Individual monitoring of immune response in Atlantic salmon Salmo salar following experimental infection with piscine myocarditis virus (PMCV), agent of cardiomyopathy syndrome (CMS)

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Abstract

Piscine myocarditis virus (PCMV) is a double-stranded RNA virus structurally similar to the *Totiviridae* family. PCMV is the causative agent of cardiomyopathy syndrome (CMS), a severe cardiac disease that affects farmed Atlantic salmon (*Salmo salar*). A recent study characterized the host immune response in infected salmon through a transcriptome immune profiling, which confirmed a high regulation of immune and anti-viral genes throughout infection with PCMV. Previously we developed a novel model based on repeated non-lethal blood sampling, enabling the individual monitoring of salmonids during an infection. In the present work, we used this model to describe the host immune response in the blood cells of Atlantic salmon after intramuscular infection with PCMV-containing tissue homogenate over a 77-day period. At the final stage heart samples were also collected to verify the PCMV load, the pathological impact of infection and to compare the transcript profiles to blood. The expression level of a range of key immune genes was determined in the blood and heart samples by real-time PCR. Results indicated selected immune genes (*mx*, *cd8α* and *γip*) were up-regulated in the heart tissue of infected animals at the terminal time point, in comparison to the non-infected fish. When analyzing the blood samples over the course of infection, a significant n up-regulation of *mx* gene was also observed. The time and number of peaks in the kinetics of expression was different between individuals. The PCMV load and CMS pathology was verified by real-time PCR and histopathology, respectively. No pathogen and no pathology could be detected during the course of the experiment except at the terminal stage (viral load by qPCR and pathology by histology). This study emphasizes the value of non-lethal monitoring for evaluating the health status of fish at early stages of infection and in the absence of clinical signs.

Keywords: Non-lethal sampling; immune response; Atlantic salmon; PMCV; 3Rs; mx; interferon

1. Introduction

Animal infectiology experiments are traditionally carried out by culling animals regularly during the course of infection. The accuracy of the description of the host response to a live pathogen or a treatment suffers from the underlying assumption that a population of animals reacts in a similar manner to each other. A novel model based on single animal analysis in which repeated non-lethal blood samples are collected and used to describe the response was developed previously for a fast-developing viral disease (Infectious Salmon Anaemia, Collet et al., 2015). In the present article we used this model for a slower developing disease Cardiomyopathy Syndrome (CMS), characterised by a severe cardiac inflammation that affects Atlantic salmon (*Salmo salar*) (Rodger and Turnbull, 2000; Garseth et al., 2017). The causative agent of CMS is the piscine myocarditis virus (PCMV), a double-stranded RNA virus structurally similar to the *Totiviridae* family (Haugland et al, 2011). A recent study characterized the host immune response in infected salmon through a transcriptome immune profiling, which confirmed a high regulation of immune and anti-viral genes throughout infection with PCMV (Timmerhaus et al, 2011). Taking into account the above findings, we applied the non-lethal blood sampling method, enabling for the first time the individual monitoring of Atlantic salmon during an infection with CMS after intramuscular infection with PCMV over a 77-day period. A range of key immune genes was monitored during infection, by real-time PCR, in both blood and terminal heart tissues. The PCMV load and CMS pathology was verified by real-time PCR and histopathology. This study confirms the importance of using a non-lethal sampling method in order to individually monitor the host response throughout infection with a chronic disease such as CMS.

