Immune gene profiles in Atlantic salmon *(salmo salar L.)* post-smolts infected with SAV3 by bath-challenge show a delayed response and lower levels of gene transcription compared to injected fish.

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Keywords

Salmonid alphavirus, pancreas disease, gene expression, interferon, bath immersion, infection route.

Highlights:

1. The route of SAV3 infection affects the innate response in Atlantic salmon post-smolts recently transferred to seawater
2. SAV3 bath immersion challenge induces lower and more sustained innate immune response compared to injection challenge
3. Recently smoltified Atlantic salmon have a poor interferon response to salmonid alphavirus.

Abstract

Salmonid alphavirus (SAV) causes pancreatic disease (PD) in salmonids in Northern Europe which results in large economic losses within the aquaculture industry. In order to better understand the underlying immune mechanisms during a SAV3 infection Atlantic salmon post-smolts were infected by either *i.m.*-injection or bath immersion and their immune responses compared. Analysis of viral loads showed that by 14 dpi *i.m.*-injected and bath immersion groups had 95.6% and 100% prevalence respectively and that both groups had developed the severe pathology typical of PD. The immune response was evaluated by using RT-qPCR to measure the transcription of innate immune genes involved in the interferon (IFN) response as well as genes associated with inflammation. Our results showed that IFNa transcription was only weakly upregulated, especially in the bath immersion group. Despite this, high levels of the IFN-stimulated genes (ISGs) such as Mx and viperin were observed. The immune response in the *i.m.*-injected group as measured by immune gene transcription was generally faster, and more pronounced than the response in the bath immersion group, especially at earlier time-points. The response in the bath immersion group started later as expected and appeared to last longer often exceeding the response in the *i.m*-injected fish at later time-points. High levels of transcription of many genes indicative of an active innate immune response were present in both groups.

Introduction

Salmonid alphavirus (SAV) also known as salmon pancreas disease virus (SPDV) causes pancreatic disease (PD) in Atlantic salmon and rainbow trout in fresh and salt water in Northern Europe. There are several sub-types (SAV1-6) which show distinct geographical distributions [1, 2]. Until recently, all Norwegian PD outbreaks were shown to be caused by

SAV3 [3]. In 2010, SAV2 was introduced to Norway and in the last few years this isotype has been shown to be responsible for an increasing number of PD outbreaks [4]. SAV is a positive sense, single stranded RNA virus that can act as an mRNA and be directly translated after entry. The 12kb genome has two open reading frames encoding 4 structural, capsid/membrane proteins (E1-3 and 6K) and 4 non-structural proteins (nsP1-4).

SAV causes inflammation and cellular necrosis in target organs, initially in exocrine pancreas followed by heart and then skeletal muscle. Mortality can be difficult to reproduce experimentally, but appears to be exacerbated by stressors such as fish transport and the handling associated with anti-lice treatment [5].

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 [6-8]. The classical IFN response promotes and maintains an anti-viral state in two steps, with the first step resulting in the production of IFN. The second step maintains an anti-viral state by stimulating the transcription of a myriad of IFN stimulated genes (ISGs) of which there are over 300 known in mammals [9]. Increased transcription of interferon in fish has been observed in SAV infections and other viral infections [10-13]. The interferon response has been studied in experimental SAV infections instigated by both injection and cohabitation [14-16].

In mammals the pathway from virus attachment and internalization to changes in gene transcription including interferon production has been well characterised [17, 18]. Since many of the same genes have teleost counterparts it should be possible to study this pathway in similar detail during SAV infection of Atlantic salmon [12]. The entry route of SAV in Atlantic salmon is unknown, but once SAV has gained access to permissive cells, molecules detecting the single stranded viral RNA (ssRNA) form the first line of defence. As a pathogen

associated molecular pattern (PAMP), viral ssRNA interacts with pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) triggering IFN production. The accompanying inflammatory response can be both beneficial and detrimental to the host. Many of the genes involved in these pathways have been characterised in salmonids and have previously been shown to be modulated during viral infections [14-16, 19]. In human alphavirus infections patients can be left with chronic polyarthralgia [20] and in fish, recovered individuals often fail to thrive and can exhibit poor fillet quality at slaughter [21]

We have recently established a bath-immersion infection model for SAV3 in Atlantic salmon in seawater [22]. This model provides both a natural route of infection and synchronisation of the time of infection, by limiting the exposure time to 6 hours. Also, since in Norway SAV3 most commonly affects Atlantic salmon during their first summer, which can be shortly after sea transfer for spring smolts, it was also relevant to examine the immune response to this virus shortly after seawater transfer.

In the study presented here, we have compared the transcription levels of a panel of innate immune genes many of which have been shown to be modulated during viral infections in fish. The immune gene transcription was compared between fish infected with SAV3 via bath-immersion and those infected by *i.m.*-injection. Our results revealed important differences in the kinetics and duration of the immune responses triggered by SAV3 infection following either bath-immersion or *i.m.*-injection.

2 Materials and Methods

Atlantic salmon post-smolts (average weight 41 g) were infected with SAV3 by *i.m.* injection (IM) with10^4 $TCID_{50}$ per fish or by bath immersion (BI) 2 weeks after transfer to seawater. Salinity was maintained at 34.5 ‰ for whole experimental period. Sea water containing virus

for the BI group was produced by shedder fish injected with 10^4 $TCID_{50}$ SAV3 per fish one week before the experiment started. A third group was injected with non-infected cell culture supernatant as a control group (CT). They were held in triplicate tanks at 12 ºC and 8 fish were sampled from each tank of 65 fish. This corresponded to 24 fish from each treatment group, at 1, 3, 7, 14, 21 and 28 dpi (Fig. 1).

The virus prepared for use in this experiment was subsequently discovered to be contaminated with infectious pancreas necrosis virus (IPNV). However, head kidney samples from BI fish were negative for IPNV RNA at all sampling points and although 25% of the IM fish were positive the Ct values were on average 36 indicating very low levels of virus. Thus, it is unlikely that the IPNV present had major effects on the interpretation of the results in this study.

More details regarding the fish, virus and experimental procedures have been described in our previous study [22].

2.1 Bath immersion dose

Seawater (1 litre) was sampled from each of the three shedder tanks on the day of bath immersion. It was filtered, concentrated and eluted in lysis buffer [22]. The SAV3 RNA measured in these seawater samples using a one-step RT-qPCR assay represents the bath immersion dose. The average Ct value of 1 litre of filtered/concentrated seawater from shedder tanks was 28, and the Ct value of 100 µl of the SAV3 stock used to inject the IM group, was 21.5. This SAV3 stock was diluted 1:100 before use, approximating a Ct value of 28. Since the fish both drink seawater and filter it through their gills during the 6 hour exposure there was probably little difference between the IM dose and the BI exposure.

2.2 Sampling and RNA extraction

Pancreas and heart tissue samples were fixed and processed for histological examination from 4 of the 8 fish sampled at 7, 14 and 21 dpi [22]. Heart and head kidney tissue samples for RT-qPCR analysis were flash frozen in liquid nitrogen and total RNA isolated using Trizol as previously published [22]. RNA concentration and quality was estimated 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.

2.3 cDNA synthesis and RT-qPCR

One-step RT-qPCR (AgPath, Ambion) was employed to detect SAV3 RNA in heart using a modified TaqMan nsP1 assay [23] with a sense probe. Heart has been previously been shown to be a target organ for SAV where the viral RNA persists longest indicating infection long after the relatively short viraemic phase and recovery of histopathological changes in the pancreas [24]

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 RT-qPCR on pooled cDNA showed that this was an optimal dilution. Assays for TLR7, TLR8a1, MyD88, MDA5, LGP2a, IRF7, IFNa, Mx, IFNγ, CXCL11-L1, IL-1β, CRFB5, IL-8 and IL-4/13A were designed for use in this study. In addition, an assay for viperin was adapted from a previously published study [14]. All primers and assay data are listed in Table 1. All head kidney cDNA samples were analysed with the above mentioned assays. Assays were designed with primers on 2 exons or where at least one primer spanned an exon boundary. Some assays were generic, such as that encoding the IFNa receptor chain CRFB5 which was designed to detect all 3 isoforms a, b and c [25]. The Mx assay detects Mx1, 2 and 3, whereas the LGP2a assay would not detect LGP2b [26]. The TLR8b isotypes (TLR8b1 and b2) were undetectable in the pooled cDNA used to screen immune assays and a further isotype of TLR8 (TLR8a2) had between 10 and100 times less transcription, which is agreement with *in vitro* studies [27]. Hence only TLR8a1 [28] was chosen for immune gene analysis in this study. Activation of both the innate immune response and of inflammatory genes has been noted previously and the genes chosen for analysis helped evaluate these important pathways. All assay products were visualized on a 3% MetaPhor® Agarose gel (Lonza) and sequenced to verify the specificity of the assay. Efficiencies were also calculated for each primer set using triplicates of a five point, 4 x dilution series of the pooled cDNA. Elongation factor 1A [29] was used for normalization and is considered the best option of several endogenous reference genes evaluated for use with Atlantis salmon during SAV infection [30] .

