidney samples from the IM-B group, which are indicative of activation of a more cell-mediated immune response. MHCIIβ is a part of the MHCII complex, which is involved in antigen

presentation and is normally expressed on antigen-presenting cells. Its expression was significantly up-regulated at 14 and 21 dpi in the IM-B group, in contrast to previous studies where regulation of MHCII mRNAs was only detected after 8 weeks in target organs during SAV3 co-habitation infection [16,33]. A similar observation of later/delayed response of MHCII expression was reported after infection with ISAV [34]. In addition, the costimulatory molecule CD40, a transmembrane glycoprotein, which is a member of the tumor necrosis factor (TNF) receptor superfamily and plays an immune-regulatory function in the adaptive response [35], was studied. CD40 was up-regulated at 7 dpi in both, the IM-A and IM-B groups, and this up-regulation was maintained for a longer time in the IM-B group. Furthermore, the cytokine IL-15, which is induced by IFN-γ in salmon leucocytes and plays a role in promoting Th1 responses and memory T cell maturation, was significantly up-regulated in the IM-B group at 14 dpi [36]. We also observed that IgM and IgT were highly up-regulated in the IM-B group. Previously our lab has shown that higher numbers of IgM^+ and MHC II^+ cells could be detected by immune-histo-chemical staining of the heart and pancreas of IM-B fish [37]. Taken together with findings presented here, this suggests that a larger population of B cells was present in the head kidney in the IM-B group.

Increased regulation of IgM and IgT genes was especially interesting since we were able to link gene expression data with the presence or absence of SAV neutralizing antibodies in individual fish. A neutralizing antibody response was clearly detected in Phase B, where only moderate increases (3-4-fold) in gene expression levels led to high titres of neutralizing antibodies. The plasma results show a lack of a neutralizing antibody response after only 2 weeks in seawater compared to the Phase B fish. Besides, many plasma samples from Phase A fish displayed a toxic reaction to the CHSE-214 cell line used in the neutralization assay, which was not seen with any of the Phase B samples. In addition, the serum results from Phase B fish support the high titres observed in plasma samples and appear more sensitive with both

444 detectable antibodies at 21 dpi and more SAV3-positive individuals. Three of the 4 BI-B fish
445 with detectable neutralizing antibodies were also shown to be SAV-positive and had a high
446 abundance of IgM mRNA (3.9-, 4- and 4.7-fold change). The results from the BI-B group are
447 more difficult to interpret due to the lower dose of virus present in the shedder water and the
448 lower prevalence of SAV positive fish detected in this group. Interestingly, very little
449 pancreatic pathology was observed in two of these fish, perhaps indicating that the immune
450 response, especially the antibody response had successfully cleared the virus. The apparent
451 lack of an antibody response in Phase A may be explained by the ability of the SAV nsP2
452 protein to suppress the host antiviral response and shut-off protein expression [38-40]. This
453 ability may be one of the factors that contribute to the poor immune response seen during SAV
454 infection in Phase A, despite the elevated abundance of several genes in fish recently
455 transferred to seawater. Thus, we can speculate that although the basal expression of some of
456 the genes studied was found to be greater in CT-A than in CT-B, fish from Phase B were able
457 to mount a more effective response that could have inhibited the SAV mediated host-shut-off
458 protein mechanism. Conversely, many innate immune genes were more highly expressed in
459 Phase B [19], but it is quite possible that the timely production of neutralizing antibodies is
460 decisive in mitigating SAV3 infection since previous studies have shown antibodies to both
461 neutralize SAV and protect against infection [41]. On the other hand, inflammatory cells and
462 MHC class II+ and IgM+ cells were observed in a greater number in the target organs in the
463 CT-B group [16,37].
464
465 In conclusion, Atlantic salmon post-smolts transferred to seawater at the same time, and
466 challenged at week 2 and 9, 7 weeks apart, displayed very different immune responses
467 following infection with SAV3. Fish adapted to seawater for an extra 7 weeks appeared to have
468 a better developed and more effective cell-mediated and humoral response against SAV3

infection, resulting in only mild histopathological changes in the pancreas and heart, and a reduced susceptibility to SAV3 infection.

Conflict of interest

The authors declare no conflict of interest.

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Abbreviations

BI bath immersion

cDNA complementary DNA

Dpi Days post infection

IFN interferon

IL Interleukin

IM Intramuscular injection

ISGs interferon-stimulated genes

MHCIIβ Major Histocompatibility Complex class II beta chain

NFκB Nuclear factor kappa-light-chain-enhancer of activated B

RIG-1 Retinoic-acid-inducible protein 1

RT-qPCR reverse transcriptase quantitative polymerase chain reaction

SOCS1 Suppressor of cytokine signaling 1

SWT seawater transfer

TCID50 50% tissue culture infective dose

TCR T cell receptor

wpt weeks post seawater transfer

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Highlights

- Neutralizing abs to SAV were detected only in salmon adapted for longer to seawater.
- Salmon adapted for longer to SW develop stronger cell-mediated immunity to SAV.
- Upon shorter adaptation to SW, salmon show IL-10 upregulation at early time points.
- SOCS1 gene was upregulated in all infected groups in both phases.
- Upon shorter adaptation to SW, SOCS1 upregulation was maintained for longer time.

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Figure legends

Fig. 1. Experimental setup and percentage prevalence of SAV-positive hearts.

A. Atlantic salmon post- smolts 2 weeks (Phase A) or 9 weeks (Phase B) post seawater-transfer (wpt) were infected with SAV3. Treatments performed in triplicate tanks consisted of intramuscular (*i.m.*) injection with non-infected cell culture supernatant (CT group), with 10^4 $TCID_{50}$ SAV3 (IM group), and bath challenge in water containing shed virus (BI group).

642 Shedder fish were *i.m.* injected with SAV3 approx. 1 week before the day of experimental
643 challenge. 8 fish per tank (24 fish per treatment group) were collected at each sampling time-
644 point: 3, 7, 14, 21 and 28 days post-infection (dpi). **B.** Bars represent mean percentage of
645 SAV3-RNA positive samples ± SEM at each time point of the IM-A (black bars), IM-B (dark
646 grey bars), BI-A (white bars) and BI-B (light grey bars) groups. N=24 for all groups and time-
647 points (except for the BI-A group at 14 dpi where n=22 and the IM-B group at 7 and 28 dpi
648 where n = 22 and 23 respectively).
649
650 **Fig. 2. Fold change in mRNA levels of IL-18 and IL-10.**
651 The y axis represents normalized, fold changes in mRNA levels for each treatment group
652 compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th percentiles
653 for each group with the median value shown by a black bar within each box. The whiskers
654 represent the maximum and minimum values for each group. Open bars represent the CT
655 group, dark grey bars the IM group and light grey bars the BI group. The left panels correspond
656 to the Phase A and the right panels correspond to the Phase B groups. Trend lines indicate
657 transcriptional changes over time; solid line IM group and dashed line the BI group. Asterisks
658 denote statistically significant differences between the infected and CT groups: * $p < 0.05$, **
659 $p < 0.01$ and *** $p < 0.001$. Lower case letters indicate statistically significant differences
660 between the IM and BI groups: ^{a} $p < 0.05$, ^{b} $p < 0.01$ and ^{c} $p < 0.001$.
661
662 **Fig. 3. Fold change in mRNA levels of RIG-1, SOCS1, and STAT1.**
663 The y axis represents normalized, fold changes in mRNA levels for each treatment group
664 compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th percentiles
665 for each group with the median value shown by a black bar within each box. The whiskers
666 represent the maximum and minimum values for each group. Open bars represent the CT

group, dark grey bars the IM group and light grey bars the BI group. The left panels correspond to the Phase A and the right panels correspond to the Phase B groups. Trend lines indicate transcriptional changes over time; solid line IM group and dashed line the BI group. Asterisks denote statistically significant differences between the infected and CT groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Lower case letters indicate statistically significant differences between the IM and BI groups: [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$.

