

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

5

6 L. J. Moore^{1*}, J. Jarungsriapisit^{1,3*}, T. O. Nilsen², S. Stefansson³, G. L. Taranger¹, C.J.
7 Secombes⁴, H. C. Morton¹ and S. Patel^{1*}

8 ¹ Institute of Marine Research, P.O. Box 1870, Nordnes, 5817 Bergen, Norway

9 ² Uni Research Environment, Uni Research, Thormøhlensgt. 49B 5006 Bergen, Norway

10 ³ Department of Biology, University of Bergen, P.O. Box 7803, 5020 Bergen, Norway

11 ⁴ Scottish Fish Immunology Research Centre, University of Aberdeen, Zoology Building,
12 Tillydrone Avenue, Aberdeen AB24 2TZ, Scotland, UK

13 *corresponding author

14 ♣ Contributed equally

15 Keywords

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

18

19 Highlights:

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

26

27 Abstract

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

45 Introduction

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

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

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

59 In humans alphavirus infections are controlled by both humoral and cellular immune
60 responses, but the innate immune response, starting with interferon (IFN) production is
61 central to controlling the acute phase [6-8]. The classical IFN response promotes and
62 maintains an anti-viral state in two steps, with the first step resulting in the production of IFN.
63 The second step maintains an anti-viral state by stimulating the transcription of a myriad of
64 IFN stimulated genes (ISGs) of which there are over 300 known in mammals [9]. Increased
65 transcription of interferon in fish has been observed in SAV infections and other viral
66 infections [10-13]. The interferon response has been studied in experimental SAV infections
67 instigated by both injection and cohabitation [14-16].

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

74 associated molecular pattern (PAMP), viral ssRNA interacts with pattern recognition
75 receptors (PRRs) such as toll-like receptors (TLRs) triggering IFN production. The
76 accompanying inflammatory response can be both beneficial and detrimental to the host.
77 Many of the genes involved in these pathways have been characterised in salmonids and have
78 previously been shown to be modulated during viral infections [14-16, 19]. In human
79 alphavirus infections patients can be left with chronic polyarthralgia [20] and in fish,
80 recovered individuals often fail to thrive and can exhibit poor fillet quality at slaughter [21]
81 We have recently established a bath-immersion infection model for SAV3 in Atlantic salmon
82 in seawater [22]. This model provides both a natural route of infection and synchronisation of
83 the time of infection, by limiting the exposure time to 6 hours. Also, since in Norway SAV3
84 most commonly affects Atlantic salmon during their first summer, which can be shortly after
85 sea transfer for spring smolts, it was also relevant to examine the immune response to this
86 virus shortly after seawater transfer.
87 In the study presented here, we have compared the transcription levels of a panel of innate
88 immune genes many of which have been shown to be modulated during viral infections in
89 fish. The immune gene transcription was compared between fish infected with SAV3 via
90 bath-immersion and those infected by *i.m.*-injection. Our results revealed important
91 differences in the kinetics and duration of the immune responses triggered by SAV3 infection
92 following either bath-immersion or *i.m.*-injection.

93 2 Materials and Methods

94
95 Atlantic salmon post-smolts (average weight 41 g) were infected with SAV3 by *i.m.* injection
96 (IM) with 10^4 TCID₅₀ per fish or by bath immersion (BI) 2 weeks after transfer to seawater.
97 Salinity was maintained at 34.5 ‰ for whole experimental period. Sea water containing virus

98 for the BI group was produced by shedder fish injected with 10^4 TCID₅₀ SAV3 per fish one
99 week before the experiment started. A third group was injected with non-infected cell culture
100 supernatant as a control group (CT). They were held in triplicate tanks at 12 °C and 8 fish
101 were sampled from each tank of 65 fish. This corresponded to 24 fish from each treatment
102 group, at 1, 3, 7, 14, 21 and 28 dpi (Fig. 1).

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

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

111 2.1 Bath immersion dose

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

120 2.2 Sampling and RNA extraction

121 Pancreas and heart tissue samples were fixed and processed for histological examination from
122 4 of the 8 fish sampled at 7, 14 and 21 dpi [22]. Heart and head kidney tissue samples for RT-
123 qPCR analysis were flash frozen in liquid nitrogen and total RNA isolated using Trizol as
124 previously published [22]. RNA concentration and quality was estimated using a Nanodrop
125 ND-1000. Five percent of the RNA samples from tissues were randomly chosen and checked
126 for integrity on a Bioanalyser (Agilent Instruments), resulting in RINs of ≥ 9 for all samples
127 tested.

128 2.3 cDNA synthesis and RT-qPCR

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

134 cDNA was transcribed from 1 µg total head kidney RNA in a 20 µl reaction using qScript™
135 SuperMix (Quanta Biosciences) including priming with both random hexamers and Oligo-
136 dT as described in the manufacturer's instructions. cDNA was diluted 1:10 before use as RT-
137 qPCR on pooled cDNA showed that this was an optimal dilution. Assays for TLR7, TLR8a1,
138 MyD88, MDA5, LGP2a, IRF7, IFN α , Mx, IFN γ , CXCL11-L1, IL-1 β , CRFB5, IL-8 and IL-
139 4/13A were designed for use in this study. In addition, an assay for viperin was adapted from
140 a previously published study [14]. All primers and assay data are listed in Table 1. All head
141 kidney cDNA samples were analysed with the above mentioned assays. Assays were
142 designed with primers on 2 exons or where at least one primer spanned an exon boundary.
143 Some assays were generic, such as that encoding the IFN α receptor chain CRFB5 which was
144 designed to detect all 3 isoforms a, b and c [25]. The Mx assay detects Mx1, 2 and 3, whereas
145 the LGP2a assay would not detect LGP2b [26]. The TLR8b isotypes (TLR8b1 and b2) were
146 undetectable in the pooled cDNA used to screen immune assays and a further isotype of
147 TLR8 (TLR8a2) had between 10 and 100 times less transcription, which is agreement with *in*
148 *vitro* studies [27]. Hence only TLR8a1 [28] was chosen for immune gene analysis in this
149 study. Activation of both the innate immune response and of inflammatory genes has been
150 noted previously and the genes chosen for analysis helped evaluate these important pathways.
151 All assay products were visualized on a 3% MetaPhor® Agarose gel (Lonza) and sequenced
152 to verify the specificity of the assay. Efficiencies were also calculated for each primer set
153 using triplicates of a five point, 4 x dilution series of the pooled cDNA. Elongation factor 1A
154 [29] was used for normalization and is considered the best option of several endogenous
155 reference genes evaluated for use with Atlantic salmon during SAV infection [30].

156 RT-qPCR was run in 384 plates using Brilliant III Ultra-Fast SYBR® Green master mix
157 (Agilent) and Applied Biosystems 7900HT Fast Real-Time PCR system in a 7 µl reaction

158 volume containing 2 μ l diluted cDNA and 400 nM of each primer. The running conditions
159 were as recommended by the manufacturer and included a melting curve analysis for each
160 run.

161

162 2.4 Data Analysis

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

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

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

175 3 Results

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

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

183 3.1 PD status

184 PD status was determined by analyzing the transcription of SAV3 RNA in heart tissue and by
185 histological examination of heart and pancreatic tissue samples. The percentage prevalence
186 was calculated from the number of fish per group at each time point that were positive for
187 SAV3 RNA in heart tissue (Fig. 2). Prevalence was higher in the IM group at 3 and 7 dpi,
188 with 12 (50%) and 21(87.5%) of 24 fish positive for SAV3 respectively, compared to only 2
189 (8.3%) and 16 (66.7%) of 24 fish in the BI group at these two early time-points (Fig. 2A).
190 Additionally, the amount of virus (SAV3 RNA) was higher in the IM group than in the BI
191 group at these time-points (Fig. 2B). By 14 dpi the prevalence in the IM and BI groups was
192 95.8% (23 of 24) and 100%, respectively (Fig. 2A). At later time-points, when all the BI
193 group fish were positive (100% prevalence), only 1-2 fish were negative in the IM group
194 (Fig. 2A). Interestingly, although both viral load and prevalence was lower in the BI group
195 than in the IM group until 14 dpi, the BI group showed significantly higher amounts of SAV3
196 at 21 and 28 dpi ($p \leq 0.05$, Fig. 2B). At 14 dpi although prevalence was maximal in both
197 groups, viral load was still lower in the BI group. Histological examination showed loss of
198 exocrine pancreatic tissue and cell infiltration at 7 and 14 dpi in the IM and BI groups,
199 respectively (Figs. 2C and D). Heart tissue showed lesions typical for PD with necrotic foci
200 present at 14 and 21 dpi in IM and BI groups respectively (Figs. 2E and F). We also noted
201 that four (3.3%) of the control fish tested positive for nsP1. This was most likely due to
202 contamination during sampling or analysis, since these fish showed no increase in immune
203 gene transcription and pre-screening prior to the start of the experiment had shown these fish
204 to be negative for both SAV and IPNV.