RT-qPCR was run in 384 plates using Brilliant III Ultra-Fast SYBR® Green master mix (Agilent) and Applied Biosystems 7900HT Fast Real-Time PCR system 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 and included a melting curve analysis for each run.

2.4 Data Analysis

The Ct values were normalized using Ct values from the elongation factor 1A assay run on the same plate for each individual (ΔCt). Fold change of transcription for each gene was calculated by subtracting normalized Ct values for each gene from control fish sampled before day 0 and used as calibrators ($2^{-\Delta\Delta Ct}$) [31]. Outliers were present in all groups, but not removed from any of the data sets for either analysis or presentation in the figures as they represent the real biological diversity of these groups.

One-way ANOVA was calculated after transforming the data (+1, $\log_{10}$) followed by Neumans Keul's post hoc test using Statistica version 12.7 to examine differences between treatments and tanks. Although these methods use averages in their calculations because of the asymmetric distribution of the data, medians were used for discussion and visual representation of the data.

Figures have been prepared using Prism 6.0 (Graphpad.com) and Excel 2013.

3 Results

Identification of differences in the infection status and in the immune response between the IM and BI groups were analysed by estimation of the SAV3 RNA in heart tissue and by measurement of 15 immune genes in head kidney tissue using RT-qPCR. There were no significant changes in the transcription of the immune genes measured between the experimental groups at 1 or 3 dpi, and therefore 1 dpi results are not shown. The elongation

factor used for normalization of transcription had an average Ct value of 18.3 at 3 dpi rising slightly to 18.5 at 28 dpi with 90% of all samples lying between 17.5 and 19.5

3.1 PD status

PD status was determined by analyzing the transcription of SAV3 RNA in heart tissue and by histological examination of heart and pancreatic tissue samples. The percentage prevalence was calculated from the number of fish per group at each time point that were positive for SAV3 RNA in heart tissue (Fig. 2). Prevalence was higher in the IM group at 3 and 7 dpi, with 12 (50%) and 21(87.5%) of 24 fish positive for SAV3 respectively, compared to only 2 (8.3%) and 16 (66.7%) of 24 fish in the BI group at these two early time-points (Fig. 2A). Additionally, the amount of virus (SAV3 RNA) was higher in the IM group than in the BI group at these time-points (Fig. 2B). By 14 dpi the prevalence in the IM and BI groups was 95.8% (23 of 24) and 100%, respectively (Fig. 2A). At later time-points, when all the BI group fish were positive (100% prevalence), only 1-2 fish were negative in the IM group (Fig. 2A). Interestingly, although both viral load and prevalence was lower in the BI group than in the IM group until 14 dpi, the BI group showed significantly higher amounts of SAV3 at 21 and 28 dpi ($p \leq 0.05$, Fig. 2B). At 14 dpi although prevalence was maximal in both groups, viral load was still lower in the BI group. Histological examination showed loss of exocrine pancreatic tissue and cell infiltration at 7 and 14 dpi in the IM and BI groups, respectively (Figs. 2C and D). Heart tissue showed lesions typical for PD with necrotic foci present at 14 and 21 dpi in IM and BI groups respectively (Figs. 2E and F). We also noted that four (3.3%) of the control fish tested positive for nsP1. This was most likely due to contamination during sampling or analysis, since these fish showed no increase in immune gene transcription and pre-screening prior to the start of the experiment had shown these fish to be negative for both SAV and IPNV.

3.2 Immune gene transcription

Head kidney samples were analysed for 15 genes associated with the innate immune response. The IM group showed peak up-regulation at 7 dpi for many genes. Conversely, the BI group failed to up-regulate relevant immune genes as quickly, but by 21 or 28 dpi when the transcription of many genes in the IM group had returned to control levels the BI group exhibited peak fold increases for many of the same genes (Figs. 3-5). Interestingly, fish negative for SAV3 in heart tissue frequently showed immune gene transcription levels in head kidney comparable to positive individuals. This phenomenon could be seen at 3 dpi in the IM group and at 7 dpi in the BI groups, when prevalence was 50% and 66.6% respectively (S.1). Since prevalence reached 100% for both groups after this early phase, all fish are included in the analyses and presentation of the immune gene results.

3.2.1 Genes encoding PRRs

Two genes encoding PRRs associated with endosomal membranes, TLR7 and TLR8a1, and two PRRs that reside in the cytosol, LGP2a and MDA5, were examined. Both TLRs were upregulated with a maximum transcription at 7 dpi in the IM group, and at 21 dpi in the BI group, although TLR7 showed a higher transcription than TLR8a1 for both groups (Fig. 3). TLR7 peaked with a 7.2-fold increase in the IM group at 7 dpi and with a 5.7-fold increase in the BI group at 21 dpi. MDA5 and LGP2a that interact with viral dsRNA in the cytoplasm showed similar patterns of transcription. LGP2a was one of the genes showing the highest fold increase in transcription, with 29 and 21-fold increases in IM at 7 dpi, and BI at 14 dpi respectively (Fig. 3). MDA5 showed more moderate fold increases of 5.8-fold at 7 dpi in the IM group and 4-fold at 21 dpi, in BI group. All these PRRs were significantly highly up-regulated in both infected groups compared to the CT group at 7, 14 and 21 dpi. Many of the genes were also significantly differently regulated between the IM and BI groups (S.3)

229 *3.2.2 MyD88 and IRF7*

230 The ubiquitous adaptor molecule MyD88, was the most highly constitutively expressed
231 immune gene examined. The transcription of MyD88 peaked at 7 dpi for the IM group and at
232 21 dpi for the BI group (Fig. 3). The downstream transcription factor, IRF7 showed a similar
233 profile to the PRRs with a maximum fold increase of 9.8 in IM at 7 dpi, and 8.5 in BI groups,
234 at 21 dpi (Fig. 3).

235 *3.2.3 Genes encoding immune-modulating proteins*

236 Genes encoding effector molecules such as viperin and Mx were the most highly upregulated
237 genes measured in this study. Some individuals in the IM group showed more than a 200-fold
238 increase at 7 dpi for viperin, while the median value was 100-fold. The maximum
239 transcription for viperin in the BI group was 35-fold at 14 and 21 dpi. Mx peaked at
240 approximately 92 and 48-fold at 7 and 14 dpi, in IM and BI groups respectively. These genes
241 also followed a pattern of maximum up-regulation of transcription at 7 dpi for the IM group
242 while the BI group had a later, lower, but sustained up-regulation of transcription of both
243 viperin and Mx. (Fig. 4).

244 IFNa as one of the main immune-modulators responsible for stimulating many ISGs was by
245 contrast only moderately increased (5.4-fold at 7 dpi in IM group) and was never more than
246 2-fold increased in the BI group.

247 *3.2.4 Genes encoding cytokines associated with the inflammatory response*

248 Genes associated with the inflammatory response IFNγ and CXCL11-L1 were more highly
249 transcribed in the IM than in the BI group. The IM group peaked at 7dpi where transcription
250 of IFNγ was increased 5.3-fold and CXCL11_L1 8.3-fold (Fig. 5). Some individuals in the BI
251 group showed high fold transcription increases of these genes at 7 and 14 dpi, but the highest
252 median values were 1.8 and 2.7-fold at 14 dpi for IFNγ and CXCL11-L1 respectively (Fig.

5). IL-1β, that we hypothesised may also be involved in the inflammatory response to SAV3, did not display increased transcription at any time point in any of the experimental groups (S.2). However, at 14 and 21 dpi there were significant differences in IL-1β transcription levels between the control group and the infected groups coinciding with severe necrosis, observed histologically in the pancreas (Figs 2C and 2D and S.3). The transcription of CRFB5 (encoding an IFN type I receptor chain), IL-8 and IL-4/13A showed negligible regulation during the sampling period in all treatment groups (S.2 and S.3).

