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Atlantic salmon adapted to seawater for 9 weeks develop a robust immune response to salmonid alphavirus upon bath challenge.

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Keywords

Salmo salar, salmonid alphavirus, inflammation, gene transcription, bath immersion, smoltification, seawater transfer, post-smolt; SAV.

Abstract

Pancreas disease (PD) caused by salmonid alphavirus (SAV) is the most serious viral disease in Norwegian aquaculture. Study of the immune response to SAV will aid preventative measures including vaccine development. The innate immune response was studied in Atlantic salmon infected by either bath immersion (BI) or by intra-muscular (*i.m.*) injection (IM) with SAV subtype 3, two and nine weeks after seawater transfer (Phases A and B respectively). Phase A results have been previously published (Moore et al. 2017) and Phase B results are presented here together with a comparison of results achieved in Phase A. There was a rapid accumulation of infected fish in the IM-B (IM Phase B) group and all fish

sampled were SAV RNA positive by 7 dpi (days post infection). In contrast, only a few SAV RNA positive (infected) fish were identified at 14, 21 and 28 dpi in the BI-B (BI Phase B) group. Differences in the transcription of several immune genes were apparent when compared between the infected fish in the IM-B and BI-B groups. Transcription of the analysed genes peaked at 7 dpi in the IM-B group and at 14 dpi in the BI-B group. However, this latter finding was difficult to interpret due to the low prevalence of SAV positive fish in this group. Additionally, fish positive for SAV RNA in the BI-B group showed higher transcription of IL-1β, IFNγ and CXCL11_L1, all genes associated with the inflammatory response, compared to the IM-B group. Histopathological changes in the heart were restricted to the IM-B group, while (immune) cell filtration into the pancreas was observed in both groups. Compared to the Phase A fish that were exposed to SAV3 two weeks after seawater transfer, the Phase B fish in the current paper, showed a higher and more sustained innate immune gene transcription in response to the SAV3 infection. In addition, the basal transcription of several innate immune genes in non-infected control fish in Phase B (CT-B) was also significantly different when compared to Phase A control fish (CT-A).

Introduction

Atlantic salmon is the most important commercial aquaculture species in Northern Europe, and increased production is hampered due to disease caused by both viruses and parasites. Pancreas disease (PD) caused by salmonid alphavirus (SAV), also known as salmon pancreas disease virus (SPDV), is the most frequent and serious viral disease in Norwegian salmon aquaculture with 137 outbreaks recorded in 2016 resulting in large economic losses [1]. Study of the underlying immune mechanisms of infection with SAV will aid preventative measures including vaccine development.

SAV affects salmonid fish in both fresh and salt water in Northern Europe and has 6 known sub-types causing PD in different geographical areas [2]. Until recently, SAV sub-type 3 caused all known outbreaks of PD in Norway [3]. In 2011, SAV2 was reported as the causative agent of PD for the first time and its introduction traced back to 2010 [4]. Despite small genetic differences between the sub-types, SAV2 has a tendency to cause a less severe disease and fewer mortalities than SAV3 in Norway [5, 6]. Fish surviving PD can be subject to down-grading of fillet quality at slaughter further compounding the financial losses caused by mortalities [7, 8]

PD is characterised by inflammation and necrosis in target tissues starting in the pancreas and followed by lesions in the heart. Skeletal muscle is also affected, but is usually only observed in field outbreaks due to the brevity of experimental infections. Mortality is isolate dependent and can be difficult to reproduce experimentally. Secondary stressors to SAV infection, such as anti-lice treatments and fish transport, have also been linked to increased mortality [6, 9].

SAV is an alphavirus and in humans, alphavirus infections are controlled by both humoral and cellular immune responses, but the innate immune response, starting with interferon (IFN) production is central to controlling the acute phase [10-12]. The classical IFN response

promotes and maintains an anti-viral state in two steps. On gaining access to cells viral RNA is recognised by several intra-cellular pattern recognition receptors (PRRs) such as LGP2a, MDA-5 and TLR7 and 8 that are also found in teleosts and signal the production of IFN [13-15]. The second step maintains the anti-viral state with the transcription of a myriad of interferon stimulated genes (ISGs) of which there are over 300 known in mammals [16]. The immune response can also lead to damaging inflammation in the affected tissues of fish not only for PD, but for other viral diseases such as HSMI and CMS [17].

Pancreas disease affects Atlantic salmon at marine sites and as such a bath immersion in seawater is the most relevant experimental model to study the immune response to SAV infection as it mimics the natural route of infection. Our own recent experiments have shown that when fish were infected with SAV3 two weeks after seawater transfer, immune responses were short-lived in *i.m.* infected fish and delayed in fish infected by bath immersion [18]. Others have also reported differences in transcription of immune genes during infection before and after transfer to seawater [19]. Even without the added pressure of a pathogen challenge salmon have shown changes in the transcription of immune genes during smoltification [20, 21]. The increased metabolic load of preparing for life at sea leaves salmon with a reduced capacity to cope with stressors including infection [19, 22, 23]

This experimental SAV3 infection was carried out 9 weeks after seawater transfer and constituted Phase B of a two-phase experiment designed to compare the immune responses of fish to SAV3 at different times after seawater transfer. In Phase B two experimentally infected groups of fish were used; the first was intra muscularly (*i.m*) injected with SAV3, and the second was bath immersed using a previously described protocol featuring a natural infection route and a defined time of infection [24]. The response to infection was evaluated by analysing RNA from head kidney tissue for the transcription of 15 different immune genes and by histological examination of pancreas and heart tissue.

2.0 Materials and Methods

2.1 Experimental design

This experiment formed Phase B of a two part experiment designed to compare immune responses to SAV3 infection at different times after transfer to seawater. The Phase B Atlantic salmon post-smolts (average weight 89 g) were transferred to seawater (34.5 ‰) at 12°C, nine weeks before the start of the experiment at Matre Research Station. They were transferred to the Institute for Marine Research (IMR) in Bergen one week before the infection experiment started (Fig.1). Three groups of these fish were held in triplicate tanks, a control group (CT-B), injected with non-infected cell culture supernatant and two groups infected with SAV3 by *i.m.* injection (IM-B) or by bath immersion (BI-B) (Fig. 1). The *i.m.* injection dose was 10^4 $TCID_{50}$ SAV3 per fish for both the experimental IM-B group and for shedder fish, which were used to produce the immersion dose. The shedders were injected one week prior to day 0 (Fig.1). These experimental procedures were identical to those used in the Phase A of this 2-part experiment (Fig. 1). The design of this experiment and all procedures were approved by the Norwegian Animal Research Authority. The average Ct value when SAV RNA was measured in 1 litre of filtered/concentrated shedder tank water was 34, which from previous experience with this infection model indicates a relatively low level of infectious virus for the BI-B group [24, 25]. However, since bath immersion was carried out for 6 hours this exposure allowed ample opportunity for infection. The SAV3 isolate used was subsequently discovered to be contaminated with infectious pancreatic necrosis virus (IPNV). All IM-B group samples were screened for IPNV RNA and 4 individuals (4.2%) were found to be positive (Cts all >36). The level of IPNV compared with the SAV was so low it is unlikely to have caused any discernable effect on the interpretation on the immune gene transcription evaluated post infection.

2.2 Sampling

Water (1 litre) was sampled from each of the three shedder tanks on the day of bath immersion and this constituted the bath immersion dose. The water was filtered/concentrated and eluted in lysis buffer [24]. Each tank contained 65 fish and 8 fish were sampled from each tank (24 from each group) at 3, 7, 14, 21, and 28 dpi. Half of each heart and head kidney tissue was sampled for RT-qPCR analysis and flash frozen in liquid nitrogen. Pancreatic tissue and the other half of the heart were sampled for histology from 4 of the 8 fish sampled from each tank at 7, 14, 21 and 28 dpi. The tissue was fixed in 10% neutral buffered formalin and processed as described previously [24]. Representative sections from 21 and 28 dpi are included to show the development of PD.

2.3 Total RNA isolation, cDNA synthesis and qPCR

Total RNA was extracted from heart and head kidney using TRIzol™, quantified and validated as described previously [24]. Random quality checks were performed on 5% of all RNA samples and showed RIN values ≥ 9. RNA extracted from heart tissue and filtered/concentrated tank water was analysed for SAV RNA using a one-step PCR (AgPath, Ambion) and detected using a TaqMan nsp-1 assay [26].

For immune gene analysis, cDNA was transcribed from 1 µg total head kidney RNA in a 20 µl reaction using qScript™ SuperMix (Quanta Biosciences) including priming with both random hexamers and oligo-dT as described in the manufacturer's instructions. cDNA was diluted 1:10 before use, as qPCR on pooled cDNA showed that this was an optimal dilution. All primers and assay data are listed in Table 1. Assays for TLR7, TLR8a1, MyD88, MDA5, LGP2a, IRF7, IFNa, Mx, IFNγ, CXCL11-L1, IL-1β, CRFB5, IL-8 and IL-4/13A were used and their design and validation described previously [18]. In addition, an assay for Viperin was adapted from a published study [27]. All head kidney cDNA samples were analysed for

the above mentioned assays. Elongation factor 1A (EF1A) [28] was used for normalization as this gene has been validated as the most useful reference gene in Atlantic salmon infected with SAV [29]

qPCR was run in 384 well plates using Brilliant III Ultra-Fast SYBR® Green master mix (Agilent) and an Applied Biosystems 7900H Fast sequence detection analyser in a 7 µl reaction volume containing 2 µl diluted cDNA and 400 nM of each primer. The running conditions were as recommended by the manufacturer including melting curve analysis for each run.

2.4 Data Analysis

The Ct values were normalized using the Ct values from the EF1A assay run on the same plate for each individual (ΔCt). Fold change of transcription for each result was calculated by subtracting the relevant mean ΔCt values obtained from calibrator fish, sampled before day 0 from the ΔCt from each result ($2^{-\Delta\Delta Ct}$) [30]. Outliers were not removed as they represent the real biological diversity within these groups.

A t -test was used to examine differences between the positive IM-B fish and CT-B fish. T-tests use averages in their calculations, but medians and ranges were used for discussion and visual representation as they more accurately portray the spread of the data. Due to the small number of positive results in the BI-B group these results were excluded from statistical analyses, but are presented as individual data points in supplementary figures with trend lines for the IM-B and CT-B groups for comparison (S4). Average transcriptions of positive and negative fish in both infected groups are also included (S3).

In addition, it was of interest to compare immune gene transcription between Phase A and Phase B fish to determine any changes in either the immune status or the immune response as assessed using the transcription of the same 15 innate immune genes. T- tests were used to

determine differences in gene transcription levels between CT-A and CT-B and between IM-A and IM-B. Figures were prepared using Prism 6.0 (Graphpad.com) and Excel 2013.