205 3.2 Immune gene transcription

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

216 3.2.1 Genes encoding PRRs

217 Two genes encoding PRRs associated with endosomal membranes, TLR7 and TLR8a1, and
218 two PRRs that reside in the cytosol, LGP2a and MDA5, were examined. Both TLRs were
219 upregulated with a maximum transcription at 7 dpi in the IM group, and at 21 dpi in the BI
220 group, although TLR7 showed a higher transcription than TLR8a1 for both groups (Fig. 3).
221 TLR7 peaked with a 7.2-fold increase in the IM group at 7 dpi and with a 5.7-fold increase in
222 the BI group at 21 dpi. MDA5 and LGP2a that interact with viral dsRNA in the cytoplasm
223 showed similar patterns of transcription. LGP2a was one of the genes showing the highest
224 fold increase in transcription, with 29 and 21-fold increases in IM at 7 dpi, and BI at 14 dpi
225 respectively (Fig. 3). MDA5 showed more moderate fold increases of 5.8-fold at 7 dpi in the
226 IM group and 4-fold at 21 dpi, in BI group. All these PRRs were significantly highly up-
227 regulated in both infected groups compared to the CT group at 7, 14 and 21 dpi. Many of the
228 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 IFN α 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 IFN γ 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 IFN γ 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 IFN γ and CXCL11-L1 respectively (Fig.

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

260 3.2.5 Magnitude of transcription

261 All the genes assayed and their relative transcription levels between the treatment groups are
262 compared using a one-way ANOVA. The transcription of IL-1 β , CRFB5, IL-8 and IL-4/13A
263 was relatively unregulated throughout the experiment in all treatment groups (S.2 and S.3).
264 At 7, 14 and 21 dpi all other genes in both infected groups were significantly upregulated
265 compared to the CT group ($p \leq 0.01$) (S.3). At 7 dpi, when IM genes were at their peak all
266 genes had significantly higher fold transcriptions than both CT and BI groups ($p \leq 0.01$,
267 except for LGP2a, $p \leq 0.05$). At 14 dpi the fold increase in transcription for both infected
268 groups was significantly higher than in the CT group ($p \leq 0.001$, except for IM vs CXCL11-
269 L1, $p \leq 0.01$). However, at 14 dpi many genes displayed similar fold changes between the
270 infected groups since the fold changes in transcription of genes in the IM group were mostly
271 decreasing and in the BI group they were mostly increasing (Figs. 3-5). Thus, at 14 dpi only
272 TLR7 showed a significant difference ($p \leq 0.001$) between the infected groups (S.3). At 21
273 dpi the transcription of genes in the BI group had significantly higher fold increases than the
274 IM group for all genes ($p \leq 0.001$). At 28 dpi some genes including, TLR7, MDA5 and IRF7
275 were still significantly more highly transcribed in both infected groups compared to the CT
276 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 IFN α , viperin, and MyD88 together with much lower transcriptions of Mx,
280 LGP2a and IFN γ 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 (IFN γ , 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.

298 4 Discussion

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

308 4.1 SAV status

309 The amount of SAV RNA present in heart tissue increased during the experimental period in
310 both infected groups, and almost 100% prevalence was apparent in heart from both groups by
311 14 dpi. Recent analyses in our laboratory indicate that the range of Ct values reported here
312 (between 20 and 30) corresponds to nsP1 copy numbers in the range 4×10^2 to 4×10^5 (data
313 not shown).

314 There was little difference in the transcription of most of the genes between positive and
315 negative fish at earlier sampling points (S.1) possibly because although the fish were
316 infected, the viral replication had not yet reached a detectable level in heart tissue. Fish which
317 tested negative by RT-qPCR could still have been viraemic, as previously demonstrated [22,
318 32]. Moreover, both infected groups reached 100% prevalence at later time-points indicating
319 that all fish were infected and were responding with individual variance.

320 A dose of 10^4 TCID₅₀ SAV3 per fish in the IM group induced maximum levels of immune
321 gene transcription at 7 dpi, whereas the maximum viral load was at 14dpi. In the BI group,
322 maximum levels of transcription were observed at 14 or 21 dpi more accurately coinciding
323 with the peak viral load at 21 dpi. The natural route of infection in the BI group shows a
324 better correlation between the maximum levels of gene transcription and the peaks of both
325 viral load and prevalence. This is supported by the higher correlation coefficients between the
326 SAV3 RNA levels and the transcription of immune genes in the BI group. Correlation
327 coefficients where $R^2 > 0.5$ were only present in the BI group. In agreement with our BI group
328 result, other studies using a cohabitation model have also concluded that the maximum
329 transcription of innate immune genes occurs at the same time as maximum viral load [16,
330 33].

331 The expression of immune genes over time in head kidney during SAV infections is dose
332 dependent. The dose of 10^4 TCID₅₀ SAV3 used in the present study produced maximal
333 transcription at 7 dpi in the IM group and 14 or 21 dpi for the BI group. When a high
334 intraperitoneal injection dose of 10^7 TCID₅₀ SAV1 was used, maximal transcription followed
335 at 3 dpi [15], whereas a cohabitation model using only 10^3 TCID₅₀ SAV3 in shedder fish took
336 3.5 weeks to observe increases in gene transcription [34]. Johansen *et al.* [34] also state that
337 the gradual increase of positive fish is typical of a cohabitation infection, whereas our study
338 clearly shows a rapid accumulation of positive fish during a bath challenge model
339 comparable to *i.m.* models. In order to evaluate the immune response in all its complexities it
340 is clearly advantageous to have an infection model with a synchronized time-of-infection
341 which is of sufficient infectivity to achieve 100% prevalence during the initial stage of
342 infection. Lower doses in an infection model cause a staggered rather than a synchronized
343 infection due to infected fish shedding virus and exposing naïve fish not infected at time zero
344 [32]. This makes it difficult to relate the immune response to the time of infection. In the

345 present study, comparing the overall transcription patterns (represented by trend-lines on the
346 figures) for both challenge models consistent patterns for most genes can be seen, indicating
347 a single synchronized point of infection (Figs. 3-6). The synchronized nature of the 2 infected
348 groups is further illustrated by the rather narrow ranges at many time-points. Thus, even
349 small fold changes in the transcription of immune genes between the experimental groups can
350 be significantly different (eg between IM and BI groups at 14 dpi for TLR7, Fig 3 and table
351 3).

352 4.2 *The anti-viral response*

353 The *i.m.* administration of the infective dose apparently triggered a much stronger initial
354 immune response with high, but transient fold increases in the transcription of many genes. In
355 the BI group, the SAV infection took longer to cause elevated transcription of many of the
356 genes. This indicates that the virus, due perhaps to the route of infection, took a longer time
357 to amplify in the host and reach the viral RNA loads necessary to trigger an immune
358 response. This delayed increase in the transcription of the immune genes in the BI group was
359 possibly the cause of the high viral loads that exceeded viral loads in the IM group at 21 and
360 28 dpi and of the typical PD histopathology seen at later time-points. Even though the
361 immune response in the IM group was relatively swift and strong, it still failed to prevent
362 disease progression and the development of the typical PD pathology,

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

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

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

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

384 Of the two endosomal PRRs measured, TLR7 was more highly expressed than TLR8a1,
385 although TLR8a1 had approximately 5 to 10-fold higher resting/constitutive transcription.
386 Conversely, it has recently been reported that SAV3 infection of TO cells, which have a
387 dendritic/macrophage-like gene expression profile, upregulated only TLR8 and TLR3 and not
388 TLR7, during SAV3 infection [40].