3.2.5 Magnitude of transcription

All the genes assayed and their relative transcription levels between the treatment groups are compared using a one-way ANOVA. The transcription of IL-1β, CRFB5, IL-8 and IL-4/13A was relatively unregulated throughout the experiment in all treatment groups (S.2 and S.3). At 7, 14 and 21 dpi all other genes in both infected groups were significantly upregulated compared to the CT group ($p \leq 0.01$) (S.3). At 7 dpi, when IM genes were at their peak all genes had significantly higher fold transcriptions than both CT and BI groups ($p \leq 0.01$, except for LGP2a, $p \leq 0.05$). At 14 dpi the fold increase in transcription for both infected groups was significantly higher than in the CT group ($p \leq 0.001$, except for IM *vs* CXCL11-L1, $p \leq 0.01$). However, at 14 dpi many genes displayed similar fold changes between the infected groups since the fold changes in transcription of genes in the IM group were mostly decreasing and in the BI group they were mostly increasing (Figs. 3-5). Thus, at 14 dpi only TLR7 showed a significant difference ($p \leq 0.001$) between the infected groups (S.3). At 21 dpi the transcription of genes in the BI group had significantly higher fold increases than the IM group for all genes ($p \leq 0.001$). At 28 dpi some genes including, TLR7, MDA5 and IRF7 were still significantly more highly transcribed in both infected groups compared to the CT group.

277 *3.2.6 Tank effects*

278 Some of the fish in one of the triplicate tanks in the IM group at 7 dpi displayed strong up-
279 regulation of IFNa, viperin, and MyD88 together with much lower transcriptions of Mx,
280 LGP2a and IFNγ compared to individuals from the other 2 replicate tanks. This is apparent
281 by the wide range of values at this time-point in the IM group (Figs. 3-5). The IM group at 7
282 dpi was the only time point where this phenomenon was present. It is possible that by
283 sampling at 3, 7 and 14 dpi a similar picture of gene transcription was present before or after
284 the 7 dpi sampling, in the other 2 replicate tanks. Also 3 of the 8 individuals sampled from
285 this tank were negative while the other 2 tanks showed 100% prevalence indicating that the
286 fish in this tank were displaying a slightly delayed disease progression. However, if this tank
287 is removed from subsequent statistical analysis only LGP2a is affected being then not
288 significantly different to the BI group at this time-point (results not shown).

289

290 ## 3.3 Correlation between viral load and immune gene transcription

291 Since the infection by SAV was driving the immune response, some positive correlation
292 between the viral load (Ct value of nsP1) and the magnitude of transcription (fold increases)
293 might be expected, for at least some of the immune genes measured. However, this was rarely
294 the case and only four genes (IFNγ, CXCL11-L1, MDA5 and Mx) at two time-points (7 and
295 21 dpi) showed a correlation of $R^2 > 0.5$ (Table 2). Interestingly, the correlation with the IM
296 group was always lower than for the corresponding gene in the BI group (Table 2) regardless
297 of how poor that correlation was.

4 Discussion

We have studied immune gene responses to the Norwegian sub-type of SAV in Atlantic salmon post-smolts, recently transferred to seawater, using a newly established bath immersion model and compared it to an *i.m.* infection model. We have measured the transcription of 15 genes involved in the innate immune response, particularly those involved in the classical interferon response leading to the transcription of many ISGs. There are clear differences in the immune gene transcription between fish infected by *i.m.* injection and those infected by bath immersion. The defined time-of-infection, the similar dose given to both infection groups and the large number of individual fish that were sampled gives this study the statistical strength to explore the mechanisms in detail.

4.1 SAV status

The amount of SAV RNA present in heart tissue increased during the experimental period in both infected groups, and almost 100% prevalence was apparent in heart from both groups by 14 dpi. Recent analyses in our laboratory indicate that the range of Ct values reported here (between 20 and 30) corresponds to nsP1 copy numbers in the range 4×10^2 to 4×10^5 (data not shown).

There was little difference in the transcription of most of the genes between positive and negative fish at earlier sampling points (S.1) possibly because although the fish were infected, the viral replication had not yet reached a detectable level in heart tissue. Fish which tested negative by RT-qPCR could still have been viraemic, as previously demonstrated [22, 32]. Moreover, both infected groups reached 100% prevalence at later time-points indicating that all fish were infected and were responding with individual variance.

A dose of 10^4 $TCID_{50}$ SAV3 per fish in the IM group induced maximum levels of immune gene transcription at 7 dpi, whereas the maximum viral load was at 14dpi. In the BI group, maximum levels of transcription were observed at 14 or 21 dpi more accurately coinciding with the peak viral load at 21 dpi. The natural route of infection in the BI group shows a better correlation between the maximum levels of gene transcription and the peaks of both viral load and prevalence. This is supported by the higher correlation coefficients between the SAV3 RNA levels and the transcription of immune genes in the BI group. Correlation coefficients where R^2 >0.5 were only present in the BI group. In agreement with our BI group result, other studies using a cohabitation model have also concluded that the maximum transcription of innate immune genes occurs at the same time as maximum viral load [16, 33].

The expression of immune genes over time in head kidney during SAV infections is dose dependent. The dose of 10^4 $TCID_{50}$ SAV3 used in the present study produced maximal transcription at 7 dpi in the IM group and 14 or 21 dpi for the BI group. When a high intraperitoneal injection dose of 10^7 $TCID_{50}$ SAV1 was used, maximal transcription followed at 3 dpi [15], whereas a cohabitation model using only 10^3 $TCID_{50}$ SAV3 in shedder fish took 3.5 weeks to observe increases in gene transcription [34]. Johansen *et al*. [34] also state that the gradual increase of positive fish is typical of a cohabitation infection, whereas our study clearly shows a rapid accumulation of positive fish during a bath challenge model comparable to *i.m.* models. In order to evaluate the immune response in all its complexities it is clearly advantageous to have an infection model with a synchronized time-of-infection which is of sufficient infectivity to achieve 100% prevalence during the initial stage of infection. Lower doses in an infection model cause a staggered rather than a synchronized infection due to infected fish shedding virus and exposing naïve fish not infected at time zero [32]. This makes it difficult to relate the immune response to the time of infection. In the

present study, comparing the overall transcription patterns (represented by trend-lines on the figures) for both challenge models consistent patterns for most genes can be seen, indicating a single synchronized point of infection (Figs. 3-6). The synchronized nature of the 2 infected groups is further illustrated by the rather narrow ranges at many time-points. Thus, even small fold changes in the transcription of immune genes between the experimental groups can be significantly different (eg between IM and BI groups at 14 dpi for TLR7, Fig 3 and table 3).

4.2 The anti-viral response

The *i.m.* administration of the infective dose apparently triggered a much stronger initial immune response with high, but transient fold increases in the transcription of many genes. In the BI group, the SAV infection took longer to cause elevated transcription of many of the genes. This indicates that the virus, due perhaps to the route of infection, took a longer time to amplify in the host and reach the viral RNA loads necessary to trigger an immune response. This delayed increase in the transcription of the immune genes in the BI group was possibly the cause of the high viral loads that exceeded viral loads in the IM group at 21 and 28 dpi and of the typical PD histopathology seen at later time-points. Even though the immune response in the IM group was relatively swift and strong, it still failed to prevent disease progression and the development of the typical PD pathology,

The magnitude of IFNa transcription in the IM group was similar to previous *in vivo* studies which also showed progression to PD [16, 35]. The negligible IFNa response in the BI group has been observed previously in cohabitation infections with SAV [14]. In a recent study, recombinant IFNa applied simultaneously with SAV3 to a TO cell culture was able to induce the rapid transcription of ISGs resulting in a 20-fold reduction of SAV3 RNA compared to cells not treated with IFNa [36]. Clearly the more rapid the induction of IFNa the better

protection the host has against SAV. Alternatively, IFNa production may be blocked or inhibited in our study since immune suppression or evasion by terrestrial alphaviruses is well documented [37, 38]. Similarly, SAV3 has recently been shown to modulate the JAK/STAT pathway *in vitro*, causing down-regulation of both Jak2 and Tyk2 (downstream signaling components of the IFN receptor) that could inhibit transcription of ISGs [36]. Salmonids also possess many other type I IFN genes including IFNb and IFNc that were not measured in the current study, but have been shown to increase more dramatically than IFNa during viral infection [39]. Hence, it is possible that these other IFNs could have been orchestrating the sustained increases of many genes seen at 21 dpi in the BI group.