Fig. 4. Fold change in mRNA levels of CD40, CD4-1, and CD8α.

The y axis represents normalized, fold changes in mRNA levels for each treatment group compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th percentiles for each group with the median value shown by a black bar within each box. The whiskers represent the maximum and minimum values for each group. Open bars represent the CT group, dark grey bars the IM group and light grey bars the BI group The left panels correspond to the Phase A and the right panels correspond to the Phase B groups. Trend lines indicate transcriptional changes over time; solid line IM group and dashed line the BI group. Asterisks denote statistically significant differences between the infected and CT groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Lower case letters indicate statistically significant differences between the IM and BI groups: [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$.

Fig. 5. Fold change in mRNA levels of MHCIIβ, NFκB, and IL-2Rβ.

The y axis represents normalized, fold changes in mRNA levels for each treatment group compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th percentiles for each group with the median value shown by a black bar within each box. The whiskers represent the maximum and minimum values for each group. Open bars represent the CT group, dark grey bars the IM group and light grey bars the BI group. The left panels correspond

to the Phase A and the right panels correspond to the Phase B groups. Trend lines indicate transcriptional changes over time; solid line IM group and dashed line the BI group. Asterisks denote statistically significant differences between the infected and CT groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Lower case letters indicate statistically significant differences between the IM and BI groups: [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$.

Fig. 6. Fold change in mRNA levels of IL-2 and IL-15.

The y axis represents normalized, foldchanges in mRNA levels for each treatment group compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th percentiles for each group with the median value shown by a black bar within each box. The whiskers represent the maximum and minimum values for each group. Open bars represent the CT group, dark grey bars the IM group and light grey bars the BI group. The left panels correspond to the Phase A and the right panels correspond to the Phase B groups. Trend lines indicate transcriptional changes over time; solid line IM group and dashed line the BI group. Asterisks denote statistically significant differences between the infected and CT groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Lower case letters indicate statistically significant differences between the IM and BI groups: [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$.

Fig. 7. Fold changes in mRNA levels of IgM and IgT.

The y axis represents normalized, fold changes in mRNA levels for each treatment group compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th percentiles for each group with the median value shown by a black bar within each box. The whiskers represent the maximum and minimum values for each group. Open bars represent the CT group, dark grey bars the IM group and light grey bars the BI group. The left panels correspond to the Phase A and the right panels correspond to the Phase B groups. Trend lines indicate

transcriptional changes over time; solid line IM group and dashed line the BI group. Asterisks denote statistically significant differences between the infected and CT groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Lower case letters indicate statistically significant differences between the IM and BI groups: [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$.

Fig. 8. Neutralizing antibody titres to SAV at 28 dpi in fish from Phase B groups.

Percentage of fish producing neutralizing antibodies to SAV in plasma samples at 28 dpi. Grey bars represent the *i.m.* injected group (IM-B) and black bars represent the bath challenged group (BI-B).

Table 1. Primer sequences. Primers employed in this study, product size, relative efficiencies and the Genbank accession numbers used for primer design or the reference for previously published assays.

Table 2. Significant differences (ANOVA on log transformed data followed by Tukey's multiple comparisons test) in gene expression at all time-points and for all genes comparing Phase A to Phase B. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Table 3. Neutralizing antibody titres. Number of fish with neutralizing antibodies in plasma and serum samples in fish from Phase B groups.

S1. Immune gene mRNA levels in control, positive and negative fish for SAV3-RNA in Phase A. Graphs show trend lines between average fold change ± SEM positive IM-A (light blue) and BI-A (yellow) and negative IM-A (dark blue), BI-A (red) and CT-A (black) groups for all genes analysed.

S2. Immune gene mRNA levels in control, positive and negative fish for SAV3-RNA in Phase B. Graphs show trend lines between average fold change ± SEM positive IM-A (light blue) and BI-A (yellow) and negative IM-A (dark blue), BI-A (red) and CT-A (black) groups for all genes analysed.

S3. Fold changes in mRNA levels between control fish in both phases.

The y axis represents normalized, fold changes in mRNA levels for each treatment group compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th percentiles for each group with the median value shown by a black bar within each box. The whiskers represent the maximum and minimum values for each group. Dark grey bars represent the CT-A group and light grey bars the CT-B group. Trend lines indicate transcriptional changes over time; solid line CT-A group and dashed line the CT-B group. Asterisks denote significant differences between the CT groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

757 Table 1

Gene	Primers sequences	Efficiency	Product size bps*	References/Genbank accession No.
CD40	Fwd: GCCCTGCCAAGAGGATGA Rv: GTCAGGCACTCTTTACTGGAACA	2,06	173	NM_0011412361
STAT1	Fwd: TGTCTGTTGGCTCAGTTGCG Rv: GAAATTGATGCTGTGGCGTCT	1,92	100	[24] / NM_001123654.1
SOCS1	Fwd: CATTCCGCTGCCAAGTAGAC Rv: CCGTGGCGACGTCCTT	1,95	127	KF699315
MHCIIβ	Fwd: CTCACTGAGCCCATGGTGTAT Rv: GAGTCCTGCCAAGGCTAAGATG	1,90	117	[25] / BT060311
EF1A	Fwd: CCCCTCCAGGACGTTTACAAA Rv: CACACGGCCCACAGGTACA	2,02	57	[26] / BG936182.1
CD8α	Fwd: CTTCAGCGAGGAGCAGATAAAC Rv: GGCTGTGGTCATTGGTGTAGTC	2,04	187	NM_001123583.1
CD4-1	Fwd: GTGGAGGTGCTACAGGTGTTTTC Rv: GGGGAGGAGCCTAAAGCG	2,00	158	EU409794.1
RIG-1	Fwd: CCTCTGCTACAGGAGCCAATA Rv: GCCGGTTGGTGCACAGAT	1,95	157	NM_001163699.1
NFκB	Fwd: GCACTACCATTTTACTGACGCA Rv: GCGTTGGGTGACTTGCTGT	2,07	188	NM_001173583.1
IL-18	Fwd: GAGCAATGCAAAGCAGATGATT Rv: GCTCCAGTGGTTTGGCAGAA	2,07	177	BT125392.1
IL-15	Fwd: GCTTCTTAATATTGAGCTGCCTGA Rv: GGCATCTGATTTTTCTATGGTACTT	2,04	146	NM_001279065.1
IL-2	Fwd: GCGGATGTAGAGAAAAGCATTG Rv: CATTCTGACGAGTCCGTTCTGAT	2,32	155	HE805272.1
IL-10	Fwd: GCTATGGACAGCATCCTGAAGTT Rv: GGTTGTTCTGCGTTCTGTTGTT	1,99	76	EF165028
IL-2Rβ	Fwd: CTCCAAGGACTGTTTTGTGTGAA Rv: GAGGTGCTCGGCTGAACTGA	1,95	242	NM_001140548.1
IgM	Fwd: CCAGTGAAGAAACAAGCGGAAT Rv: CCTCATCCATTTGATTGTGTGTGTA	2,02	157	S48652
IgT	Fwd: CAACAAAGTCACTGTCACCTGGAA Rv: CCGTCAGCGGTTCTGTTTTG	2,10	212	GQ907003.1