389 It has been suggested that the cytosolic viral RNA sensing molecules (LGP2a and MDA5) act
390 in parallel and do not compete allowing high levels of both during a viral infection [26].

391 However, in this study the transcription of LGP2a was much higher than that for MDA5 in
392 both infected groups at all time-points, suggesting that MDA5 was either inhibited by LGP2a

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

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

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

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

421 Infections with SAV do lead to the production of neutralizing antibodies that both protect and
422 clear viraemia, [47, 48], but the delayed nature of the adaptive response in ectothermic
423 teleosts makes the innate response pivotal in immune defence. Furthermore, *in vitro*
424 experiments with CHIKV [49] have demonstrated that high transcription of host ISGs are not
425 translated into increased levels of the corresponding proteins and a similar mechanism could
426 account for the severe pathology seen in the present study. There are few studies addressing
427 the teleost response to viral infection at a protein level. Braceland *et al.* [50] have analysed
428 sera of PD infected individuals, but not immune parameters. Measurement of neutralizing
429 antibodies is both relevant and widespread [51-54], and the presence of Mx protein has been
430 semi-quantitatively analysed in heart during SAV1 infection using immuno-histochemistry
431 [15], but clearly there is a dearth of quantitative protein analysis of innate immune effectors
432 such as Mx and viperin in teleosts.

433 4.3 Smoltification status

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

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

450 4.4 Summary

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

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

472

473 **Acknowledgements**

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

480 **Abbreviations**

481 cDNA complementary DNA

482 IFN Interferon

483 IPNV Infectious pancreas necrosis virus

484 ISG Interferon stimulated genes

485 RT-qPCR reverse transcriptase quantitative polymerase chain reaction

486 PPR pattern recognition receptor

- 487 PAMP pathogen associated molecular pattern
- 488 SAV salmonid alphavirus
- 489 TCID₅₀ 50% tissue culture infective dose
- 490 TLR toll-like receptor

491 **References**

- 492 [1] D.A. Graham, E. Fringuelli, H.M. Rowley, D. Cockerill, D.I. Cox, T. Turnbull,
493 Geographical distribution of salmonid alphavirus subtypes in marine farmed Atlantic salmon,
494 *Salmo salar* L., in Scotland and Ireland, J Fish Dis 35 (2012) 755-765.
- 495 [2] E. Fringuelli, H.M. Rowley, J.C. Wilson, R. Hunter, H. Rodger, D.A. Graham,
496 Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses
497 (SAV) based on partial E2 and nsP3 gene nucleotide sequences, J Fish Dis 31(11) (2008)
498 811-823.
- 499 [3] K. Hodneland, A. Bratland, K.E. Christie, C. Endresen, A. Nylund, New subtype of
500 salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon *Salmo salar* and rainbow
501 trout *Oncorhynchus mykiss* in Norway, Dis Aquat Organ 66(2) (2005) 113-120.
- 502 [4] M.J. Hjortaa, H.R. Skjelstad, T. Taksdal, A.B. Olsen, R. Johansen, B. Bang-Jensen, I.
503 Orpetveit, H. Sindre, The first detections of subtype 2-related salmonid alphavirus (SAV2) in
504 Atlantic salmon, *Salmo salar* L., in Norway, J Fish Dis 36(1) (2013) 71-74.
- 505 [5] T. Svåsand, Ø. Karlsen, B.O. Kvamme, L.H. Stien, G.L. Taranger and K.K. Boxaspen,
506 Risikovurdering av norsk fiskeoppdrett 2016. (Risk assessment of Norwegian Aquaculture),
507 Fisken og Havet 2 (2016) 132-133.
- 508 [6] C.L. Gardner, C.W. Burke, S.T. Higgs, W.B. Klimstra, K.D. Ryman, Interferon-
509 alpha/beta deficiency greatly exacerbates arthritogenic disease in mice infected with wild-
510 type chikungunya virus but not with the cell culture-adapted live-attenuated 181/25 vaccine
511 candidate, Virology 425(2) (2012) 103-12.
- 512 [7] N. Wauquier, P. Becquart, D. Nkoghe, C. Padilla, A. Ndjoi-Mbiguino, E.M. Leroy, The
513 acute phase of Chikungunya virus infection in humans is associated with strong innate

- 514 immunity and T CD8 cell activation, *The Journal of infectious diseases* 204(1) (2011) 115-
515 23.
- 516 [8] Y. Zhang, C.W. Burke, K.D. Ryman, W.B. Klimstra, Identification and characterization
517 of interferon-induced proteins that inhibit alphavirus replication, *J Virol* 81(20) (2007)
518 11246-55.
- 519 [9] S. Karki, M.M. Li, J.W. Schoggins, S. Tian, C.M. Rice, M.R. MacDonald, Multiple
520 interferon stimulated genes synergize with the zinc finger antiviral protein to mediate anti-
521 alphavirus activity, *Plos One* 7(5) (2012) 1-13.
- 522 [10] S.M. Jørgensen, D.L. Hetland, C.M. Press, U. Grimholt, T. Gjølven, Effect of early
523 infectious salmon anaemia virus (ISAV) infection on expression of MHC pathway genes and
524 type I and II interferon in Atlantic salmon (*Salmo salar* L.) tissues, *Fish & shellfish*
525 *immunology* 23(3) (2007) 576-88.
- 526 [11] A. Krasnov, G. Timmerhaus, B.L. Schiotz, J. Torgersen, S. Afanasyev, D. Iliev, J.
527 Jørgensen, H. Takle, S.M. Jørgensen, Genomic survey of early responses to viruses in
528 Atlantic salmon, *Salmo salar* L, *Molecular Immunology* 49(1-2) (2011) 163-74.
- 529 [12] C. Langevin, E. Aleksejeva, G. Passoni, N. Palha, J.P. Levraud, P. Boudinot, The
530 antiviral innate immune response in fish: evolution and conservation of the IFN system,
531 *Journal of molecular biology* 425(24) (2013) 4904-20.
- 532 [13] M.K. Purcell, K.J. Laing, J.R. Winton, Immunity to fish rhabdoviruses, *Viruses* 4(1)
533 (2012) 140-66.
- 534 [14] S. Grove, L. Austbo, K. Hodneland, P. Frost, M. Lovoll, M. McLoughlin, H.L. Thim, S.
535 Braaen, M. König, M. Syed, J.B. Jørgensen, E. Rimstad, Immune parameters correlating with
536 reduced susceptibility to pancreas disease in experimentally challenged Atlantic salmon
537 (*Salmo salar*), *Fish & shellfish immunology* 34(3) (2013) 789-98.