3.0 Results

3.1 SAV infection and PD status

SAV viral loads and prevalence in these groups of infected fish from both Phase A and Phase B of this experiment have been reported previously [24]. PD status was determined by analyzing heart tissue for the abundance of SAV RNA and by the histological examination of heart and pancreatic tissue. Prevalence in the IM-B group accumulated quickly with 21 of 22 fish positive at 7 dpi and it remained almost 100% at all later time-points (Fig. 2A). Prevalence was plotted as a percentage of fish analysed to take account of samples being unavailable for technical reasons (samples lost or unsuitable). The BI-B group had only 4, 5 and 7 positive fish at 14, 21 and 28 dpi respectively and the viral loads in these fish were similar to the IM-B group at 14 and 28 dpi (Fig. 2B). At 21 dpi the positive BI-B group individuals had relatively low viral loads. In the IM-B group where prevalence increased rapidly to 100 %, the viral load also increased up to 14 dpi after which it decreased, falling to 7 dpi levels at 28 dpi (Fig. 2B). The histopathology in the pancreas showed moderate changes in the IM-B group at 21 dpi and relatively low scores in BI-B fish at 28 dpi, in the presence of many mononuclear cells (Figs. 2C and 2D). Both infected groups showed low scores for typical PD histopathology of the heart (Figs. 2E and F).

Prior to the start of this experiment the fish were screened and found to be negative for SAV and PRV RNA. Despite this two fish in the CT-B group tested positive for SAV RNA (both at 28 dpi with nsp-1 Ct values of 27 and 30). This most likely resulted from cross-contamination during sampling or analysis. In addition, these fish did not show any

discernable anti-viral immune responses further suggesting that the positive SAV result was due to cross-contamination.

3.2 Immune gene transcription

The immune responses observed in groups of fish infected after 2 weeks in seawater (Phase A) have been previously reported [18]. Similarly head kidney samples from all the fish sampled at all time-points (Phase B) were analyzed for 15 genes associated with the innate immune response. However, only the data from fish positive for SAV RNA in heart have been included in the analyses and in the figures for both infected groups since so few BI group fish became infected. The individual values for positive and negative fish at 2 decisive time-points are shown in S1, (3 dpi for IM group) and S2 (28 dpi for the BI group). To further illustrate the validity of excluding the considerable number of negative fish in the BI group, graphs showing the average transcription for positive and negative fish in both infected groups are shown in S3. The negative fish in the BI-B group trended with the CT-B group at all time points. Whereas the negative fish in the IM-B group at 3 and 7 dpi trended with the positive individuals for several immune genes (S3) and 100% prevalence was attained in this group at 14 dpi.

3.2.1 Genes encoding PRRs

Two genes encoding PRRs associated with endosomal membranes, TLR7 and TLR8a1 were examined. Both TLRs were upregulated with median transcriptions of 17-fold (TLR7) and 5.7-fold (TLR8a1) at 7 dpi in the IM-B group. All time-points with positive fish in the BI-B group showed greater median fold increases for TLR7 and TLR8a1 than the IM-B group (Fig. 3 and S4). Additionally, TLR7 showed approximately twice the fold increase in transcription in both infected groups compared to TLR8a1 (Fig. 3). For MDA5 and LGP2a that interact with viral dsRNA in the cytoplasm, MDA5 transcription followed a similar

pattern to the TLRs. Whereas LGP2a, one of the most highly transcribed genes examined, peaked at 7 dpi in the IM-B groups and at 21 dpi in the BI-B group (Fig. 3).

MyD88, was the most highly constitutively transcribed immune gene examined and displayed only moderate fold increases (usually less than 5). It peaked at 7 dpi for IM-B and at 14 dpi for the BI-B group (Fig. 3). IRF7, a central immune response regulator showed a similar profile to the PRRs with a median fold increase of 11.3 and 12.8, in IM-B and BI-B groups, at 7 and 14 dpi respectively (Fig. 3).

3.2.2 Genes encoding immune-modulating proteins

Genes encoding effector molecules such as viperin and Mx were the most highly transcribed genes measured in this study (Fig. 4). Two individuals showed more than a 400-fold increase for viperin in the IM-B group at 7 dpi, but the median value was 155 fold. Similarly, in the BI-B group some individuals at each time-point had very high transcription levels compared to the median (Figs. 3-5 and S4)

IFNa as one of the main immune-modulators responsible for stimulating a myriad of interferon stimulated genes (ISGs) was up-regulated in both infected groups, where 8 and 9-fold increases were observed at 7 and 14 dpi in IM-B and BI-B groups respectively (Fig. 4). In contrast, the gene encoding its cellular receptor, CRFB5 was only up-regulated 4 fold at 21 dpi in the BI-B group in 3 of 5 SAV positive individuals (Fig. 4 and S4)

3.2.3 Genes encoding inflammatory cytokines

IFNγ and CXCL11_L1 were up-regulated in both infected groups, with the highest fold increase in the BI-B group peaking at 21 dpi and 14 dpi respectively, compared to 7 dpi for both gene transcripts in the IM-B group (Fig. 4 and S4). Other interleukins showed little or no up-regulation in the IM-B group. Conversely, IL-1β, IL-8 and IL-4/13A showed clear

231 increases in transcription in the same 3 SAV positive fish showing an increased transcription
232 of CRFB5 in the BI-B group at 21 dpi (Fig. 5 and S4).

233 *3.2.4 Magnitude of transcription*

234 Fish in the IM-B group that were negative for SAV RNA at 3 dpi frequently showed
235 comparable immune gene transcription to positive individuals probably because prevalence
236 reached 100% for the IM-B group shortly afterwards (S1). In contrast, the median fold
237 increases were always higher in the 7 positive individuals in the BI-B group at 28 dpi
238 compared to the negative individuals in this group at this time-point (S2).

239 Viperin and Mx were the most highly up-regulated genes. The IM-B group with 100%
240 prevalence at 7 dpi also showed peak gene transcription for all genes assayed at this time-
241 point decreasing thereafter and mostly returning to 3 dpi levels by 21 dpi. Most genes in the
242 IM-B group had significantly higher transcription levels at 7 and 14 dpi compared to the CT-
243 B group, except CRFB5, IL-8, IL-1β and IL-4/13A (Table 2). At all other time-points there
244 were almost no significant differences in transcription between the CT-B and the IM-B
245 groups for any of the genes assayed (Table 2). The BI-B group with few positive individuals
246 exhibited large ranges of fold increases, but median values followed the IM-B response for
247 many genes (Figs. 3-5, S4). The exceptions were CRFB5, IL-1β, IL-4/13A and IL-8 where
248 the median values were significantly increased at 21 dpi in the BI group and were clearly
249 accounted for by the same 3 individuals (S4). Also positive individuals at both 21 and 28 dpi
250 in the BI group clearly showed different gene profiles (S4). This is illustrated by a heat map
251 of fold changes in the transcription of all genes for the seven BI-B fish that tested positive for
252 SAV RNA at 28 dpi, where individual fish have very different immune response profiles
253 (Fig. 6). Individuals 3 and 5 show high responses of many genes whereas individuals 4, 6 and
254 7 show only a few raised inflammatory genes, while individuals 1 and 2 appear unresponsive
255 for all genes by comparison. Interestingly, the viral load in heart (nsp-1 Ct value) appears to

be unrelated to the immune gene transcription pattern assessed in kidney tissue despite the systemic nature of PD (Fig. 6)

3.3 Smoltification status, and effect of adaptation time in seawater

The fish in these experimental groups had been transferred to seawater 9 weeks before infection and therefore sodium potassium ATPase (NKA) levels were not evaluated as it was assumed they had developed good seawater tolerance. In fact fish from the same production batch challenged only 2 weeks after seawater transfer (Phase A) were evaluated and found to have acceptable NKA activities with no influence of SAV infection in gill tissue [24]. However, the effect of this extra time in seawater on the basal transcription of immune genes compared to fish transferred only 2 weeks before challenge [18] was interesting. The transcription of viperin, IFNa, MyD88, TLR7, TLR8a1 and IRF7 was significantly increased in CT-B fish compared to CT-A fish (Fig. 7A). Conversely, the transcription of some inflammatory genes (IL-8, IL-1β, IL-4/13A and IFNγ) was higher in the CT-A fish (Fig. 7A).

In addition, when the IM-A and IM-B groups were compared it was found that IM-B fish had significantly higher responses for viperin, IFNa, MyD88, TLR7, TLR8a1 and IRF7 at 7 and 14 dpi (Fig. 7B and Table 3). Whereas, several genes had significantly lower transcription in IM-B compared to IM-A including IL-1β, IFNγ, LGP2a and Mx at 7 and 14 dpi (Fig. 7B and Table 3). Unfortunately, there were too few SAV positive fish in the BI-B group to make any meaningful comparison with BI-A fish.

4.0 Discussion

4.1 SAV infection and PD status

Fish in both IM-B and BI-B groups became infected with SAV. Fish in the BI-B group were infected to a much lesser extent which can be partly explained by exposure to a reduced

amount of SAV in the shedder water compared to the IM-B group that were injected. The relative paucity of virus produced by the shedder fish was probably due to their smoltification status which has been discussed previously [24]. As the shedder fish and the BI-B group fish were from the same batch of fish the effect on the BI-B group was two-fold, since not only did the shedder fish produce less virus, but the BI-B fish were less susceptible [24]. Conversely, although the IM-B group were also from the same batch as the BI-B group, their dose was injected and all these fish became infected and developed PD.

The fish negative for SAV in heart tissue in the IM-B group at 3 dpi that exhibited some immune gene transcription (S1) was surprising since the nsp-1 assay for SAV RNA is very sensitive, but these individuals may have had viraemia. At 28 dpi the immune gene transcription in the BI-B group was more complex. Some of the seven SAV positive fish at this time-point probably represent naïve fish infected after day 0, for example: infection that resulted from shedding activity of the fish that were infected on day 0. Fish showing high viral loads at 28 dpi in the BI-B group were possibly more recently infected compared to IM-B fish (injected on day 0) whose viral loads peaked at 14 dpi and were decreasing by 28 dpi (Fig. 2B). The possibly delayed infection of some of the 7 positive fish at 28 dpi in the BI-B group is also illustrated not only by the range of viral loads in the heart tissue, but also by the relatively large ranges of immune gene transcriptions. Conversely, at 21 dpi the viral loads in in the BI-B group were lower than at either 14 or 28 dpi, perhaps indicating the beginning of a second round of infection in this group. This hypothesis demonstrates the infectious nature of SAV since the exposure was to very few positive fish (prevalence 16 % at 14 dpi and undetectable at 7 dpi) and the fish density was decreasing throughout the experiment due to sampling.

4.2 Anti-viral immune response

Genes involved in early innate responses; TLRs, MDA5, MyD88, IRF7 and even IFNa and Viperin were expressed in synchrony in the IM-B and BI-B groups. In contrast, the BI-A group showed a delayed transcription of these genes compared to IM-A fish [18]. Many of the genes showed a pattern of maximum transcription at 7 dpi in the IM-B and at 14 dpi in the BI-B group. Had there been positive fish sampled at 7 dpi the BI-B group they may well also have shown peak transcription at 7 dpi, as in the IM-B group. In fact the median trend lines for several immune genes of these 2 groups of infected fish (Figs. 3-5) suggest that there could have been infected fish (SAV positive) at 7 dpi in the BI-B group. The prevalence was probably too low to be detected by sampling only 8 fish from each tank (from 50 remaining fish at this time-point). This result is in clear contrast to the BI-A fish, where although prevalence reached 100% at 14 dpi, peak transcription of immune genes was at 21 dpi [18]. This could indicate that at later times after seawater transfer the natural infection route in the BI-B group resulted in a faster response time with peak immune gene transcription following the IM-B group and peaking earlier than in the BI-A group. This is further illustrated by the increased transcription of IFNa, MyD88 and TLR8a1 in several negative fish at 7 dpi in the BI-B group (data not shown). Similarly, the negative fish in the IM-B group at 7 dpi showed comparable transcription of innate immune genes to the positive fish at this time-point (S3).