758 *bps = base pairs

759

Table 2

		3 dpi			7 dpi			14 dpi			21 dpi			28 dpi		
Gene Assay	**Treatment**	**CT-B**	**IM-B**	**BI-B**	**CT-B**	**IM-B**	**BI-B**	**CT-B**	**IM-B**	**BI-B**	**CT-B**	**IM-B**	**BI-B**	**CT-B**	**IM-B**	**BI-B**
CD40	**CT-A**	*	*	**					***					*		
	IM-A				*				*					*		
	BI-A												**			
CD4-1	**CT-A**	***	*	**										***	***	***
	IM-A	**		*							*	*	*	***	***	***
	BI-A	*						*	*	**	**	***	***	***	***	***
CD8α	**CT-A**	**	*	*										***	**	**
	IM-A	*												***		*
	BI-A															
MHCIIβ	**CT-A**					**	*	***	***	***		***				*
	IM-A				*	***	**	**	***	**		***			*	**
	BI-A					*		**	***	**		***			*	**
NFκβ	**CT-A**													**	*	*
	IM-A	*										*	**	**	*	*
	BI-A				*		*	**				*	**			
IL-2Rβ	**CT-A**															*
	IM-A															
	BI-A															
IgM	**CT-A**				**	***	***	**	***	**		***				
	IM-A				*	***	**		***	***	**	***		***		
	BI-A					***	**		***		**		***	**		
IgT	**CT-A**					**			***							
	IM-A										***	**	***			
	BI-A					**					***		***			
IL-2	**CT-A**															*
	IM-A															
	BI-A															
IL-10	**CT-A**								***	**						
	IM-A				***	***	***	*								
	BI-A							***			***	**	***	*		
IL-15	**CT-A**								***					***	*	**
	IM-A								***					*		
	BI-A								*							
IL-18	**CT-A**				*				***							
	IM-A				***	**	**		***							
	BI-A				**				***							
RIG-1	**CT-A**					**	*		***			**		***	***	***
	IM-A				***	***	***	***		***	**	***	***	***	***	***
	BI-A				**			***		***	***	***	***	***	***	***
SOCS1	**CT-A**	*	***	***					***					***	***	***
	IM-A		*	**	***	**	***	***	*	***	**	***		***	***	**
	BI-A			*	***		**	***	***	***	***	***	***	***	***	***
STAT1	**CT-A**					***			***							
	IM-A				***		*									
	BI-A							*			***	***	***			

Table 3

Group	Sample	Titre				Total
		1:20	1:30	1:40	<1:40	
IM-B 21 dpi	Plasma					**0**
	Sera	2	1			**3**
BI-B 21 dpi	Plasma					**0**
	Sera					**0**
IM-B 28 dpi	Plasma	2	3	3	4	**12**
	Sera	2	4	3	5	**14**
BI-B 28 dpi	Plasma			1	1	**2**
	Sera		3		1	**4**

Fig. 1

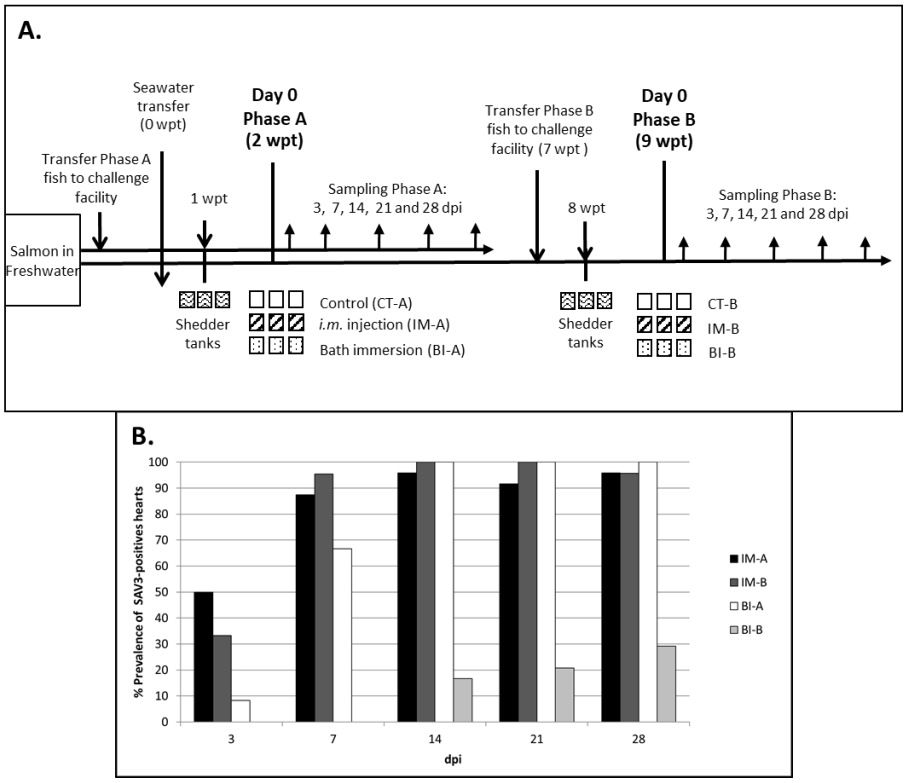

A.
Seawater transfer (0 wpt)
Day 0 Phase A (2 wpt)
Transfer Phase B fish to challenge facility (7 wpt)
Day 0 Phase B (9 wpt)
Transfer Phase A fish to challenge facility
1 wpt
Sampling Phase A: 3, 7, 14, 21 and 28 dpi
8 wpt
Sampling Phase B: 3, 7, 14, 21 and 28 dpi
Salmon in Freshwater
Shedder tanks
Control (CT-A)
i.m. injection (IM-A)
Bath immersion (BI-A)
Shedder tanks
CT-B
IM-B
BI-B
B.
% Prevalence of SAV3-positives hearts
100
90
80
70
60
50
40
30
20
10
0
3
7
14
21
28
dpi
IM-A
IM-B
BI-A
BI-B