- 538 [15] T.K. Herath, K.D. Thompson, A. Adams, R.H. Richards, Interferon-mediated host
539 response in experimentally induced salmonid alphavirus 1 infection in Atlantic salmon
540 (*Salmo salar* L.), Veterinary immunology and immunopathology 155 (2013).9-20
- 541 [16] C. Xu, T.C. Guo, S. Mutoloki, O. Haugland, O. Evensen, Gene expression studies of
542 host response to Salmonid alphavirus subtype 3 experimental infections in Atlantic salmon,
543 Vet Res 43 (2012).
- 544 [17] K.T. Chow, M. Gale Jr, SnapShot: Interferon Signaling, Cell 163(7) (2015) 1808-
545 1808.e1.
- 546 [18] T. Kawai, S. Akira, Innate immune recognition of viral infection, Nature immunology
547 7(2) (2006) 131-137.
- 548 [19] L.-H. Johansen, M.K. Dahle, Ø. Wessel, G. Timmerhaus, M. Løvoll, M. Røsæg, S.M.
549 Jørgensen, E. Rimstad, A. Krasnov, Differences in gene expression in Atlantic salmon parr
550 and smolt after challenge with Piscine orthoreovirus (PRV), Molecular Immunology 73
551 (2016) 138-150.
- 552 [20] J. Hoarau, M.C. Jaffar Bandjee, P. Krejbich Trotot, T. Das, G. Li-Pat-Yuen, B. Dassa,
553 M. Denizot, E. Guichard, A. Ribera, T. Henni, F. Tallet, M.P. Moiton, B.A. Gauzere, S.
554 Bruniquet, Z. Jaffar Bandjee, P. Morbidelli, G. Martigny, M. Jolivet, F. Gay, M. Grandadam,
555 H. Tolou, V. Vieillard, P. Debre, B. Autran, P. Gasque, Persistent chronic inflammation and
556 infection by Chikungunya arthritogenic alphavirus in spite of a robust host immune response,
557 Journal of immunology 184(10) (2010) 5914-27.
- 558 [21] T. Taksdal, J. Wiik-Nielsen, S. Birkeland, P. Dalgaard, T. Mørkøre, Quality of raw and
559 smoked fillets from clinically healthy Atlantic salmon, *Salmo salar* L., following an outbreak
560 of pancreas disease (PD), J Fish Dis 35(12) (2012) 897-906.
- 561 [22] J. Jarungsriapisit, L.J. Moore, G.L. Taranger, T.O. Nilsen, H.C. Morton, I.U. Fiksdal, S.
562 Stefansson, P.G. Fjellidal, Ø. Evensen, S. Patel, Atlantic salmon (*Salmo salar* L.) post-smolts

- 563 challenged two or nine weeks after seawater-transfer show differences in their susceptibility
564 to salmonid alphavirus subtype 3 (SAV3), *Virology* 13(1) (2016) 1-14.
- 565 [23] K. Hodneland, C. Endresen, Sensitive and specific detection of Salmonid alphavirus
566 using real-time PCR (TaqMan), *J Virol Methods* 131(2) (2006) 184-92.
- 567 [24] L. Andersen, A. Bratland, K. Hodneland, A. Nylund, Tissue tropism of salmonid
568 alphaviruses (subtypes SAV1 and SAV3) in experimentally challenged Atlantic salmon
569 (*Salmo salar* L.), *Arch Virol* 152(10) (2007) 1871-1883.
- 570 [25] B. Sun, L. Greiner-Tollersrud, B.F. Koop, B. Robertsen, Atlantic salmon possesses two
571 clusters of type I interferon receptor genes on different chromosomes, which allows for a
572 larger repertoire of interferon receptors than in zebrafish and mammals, *Dev Comp Immunol*
573 47(2) (2014) 275-86.
- 574 [26] M. Chang, B. Collet, P. Nie, K. Lester, S. Campbell, C.J. Secombes, J. Zou, Expression
575 and functional characterization of the RIG-I-like receptors MDA5 and LGP2 in Rainbow
576 trout (*Oncorhynchus mykiss*), *J Virol* 85(16) (2011) 8403-12.
- 577 [27] P.T. Lee, J. Zou, J.W. Holland, S.A. Martin, T. Kanellos, C.J. Secombes, Identification
578 and characterization of TLR7, TLR8a2, TLR8b1 and TLR8b2 genes in Atlantic salmon
579 (*Salmo salar*), *Dev Comp Immunol* 41(2) (2013) 295-305.
- 580 [28] I. Skjæveland, D.B. Iliev, G. Strandskog, J.B. Jorgensen, Identification and
581 characterization of TLR8 and MyD88 homologs in Atlantic salmon (*Salmo salar*), *Dev Comp*
582 *Immunol* 33(9) (2009) 1011-7.
- 583 [29] P.A. Olsvik, K.K. Lie, A.-E.O. Jordal, T.O. Nilsen, I. Hordvik, Evaluation of potential
584 reference genes in real-time RT-PCR studies of Atlantic salmon, *BMC Molecular Biology*
585 6(1) (2005) 1-9.

- 586 [30] M. Lovøll, L. Austbo, J.B. Jorgensen, E. Rimstad, P. Frost, Transcription of reference
587 genes used for quantitative RT-PCR in Atlantic salmon is affected by viral infection, *Vet Res*
588 42 (2011) 8-13.
- 589 [31] A. Biosystems, Guide to Performing Relative Quantitation of Gene Expression Using
590 Real-Time Quantitative PCR, (2008) 52-59.
- 591 [32] J. Jarungsriapisit, Moore, L. J., Maehle, S. Skaar, C., Einen, A.C. B., Fiksdal, I., Morton,
592 H. C., Stefansson, S., Taranger, G. L. and Patel S., Relationship between viral dose and
593 outcome of infection in Atlantic salmon, *Salmo salar* L., post-smolts bath-challenged with
594 salmonid alphavirus subtype 3, *J. Vet .Res.* 47 (1), 2016, 102.
- 595 [33] Z. Heidari, J. Tinsley, R. Bickerdike, M.F. McLoughlin, J. Zou, S.A. Martin, Antiviral
596 and metabolic gene expression responses to viral infection in Atlantic salmon (*Salmo salar*),
597 *Fish & shellfish immunology* 42(2) (2014) 297-305.
- 598 [34] L.-H. Johansen, H.L. Thim, S.M. Jørgensen, S. Afanasyev, G. Strandskog, T. Taksdal,
599 K. Fremmerlid, M. McLoughlin, J.B. Jørgensen, A. Krasnov, Comparison of transcriptomic
600 responses to pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) in
601 heart of Atlantic salmon (*Salmo salar* L), *Fish Shellfish Immun* 46(2) (2015) 612-623.
- 602 [35] T.K. Herath, J.E. Bron, K.D. Thompson, J.B. Taggart, A. Adams, J.H. Ireland, R.H.
603 Richards, Transcriptomic analysis of the host response to early stage salmonid alphavirus
604 (SAV-1) infection in Atlantic salmon *Salmo salar* L, *Fish & shellfish immunology* 32(5)
605 (2012) 796-807.
- 606 [36] C. Xu, Ø. Evensen, H.M. Munang'andu, A de novo transcriptome analysis shows that
607 modulation of the JAK-STAT signaling pathway by salmonid alphavirus subtype 3 favors
608 virus replication in macrophage/dendritic-like TO-cells, *BMC genomics* 17(1) (2016) 1-17.

- 609 [37] I. Akhrymuk, S.V. Kulemzin, E.I. Frolova, Evasion of the innate immune response: the
610 Old World alphavirus nsP2 protein induces rapid degradation of Rpb1, a catalytic subunit of
611 RNA polymerase II, *J Virol* 86(13) (2012) 7180-91.
- 612 [38] P.V. Aguilar, S.C. Weaver, C.F. Basler, Capsid protein of eastern equine encephalitis
613 virus inhibits host cell gene expression, *J Virol* 81(8) (2007) 3866-76.
- 614 [39] J. Zou, B. Gorgoglione, N.G. Taylor, T. Summathed, P.T. Lee, A. Panigrahi, C. Genet,
615 Y.M. Chen, T.Y. Chen, M. Ul Hassan, S.M. Mughal, P. Boudinot, C.J. Secombes, Salmonids
616 have an extraordinary complex type I IFN system: characterization of the IFN locus in
617 rainbow trout *oncorhynchus mykiss* reveals two novel IFN subgroups, *Journal of*
618 *immunology* 193(5) (2014) 2273-86.
- 619 [40] C. Zu, Ø. Evensen, H. Mweemba Munang'andu, De Novo Transcriptome Analysis
620 Shows That SAV-3 Infection Upregulates Pattern Recognition Receptors of the Endosomal
621 Toll-Like and RIG-I-Like Receptor Signaling Pathways in Macrophage/Dendritic Like TO-
622 Cells, *Viruses* 8(4) (2015) 114-130.
- 623 [41] C. Xu, O. Evensen, H. Munang'andu, De novo assembly and transcriptome analysis of
624 Atlantic salmon macrophage/dendritic-like TO cells following type I IFN treatment and
625 Salmonid alphavirus subtype-3 infection, *BMC genomics* 16(1) (2015) 96.
- 626 [42] C. Xu, T.C. Guo, S. Mutoloki, O. Haugland, I.S. Marjara, O. Evensen, Alpha interferon
627 and not gamma interferon inhibits salmonid alphavirus subtype 3 replication in vitro, *J Virol*
628 84(17) (2010) 8903-12.
- 629 [43] S.K. Gahlawat, A.E. Ellis, B. Collet, Expression of interferon and interferon induced
630 genes in Atlantic salmon *Salmo salar* cell lines SHK-1 and TO following infection with
631 Salmon AlphaVirus SAV, *Fish & shellfish immunology* 26(4) (2009) 672-5.