The induction of IRF7 is linked to IFN production and since IRF7 was highly expressed by both infected groups in this study, the IFN production could be via this pathway. However, IFN transcription in the IM group was transient (dropping at 14 dpi) despite high transcription of PRRs and IRF7 at this time-point, suggesting inhibition by viral mechanisms.

The IFN receptor gene CRFB5 displayed only minor changes in transcription in this study. This has also been observed for the IFN receptor 2 gene (IFNR2) [35].

Of the two endosomal PRRs measured, TLR7 was more highly expressed than TLR8a1, although TLR8a1 had approximately 5 to10-fold higher resting/constitutive transcription. Conversely, it has recently been reported that SAV3 infection of TO cells, which have a dendritic/macrophage-like gene expression profile, upregulated only TLR8 and TLR3 and not TLR7, during SAV3 infection [40].

It has been suggested that the cytosolic viral RNA sensing molecules (LGP2a and MDA5) act in parallel and do not compete allowing high levels of both during a viral infection [26]. However, in this study the transcription of LGP2a was much higher than that for MDA5 in both infected groups at all time-points, suggesting that MDA5 was either inhibited by LGP2a

or did not interact with SAV3 RNA sufficiently to cause up-regulation. The latter explanation seems unlikely since MDA5 had the strongest correlation with levels of SAV3 RNA of all the immune genes studied. Similarly, in a previous study LGP2a exhibited higher fold increases in transcription than MDA5 in response to IFN and SAV *in vitro* [41].

The two key molecules that might have been expected to protect against PD, viperin and Mx were both highly, but transiently expressed in the IM group. Conversely, transcription of both Mx and viperin was moderately increased in the BI group compared to the IM group. Grove *et al.* [14] showed that fish relatively resistant to ISAV had a significantly higher constitutive expression of many relevant genes in head kidney such as viperin, Mx, TLR8, CXCL11-L1 and IFNa in a cohabitation experiment using SAV3. This is in agreement with *in vitro* experiments where IFNa was only found to be protective if present before infection [42, 43]. Thus despite a rapid induction of these effector genes in the IM group, in this study, it was apparently too late to control the virus sufficiently to prevent disease development. ISG induction of both Mx and viperin has also been reported in fish cell lines without IFN involvement [44] a mechanism that could account for the relatively high levels of these 2 transcripts in the absence of a robust IFN response in the present study.

Due to the severe necrosis seen histologically in pancreas and heart especially at later time-points, inflammatory genes were considered of interest. There was increased transcription of IFNγ and CXCL11-L1, but relatively little for IL-1β similar to salmon infected ISAV or IPNV [45]. IFNγ causes transcription of CXCL11-L1 and the regulation of these genes in this study showed similar profiles which is comparable to earlier studies [14]. In addition, although PD is a systemic disease, this could also be due to local effects since the inflammation is occurring in heart and pancreas, while these immune genes were measured in head kidney.

There was minimal regulation of IL-4/13A in this study. In a recent study Wang *et al.* found that IL-4/13B was more actively transcribed during infections while IL-4/13A had a higher constitutive expression perhaps explaining why there was minimal regulation of IL-4/13A in the present study [46].

Infections with SAV do lead to the production of neutralizing antibodies that both protect and clear viraemia, [47, 48], but the delayed nature of the adaptive response in ectothermic teleosts makes the innate response pivotal in immune defence. Furthermore, *in vitro* experiments with CHIKV [49] have demonstrated that high transcription of host ISGs are not translated into increased levels of the corresponding proteins and a similar mechanism could account for the severe pathology seen in the present study. There are few studies addressing the teleost response to viral infection at a protein level. Braceland *et al.* [50] have analysed sera of PD infected individuals, but not immune parameters. Measurement of neutralizing antibodies is both relevant and widespread [51-54], and the presence of Mx protein has been semi-quantitatively analysed in heart during SAV1 infection using immuno-histochemistry [15], but clearly there is a dearth of quantitative protein analysis of innate immune effectors such as Mx and viperin in teleosts.

4.3 Smoltification status

The fish infected with SAV3 in this study had recently been transferred to seawater (experiment start 2 wpt) and therefore their immune responses could conceivably have been compromised due in part to the osmotic challenges of adapting to a new life in seawater. Changes in both immune cells and antibody levels associated with smoltification have been previously reported [55, 56]. There is also evidence that during smoltification fish have raised transcription levels of both IFNa and Mx that could protect smolts from virus infection during this period [57]. However, these authors also reported that these increases are negated shortly

after seawater transfer, and if present, were clearly not able to alleviate infection in the present study. Gill ATPase levels were measured in 12 fish from each time-point and each group and were within the expected range [22] indicating these groups of fish were good post-smolts. Differences in susceptibility and immune gene transcription have been noted between parr and smolts for other viruses such as *piscine orthoreovirus* [19] and ISAV [58]. Very recently a massive down-regulation of immune genes has been reported immediately following seawater transfer [59]. Thus, it cannot be ruled out that the stress involved in maintaining osmotic parameters may be one of the contributing factors to the poor immune response seen during these SAV3 infections.

4.4 Summary

There are clear temporal differences in the immune response between these two infection challenge models. Fish in both infected groups developed typical PD pathology and high SAV3 levels. By 14 dpi almost all fish in both the infected groups were positive for SAV3. Histological examination of heart and pancreas showed typical PD histopathology with a delay of approximately 1 week for similar pathology to be observed in the BI group. None of the immune genes in either infected group showed biologically significant increases in transcription until 7 dpi. In the IM group, most of the immune genes evaluated showed a faster, more pronounced, but transient response. Conversely, in the BI group, immune gene transcription exhibited a slower, less pronounced, but more prolonged response, often exceeding the IM response at the later time-points for the same genes. Therefore, the bath immersion model more closely representing the natural route of infection and using an appropriate exposure to SAV for a defined time period is a useful model in which to study the immune response to SAV in salmon. We have measured the transcription of genes involved in the pathways leading to interferon secretion and the production of ISGs, but these are

difficult to compare to previous studies due to differences in both dose and experimental design. It is apparent that the immune response in these groups of infected fish was insufficient to prevent the development of PD and it is likely that the recent transfer to seawater also compromised their immune responses. To further elucidate immune responses during SAV infections the investigation of protein levels for some of these immune genes is needed. Additionally, it will be of great interest to examine the humoral and cellular adaptive response in these groups of infected fish.

Acknowledgements

This research was funded by the Research Council of Norway, Research grant # 224885/E40. The following people are thanked for their expert technical assistance and help during sampling; Ann Catherine Bårdsgjære Einen, Stig Mæhle, Ingrid Fiksdal and Miriam Castillo Furné. Thanks also to Ivar Helge Matre at Matre Research Station, IMR for the production of fish and Joachim Nordbø for fish husbandry and help with sampling. Øystein Evensen, Norwegian University of Life Sciences, is acknowledged for providing the SAV3 isolate.

Abbreviations

cDNA complementary DNA

IFN Interferon

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

SAV salmonid alphavirus

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

TLR toll-like receptor

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686

Figure legends

Fig. 1 Experimental set-up.

All experimental groups of fish were transferred to seawater 1 week before *i.m*. injection of the shedder fish. On the day the experiment started, (0 dpi or 2 wpt, weeks post seawater transfer) the CT group was *i.m.* injected with non-infected cell culture supernatant, the IM group was *i.m* injected with 10^4 $TCID_{50}$ SAV3, similarly to the shedders and the BI 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 treatment group) was carried out at 1, 3, 7, 14, 21 and 28 dpi.

Fig. 2 PD status of the infected groups

A. Percentage prevalence of SAV3 RNA in IM (dark grey bars) and BI (light grey bars) groups at all time-points. Numbers above the columns indicate the number of positive fish per group where prevalence was less than 100%, n = 24 for all group and time-points (except for BI at 14 dpi n = 22). **B.** Average ± SE, Ct values of nsp-1 assay plotted in reverse, represent viral load, in IM group (solid line) and BI group (dashed line) at each time point. Asterisks (*) indicate significant differences in viral load (Ct value) between the 2 groups ($p \leq 0.05$). **C and D.** Histological sections of pancreatic tissue for IM fish at 7 dpi (C) and for BI fish at 14 dpi (D) showing loss of exocrine pancreas tissue and necrosis (►). Bar = 50µm. **E and F**. Histological sections of heart tissue for IM fish at 14 dpi (E) and for BI fish at 21 dpi (F) showing necrotic cardiomyocytes (Δ). Bar = 50µm

Fig 3. Innate gene transcription.