Fig. 2

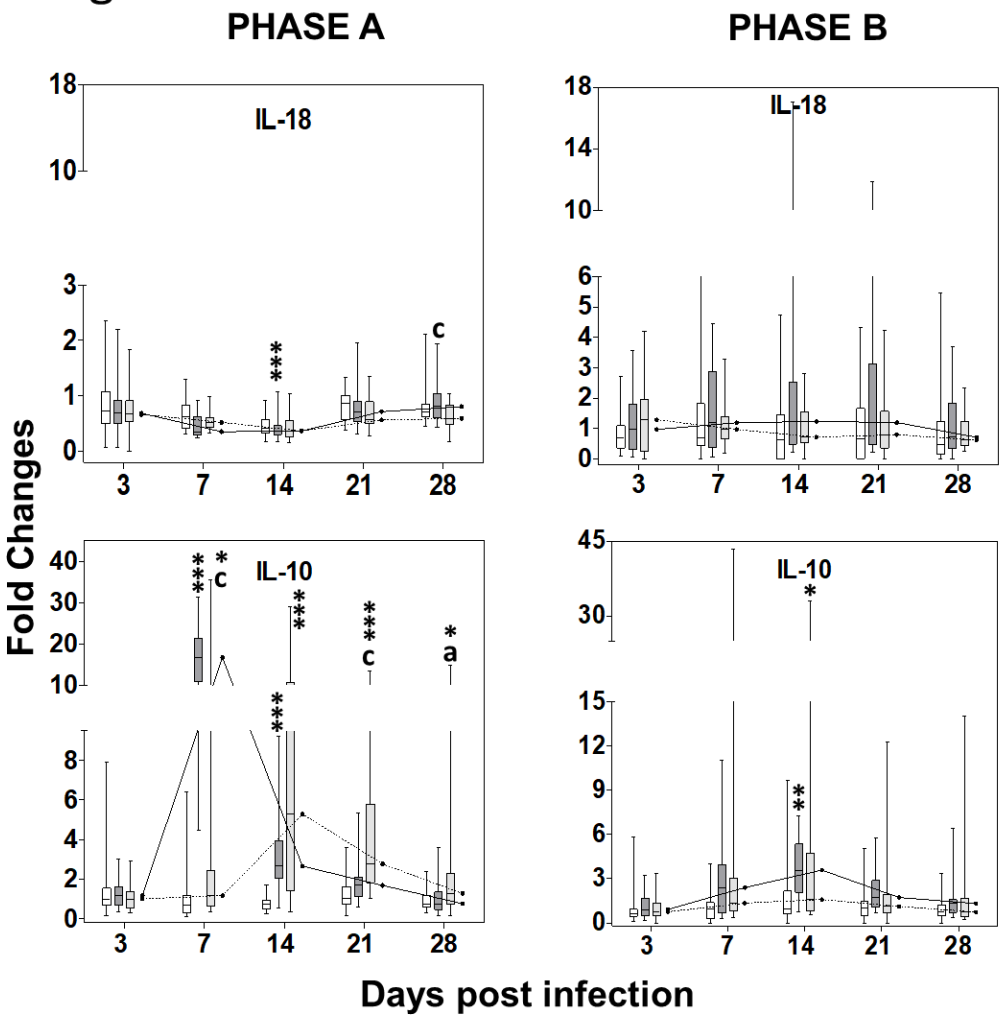

PHASE A
PHASE B
IL-18
IL-10
Fold Changes
Days post infection
3
7
14
21
28

Fig. 3

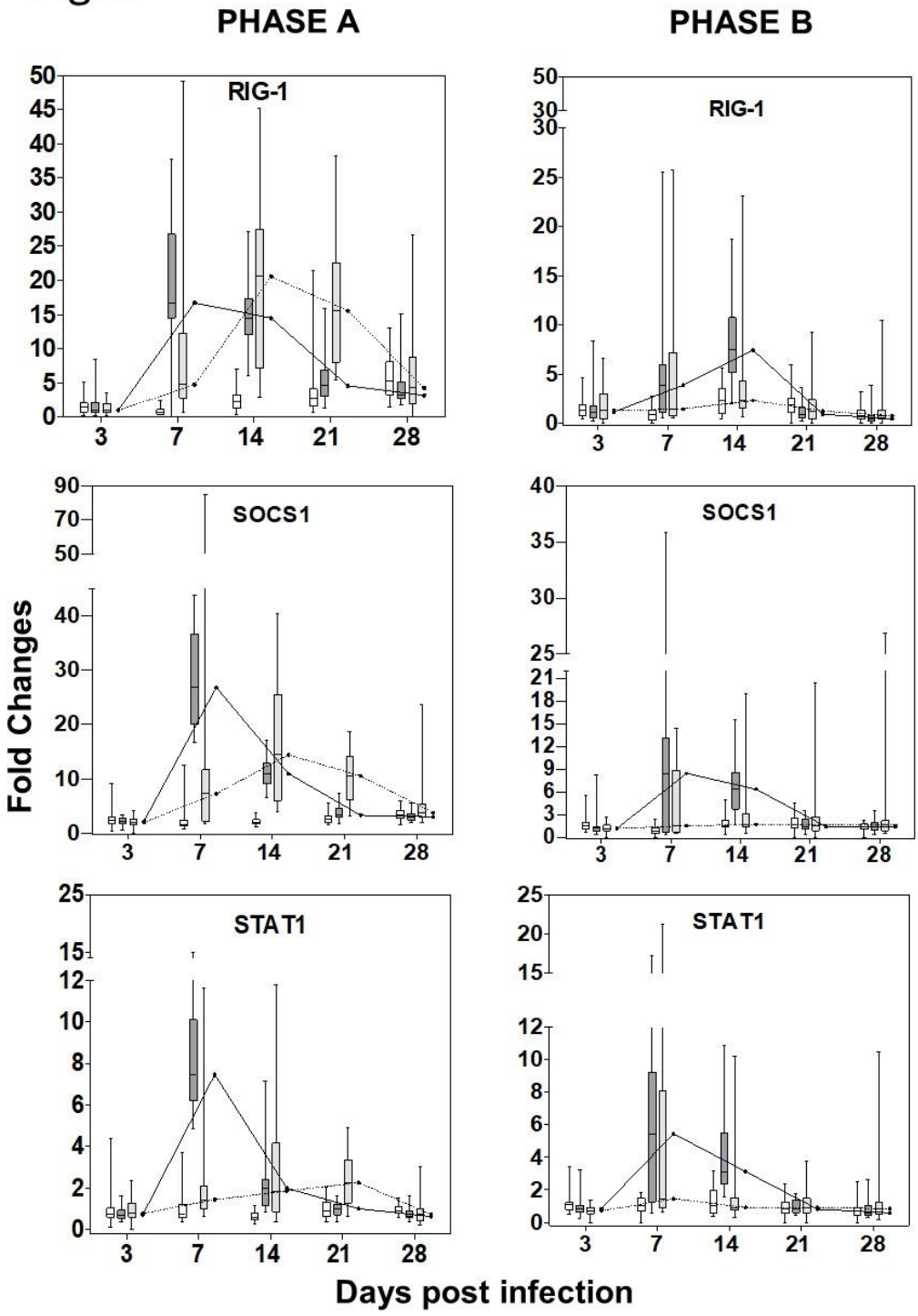

PHASE A
PHASE B
RIG-1
RIG-1
SOCS1
SOCS1
STAT1
STAT1
Fold Changes
Days post infection
3
7
14
21
28