The transcription of the TLRs analysed showed greater fold increases in TLR7 than for TLR8a1, however TLR8a1 has between 5 and 10 times higher constitutive transcription levels meaning the transcription of these two TLRs in infected groups were comparable. Similarly, for the cytosolic PRRs, MDA5 showed only a 6.5 fold increase in both infected groups compared to LGP2a that showed 11 and 35 maximal fold increases in the IM-B and BI-B groups, respectively. However, MDA5 has a 4-5 times higher resting transcription level compared to LGP2a, that could also indicate more equal levels of these two transcripts. These

observations indicate that fold increases alone are not the best way of reporting immune responses.

The IFN response was relatively robust in both infected groups of Phase B fish, with high transcription of IFNa at 7 and 14 dpi in the IM-A fish group, whereas positive fish in the BI-B group had high IFNa transcription at 14 and 28 dpi. By contrast, the Phase A fish have previously shown both a transient IFNa transcription in IM-A fish and a very weak transcription of IFNa in BI-A fish [18]. The more sustained IFNa response in the Phase B trial could be a result of higher TLR transcription that was approximately twice that seen for TLR7 and TLR8a1, in Phase A fish [18]. However, this apparently improved IFNa response is not further reflected in higher transcriptions of ISGs, since although viperin transcription is similar, Mx transcription was half of that in Phase A fish. Clearly the ISG levels needed to effectively fight the infection are beyond the capability of most individuals, or that the transcription seen for these genes is not correlated with production of functional anti-viral proteins [31]. In fact, it has been demonstrated that only when IFNa (recombinant protein) is added (*in vitro*) before infection [32] or occurs naturally at higher basal levels (in a viral resistant salmon strain) [33] is SAV susceptibility reduced. Further, the natural increase in cortisol during smoltification and after seawater transfer [34] may have reduced the ability of these fish to mount an immune response during the early seawater stage (Phase A). Reduced circulating levels of serum proteins and IgM have been reported in smolts in freshwater and after seawater transfer and have been taken as indicators of reduced immune competence of Atlantic salmon smolts [35].

It has also been demonstrated that SAV can inhibit signal transduction via the JAK/STAT pathway [36] and can also increase the transcription of SOCS1 an inhibitor of cytokine signalling [37]. Such a survival strategy for the virus could result in reduced ISG production including the lower Mx transcription observed in the IM-B group. Viperin could conceivably

remain unaffected due to an alternative mechanism for viperin production during the innate immune response via IRF1 or IFR3 [38], which has been studied in experiments with terrestrial alphaviruses [39].

Interestingly, at 21 dpi some of the BI-B positive fish showed a spike in transcription of genes associated with the inflammatory response, such as IL-1β, IL-8, IFNγ and also IL-4/13A. The inflammatory response could not be verified histologically since only 1 of the 3 fish showing high fold increase of these genes was sampled for histology. Additionally, despite the systemic nature of SAV infection there appears to be no correlation between either viral loads or immune responses (measured in heart and head kidney respectively) and histopathology in the heart or pancreas. However, compared to the Phase A fish, both infected groups from Phase B exhibited lower necrosis of exocrine pancreatic tissue and similar cell infiltration and inflammation in pancreas sections, but only the BI-B fish showed increases in the transcription of inflammatory genes.

The magnitude of transcription in Phase B was similar for many innate immune genes in both infected groups and the BI-B fish gene transcription was largely in synchrony with the IM-B fish. The notable exceptions were the inflammatory genes assayed at 21 dpi in some of the positive fish from the BI-B group. Although all these fish were able to eventually overcome the virus, indicated by the declining SAV RNA levels at 28 dpi, they still developed the clinical signs of PD. The BI-B group showed clear indications of multiple infection points probably due to the low prevalence of infection at the beginning of the experiment and the plethora of naïve fish remaining.

4.3 Effect of time after seawater transfer

The injection of SAV3 into the salmon transferred to seawater 9 weeks earlier (Phase B) resulted in relatively little shedding of infectious virus, reducing the bath immersion dose

[24]. When transcription levels of the immune genes were compared in the control groups (CT-A and CT- B), CT-B fish had significantly higher transcription of several key components of the innate immune response. Viperin, IFNa, TLR7, TLR8a1 and IRF7 had between 2 and 4 times the basal transcription at all time-points compared to CT-A fish (Fig.7A). This suggests that the longer adaptation to seawater allows more energy to be diverted into maintaining immune parameters that may have been down-regulated directly after seawater transfer. Previously, salmon immune genes have been shown to be severely repressed by seawater transfer with no recovery detected for at least 3 weeks post transfer [23]. The significantly higher response of viperin, IFNa, TLR7, TLR8a1 and IRF7 in the IM-B group at 7 and 14 dpi (Fig.7B) was presumably also beneficial in clearing the virus and reducing pathology, indicated by a lack of typical heart pathology in this group. For example, the IM-B group had a higher and more sustained IFN response compared to the transitory response in IM-A fish [18].

This study indicates that smolts fully acclimatized to seawater have not only an increased immune response, but a different one that is accompanied by a higher basal transcription of several innate immune genes. Parr showing morphological signs of smoltification showed increased susceptibility to another viral disease, ISAV [40] and parr compared to smolts recently transferred to seawater showed both lower viral loads (*piscine orthoreovirus*) and increased basal levels of innate immune genes [19]. It could therefore be suggested that after nine weeks in seawater immune gene transcription was returning to pre-transfer (parr) levels and that a compromised immune response is not a result of living in seawater *per se*, but rather the length of time in seawater.

The apparent reduced transcription of inflammatory genes (IFNγ, IL-8 and IL-1β) in CT-B fish (Fig. 7A) could be a result of CT-A fish being stimulated when initially exposed to the plethora of environmental bacteria present in seawater causing raised trancription of these

genes. Also the reduction of inflammatory gene transcription (IFNγ, IL-8 and IL-1β) in IM-B compared to IM-A fish may indicate that these fish had better control of their immune response and didn't exhibit an inappropriate transcription of inflammatory genes.

This is the first study where two groups of fish from the same production batch were challenged at different times after seawater transfer and therefore allows direct comparison in their immune response to a viral infection. Both IM-A and CT-A groups showed reduced transcription of innate immune genes compared to the equivalent groups 7 weeks later (Phase B). This comparison clearly implicates time after seawater transfer as a factor for consideration if fish are to mount and maintain a more effective immune response. After nine weeks in seawater the fish in this study were able to mount a more robust and appropriate immune response and thus reduce pathology.

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Abbreviations

cDNA complementary DNA

CMS cardiomyopathy syndrome

IFN Interferon

i.m. intra-muscular

HSMI heart and skeletal muscle inflammation

IPNV Infectious pancreas necrosis virus

ISG Interferon stimulated genes

RT-qPCR reverse transcriptase quantitative polymerase chain reaction

PPR pattern recognition receptor

PAMP pathogen associated molecular pattern

PD pancreas disease

SAV salmonid alphavirus

SPDV salmonid pancreas disease virus

$TCID_{50}$ 50% tissue culture infective dose

TLR toll-like receptor

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570

Figure legends

Fig. 1 Experimental set-up.

All fish were from same production batch and all were transferred to seawater at the same time. The Phase B groups are presented here. The CT-B group was *i.m.* injected with non-infected cell culture supernatant, the IM-B group was *i.m* injected with 10^4 $TCID_{50}$ SAV3, similarly to the shedders and the BI-B group was bathed in water containing shed virus from the shedder fish (shedder water). The experiment was performed in triplicate tanks for all treatment groups, 65 fish in each tank. Sampling of 8 fish per tank (24 fish per group) was carried out at 3, 7, 14, 21 and 28 dpi.

Fig. 2 PD status of the infected groups

A. Percentage prevalence of SAV RNA measured in heart tissue in IM-B (black bars) and BI-B (dark grey bars) groups at all time-points. n = 24 for both groups and all time-points (except for the IM-B group at 7 and 28 dpi where n = 22 and 23 respectively). **B.** Ct values for nsp-1 assay in all fish positive for SAV RNA plotted in reverse, representing viral load, in IM-B group (O) and BI-B group (►) at each time point. **C to F.** Histological sections of IM-B fish at 21 dpi (C-pancreas, E-heart) and for BI-B fish at 28 dpi (D-pancreas, F-heart). Bar = 50μm

Fig 3. Transcription of PRRs and early innate genes.

The figure shows the transcription of PRRs; TLR7, TLR8a1, MDA5, LGP2a and the early innate genes for MyD88 and IRF7 in head kidney tissue at 3, 7, 14, 21 and 28 dpi. The y axis

represents normalized, fold transcription increases 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 in this box. The whiskers represent the maximum and minimum values for each group. Open bars represent the CT-B group, dark grey bars the IM-B group and light grey bars the BI-B group. Trend lines indicate transcriptional changes over time; solid line IM-B group and dashed line the BI-B group. Asterisks denote statistical significant differences between the IM-B and CT-B groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig 4. Transcription of immune modulators and IFNs.

The figure shows the transcription of interferon and one of its cell receptor components (CRFB5), Viperin, Mx IFNγ and CXCL11_L1 in head kidney tissue at 3, 7, 14, 21 and 28 dpi. The y axis represents normalized, fold transcription increases 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 in this box. The whiskers represent the maximum and minimum values for each group. Open bars represent the CT-B group, dark grey bars the IM-B group and light grey bars the BI-B group. Trend lines indicate transcriptional changes over time; solid line IM-B group and dashed line the BI-B group. Asterisks denote statistically significant differences between the IM-B and CT-B groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

Fig 5. Transcription of interleukin genes

The figure shows the transcription of 3 interleukins, IL-1β, IL-8 and IL4/13A in head kidney tissue at 3, 7, 14, 21 and 28 dpi. The y axis represents normalized, fold transcription increases 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 in this box. The whiskers represent the maximum and minimum values for each group. Open bars represent the CT-B group, dark grey bars the IM-B group and light grey bars the BI-B group. Trend lines indicate transcriptional changes over time; solid line IM-B group and dashed line the BI-B group. Asterisks denote statistically significant differences between the

IM-B and CT-B groups: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Vertical scales have been kept constant to allow comparison between genes.

Fig 6. Heat map of gene transcription in positive individuals at 28 dpi in the BI-B group

The relative transcription of all immune genes assayed shows the range of immune responses in the individuals positive for SAV RNA in head kidney at this time-point indicating that these fish were at different stages of their immune response and further suggesting they had been infected at different time points after the initial bath immersion on Day 0. Blue through yellow to orange and red denotes low to high fold transcription.