2. Material and methods

2.1. Experimental design

This study was carried out in strict accordance with the UK Animals (Scientific Procedures) Act 1986 (ASPA) under project licence PPL3965. The protocol was approved by the Marine Scotland Ethical Review Committee. All procedures were performed under MS222 anaesthesia, and all efforts were made to minimise suffering. Twenty PIT tagged Atlantic salmon *Salmo salar* were provided by Landcatch Natural Selection (Hendrix-Genetics), transported to the Aquarium Facility at Marine Scotland and divided equally into two circular 1 m^3 tanks. The fish were screened for common pathogens, viral hemorrhagic septicaemia virus, infectious pancreatic necrosis virus, infectious salmon anemia virus, infectious hematopoietic necrosis virus, and Aeromonas salmonicida using standard methods in the laboratory (Collet et al. 2015). They were kept under natural photoperiod (October), sea water salinity 37 ‰ and at 10 °C. They were fed once a day with pellets (EWOS). After a week of acclimation, all the fish were anaesthetised, weighed (average weight 1,109 ± 167 g) and injected intra-muscularly with 100 µl heart tissue homogenate from salmon originating from a clinical case of CMS on the north-west coast of Norway and diagnosed with a piscine myocarditis virus infection by real-time PCR and with typical histo-morphological heart changes (N=10, 1 tank) or 100 µl tissue homogenate from healthy salmon (N=10, 1 tank). The PMCV-positive homogenate was tested earlier to yield high levels of PMCV genome in heart tested by real-time PCR (Haugland et al. 2010). The sampling model was as described by Collet et al. (2015). Immediately before injection, a small blood sample (150 µl) was collected from the caudal vein (Pre-infection bleed). Subsequently, non-lethal blood samples were collected repeatedly at 4, 8, 12, 16, 28, 35, 42, 49, 56, 63, 70, and 77 days post infection (dpi). At 77 dpi all the fish were killed by exposure to an overdose of anaesthetic and blood collected immediately. For all samples, the blood was centrifuged for 30 sec at 10,000g to separate blood cells and plasma, and stored at -80°C until processed further. Heart tissue was dissected and half was stored in 750 µl RNAlater (Sigma) at -80°C for subsequent viral load analysis and the other half was fixed in 10% phosphate buffered formalin, embedded in paraffin

wax, sectioned at 3-4 micrometres and stained with haematoxylin and eosin for histopathology analysis.

2.2. RNA extraction, cDNA synthesis and real-time PCR

RNA purification and cDNA synthesis from whole blood cells and heart tissue was carried out using a method described by Collet et al. (2015). Real-time PCR analysis was performed for the house keeping gene ef1α, PCMV using forward primer 5'-TTCCAAACAATTCGAGAAGCG-3', reverse primer 5'-ACCTGCCATTTTCCCCTCTT-3' and Taqman probe 5'-6FAM-CCGGGTAAAGTATTTGCGTC-3'. (amplicon 141 nt, accession number NC_015639), and immune genes (*mx*, *cd8α* and *yip*), as described by Collet et al. (2015).

2.3. Histopathology analysis.

Heart samples (n=18) collected at 77 days post challenge were examined for pathological changes by light microscopy. Histopathological changes in heart sections were scored as described by Haugland et al. (2011), and recorded on a visual analogue scale (0-4).

2.4 Statistical analysis

The histopathological scores for the non-infected and infected groups were analyzed by a Kruskal-Wallis test, with $p < 0.05$ considered statistically significant when comparing groups. The comparisons of the gene expression levels between infected and control groups at the terminal time-point (heart tissues) was carried out using a T-test. Statistical significance was taken as a *P* value of <0.05.

The set of data generated from repeated blood sampling was analysed using a method developed in R software package by Collet et al. (2015).

73 *3. Results and discussion*

74 When analysing the expression of *mx*, *cd8α* and *γip* genes in the heart tissue of Atlantic
75 salmon, results showed a significant upregulation ($P < 0.05$) of all genes in the PCMV infected group
76 (in comparison to the non-infected group) at the terminal time point (77-days post infection) (Figure
77 1A). Moreover, at day 77 qPCR detected PMCV with Cp values ranging from 16.57 to 28.17, with an
78 average Cp of 21.70. None of the un-infected control fish showed any sign of amplification.
79 Expression analysis of the PCMV load in both infected and non-infected groups is shown in Figure
80 1B ($p < 0.05$). No amplification of PMCV occurred from blood cells cDNA collected from fish during
81 the infection (results not shown). To confirm the expression analysis data, heart tissues from the
82 terminal time-point were also subjected to histopathological examination (Figures 1C-D). Results
83 confirmed that control fish (Fish 1 to 10, samples examined from 9 fish) had an overall score of 1 or
84 below representing non-specific inflammatory changes in the heart tissue (Figure 1C). The infected
85 group (Fish 11 to 20, n=9 examined) had scores that went up to 3 (Table 1). In more detail, control
86 fish revealed a mild myocarditis typified by sub-endothelial inflammation of inflammatory cells, with
87 no degenerative changes observed (Figure 1C), and considered a non-specific inflammation of
88 unknown cause. When analysing the spongious part of the cardiac ventricle of PCMV-infected
89 Atlantic salmon (Figure 1D), diffuse infiltration of cardiomyocytes with inflammatory cells was seen,
90 dominated by lymphocytes and some macrophages. Inflammatory cells were seen in the
91 sarcoplasm, beneath endothelial cells and attached to the endothelium. A distinct degeneration of
92 cardiomyocytes was also observed (Figure 1D, 1B). The score difference between controls and
93 infected fish was analysed by the Kruskal-Wallis rank test and gave p=0.034 and p=0.021 for atrium
94 and ventricle, respectively.