- 632 [44] S.J. DeWitte-Orr, J.-A.C. Leong, N.C. Bols, Induction of antiviral genes, Mx and vig-1,
633 by dsRNA and Chum salmon reovirus in rainbow trout monocyte/macrophage and fibroblast
634 cell lines, *Fish Shellfish Immun* 23(3) (2007) 670-682.
- 635 [45] A.J.A. McBeath, M. Snow, C.J. Secombes, A.E. Ellis, B. Collet, Expression kinetics of
636 interferon and interferon-induced genes in Atlantic salmon (*Salmo salar*) following infection
637 with infectious pancreatic necrosis virus and infectious salmon anaemia virus, *Fish Shellfish*
638 *Immun* 22 (2007).230-241
- 639 [46] T. Wang, Johansson, P. Abós, B. Holt, A. Tafalla, C. Jiang, Y. Wang, A. Xu, Q. Qi, Z.
640 Huang, W. Costa, M.M. Diaz-Rosales, P. Holland, J.W, Secombes, C. J., First in-depth
641 analysis of the novel Th2-type cytokines in salmonid fish reveals distinct patterns of
642 expression and modulation but overlapping bioactivities, *Oncotarget* 7(10) (2016) 10917-
643 10946.
- 644 [47] D.A. Graham, H.R. Rowley, P. Frost, Cross-neutralization studies with salmonid
645 alphavirus subtype 1-6 strains: results with sera from experimental studies and natural
646 infections, *J Fish Dis* 37 (2014) 683-691.
- 647 [48] G. Houghton, A.E. Ellis, Pancreas disease in Atlantic salmon: serum neutralisation and
648 passive immunisation, *Fish Shellfish Immun* 6 (1996) 465-472.
- 649 [49] L.K. White, T. Sali, D. Alvarado, E. Gatti, P. Pierre, D. Streblov, V.R. DeFilippis,
650 Chikungunya Virus Induces IPS-1-Dependent Innate Immune Activation and Protein Kinase
651 R-Independent Translational Shutoff, *J Virol* 85(1) (2011) 606-620.
- 652 [50] M. Braceland, R. Bickerdike, J. Tinsley, D. Cockerill, M.F. Mcloughlin, D.A. Graham,
653 R.J. Burchmore, W. Weir, C. Wallace, P.D. Eckersall, The serum proteome of Atlantic
654 salmon, *Salmo salar*, during pancreas disease (PD) following infection with salmonid
655 alphavirus subtype 3 (SAV3), *J Proteom* 94 (2013) 423-436.

- 656 [51] K.E. Christie, D.A. Graham, M.F. McLoughlin, S. Villoing, D. Todd, D. Knappskog,
657 Experimental infection of Atlantic salmon *Salmo salar* pre-smolts by *i.p.* injection with new
658 Irish and Norwegian salmonid alphavirus (SAV) isolates: a comparative study, *Dis Aquat*
659 *Organ* 75(1) (2007) 13-22.
- 660 [52] D.A. Graham, P. Frost, K. McLaughlin, H.M. Rowley, I. Gabestad, A. Gordon, M.F.
661 McLoughlin, A comparative study of marine salmonid alphavirus subtypes 1-6 using an
662 experimental cohabitation challenge model, *J Fish Dis* 34(4) (2011) 273-286.
- 663 [53] D.A. Graham, V.A. Jewhurst, H.M. Rowley, M.F. McLoughlin, H. Rodger, D. Todd,
664 Longitudinal serological surveys of Atlantic salmon, *Salmo salar* L., using a rapid
665 immunoperoxidase-based neutralization assay for salmonid alphavirus, *J Fish Dis* 28(6)
666 (2005) 373-379.
- 667 [54] M.F. McLoughlin, Rowley H. M. and Doherty, C. E., A serological survey of salmon
668 pancreas disease virus (SPDV) antibodies in farmed Atlantic salmon, *Salmo salar*.L., *J Fish*
669 *Dis* 21 (1998) 305-307.
- 670 [55] G.O. Melingen, S.O. Stefansson, A. Berg, H.I. Wergeland, Changes in Serum-Protein
671 and Igm Concentration during Smolting and Early Post-Smolt Period in Vaccinated and
672 Unvaccinated Atlantic Salmon (*Salmo salar* L), *Fish Shellfish Immun* 5(3) (1995) 211-221.
- 673 [56] E.F. Pettersen, M. Ulvenes, G.O. Melingen, H.I. Wergeland, Peripheral blood and head
674 kidney leucocyte populations during out-of-season (0+) parr-smolt transformation and
675 seawater transfer of Atlantic salmon (*Salmo salar* L.), *Fish Shellfish Immun* 15(5) (2003)
676 373-385.
- 677 [57] B.K. Das, B. Collet, M. Snow, A.E. Ellis, Expression of interferon type I and II, Mx and
678 gamma IP genes in the kidney of Atlantic salmon, *Salmo salar*, is induced during smolting,
679 *Fish Shellfish Immun* 23(3) (2007) 514-20.

- 680 [58] K.A. Glover, C. Skår, K.E. Christie, J. Glette, H. Rudra, Ø. Skaala, Size-dependent
681 susceptibility to infectious salmon anemia virus (ISAV) in Atlantic salmon (*Salmo salar* L.)
682 of farm, hybrid and wild parentage, *Aquaculture* 254 (2006), 82-91
- 683 [59] L.-H. Johansson, Timmerhaus, G. , Afanasyev, S., Jørgensen, S. M. and Krasnov, A,
684 Smoltification and seawater transfer of Atlantic salmon (*Salmo salar* L.) is associated with
685 systemic repression of the immune transcriptome., *Fish Shellfish Immun* 58, (2016),.33-41
- 686

687 **Figure legends**

688 **Fig. 1 Experimental set-up.**

689 All experimental groups of fish were transferred to seawater 1 week before *i.m.* injection of
 690 the shedder fish. On the day the experiment started, (0 dpi or 2 wpt, weeks post seawater
 691 transfer) the CT group was *i.m.* injected with non-infected cell culture supernatant, the IM
 692 group was *i.m.* injected with 10^4 TCID₅₀ SAV3, similarly to the shedders and the BI group
 693 was bathed in water containing shed virus from the shedder fish (shedder water). The
 694 experiment was performed in triplicate tanks for all treatment groups, 65 fish in each tank.
 695 Sampling of 8 fish per tank (24 fish per treatment group) was carried out at 1, 3, 7, 14, 21 and
 696 28 dpi.

697

698 **Fig. 2 PD status of the infected groups**

699 **A.** Percentage prevalence of SAV3 RNA in IM (dark grey bars) and BI (light grey bars)
 700 groups at all time-points. Numbers above the columns indicate the number of positive fish
 701 per group where prevalence was less than 100%, n = 24 for all group and time-points (except
 702 for BI at 14 dpi n = 22). **B.** Average \pm SE, Ct values of nsp-1 assay plotted in reverse,
 703 represent viral load, in IM group (solid line) and BI group (dashed line) at each time point.
 704 Asterisks (*) indicate significant differences in viral load (Ct value) between the 2 groups (p
 705 ≤ 0.05). **C and D.** Histological sections of pancreatic tissue for IM fish at 7 dpi (C) and for BI
 706 fish at 14 dpi (D) showing loss of exocrine pancreas tissue and necrosis (►). Bar = 50 μ m. **E**
 707 **and F.** Histological sections of heart tissue for IM fish at 14 dpi (E) and for BI fish at 21 dpi
 708 (F) showing necrotic cardiomyocytes (Δ). Bar = 50 μ m

709

710 **Fig 3. Innate gene transcription.**

711 The y axis represents normalized, fold transcription increase for each treatment group
712 compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th
713 percentiles for each group with the median value shown by a black bar in this box. The
714 whiskers represent the maximum and minimum values for each group. Open bars represent
715 control fish, dark grey bars the IM group and light grey bars the BI group. Trend lines
716 indicate transcriptional changes over time; solid line IM group and dashed line the BI group.
717 Vertical scales have been kept constant as far as possible to allow comparison between genes.
718 Statistically significant differences between the means of the experimental groups ($p < 0.05$)
719 are indicated by lower case letters in a column to the left of each time-point. Lower case
720 letters denote the CT group, lower case, italic letters the IM groups and lower case,
721 underlined letters the BI group.