The y axis represents normalized, fold transcription increase 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 control fish, dark grey bars the IM group and light grey bars the BI group. Trend lines indicate transcriptional changes over time; solid line IM group and dashed line the BI group. Vertical scales have been kept constant as far as possible to allow comparison between genes. Statistically significant differences between the means of the experimental groups ($p < 0.05$) are indicated by lower case letters in a column to the left of each time-point. Lower case letters denote the CT group, lower case, italic letters the IM groups and lower case, underlined letters the BI group.

Fig 4. Transcription of IFNa and effector genes, viperin and Mx

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 control fish, dark grey bars the IM group and light grey bars the BI group. Trend lines indicate transcriptional changes over time; solid line IM group and dashed line the BI group.

Statistically significant differences between the means of the experimental groups ($p < 0.05$) are indicated by lower case letters in a column to the left of each time-point. Lower case

letters denote the CT group, lower case, italic letters the IM groups and lower case, underlined letters the BI group

Fig 5. Transcription of cytokine genes associated with the inflammatory response.

Vertical scales represent normalized, fold transcription increases for each treatment group compared to calibrator fish sampled before day 0. Boxes represent the 25^{th} and 75^{th} 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 control fish, dark grey bars the IM group and light grey bars the BI group. Trend lines indicate transcriptional changes over time; solid line IM group and dashed line the BI group. Vertical scales have been kept constant as far as possible to allow comparison between genes.

Statistically significant differences between the means of the experimental groups ($p < 0.05$) are indicated by lower case letters in a column to the left of each time-point. Lower case letters denote the CT group, lower case, italic letters the IM groups and lower case, underlined letters the BI group

S.1 Immune genes in Positive and Negative fish

Transcription of immune genes (fold change in transcription) of all individuals at 3 dpi in the IM group and of all individuals at 7 dpi in the BI group. At these time-points prevalence was 50% in the IM group and 66% in the BI group and allows comparison of immune gene transcription between individuals positive or negative for SAV RNA. IL-8, IL-4/13A and CRFB5 were only very slightly regulated and are therefore omitted for clarity. 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.

S.2 Genes showing relatively little change in transcription

Fold change in transcription of CRFB5, IL-8, IL-4/13A and IL-1β. The y axis represents normalized, fold transcription for each treatment group compared to calibrator fish sampled before day 0. Boxes represent the 25^{th} and 75^{th} 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 control fish, dark grey bars the IM group and light grey bars the BI group. Trend lines indicate transcription over time; solid line IM group and dashed line the BI group. Vertical scales have been kept constant as far as possible to allow comparison between genes. Statistically significant differences between groups are shown in table 3.

Table 1 Primers

Primers used in the analysis of immune genes together with their amplicon sizes, relative efficiencies and the Genebank accession number used 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 [14]
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
CRFB5	CACCCAGGGCTCCATGAA	CACCAGGTTGTTGCTAGAGT	132	2.03	KF97645860
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
ELF1a	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	57	2.02	Olsvik 2006 [19]

rainbow trout* and corresponding genomic sequence from Atlantic salmon AGKD03005035.1

Table 2 Correlation coefficients

Correlation coefficients between the fold increase in transcription for the different immune genes and the viral load (Ct value for nsp-1). All correlation coefficients where $R^2 > 0.5$ were in the BI group and are shown together with the corresponding R^2 for the IM group for the same gene and sampling time-point (dpi).

Assay	dpi	R^2 BI group	R^2 IM group
IFNγ	7	0.63	0.04
CXCL11_L1	7	0.57	0.04
MDA5	7	0.56	0.24
MDA5	21	0.57	0.22
Mx	21	0.51	0.14

Table 3 Significant differences

The data for each gene and time-point and for all fish was transformed (+1, Log_{10}). One-way ANOVA and Post Hoc Neuman Keul's was applied to the data. The table shows all significant differences between treatment groups at each sampling point and for each gene assayed: - no significant difference, $p < 0.05$ grey, $p < 0.01$ black and $p < 0.001$ **bold**

		7 dpi			14 dpi			21 dpi			28 dpi		
Gene assay	**Treatment**	**CT**	**IM**	**BI**	**CT**	**IM**	**BI**	**CT**	**IM**	**BI**	**CT**	**IM**	**BI**
TLR7	**control (CT)**		**.0001**	**.0001**		**.0001**	**.0001**		**.0001**	**.0001**		-	**.0001**
	injection (IM)	**.0001**		**.0001**	**.0001**		**.0002**	**.0001**		.0001	-		**.0001**
	Bath (BI)	**.0001**	**.0001**		**.0001**	**.0002**		**.0001**	**.0001**		**.0001**	**.0001**	
TLR8a1	**control (CT)**		**.0001**	**.0001**		**.0001**	**.0001**		**.0002**	**.0001**		-	.0013
	injection (IM)	**.0001**		**.0002**	**.0001**		-	**.0002**		**.0001**	-		.0268
	Bath (BI)	**.0001**	**.0002**		**.0001**	-		**.0001**	**.0001**		.0013	.0268	
MDA5	**control (CT)**		**.0001**	**.0001**		**.0001**	**.0001**		.0023	**.0001**		-	**.0005**
	injection (IM)	**.0001**		**.0001**	**.0001**		-	.0023		**.0001**	-		**.0002**
	Bath (BI)	**.0001**	**.0001**		**.0001**	-		**.0001**	**.0001**		**.0005**	**.0002**	
LGP2a	**control (CT)**		**.0001**	.0039		**.0001**	**.0001**		.0035	**.0001**		-	-
	injection (IM)	**.0001**		.0186	**.0001**		-	.0035		**.0001**	-		-
	Bath (BI)	.0039	.0186		**.0001**	-		**.0001**	**.0001**		-	-	
MyD88	**control (CT)**		**.0001**	-		**.0001**	**.0001**		**.0001**	**.0001**		-	-
	injection (IM)	**.0001**		**.0006**	**.0001**		-	**.0001**		**.0006**	-		-
	Bath (BI)	-	**.0006**		**.0001**	-		**.0001**	**.0006**		-	-	
IRF7	**control (CT)**		**.0001**	**.0001**		**.0001**	**.0001**		**.0001**	**.0001**		-	**.0001**
	injection (IM)	**.0001**		**.0001**	**.0001**		.0031	**.0001**		**.0001**	-		**.0001**
	Bath (BI)	**.0001**	**.0001**		**.0001**	.0031		**.0001**	**.0001**		**.0001**	**.0001**	
IFNa	**control (CT)**	**.0001**	**.0001**	-		**.0001**	**.0001**		.0037	**.0001**		-	.0018
	injection (IM)			**.0001**	**.0001**		-	.0037		**.0001**	-		**.0008**
	Bath (BI)	-	**.0001**		**.0001**	-		**.0001**	**.0001**		.0018	**.0008**	
Viperin	**control (CT)**		**.0001**	**.0001**		**.0001**	**.0001**		.0011	**.0001**		-	.0098
	injection (IM)	**.0001**		**.0001**	**.0001**		-	.0011		**.0001**	-		**.0005**
	Bath (BI)	**.0001**	**.0001**		**.0001**	-		**.0001**	**.0001**		.0098	**.0005**	
Mx	**control (CT)**		**.0001**	.0013		**.0001**	**.0001**		**.0001**	**.0001**		-	.0035
	injection (IM)	**.0001**		.0018	**.0001**		.0402	**.0001**		**.0001**	-		.0023
	Bath (BI)	.0013	.0018		**.0001**	.0402		**.0001**	**.0001**		.0035	.0023	
IFNγ	**control (CT)**		**.0001**	.0365		.0002	**.0001**		-	**.0001**		-	-
	injection (IM)	**.0001**		**.0001**	.0002		.0093	-		**.0001**	-		-

	Bath (BI)	.0365	**.0001**		**.0001**	.0093		**.0001**	**.0001**		-	-		
CXCL-10	**control (CT)**		**.0001**	-		.0016	**.0001**		-	**.0001**		.0041	.0081	
	injection (IM)	**.0001**		.0026	.0016		.0020	-		**.0001**	.0041		-	
	Bath (BI)	-	.0026		**.0001**	.0020		**.0001**	**.0001**		.0081	-		
IL-1β	**control (CT)**		-	-		**.0001**	**.0002**		.0032	**.0001**		-	.0492	
	injection (IM)	-		-	**.0001**		-	.0032		.0137	-		.0395	
	Bath (BI)	-	-		**.0002**	-		**.0001**	.0137		.0492	.0395		
CRFB5	**control (CT)**		-	-		-	-		-	.0049		-	-	
	injection (IM)	-		-	-		-	-		-	-		-	
	Bath (BI)	-	-		-	-		.0049	-		-	-		
IL-8	**control (CT)**		-	-		**.0002**	.0045		.0337	**.0002**		-	-	
	injection (IM)	-		-	**.0002**		-	.0337		.0313	-		-	
	Bath (BI)	-	-		.0045	-		**.0002**	.0313		-	-		
IL4_13A	**control (CT)**		-	-		-	-		**.0001**	**.0001**		-	-	
	injection (IM)	-		-	-		.0444	**.0001**		-	-		-	
	Bath (BI)	-	-		-	.0444		**.0001**	-		-	-		