Fig 7. Changes in gene transcription in head kidney between Phases A and B in the CT and IM groups

A. Average differences in basal gene transcription between CT-A and CT-B groups (including all time-points). **B.** Average differences in transcription between IM-A and IM.B groups at 7 dpi (black bars) and 14 dpi (grey bars). Differences in gene transcription between Phases A and B are shown as fold changes minus 1, thus showing transcription greater in Phase B as positive values and transcription greater in Phase A as negative values. Gene transcriptions significantly higher in Phase B compared to Phase A are marked ** and *** denoting p values of < 0.01 and < 0.001 respectively. Gene transcriptions significantly higher in Phase A compared to Phase B are denoted by ° or °°° with p values of < 0.05 and <0.001 respectively.

S1. All individual IM-B fish at 3 dpi. Positive and Negative fish

Transcription of immune genes (fold increases) of all IM-B group individuals at 3 dpi in head kidney. At this time-point prevalence was 36% and allows the comparison of immune gene

transcription between individuals positive or negative for SAV RNA. The black bars represent the median value for each group. The y axis is a Log_{10} scale to render the individual data points more visible.

S2. All individual BI-B fish at 28 dpi. Positive and Negative fish

Transcription of immune genes (fold increases) of all BI-B group individuals at 28 dpi in head kidney. At this time-point prevalence was 29% and allows the comparison of immune gene transcription between individuals positive or negative for SAV RNA. The black bars represent the median value for each group. The y axis is a Log_{10} scale to render the individual data points more visible.

S3. Average immune gene transcription in all Phase B fish positive or negative for SAV RNA

Graphs show the average fold change ± SEM in immune gene transcription in head kidney for fish in all groups in Phase B for all genes assayed. Both positive IM-B (red) and BI-B (blue) and negative IM-B (dark red), BI-B (light blue) and CT-B (black) groups are represented on all graphs.

S4. Individual data points for BI-B SAV3 RNA positive individuals

These graphs show the same data as in Figs. 3-5, but show all positive individuals as open circles in the BI-B group for each gene assayed with a black bar showing the median. Median values for CT-B and IM-B groups at each time-point are joined by dashed and solid lines respectively for comparison.

670 **Table 1 Primers**

671 Primers used in the analysis of immune genes together with their amplicon sizes, relative efficiencies and the Genebank accession number used
672 for primer design or the reference for previously published assays.

Target gene	Forward primer 5'-3'	Reverse primer 5'-3'	Amplicon length (bps)	Efficiency	Reference/Genebank accession No.
Viperin	AGCAATGGCAGCATGATCAG	TGGTTGGTGTCCTCGTCAAAG	101	2.03	Grove 2013 [22]
IFNa	CCTGTGTATCACCTGCCATGAA	GCCTGTGCACTGTAGTTCATTT	100	1.95	NM_001123710
MyD88	CGTGGATAGAAAAGACGTTGTG	CAGGGTGATGCCTTGTCTTT	152	2.07	EF672332
TLR7	CGCATGACGAGGTCAGAAT	GTCCTCTCTCAGTGCAATCTA	172	1.99	HF97058
TLR8a1	GGCTTTCAAAATCTCACAAGGAA	CCTTAATGTCACATGGAAAGT	150	1.93	NP_001155165
IRF7	GGACTCAAACGACCCCCATA	GGTTCAGGTCTAGGTGGTTCAA	194	2.10	NM_001136548
MDA5	CTCGTGAACTACTCAAGAGAATCG	CCTGGCTCATCTATCAAGTTAT	145	1.98	NM_001195179*
CXCL11_L1	GCTCCATTTGCCAAGAAAA	GGCACTGACTCAACTGTGGTAA	162	2.04	BT049408
CRFBa,b,c	CACCCAGGGCTCCATGAA	CACCAGGTTGTTGCTAGAGT	132	2.03	KF976458/59/60
IL-8	GAGGATTTCTAGTAGGATCATCT	ATGAGTCTACCAATTCGTCTGC	134	1.91	NM_001140710
IL-1β	GAGAGGTTAAAGGGTGGCGA	TGCTTCCCTCCTGCTCGTAG	145	1.89	NM_001123582
IL4_13A	CCGACATCTGAGGGTTTACAA	GCATTGTGTGGAGTTGGTGTA	170	2.06	AB574339
IFNγ	GGTCCACTATAAGATCTCCAAGGA	CTGGCAAGATACTCCGATACAC	133	2.00	AY795563
LGP2a	GACCCAGAATGAGCAGAAGGA	CACCACAGAGTAAACGCTGTCACT	198	1.96	NM_001140177
Mx	GGTGGTTGTGCCATGCAA	TGGTCAGGATGCCTAATGTC	100	2.02	U66475/6
EF1A	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	57	2.02	Olsvik 2006 [23]

rainbow trout* and corresponding genomic sequence from Atlantic salmon AGKD03005035

673

Table 2 Significant increases (t tests) in gene transcription in IM-B fish at all time-points and for all genes compared to the CT-B group. Asterisks * denote significantly higher transcription.

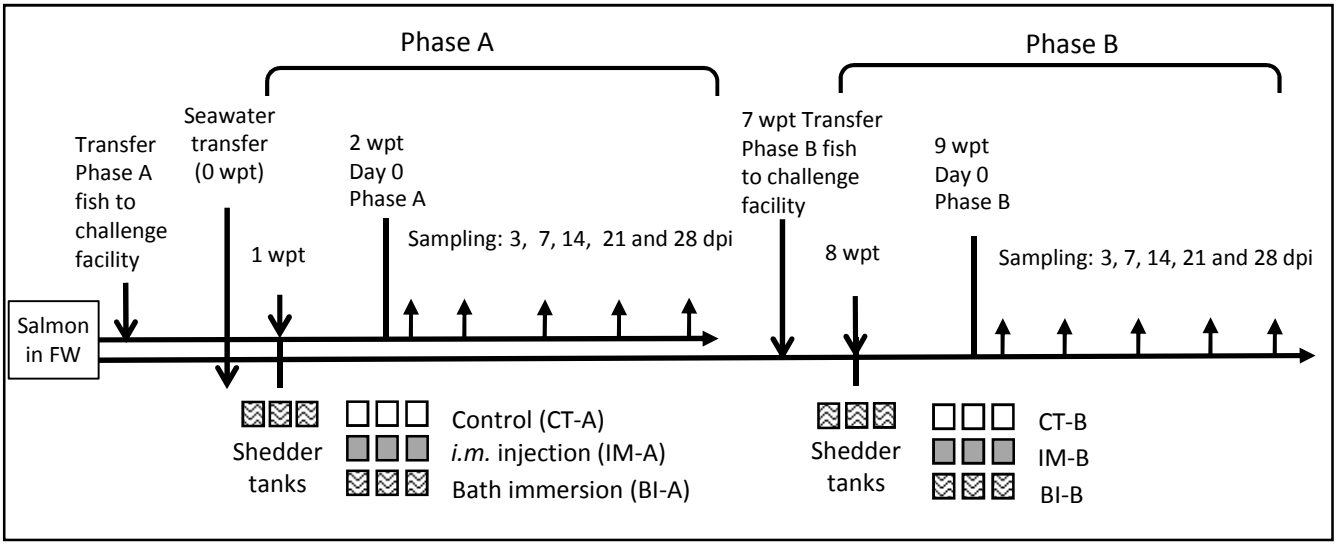
	Viperin	IFNa	MyD88	TLR7	TLR8a1	IRF7	MDA5	CXCL11_L1	CRFB5	IL-8	IL-1β	IL-4/13A	IFNγ	LGP2a	Mx
3 dpi	-	-	*	-	*	-	-	-	-	-	-	-	-	-	-
7 dpi	***	***	***	***	***	***	***	***	-	-	*	-	***	***	***
14 dpi	***	***	**	***	***	***	***	***	*	-	-	-	***	**	***
21 dpi	-	-	-	-	*	**	-	-	-	-	-	-	-	-	-
28 dpi	*	-	-	-	-	-	*	-	-	-	-	-	-	**	**

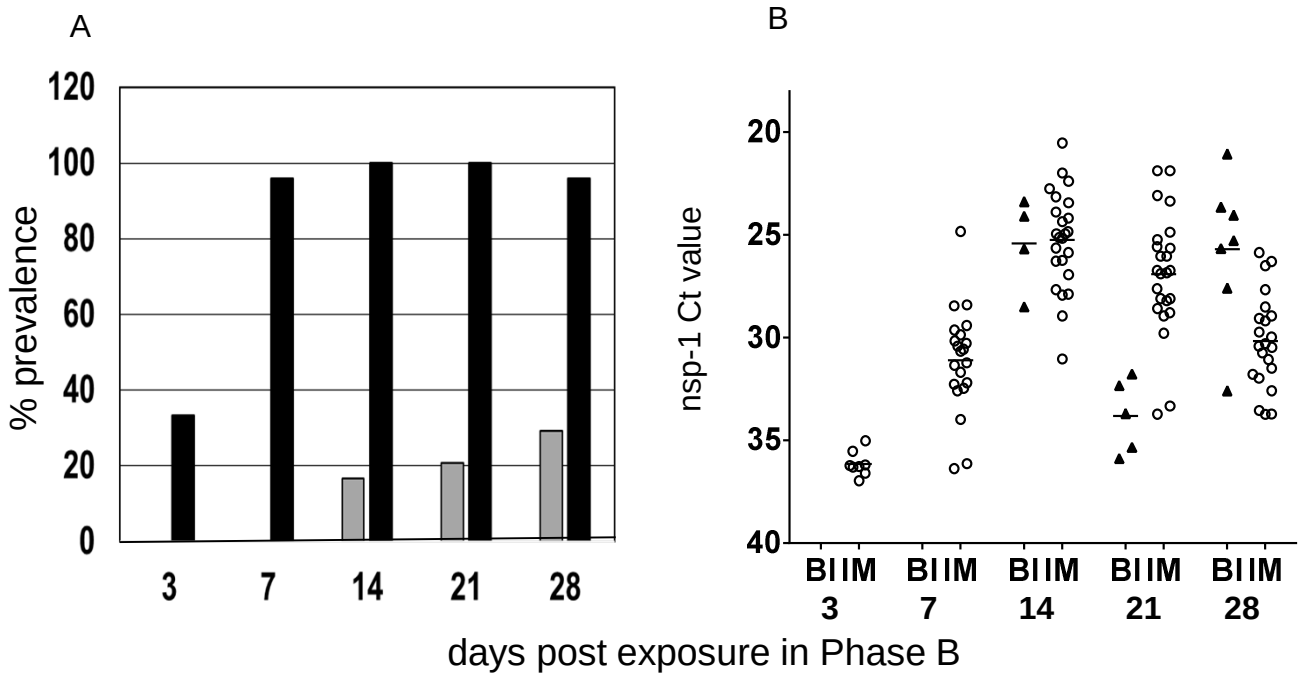
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

Table 3 Significant differences (t tests) in the gene transcription of the IM group in Phases A and B. Asterisks * denote significantly higher transcription in IM-B fish whereas circles ◦ denote significantly higher transcription in IM-A fish