722

723 **Fig 4. Transcription of IFN α and effector genes, viperin and Mx**

724 The y axis represents normalized, fold transcription increases for each treatment group
725 compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th
726 percentiles for each group with the median value shown by a black bar in this box. The
727 whiskers represent the maximum and minimum values for each group. Open bars represent
728 control fish, dark grey bars the IM group and light grey bars the BI group. Trend lines
729 indicate transcriptional changes over time; solid line IM group and dashed line the BI group.
730 Statistically significant differences between the means of the experimental groups ($p < 0.05$)
731 are indicated by lower case letters in a column to the left of each time-point. Lower case

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

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

735 Vertical scales represent normalized, fold transcription increases for each treatment group
736 compared to calibrator fish sampled before day 0. Boxes represent the 25th and 75th
737 percentiles for each group with the median value shown by a black bar in this box. The
738 whiskers represent the maximum and minimum values for each group. Open bars represent
739 control fish, dark grey bars the IM group and light grey bars the BI group. Trend lines
740 indicate transcriptional changes over time; solid line IM group and dashed line the BI group.
741 Vertical scales have been kept constant as far as possible to allow comparison between genes.
742 Statistically significant differences between the means of the experimental groups ($p < 0.05$)
743 are indicated by lower case letters in a column to the left of each time-point. Lower case
744 letters denote the CT group, lower case, italic letters the IM groups and lower case,
745 underlined letters the BI group

746

747 **S.1 Immune genes in Positive and Negative fish**

748 Transcription of immune genes (fold change in transcription) of all individuals at 3 dpi in the
749 IM group and of all individuals at 7 dpi in the BI group. At these time-points prevalence was
750 50% in the IM group and 66% in the BI group and allows comparison of immune gene
751 transcription between individuals positive or negative for SAV RNA. IL-8, IL-4/13A and
752 CRFB5 were only very slightly regulated and are therefore omitted for clarity. The black bars
753 represent the median value for each group. The y axis is a Log₁₀ scale to render the individual
754 data points more visible.

755

756

757 S.2 Genes showing relatively little change in transcription

758 Fold change in transcription of CRFB5, IL-8, IL-4/13A and IL-1 β . The y axis represents
759 normalized, fold transcription for each treatment group compared to calibrator fish sampled
760 before day 0. Boxes represent the 25th and 75th percentiles for each group with the median
761 value shown by a black bar in this box. The whiskers represent the maximum and minimum
762 values for each group. Open bars represent control fish, dark grey bars the IM group and light
763 grey bars the BI group. Trend lines indicate transcription over time; solid line IM group and
764 dashed line the BI group. Vertical scales have been kept constant as far as possible to allow
765 comparison between genes. Statistically significant differences between groups are shown in
766 table 3.

767

768

1 **Table 1 Primers**

- 2 Primers used in the analysis of immune genes together with their amplicon sizes, relative efficiencies and the Genebank accession number used
 3 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 | TGGTTGGTGTCTCGTCAAAG | 101 | 2.03 | Grove 2013 [14] |
| IFNα | CCTGTGTATCACCTGCCATGAA | GCCTGTGCACTGTAGTTCATTT | 100 | 1.95 | NM_001123710 |
| MyD88 | CGTGGATAGAAAAGACGTTGTG | CAGGGTGATGCCTTGTCTTT | 152 | 2.07 | EF672332 |
| TLR7 | CGCATGACGAGGTCAGAAT | GTCCTCTCTCAGTGCAATCTA | 172 | 1.99 | HF97058 |
| TLR8a1 | GGCTTTCAAATCTCACAAGGAA | CCTTAATGTCACATGGAAAGT | 150 | 1.93 | NP_001155165 |
| IRF7 | GGACTCAAACGACCCCATATA | GGTTCAGGTCTAGGTGGTTCAA | 194 | 2.10 | NM_001136548 |
| MDA5 | CTCGTGAAGTACTCAAGAGAATCG | 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 | CACACGCCCCACAGGTACA | 57 | 2.02 | Olsvik 2006 [19] |

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

5 **Table 2 Correlation coefficients**

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

10

| Assay | dpi | R^2 BI group | R^2 IM group |
|--------------|------------|--------------------------------------|--------------------------------------|
| IFN γ | 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 |

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30 **Table 3 Significant differences**

31 The data for each gene and time-point and for all fish was transformed (+1, Log₁₀). One-way
32 ANOVA and Post Hoc Neuman Keul's was applied to the data. The table shows all
33 significant differences between treatment groups at each sampling point and for each gene
34 assayed: - no significant difference, $p < 0.05$ grey, $p < 0.01$ black and $p < 0.001$ **bold**

| Gene assay | Treatment | 7 dpi | | | 14 dpi | | | 21 dpi | | | 28 dpi | | |
|--------------|----------------|-------|-------|-------|--------|-------|-------|--------|-------|-------|--------|-------|-------|
| | | 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 | |
| IFN γ | 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