Fig. 1

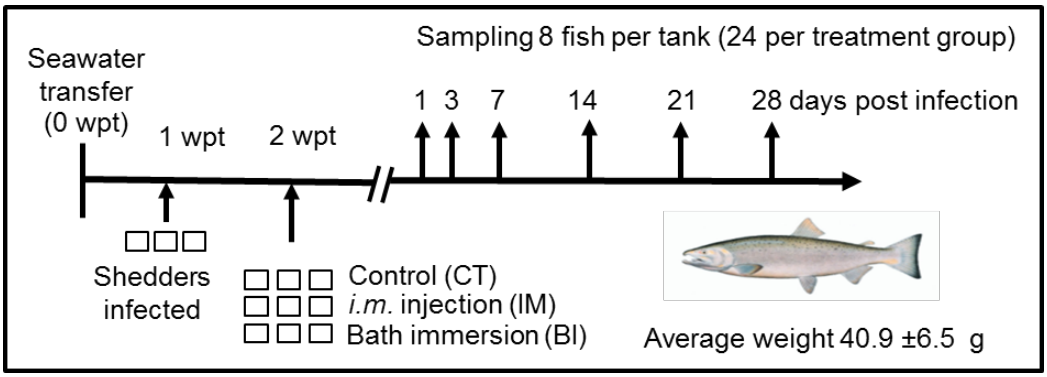

Sampling 8 fish per tank (24 per treatment group)
Seawater transfer (0 wpt)
1 wpt
2 wpt
1 3 7 14 21 28 days post infection
Shedders infected
Control (CT)
i.m. injection (IM)
Bath immersion (BI)
Average weight 40.9 ±6.5 g

Fig. 2

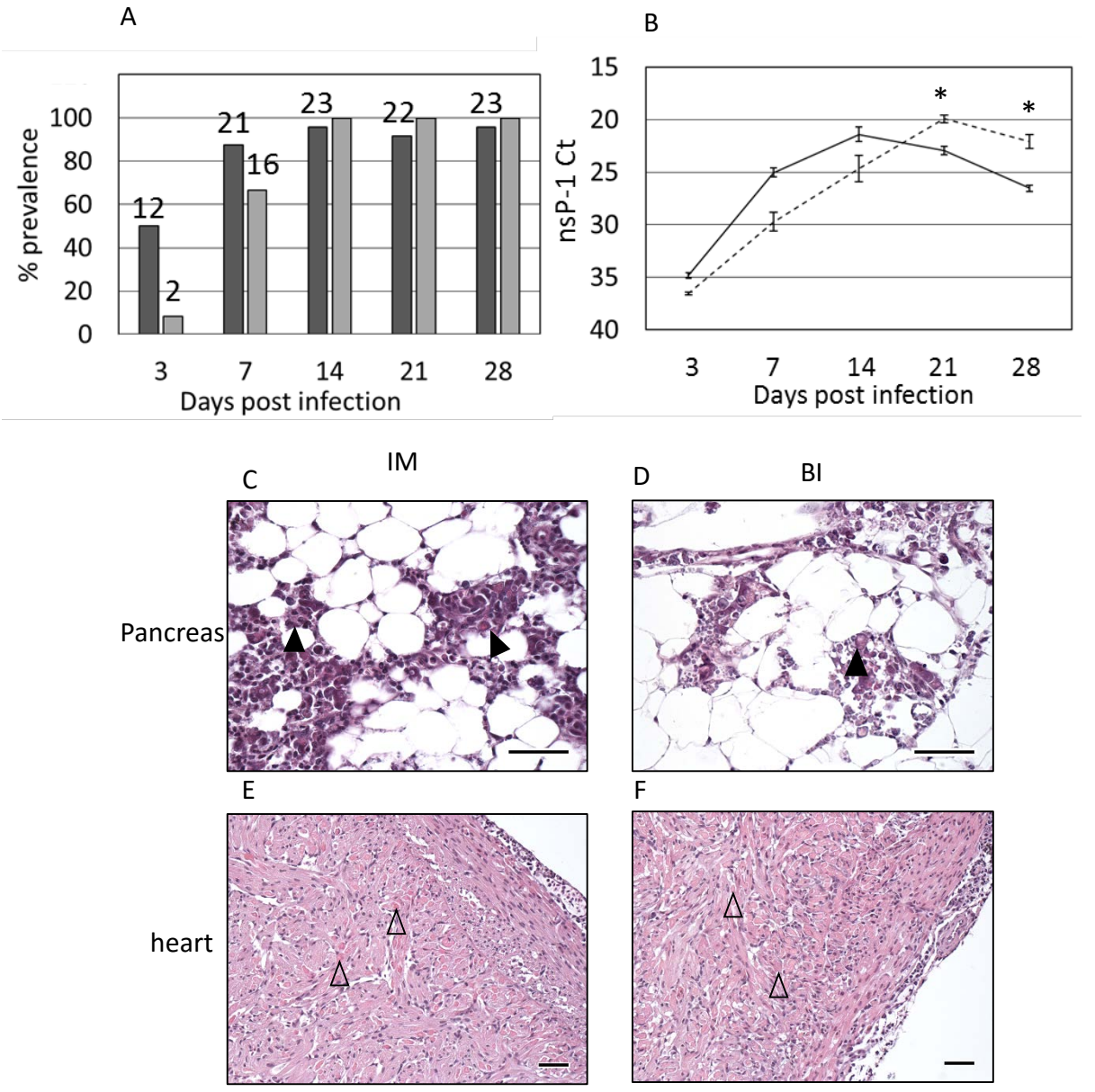

A
% prevalence
100
80
60
40
20
0
12
2
21
16
23
22
23
3
7
14
21
28
Days post infection
B
nsP-1 Ct
15
20
25
30
35
40
*
*
3
7
14
21
28
Days post infection
IM
BI
C
D
Pancreas
E
F
heart