Fig. 4

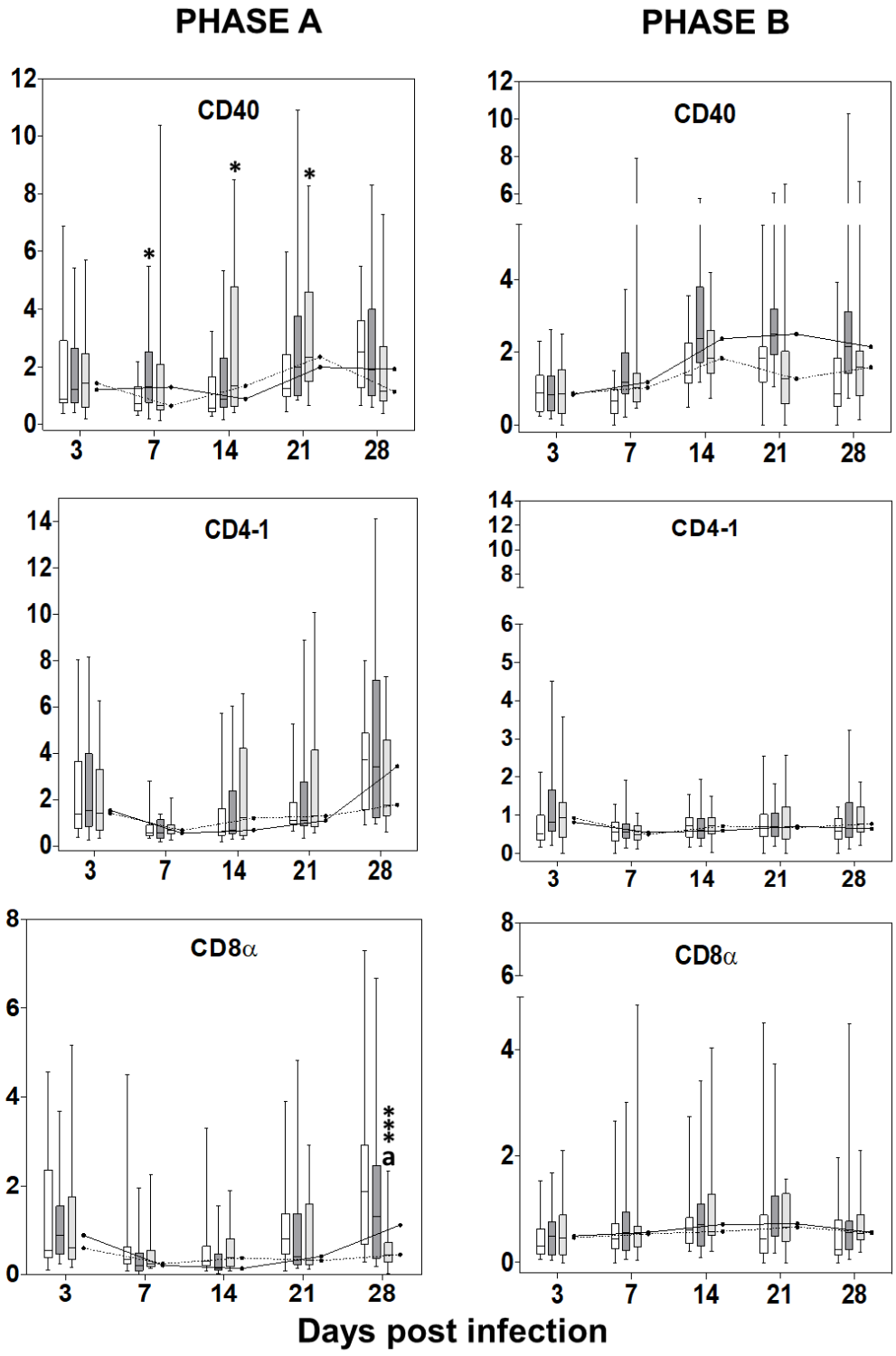

PHASE A
PHASE B
CD40
CD40
CD4-1
CD4-1
CD8α
CD8α
*
*
*
***
a
3
7
14
21
28
Days post infection

Fig. 5

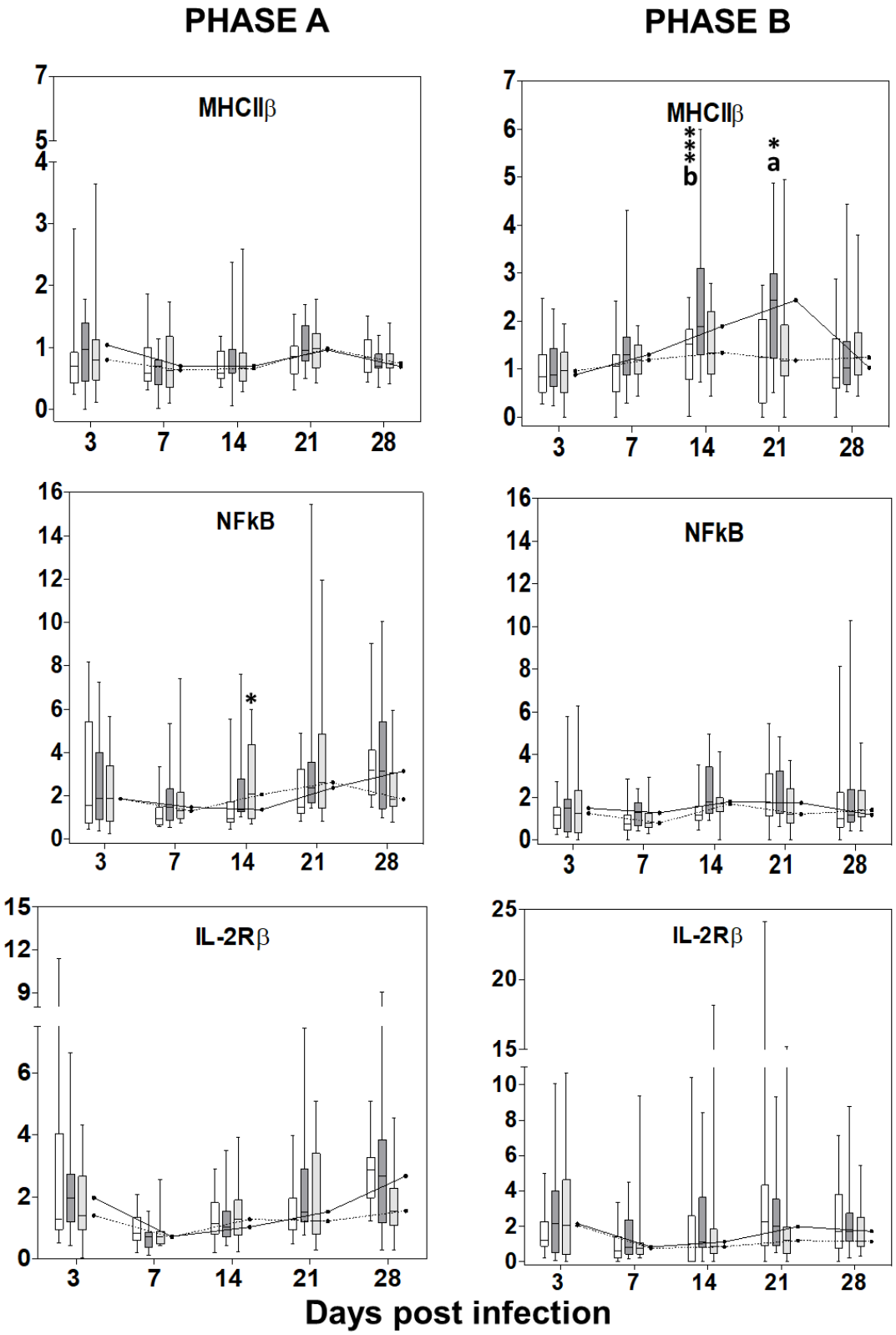

PHASE A
PHASE B
MHCIIβ
MHCIIβ
***
b
*
a
NFkB
NFkB
*
IL-2Rβ
IL-2Rβ
3
7
14
21
28
Days post infection