95 During the 77-day infection period, small blood samples were collected before the infection
96 (pre-infection bleed, Day 0) and at different time points post infection (Days 4, 8, 12, 16, 28, 35, 42,
97 49, 56, 63, 70 and 77). From the expression data obtained using the terminal heart tissues, we

98 observed that *mx* showed a high expression at day 77 post-infection by real-time PCR. In the blood
99 cells *mx* gene was significantly induced. Figure 2A shows the individual kinetics of *mx* expression in
100 the 10 control and 10 infected fish over the time of infection. In spite of a large variability between
101 individuals, the majority of the gene expression levels in the infected animals were above those in
102 the un-infected control animals. The overall effect of infection on the *mx* gene transcript level was
103 statistically significant as analysed according to the method outlined in Collet et al. (2015) . A
104 detailed look at the individual kinetics (Figure 2B) reveals that some animals had a peak of *mx*
105 expression at 8 dpi (Fish 20, Figure 2B), some at 8 and 50 dpi (Fish 12, Figure 2B), indicating that
106 animals under controlled experimental infection (by injection) are not synchronous. The lethal
107 sampling procedure used traditionally in fish infectiology is unable to resolve this individual
108 variability.

109 This is the first time that individual fish have been monitored during experimental infection
110 with material containing the infectious agent for Cardiomyopathy Syndrome, PMCV. We have
111 previously developed a non-lethal sampling method for salmonid fish (Urquhart et al., 2016) and
112 applied it to viral (Collet et al., 2015), bacterial (Monte et al., 2016) and ectoparasitic (Chance et al.,
113 2018) infection models. Monitoring the same animal over time allows an improvement in the data
114 output by considering the inter-individual variability and with the use of fewer animals (Hall et al.,
115 2018).

116 The present infection model concerns the Cardiomyopathy Syndrome, a slow progressing
117 disease not leading to any mortality. Although we cannot be guaranteed that the infected fish do
118 not have an additional ubiquitous viral infection, the terminal viral load in the heart tissue is high
119 with an average Ct value of 21.7 and the clinical signs, as assessed by histopathology examination,
120 are characteristic of CMS.

121 The resolution of the sampling regime was made up of 12 time points every four days within
122 the first two weeks and then every week for 11 weeks. Timmerhaus et al. (2011, 2012) used a lethal

123 experimental challenge with sampling at weeks 2, 4, 6, 8, 9, 10, 11 and 12 and the most severe
124 histopathology conditions were detected at weeks 8-9. The highest viral load detected was between
125 weeks 8-10 in the heart tissue. PMCV could be detected in blood cells but this was the lowest viral
126 load found compared with heart, spleen or head kidney tissues. CD8 beta chain expression level was
127 found at a later stage of infection in agreement with our findings of CD8 alpha in the heart tissue,
128 assuming co-expression of the two chains alpha and beta. These kinetic of infection were in
129 agreement with Haugland et al. (2011).

130 In the blood, the virus was detected at 4- and 8-weeks post-infection in the Timmerhaus et
131 al. (2011) study but only at low levels compared to those found in the spleen or heart tissues for the
132 same time points. In the present study, we could no detect any virus in the whole blood cells by
133 qPCR at any of the time points. This may be explained by a difference in the sensitivity of the qPCR
134 assays used and/or in the amount of material analysed. Alternatively, the virus may have replicated
135 at early stage in a tissue responsible for a release of interferon in the blood stream, in turn inducing
136 *mx* gene in some blood cells.