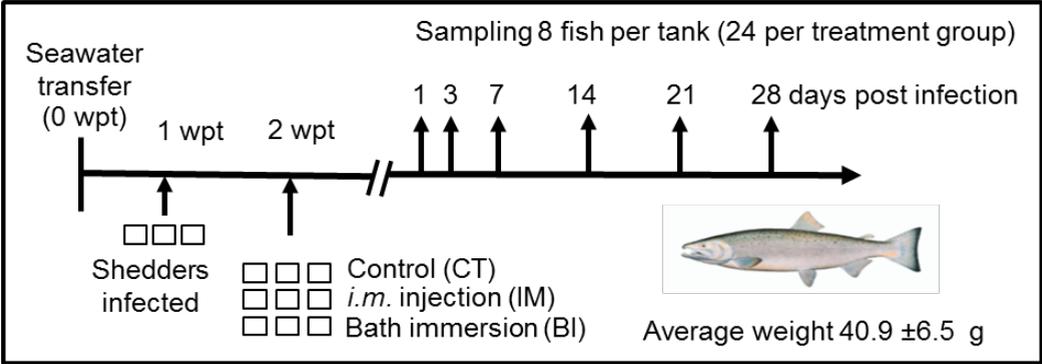


Fig. 2

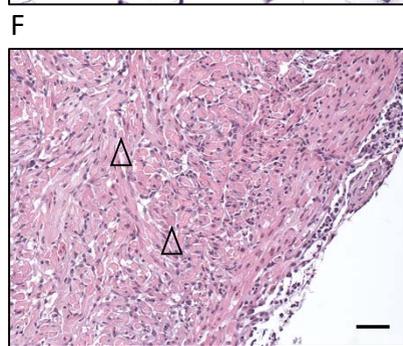
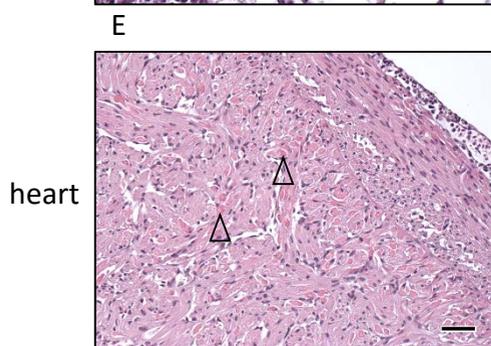
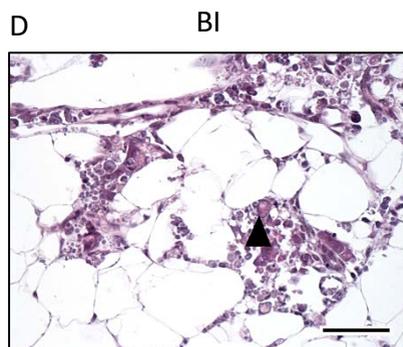
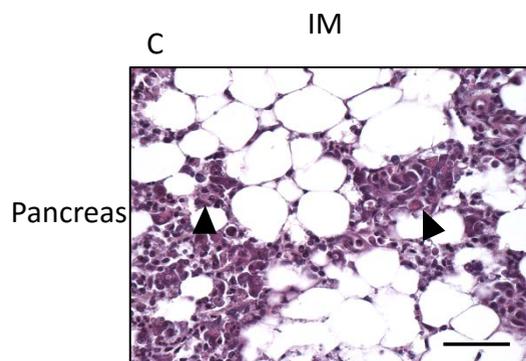
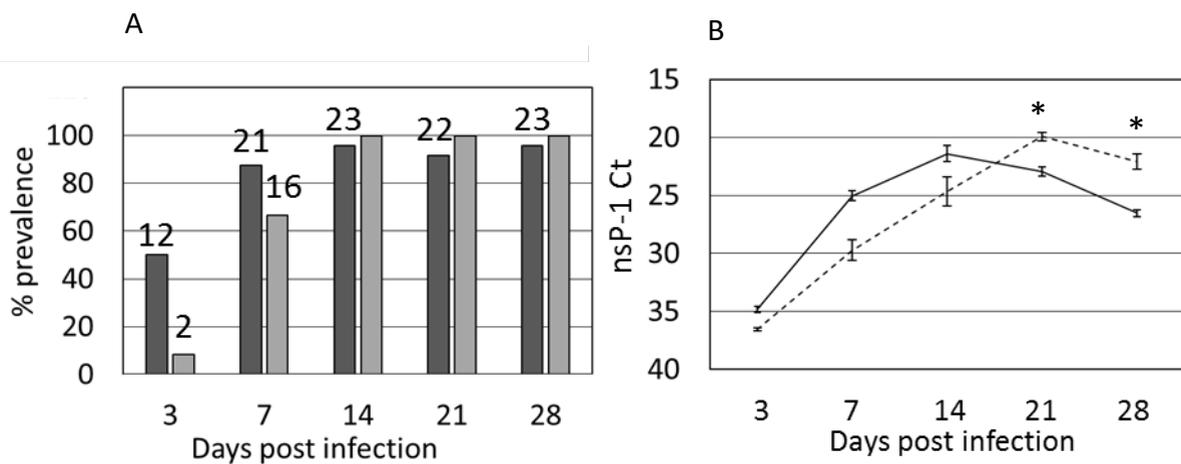


Fig.3

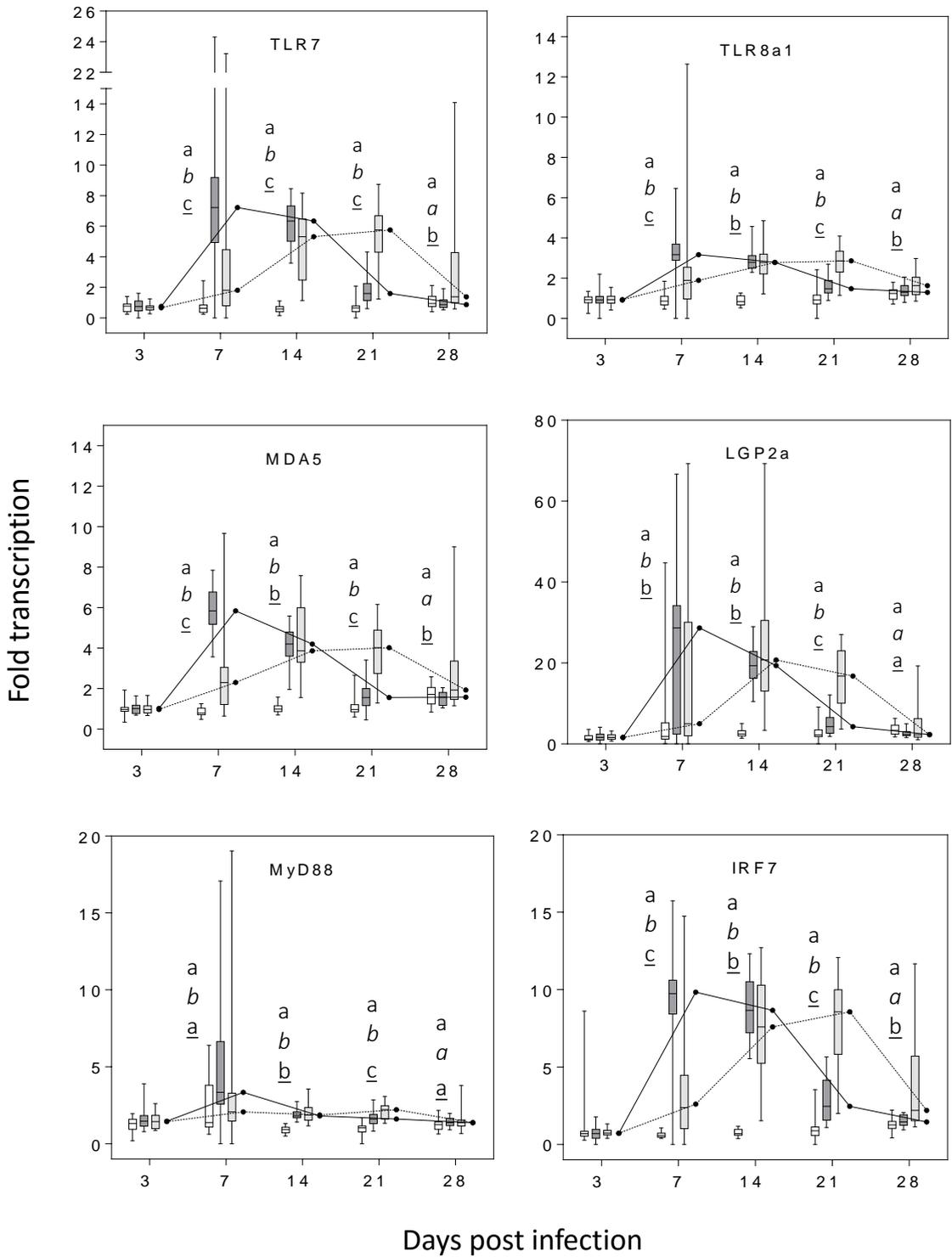
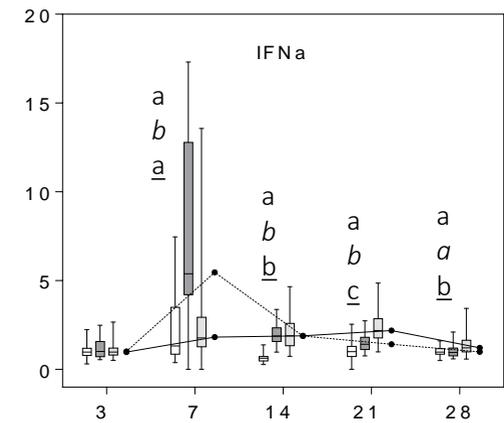
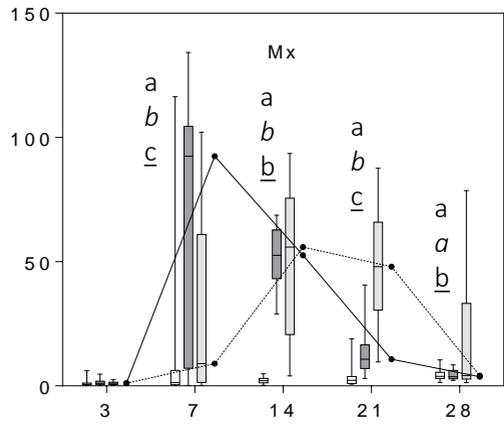
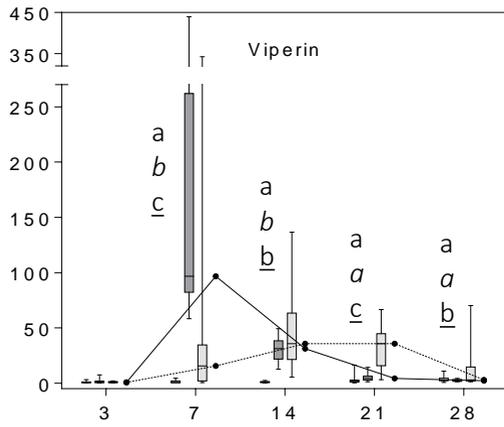