Fig. 6

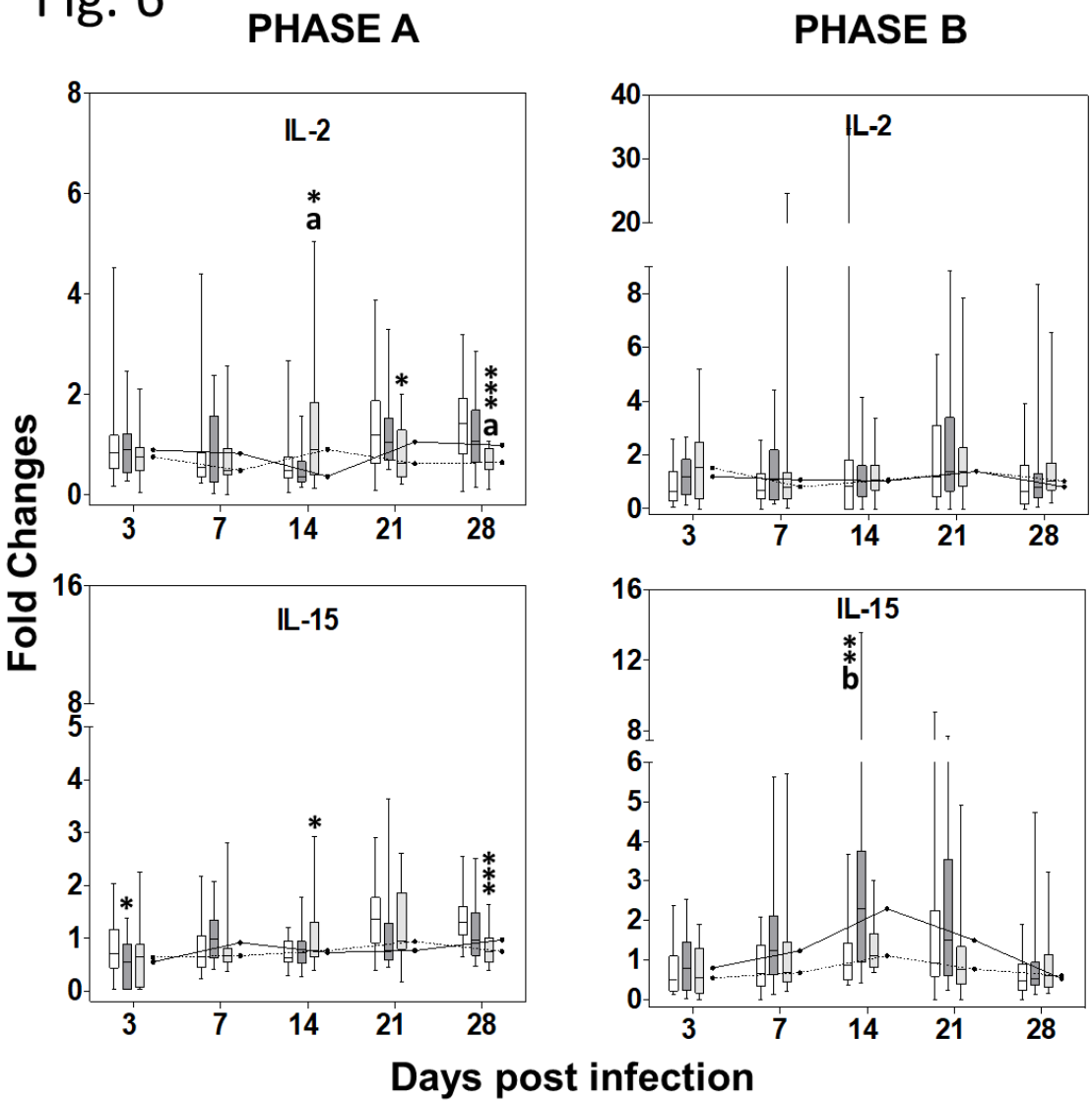

PHASE A
PHASE B
IL-2
IL-15
IL-2
IL-15
Fold Changes
Days post infection
3
7
14
21
28
a
b