137 We demonstrate here the possibility to monitor the immune response of infected individual
138 animals under experimental conditions in a situation where the pathogen could not be detected,
139 the animals did not show any external sign of disease and no mortality occurred. The final lethal
140 sampling revealed a high viral load and a clear development of CMS symptoms. Nevertheless, the
141 immune response was evident from a very early time point and could be monitored by qPCR in
142 blood cells acquired non-lethally. The viraemia phase is most common route of dissemination of
143 viral infection within the organism (Baron et al., 1996) and is often preclinical. If viremia correlates
144 well to the intensity of later clinical symptoms, this can help to predict the intensity of a viral
145 infection before the onset of clinical signs. Therefore, early blood immune parameters can be
146 valuable welfare indicators, in addition to the evident robustness of individual longitudinal data
147 made possible by the non-lethal sampling method. It is important to note that the route of infection

by injection was chosen to secure a successful outcome of infection, and thus the high prevalence it provides, especially for a small group of ten animals. However, cohabitation challenge would be preferable and would mimic natural infection processes/routes but with the limited horizontal spread of infection under experimental conditions (Haugland et al., 2011) would require a larger number of animals being included. In addition to the benefit from an animal welfare perspective (use of fewer animals), this infectiology method also allows for ~~provides improved data outputs, access to inter-individual variability,~~ rigorous validation of the Koch's postulate. ~~and prediction of immune parameter correlates~~. Ultimately this model could be used to assess, through the immune response, livestock' health status at the pre-clinical stage of infection.

158 **Acknowledgements**

159 This work was funded by the National Centre for the Replacement, Refinement and
160 Reduction of Animals in Research (NC3Rs, grant G1100675). Øystein Evensen received funding from
161 Research Council grant no. 267807, and Norwegian Seafood Research Fund grant no. 901179. The
162 authors are grateful to the Marine Scotland aquarium staff (Ben Williamson, Louise Feehan and
163 Mark Paterson) for help in the experimental work. Hendrix-Genetix (formerly Landcatch Natural
164 Selection) is also acknowledged for providing PIT tagged fish for the purpose of this project.

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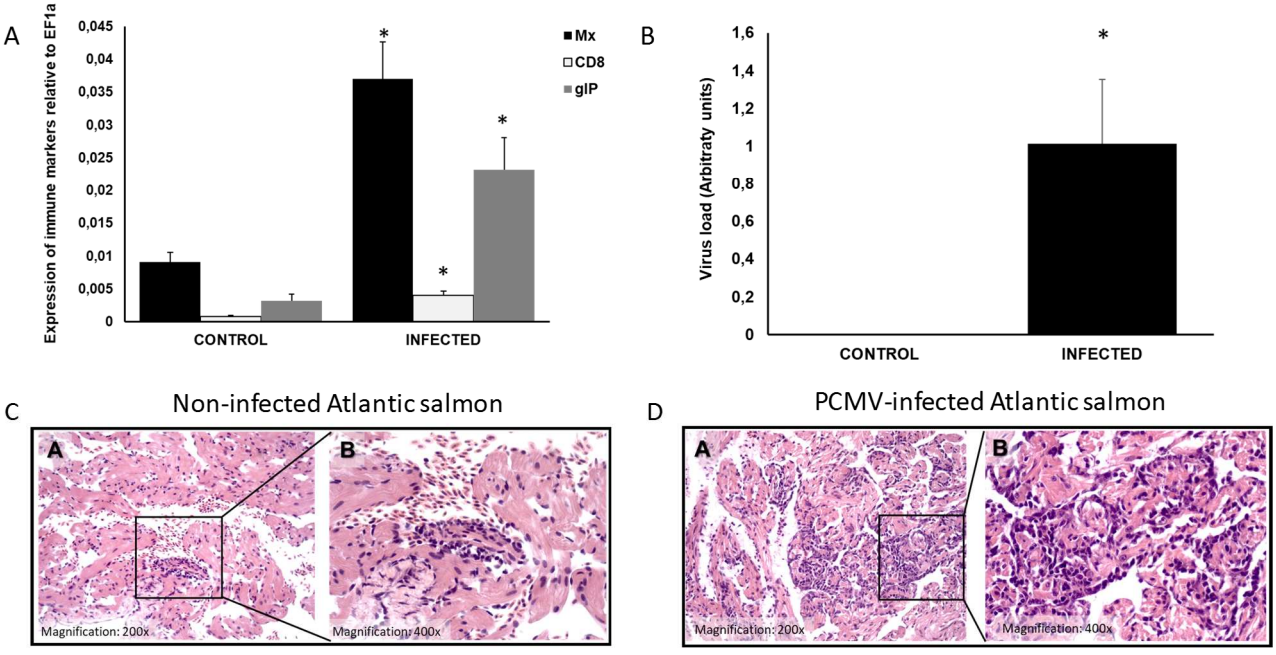
231 **Figure legends**