	Viperin	IFNa	MyD88	TLR7	TLR8a1	IRF7	MDA5	CXCL11_L1	CRFB5	IL-8	IL-1β	IL-4/13A	IFNγ	LGP2a	Mx
3dpi	*	-	-	-	***	***	-	-	◦	◦◦◦	◦◦	-	◦◦◦	-	-
7 dpi	**	***	**	***	***	***	-	-	-	-	◦◦◦	-	◦◦◦	◦◦◦	◦◦◦
14 dpi	***	***	***	***	***	***	-	-	◦◦◦	-	◦◦◦	-	◦◦◦	◦◦◦	◦◦◦
21 dpi	-	**	-	-	-	*	◦	**	-	-	-	-	-	-	-
28 dpi	*	**	-	-	**	-	◦◦◦	-	◦	-	-	-	◦	◦◦◦	◦◦◦

*, ◦ $p < 0.05$, **, ◦◦ $p < 0.01$ and ***,◦◦◦ $p < 0.001$


Phase A
Phase B
Transfer Phase A fish to challenge facility
Seawater transfer (0 wpt)
1 wpt
2 wpt Day 0 Phase A
Sampling: 3, 7, 14, 21 and 28 dpi
7 wpt Transfer Phase B fish to challenge facility
8 wpt
9 wpt Day 0 Phase B
Sampling: 3, 7, 14, 21 and 28 dpi
Salmon in FW
Shedder tanks
Control (CT-A)
i.m. injection (IM-A)
Bath immersion (BI-A)
Shedder tanks
CT-B
IM-B
BI-B


A
120
100
80
60
40
20
0
% prevalence
3
7
14
21
28
B
nsp-1 Ct value
20
25
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40
BI
IM
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21
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days post exposure in Phase B

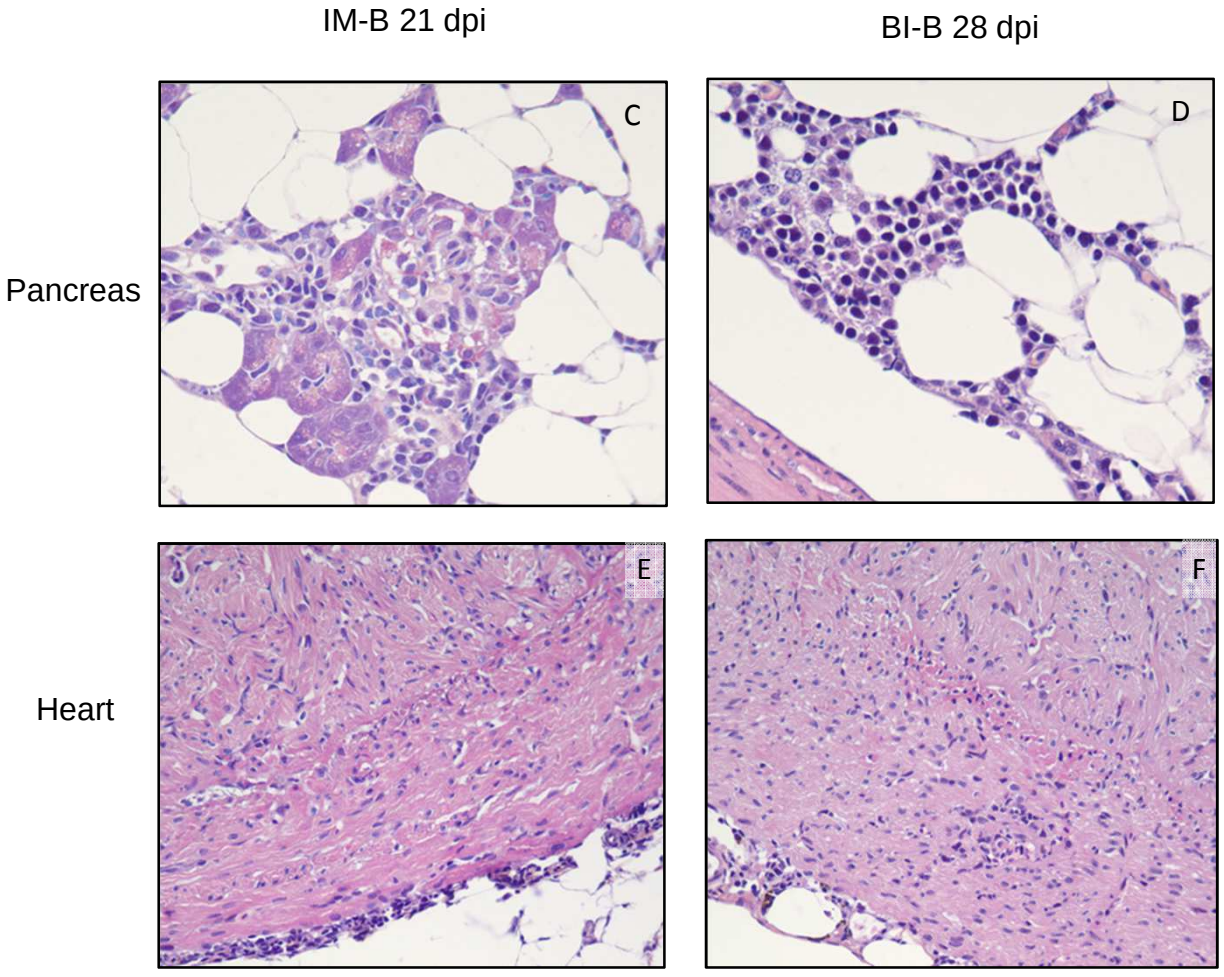
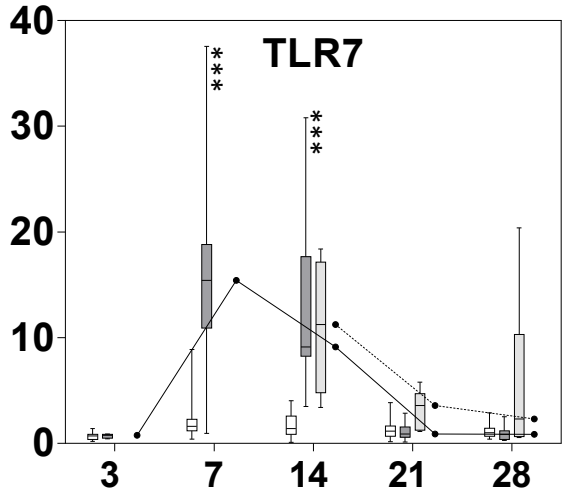

IM-B 21 dpi
BI-B 28 dpi
Pancreas
Heart
C
D
E
F

Fig 3


TLR7
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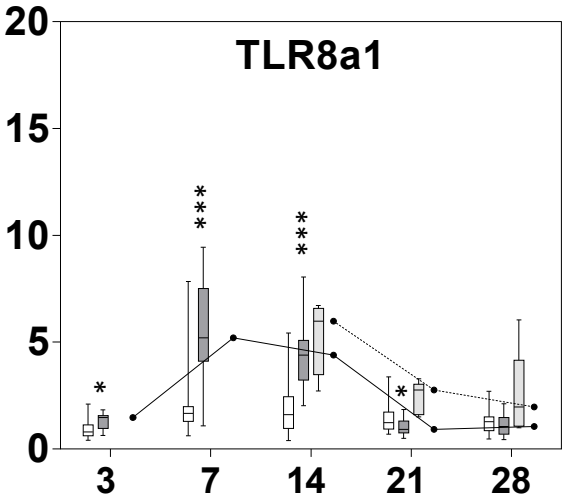
TLR8a1
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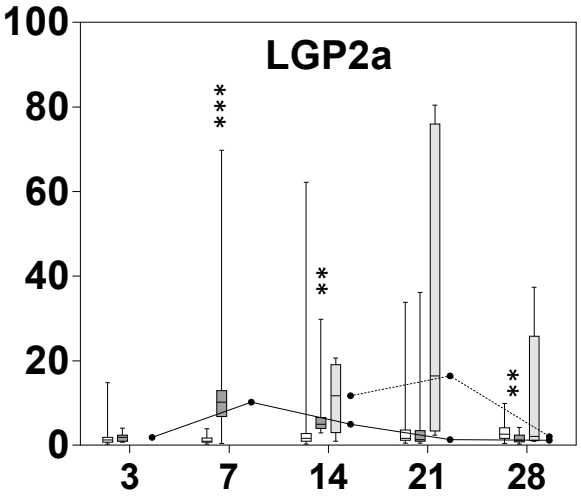
21
28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21
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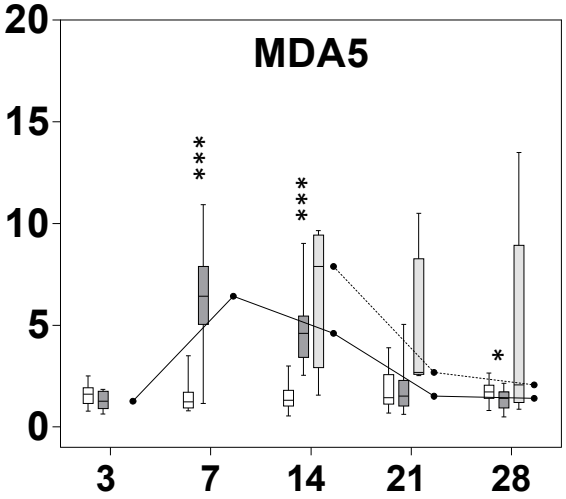
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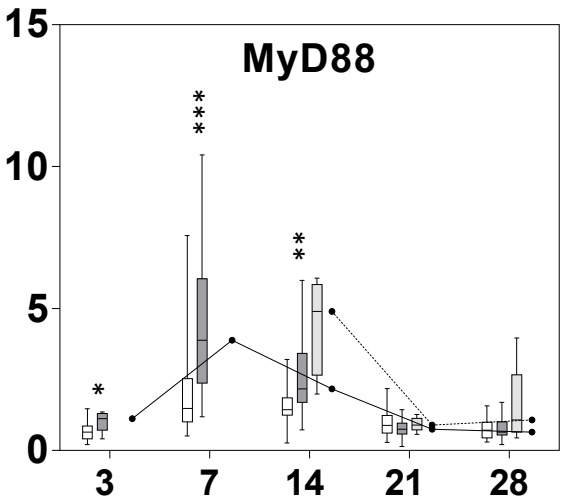
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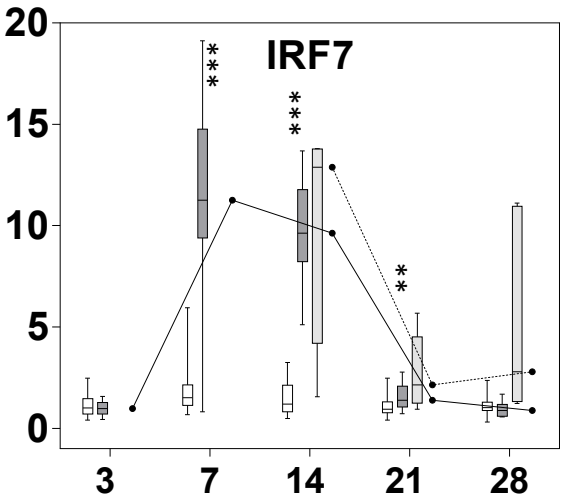
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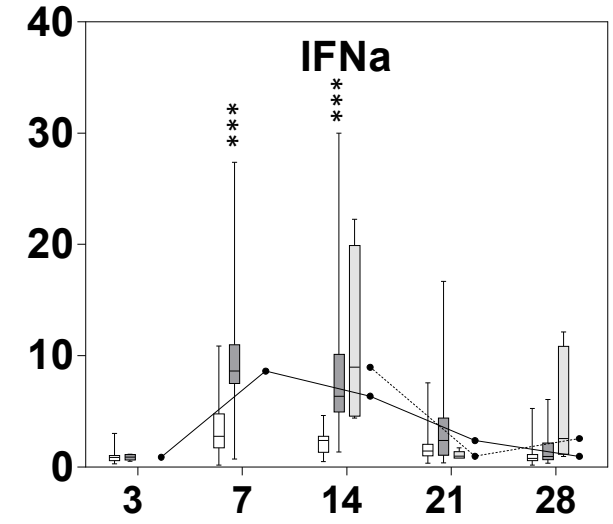
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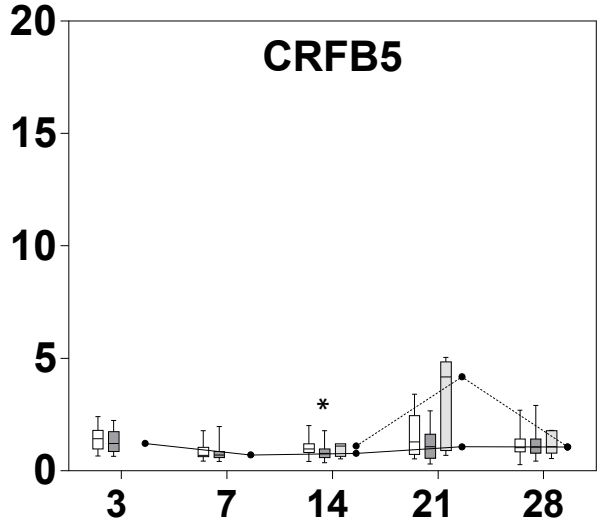
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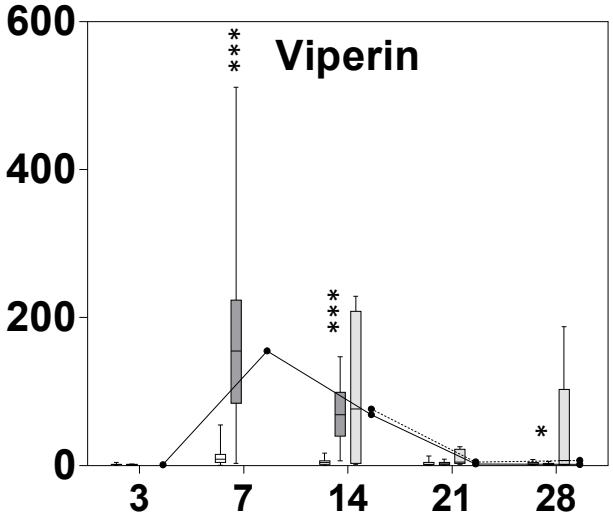
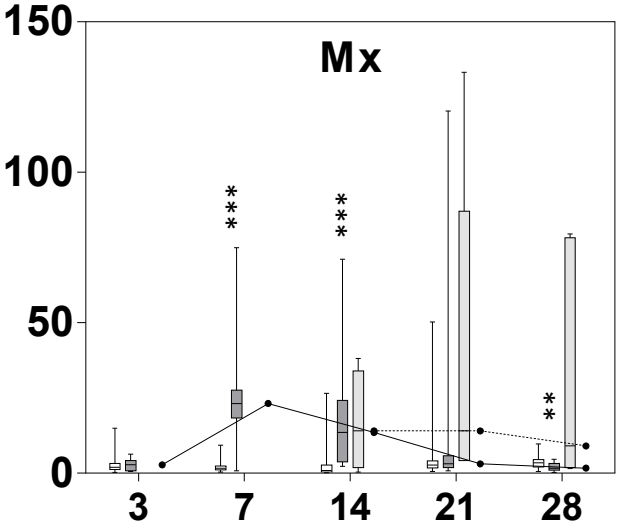