Fig.3

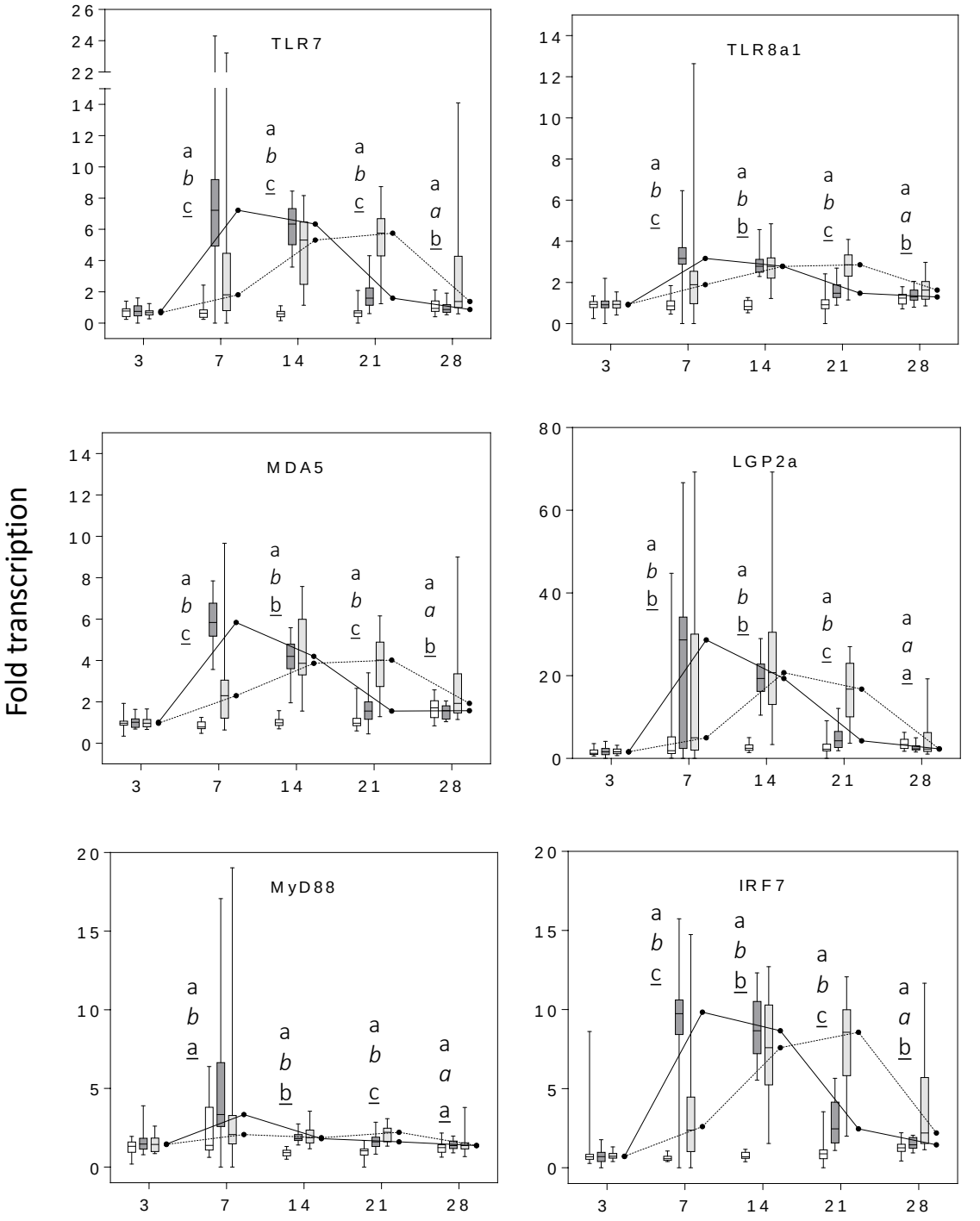

TLR7
TLR8a1
MDA5
LGP2a
MyD88
IRF7
Fold transcription
Days post infection
3
7
14
21
28

Fig. 4

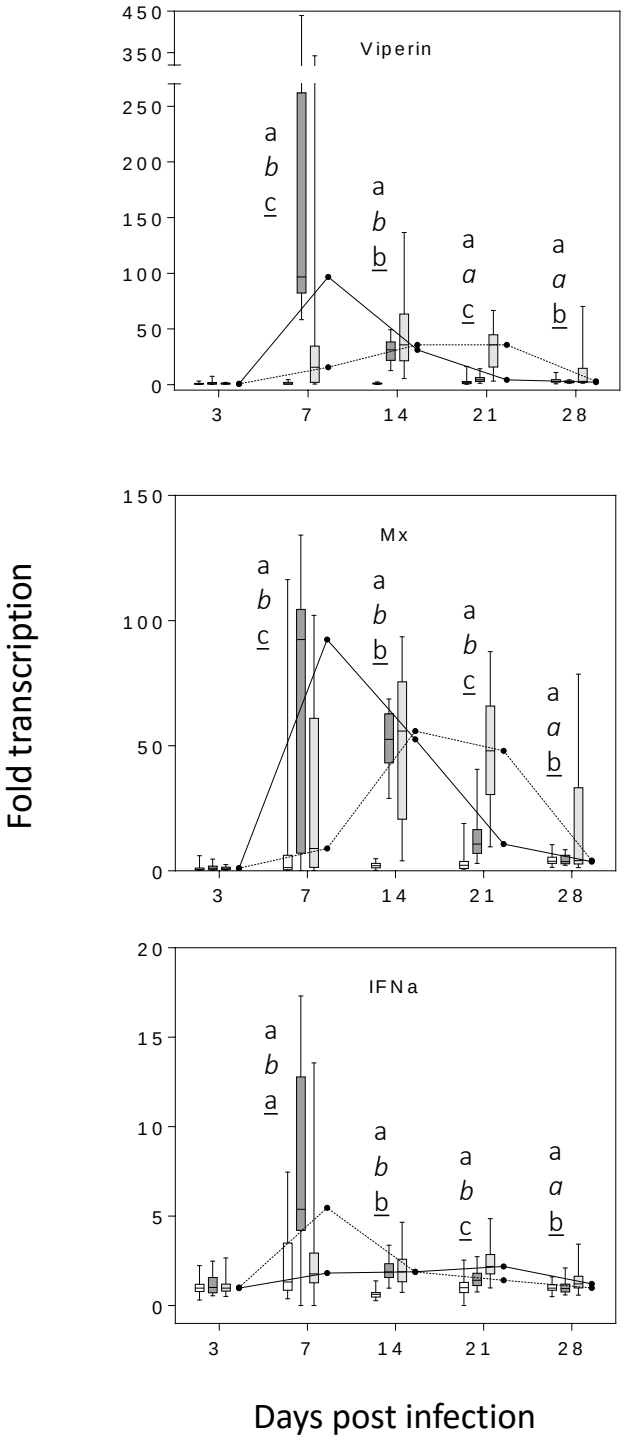
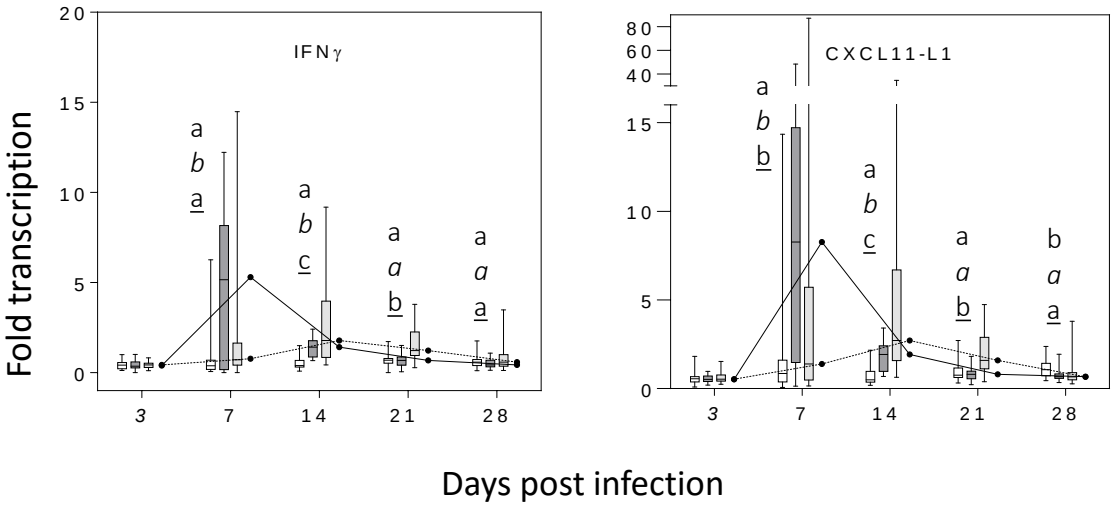
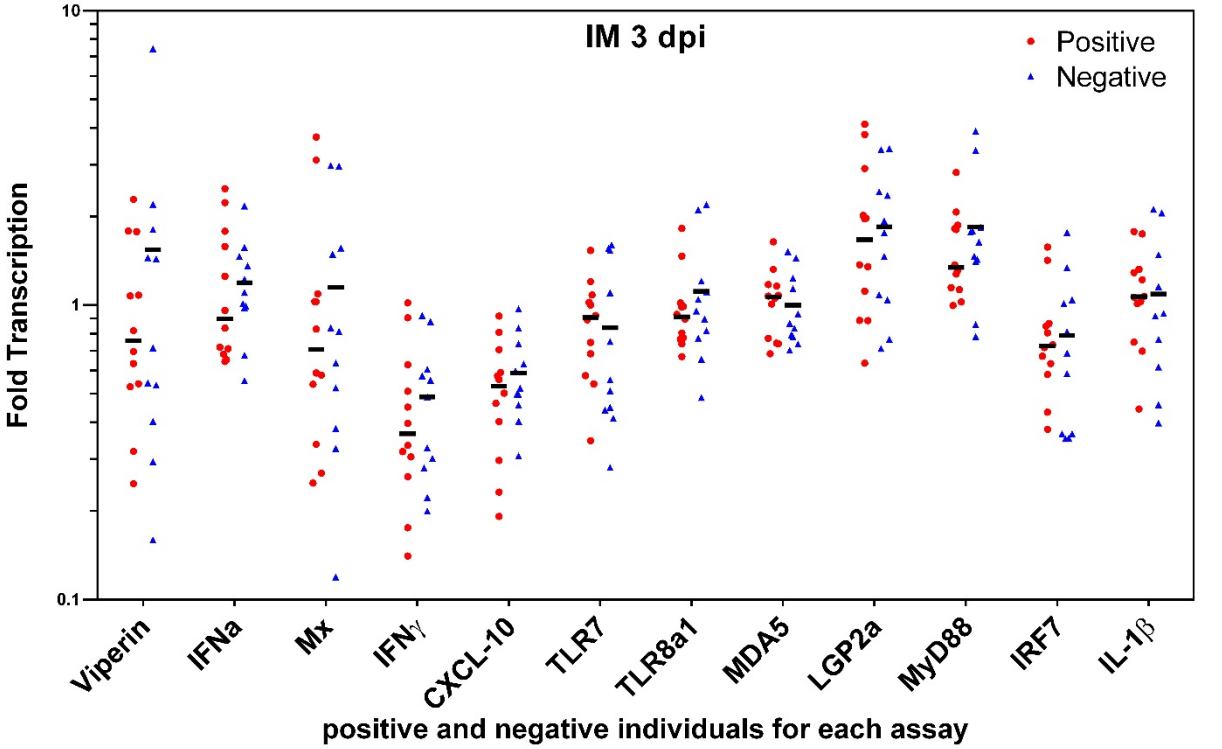
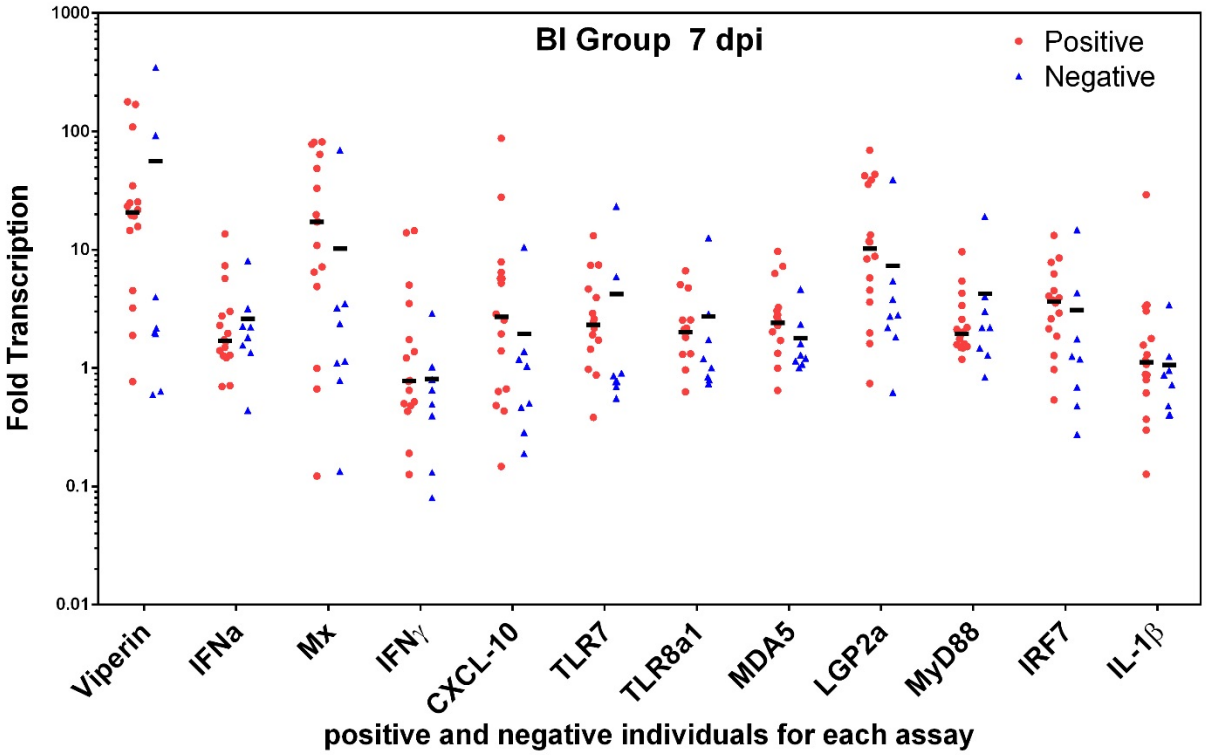