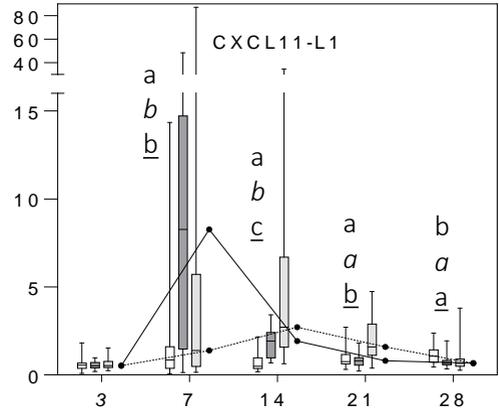
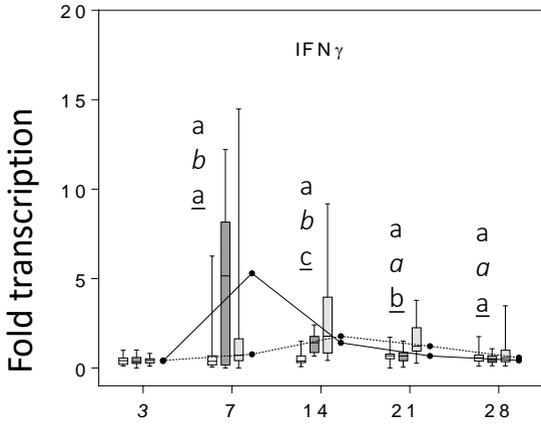
Fig. 4



Days post infection

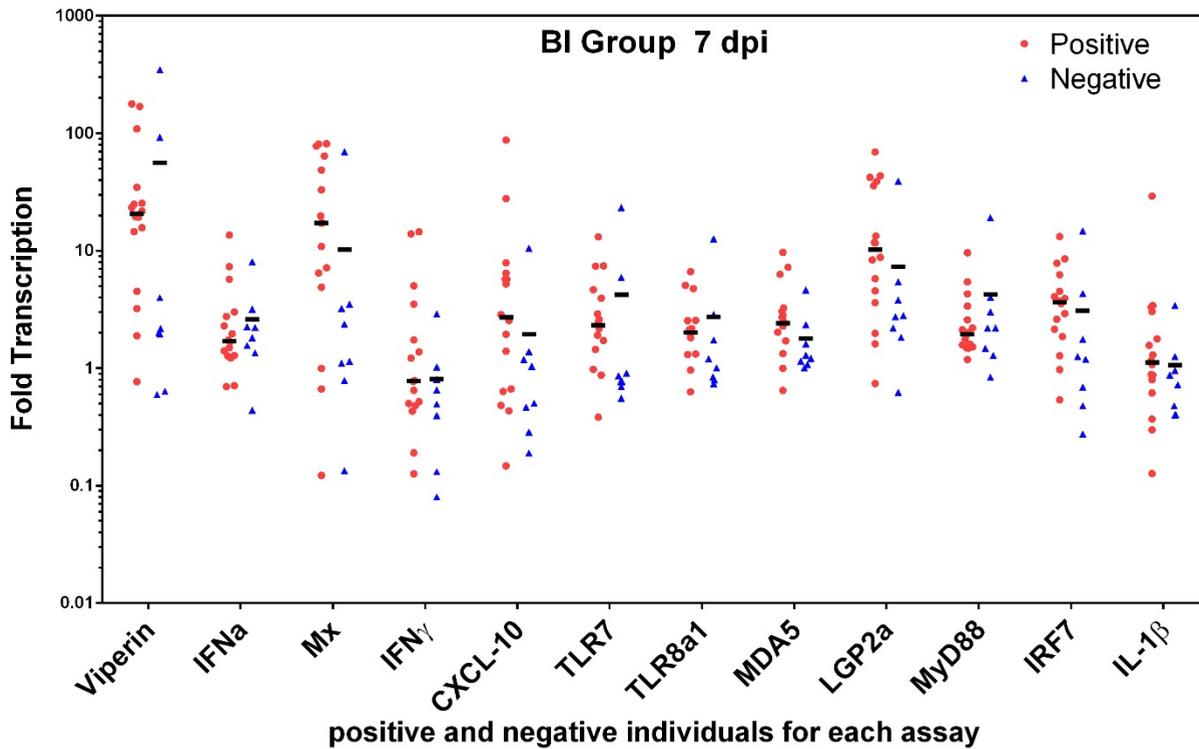
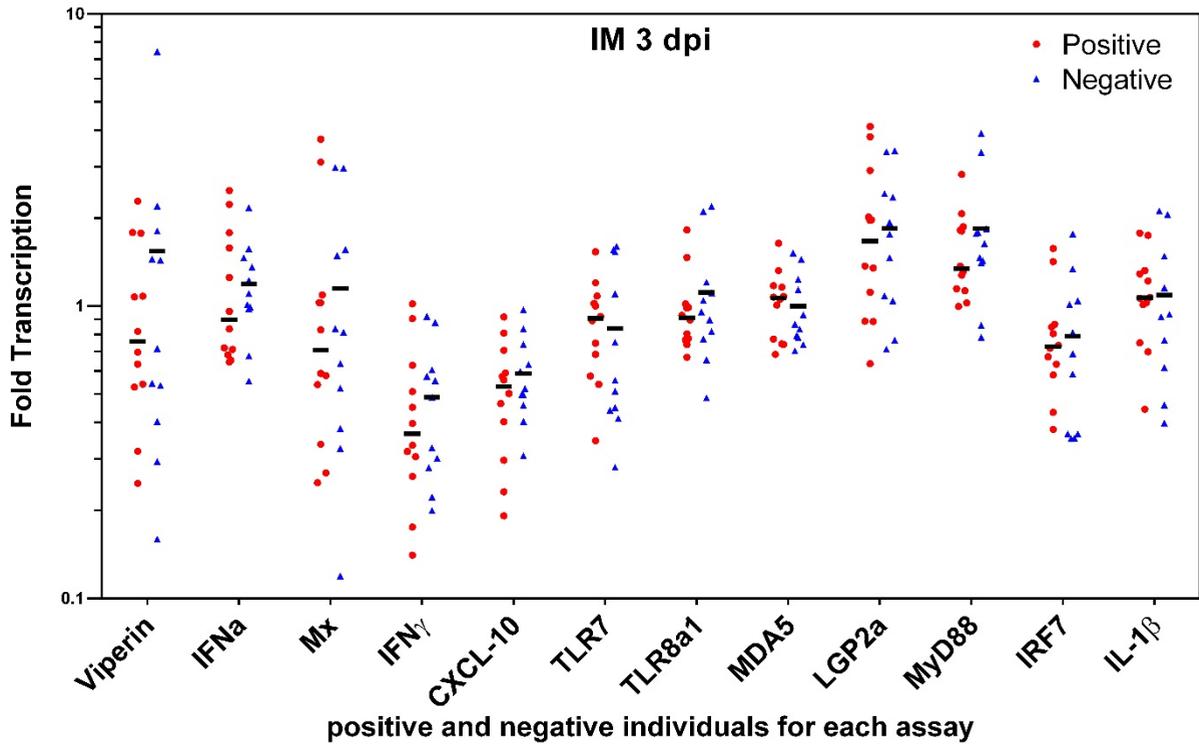
Fold transcription

Fig. 5



Days post infection

S.1



S.2