Viperin
600
400
200
0
3
7
14
21
28


Mx
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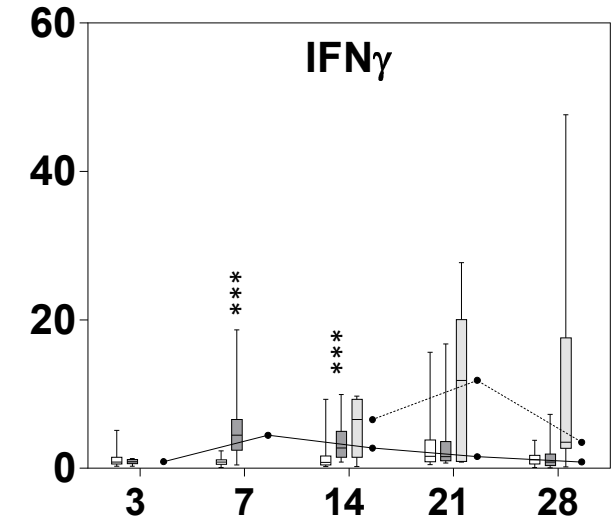
IFNγ
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3
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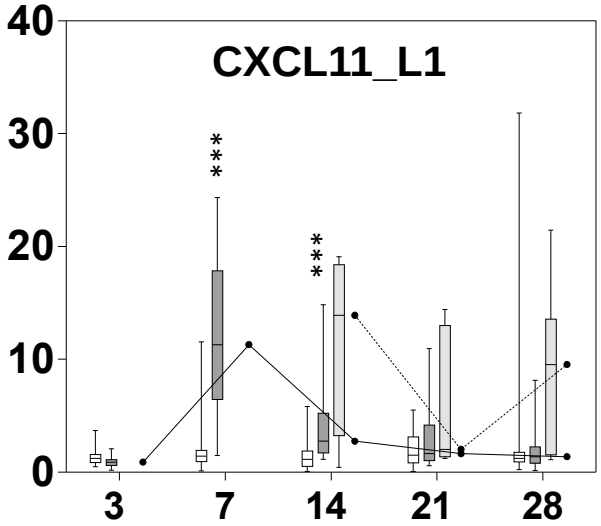
CXCL11_L1
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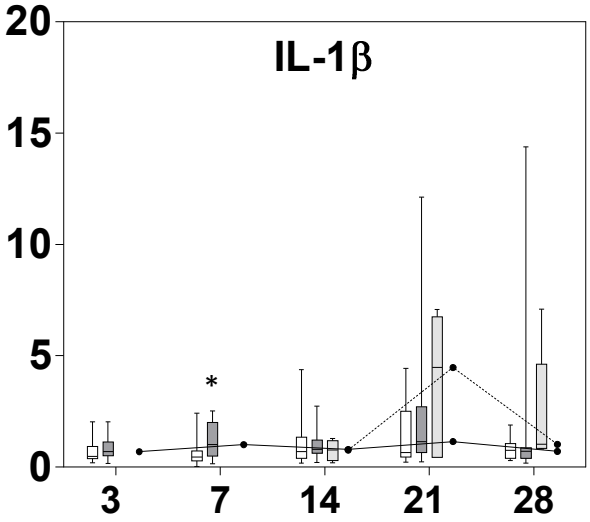
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*
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21
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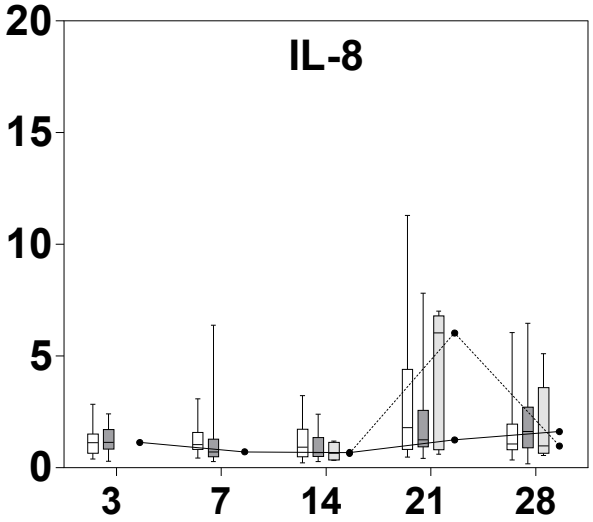
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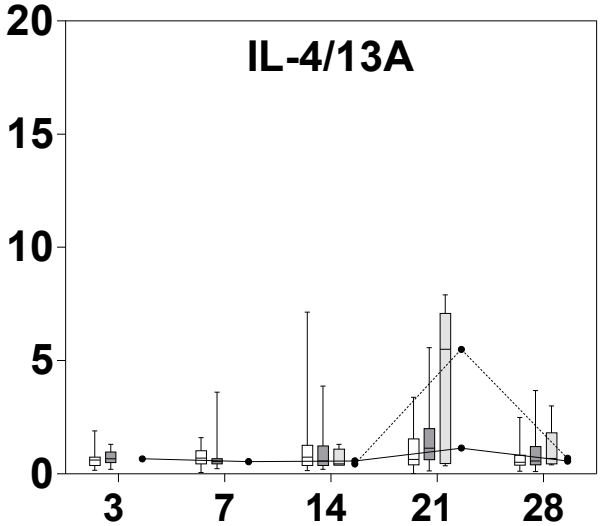
21
28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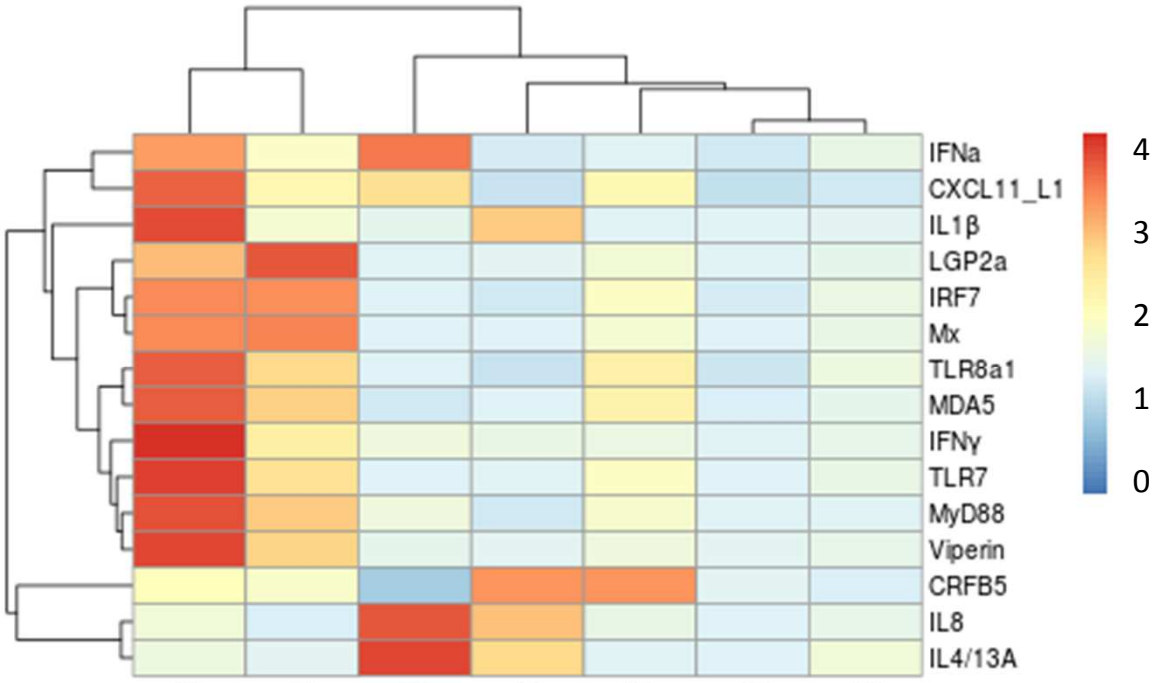
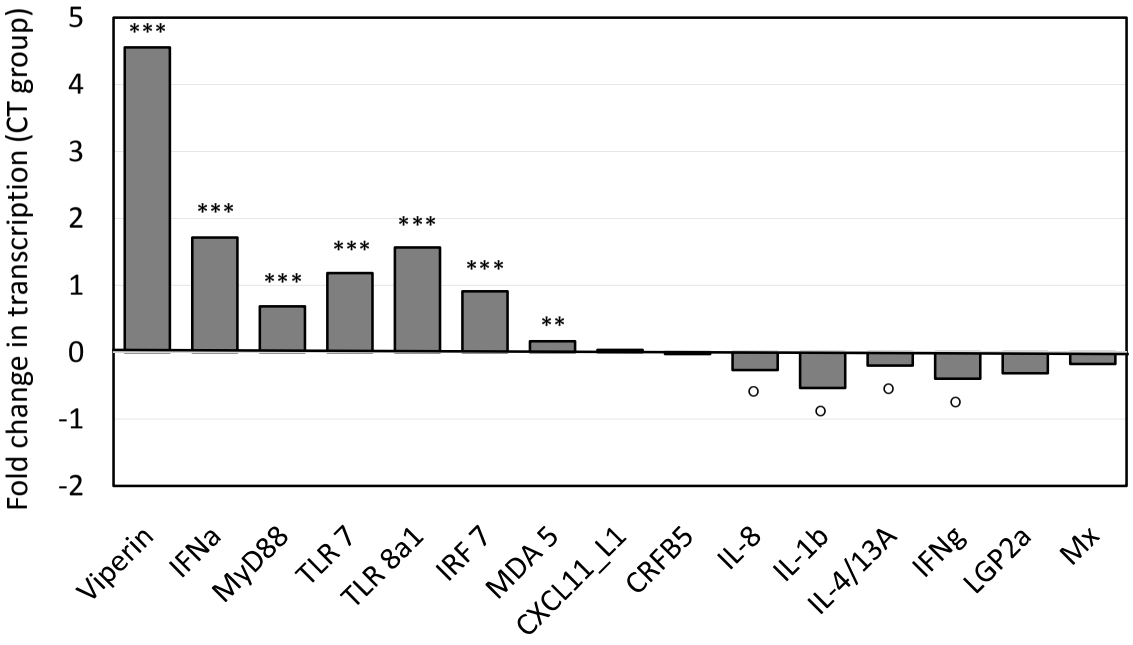
3
7
14
21
28