Viperin
Mx
IFNa
Fold transcription
Days post infection
3
7
14
21
28

Fig. 5

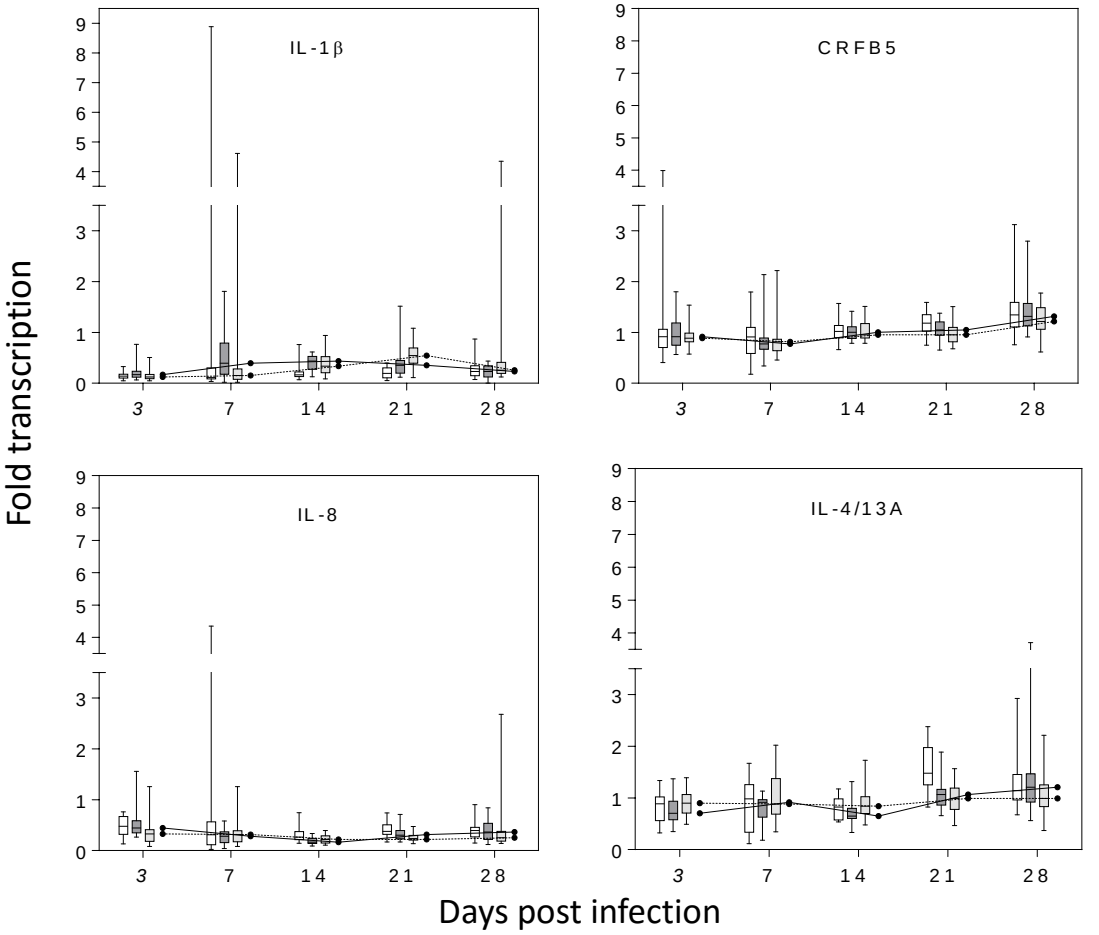

IFNγ
CXCL11-L1
Fold transcription
Days post infection
3
7
14
21
28

S.1


IM 3 dpi
Positive
Negative
Fold Transcription
10
1
0.1
Viperin
IFNa
Mx
IFNγ
CXCL-10
TLR7
TLR8a1
MDA5
LGP2a
MyD88
IRF7
IL-1β
positive and negative individuals for each assay


BI Group 7 dpi
Positive
Negative
Fold Transcription
1000
100
10
1
0.1
0.01
Viperin
IFNa
Mx
IFNγ
CXCL-10
TLR7
TLR8a1
MDA5
LGP2a
MyD88
IRF7
IL-1β
positive and negative individuals for each assay

S.2


IL-1β
CRFB5
IL-8
IL-4/13A
Fold transcription
Days post infection
3
7
14
21
28