Fig. 7

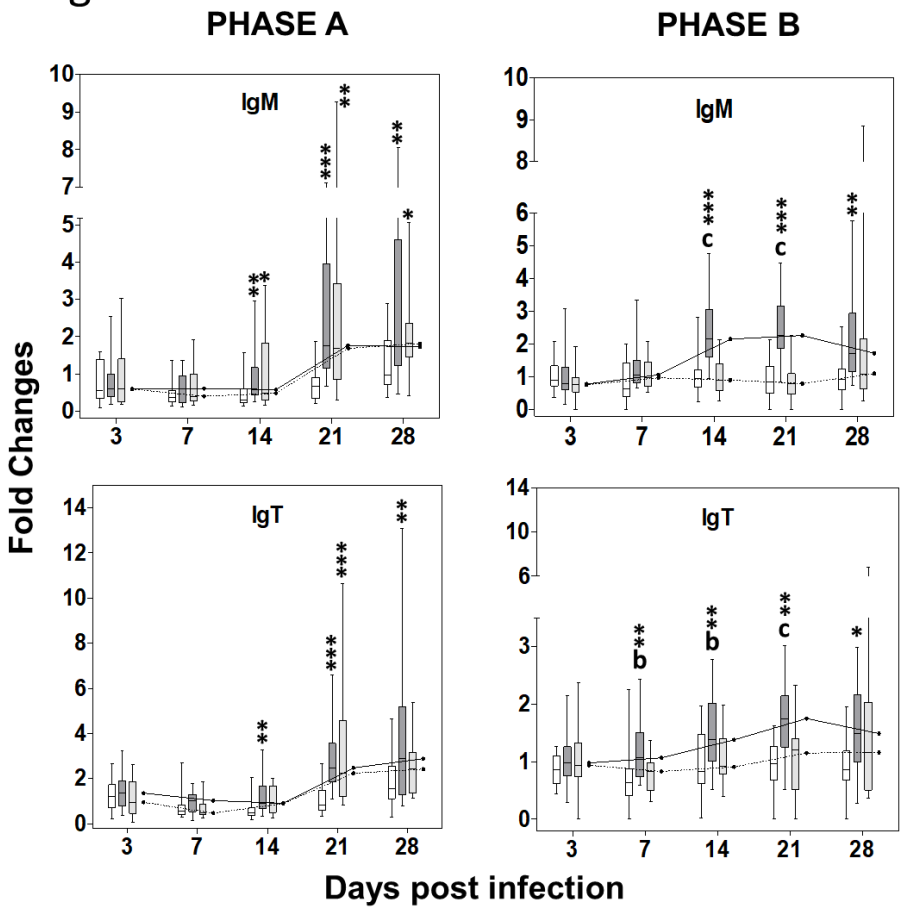
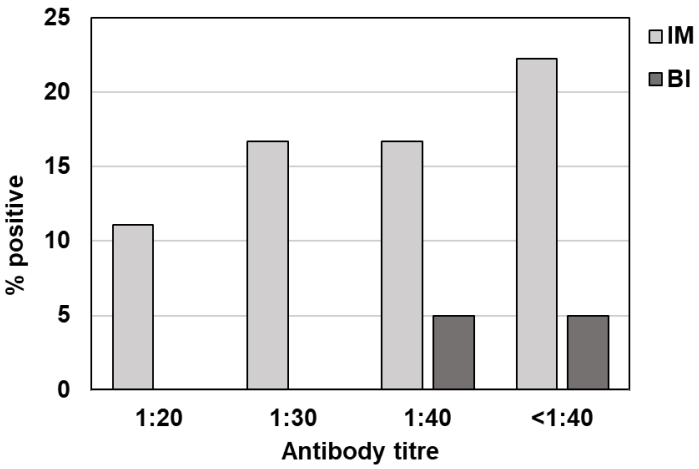
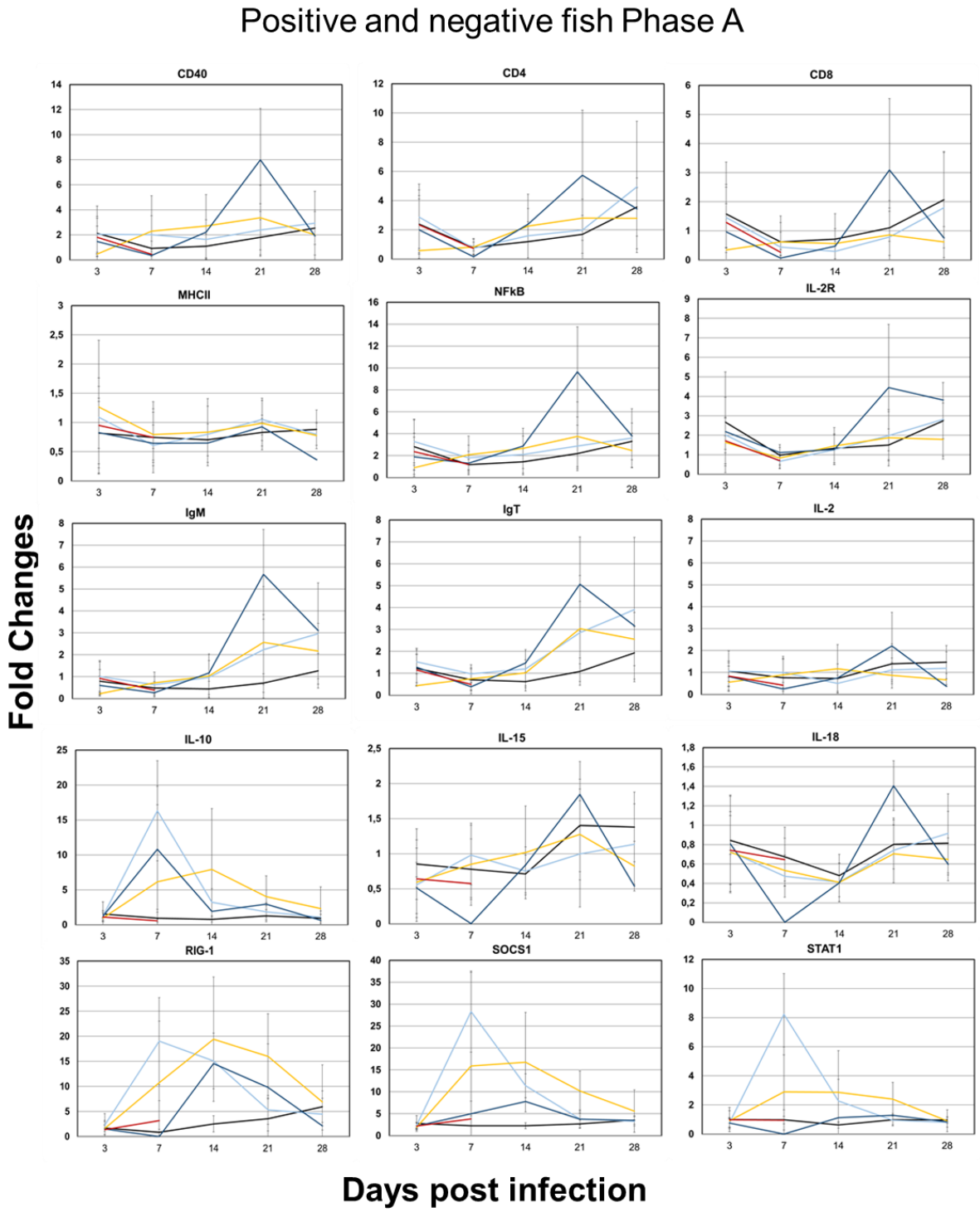