232 **Figure 1. Analysis of tissues collected at the final time-point (77 days post-infection) from non-**
233 **infected individuals (control group) and piscine myocarditis virus (PCMV) infected individuals**
234 **(infected group). A.** Expression analysis of selected immune genes (*mx*, *cd8* and *γip*) in Atlantic
235 salmon infected or non-infected (control) with PCMV pathogen 77-days post-infection. RNA was
236 collected for real-time PCR expression analysis and results are expressed as averages (n= 10 fish per
237 group). Statistically significant results between infected and non-infected groups are indicated by
238 an asterisk (*), where $P < 0.05$. **B.** Viral load was detected by performing a qPCR in the heart of the
239 non-infected and infected salmon, quantifying PCMV and normalising it to the house-keeping gene
240 EF1a. Statistically significant results (paired t test) between infected and non-infected groups are
241 indicated by an asterisk (*), where $P < 0.05$. **C.** Cardiac ventricle of non-infected salmon at day 77
242 post-infection showing a non-specific inflammatory focus with mild endothelial cell reaction (HE
243 stain). **D.** Cardiac ventricle of a PCMV-infected salmon at day 77 post-infection, showing strong
244 inflammatory reaction with infiltration of lymphocytes and some macrophages in the
245 cardiomyocytes (sarcoplasma) and with strong endothelial hypertrophy and hyperplasia (HE stain).
246 E. Summary of atrium (a) and ventricle (v) scores in the control group and in the infected fish. Atrium
247 shows the highest score while the percent non-affected fish (score of 0) is highest in the atrium
248 compared to ventricle. By Kruskal-Wallis rank test atrium and ventricle scores of infected fish were
249 significantly higher than controls, 0.034 and 0.021, respectively.

250 **Figure 2. Individual mx gene expression level in the cells of blood collected from non-infected and**
251 **PCMV-infected Atlantic salmon over a 77-day infection gene expression.** Data represent the
252 individual fold change relative to pre-infection (day 0) (1 line = 1 animal) in n=10 (blue lines) non-
253 infected and n = 10 (red lines) infected animals (A) or in two (Fish 12 and 20) infected animals (B).
254 Some of the kinetics are incomplete as a discrete number of samples were lost.