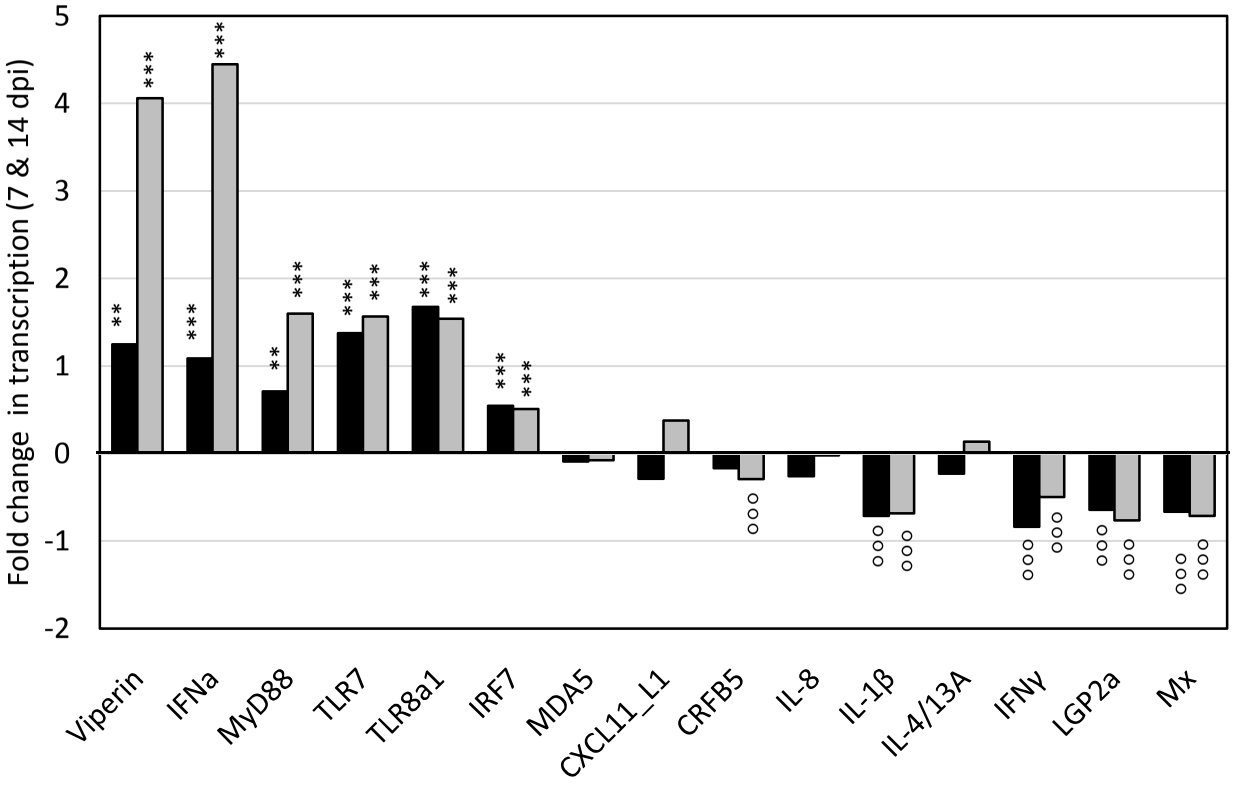


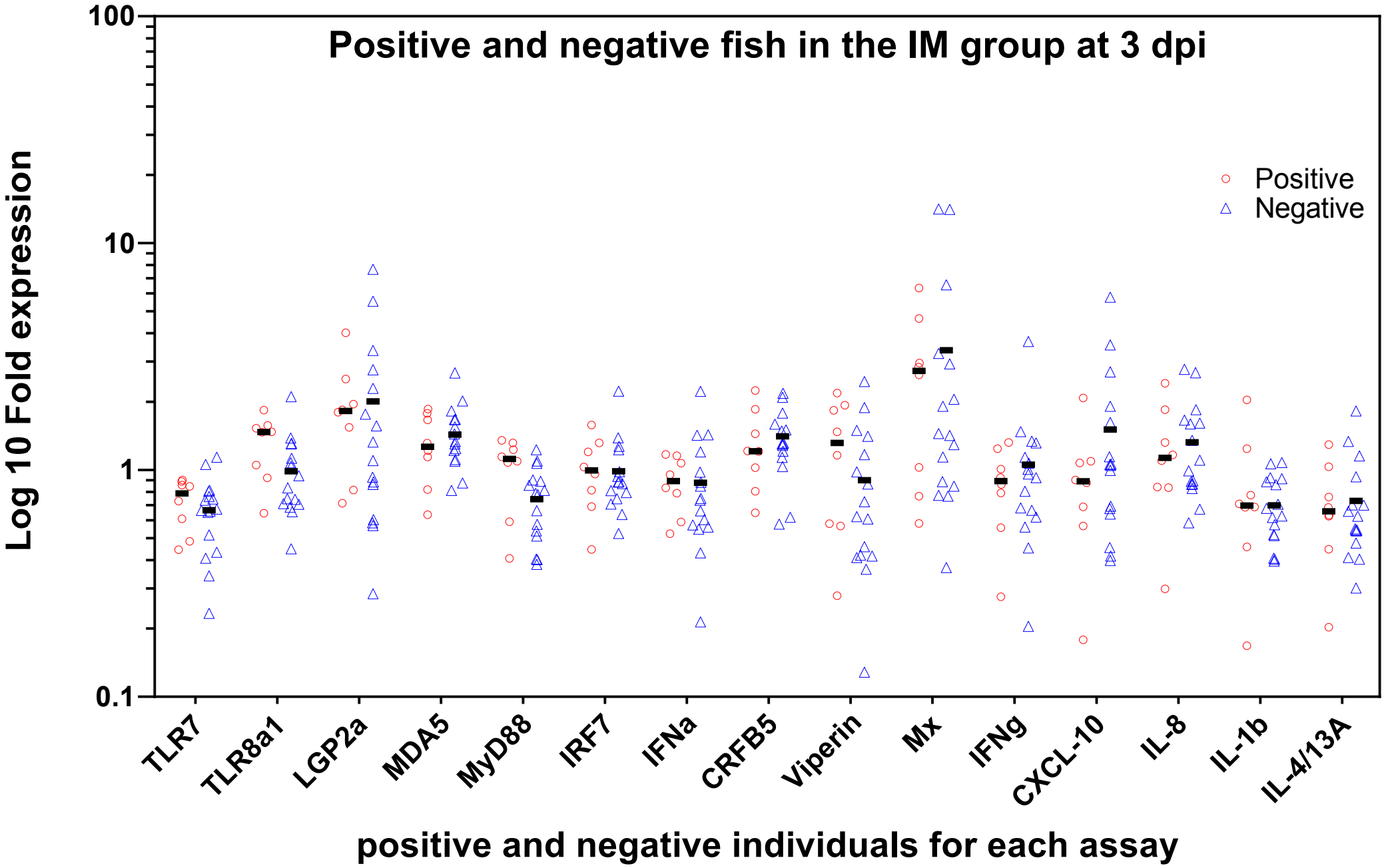
Individual	3	5	4	7	6	1	2
nsp-1 Ct	**23.6**	**24**	**25.7**	**27.6**	**32.6**	**25.3**	**23.6**