PHASE A
PHASE B
IgM
IgT
Fold Changes
Days post infection
3
7
14
21
28

Fig. 8


IM
BI
% positive
1:20
1:30
1:40
<1:40
Antibody titre

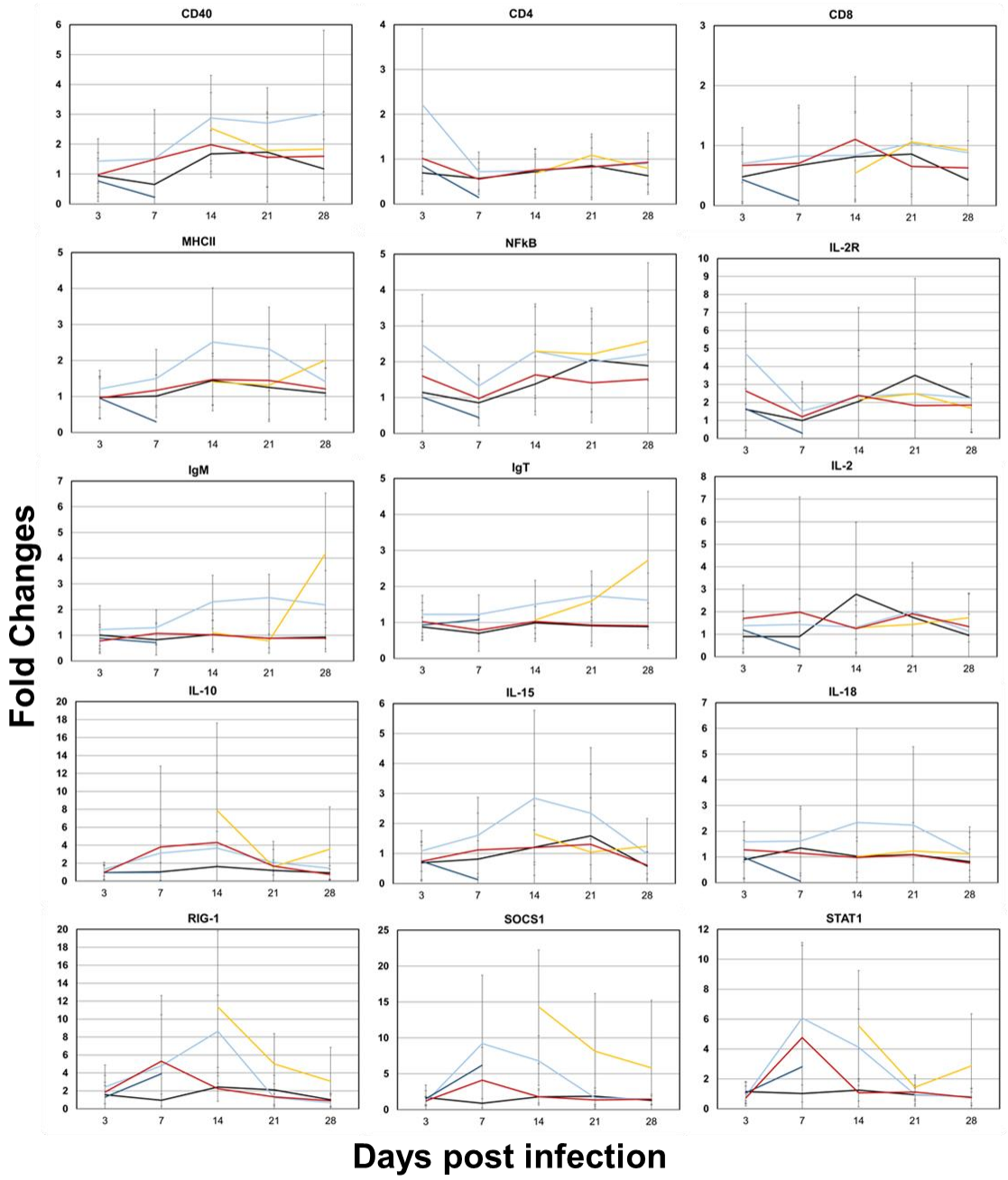
S1



S1. Immune gene mRNA levels in control, positive and negative fish for SAV3-RNA in Phase A. Graphs show trend lines between average fold change ± SEM positive IM-A (light blue) and BI-A (yellow) and negative IM-A (dark blue), BI-A (red) and CT-A (black) groups for all genes analysed.

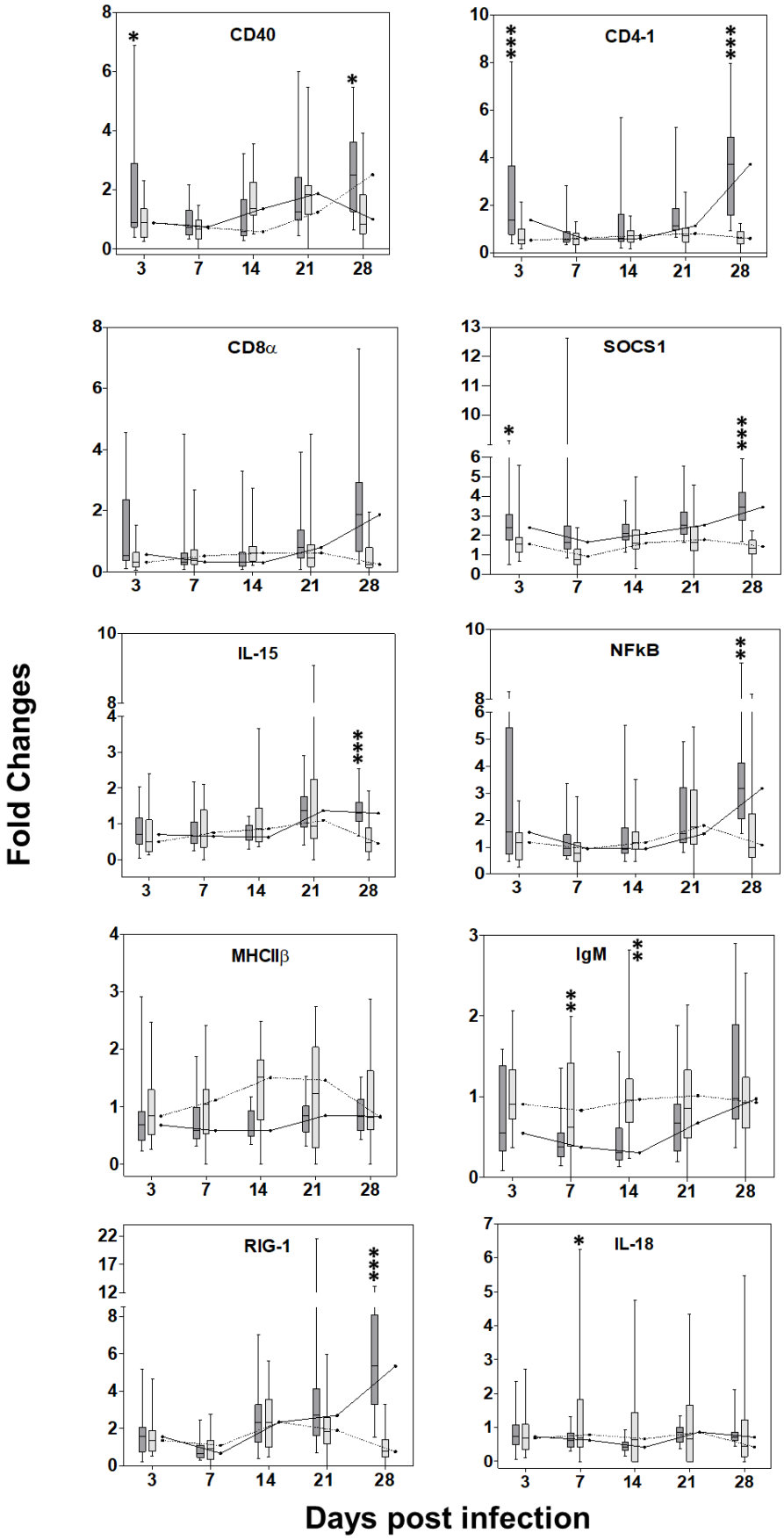
S2

Positive and negative fish Phase B



S2. Immune gene mRNA levels in control, positive and negative fish for SAV3-RNA in Phase B. Graphs show trend lines between average fold change ± SEM positive IM-A (light blue) and BI-A (yellow) and negative IM-A (dark blue), BI-A (red) and CT-A (black) groups for all genes analysed.

S3


CD40
CD4-1
CD8α
SOCS1
IL-15
NFkB
MHCIIβ
IgM
RIG-1
IL-18
Fold Changes
Days post infection