Figure 1



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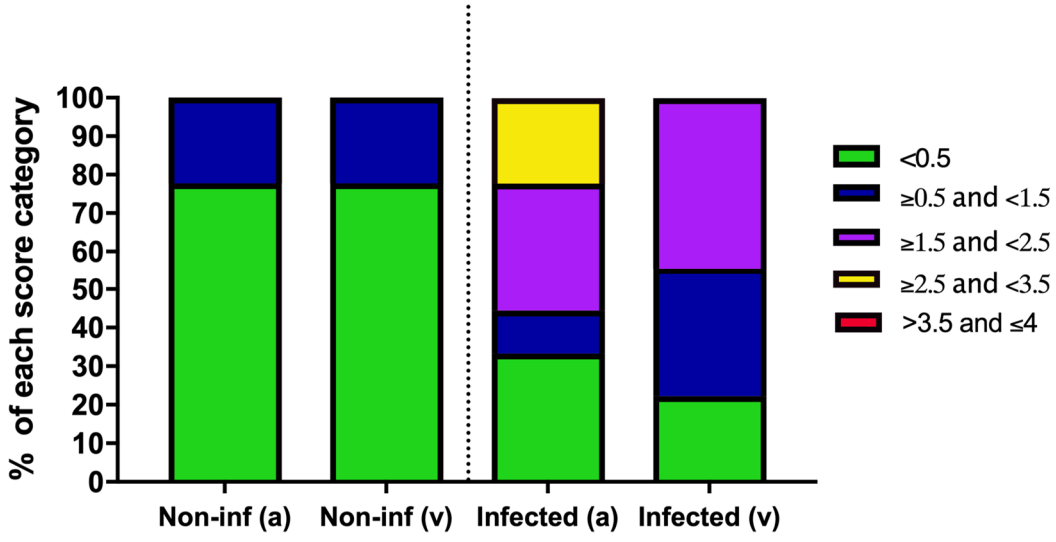
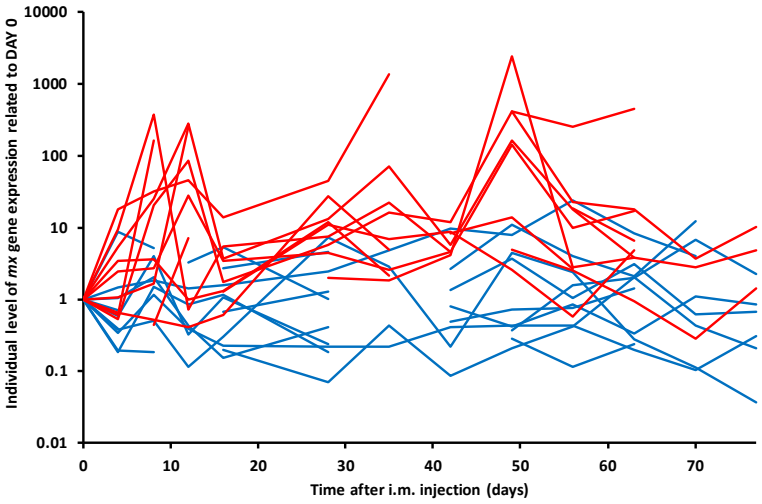


257

258

Figure 2

A


Individual level of *mx* gene expression related to DAY 0
10000
1000
100
10
1
0.1
0.01
0
10
20
30
40
50
60
70
Time after i.m. injection (days)

B

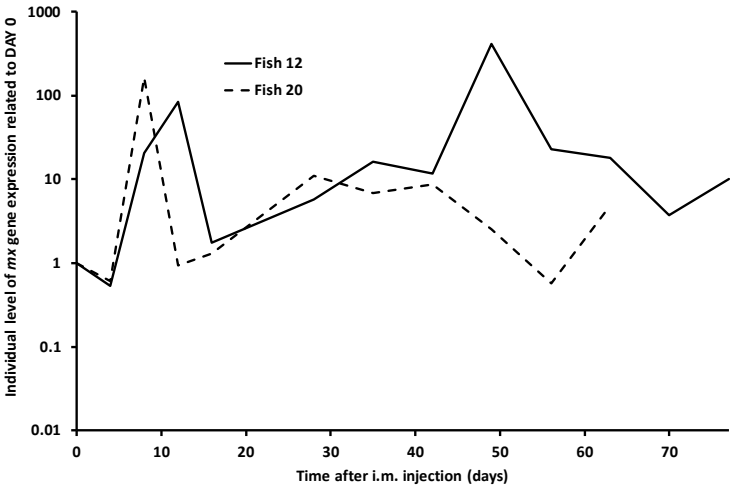

Individual level of *mx* gene expression related to DAY 0
1000
100
10
1
0.1
0.01
Fish 12
Fish 20
0
10
20
30
40
50
60
70
Time after i.m. injection (days)

Table 1. Scoring histopathological examination in heart

Fish ID	Atrium	Ventricle	Epicard
1	0	0	0
2	0	0	0
3	1	1	1
4	0	0	0
5	1	1.2	0
6			
7	0	0	0
8	0.5	0.5	0
9	0	0	0
10	0	0	0
11	0	0.7	0
12	2.3	2	0
13	2	1.3	0
14	3	2.2	0
15	2.2	2	0
16			
17	0	1	0
18	0	0	0
19	2.5	2.4	0
20	1.2	0	0

Note: Fish 1 to 10 are non-infected fish (control), Fish 11 to 20 are PCMV-infected fish