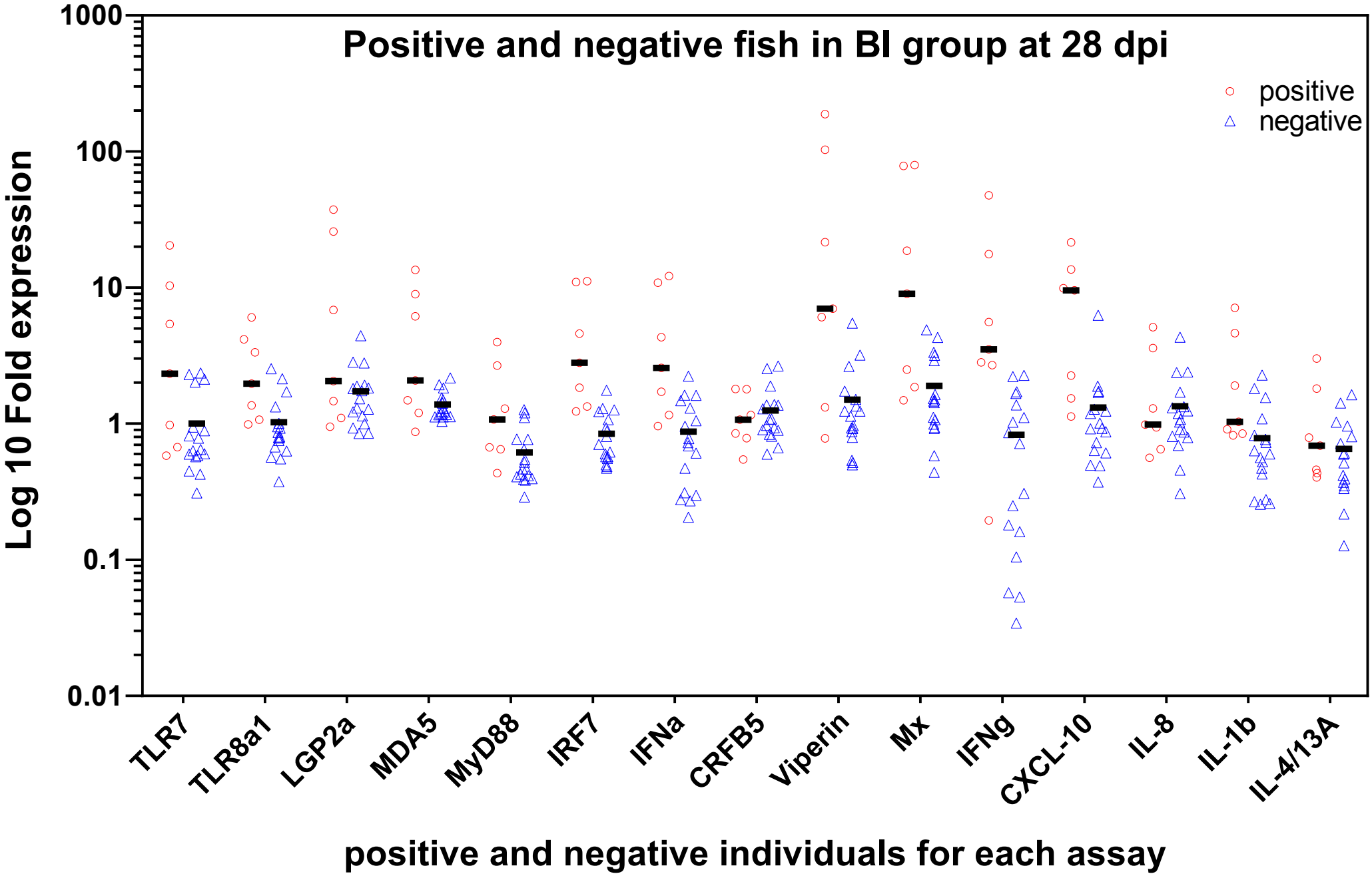
A



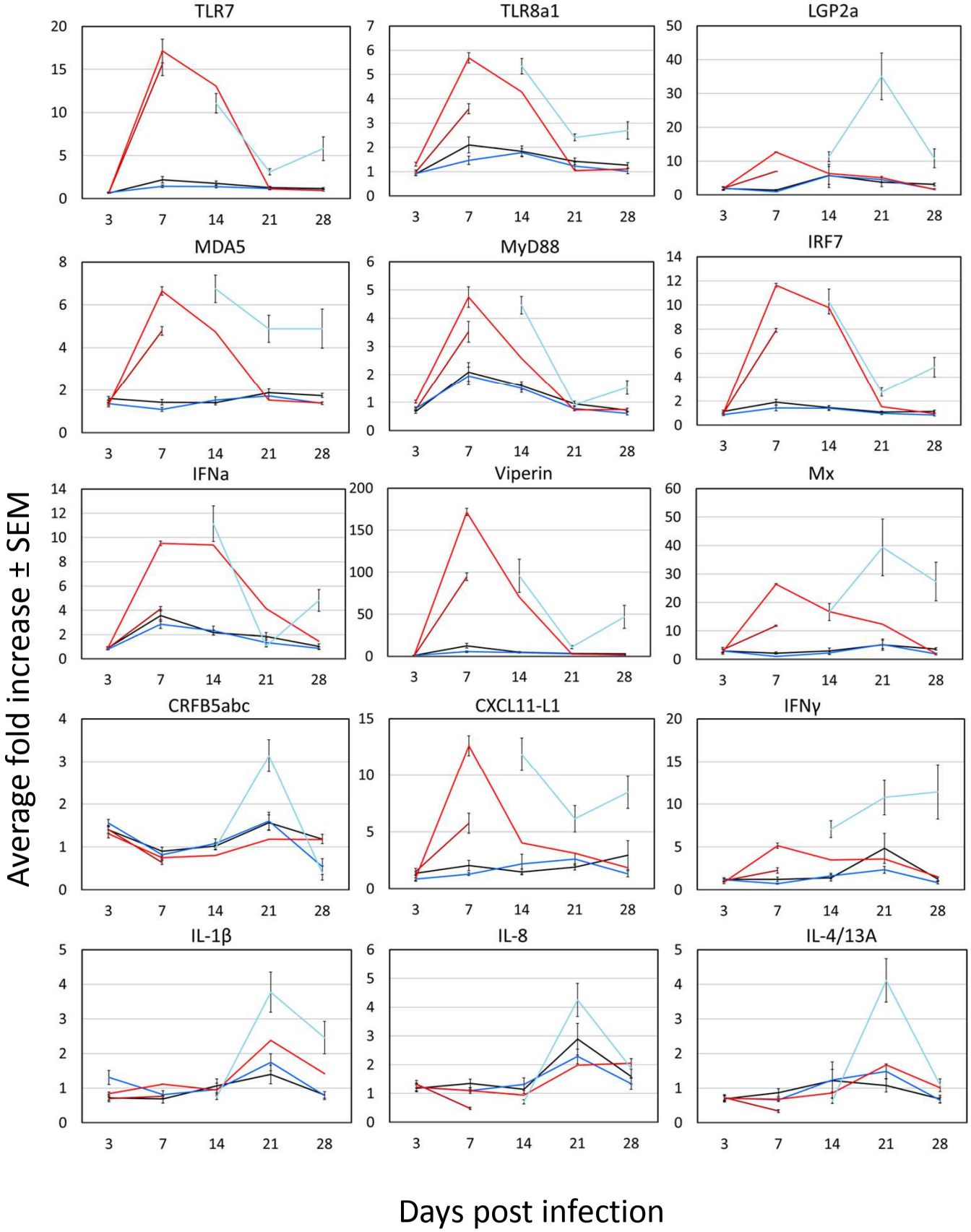
B

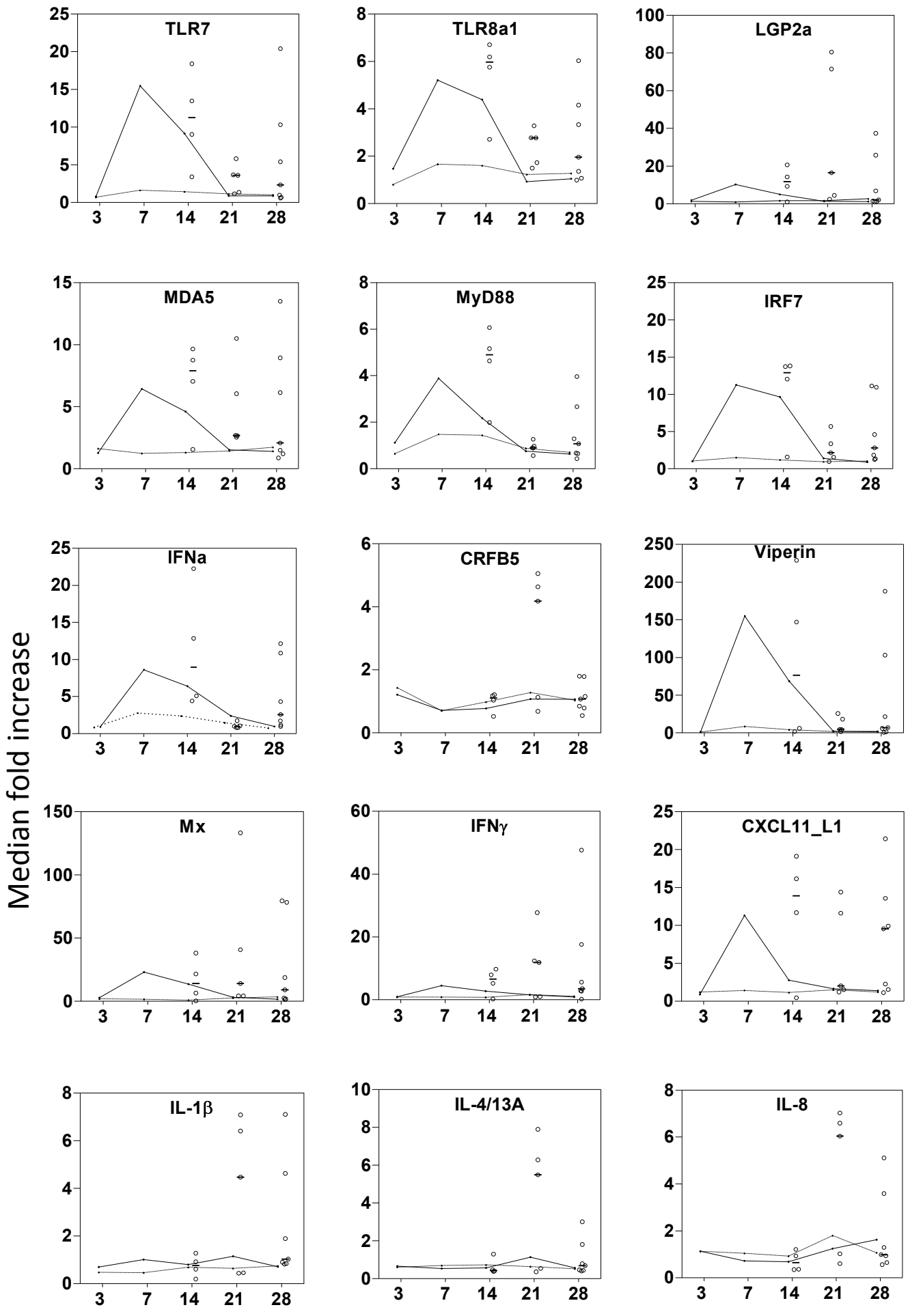






S.2




TLR7
TLR8a1
LGP2a
MDA5
MyD88
IRF7
IFNa
CRFB5
Viperin
Mx
IFNγ
CXCL11_L1
IL-1β
IL-4/13A
IL-8
Median fold increase
Days post infection

Highlights

1. Salmon adapted to seawater for longer time have a higher and longer interferon response to SAV
2. The immune response after bath immersed challenge follows that for a *i.m.* challenge in fish transferred to seawater 9 weeks earlier.
3. Non- infected control fish adapted for longer time in seawater have higher basal transcription of several immune genes compared to fish recently transferred to seawater.
4. After 9 weeks in seawater salmon can maintain a good immune response for longer period