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Title: 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).

3. Results and discussion

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

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

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

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

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

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

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

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

We demonstrate here the possibility to monitor the immune response of infected individual animals under experimental conditions in a situation where the pathogen could not be detected, the animals did not show any external sign of disease and no mortality occurred. The final lethal sampling revealed a high viral load and a clear development of CMS symptoms. Nevertheless, the immune response was evident from a very early time point and could be monitored by qPCR in blood cells acquired non-lethally. The viraemia phase is most common route of dissemination of viral infection within the organism (Baron et al., 1996) and is often preclinical. If viremia correlates well to the intensity of later clinical symptoms, this can help to predict the intensity of a viral infection before the onset of clinical signs. Therefore, early blood immune parameters can be valuable welfare indicators, in addition to the evident robustness of individual longitudinal data 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 rigorous validation of the Koch's postulate. Ultimately this model could be used to assess, through the immune response, livestock' health status at the pre-clinical stage of infection.

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References

1. Baron, S., Fons, M., Albrecht, T., 1996. Chapter 45: Viral pathogenesis, in: Baron, S. (Ed.), Medical microbiology, The University of Texas Medical Branch, Galveston, pp. 1-16.

2. Bruno D, Collet B, Turnbull A, Kilburn R, Walker A, Gubbins M et al. Examination of diagnostic methods for *Renibacterium salmoninarum* causing bacterial kidney disease (BKD) in the UK. Aquaculture 2007;269: 114-122.
3. Ellis AE, Cavaco A, Petrie A, Lockhart K, Snow M, Collet B. Histology, immunocytochemistry and qRT-PCR analysis of Atlantic salmon, *Salmo salar* L., post-smolts following infection with infectious pancreatic necrosis virus (IPNV). J Fish Dis. 2010;33: 803–818.
4. Collet B, Urquhart K, Monte M, Collins C, Secombes C, Hall M. (2015). Individual monitoring of immune response in Atlantic salmon Salmo salar following experimental infection with Infectious Salmon Anaemia Virus (ISAV). Plos One. 10(9): e0137767. https://doi.org/10.1371/journal.pone.0137767
5. Collet B, Ganne G, Bird S, Collins C. Isolation and expression profile of a gene encoding for the Signal Transducer and Activator of Transcription STAT-2 in Atlantic salmon (*Salmo salar*). Dev Comp Immunol. 2009;33: 821-829.

6. Collet B, Bain N, Prevost S, Besinque G, McBeath A, Snow M et al. Atlantic salmon *Salmo salar* Signal Transducer and Activator of Transcription STAT-1: a contrasting kinetics of expression upon ISAV or IPNV infection. Fish Shellfish Immunol. 2008;25: 861-867.
7. Collet B, Collins C. Comparative gene expression profile in two Atlantic salmon cell lines TO and SHK-1. Vet Immunol Immunopathol. 2009;130: 92-95.
8. Hall, L.M., Collins, C., Collet, B., 2018. The potential benefits of repeated measure experiments for fish disease-challenge host-pathogen investigations. Fish Shellfish Immunol. 85, 126–131. https://doi.org/10.1016/j.fsi.2018.01.033
9. Haugland, O., Mikalsen, A.B., Nilsen, P., Lindmo, K., Thu, B.J., Eliassen, T.M., Roos, N., Rode, M., Evensen, O., 2011. Cardiomyopathy syndrome of atlantic salmon (Salmo salar L.) is caused by a double-stranded RNA virus of the Totiviridae family. J. Virol. 85, 5275–86. https://doi.org/10.1128/JVI.02154-10
10. McBeath AJA, Snow M, Secombes CJ, Ellis AE, Collet B. Expression kinetics of interferon and interferon-induced genes in Atlantic salmon following infection with IPNV and ISAV. Fish Shellfish Immunol. 2007;22: 230-241.
11. Chance, R.J., Cameron, G.A., Fordyce, M., Noguera, P., Wang, T., Collins, C., Secombes, C.J., Collet, B., 2018. Effects of repeated anaesthesia on gill and general health of Atlantic salmon, *Salmo salar*. J. Fish Biol. https://doi.org/10.1111/jfb.13803
12. Collet B. Innate immune responses to viral infections of salmonid fish. Dev Comp Immunol. 2014;43: 160-173.
13. Collet, B., Urquhart, K., Monte, M., Collins, C., Perez, S.G., Secombes, C.J., Hall, M., 2015. Individual monitoring of immune response in Atlantic Salmon *Salmo Salar* following experimental infection with infectious salmon anaemia virus (ISAV). PLoS One 10. https://doi.org/10.1371/journal.pone.0137767

14. Collet B, Urquhart K, Noguera P, Larsen K, Lester K, Smail D et al. A method to measure an indicator of viraemia in Atlantic salmon using a reporter cell line. J Virol Meth. 2013;191: 113-117.

15. Garseth, Å.H., Fritsvold, C., Svendsen, J.C., Bang Jensen, B., Mikalsen, A.B., 2018. Cardiomyopathy syndrome in Atlantic salmon *Salmo salar* L.: A review of the current state of knowledge. J. Fish Dis. 41, 11–26. https://doi.org/10.1111/jfd.12735

16. Martinez-Rubio L, Evensen O, Krasnov A, Jørgensen SM, Wadsworth S, Ruohonen K, Vecino JLG, Tocher DR. 2014. Effects of functional feeds on the lipid composition, transcriptomic responses and pathology in heart of Atlantic salmon (*Salmo salar* L.) before and after experimental challenge with Piscine Myocarditis Virus (PMCV). BMC Genomics 15(1):462.

17. Monte Milena, Urquhart Katy, Secombes Christopher J, Collet B, Monte, M.M., Urquhart, K., Secombes, C.J., Collet, B., 2016. Individual monitoring of immune responses in rainbow trout after cohabitation and intraperitoneal injection challenge with Yersinia ruckeri. Fish Shellfish Immunol. 55, 469–478. https://doi.org/10.1016/j.fsi.2016.05.041

18. Rodger, H., Turnbull, T., 2000. Cardiomyopathy syndrome in farmed Scottish salmon. Vet. Rec. 146, 500–501. https://doi.org/10.1136/vr.146.17.500

19. Timmerhaus, G., Krasnov, A., Nilsen, P., Alarcon, M., Afanasyev, S., Rode, M., Takle, H., Jørgensen, S.M., 2011. Transcriptome profiling of immune responses to cardiomyopathy syndrome (CMS) in Atlantic salmon. BMC Genomics 12, 459. https://doi.org/10.1186/1471-2164-12-459

20. Timmerhaus, G., Krasnov, A., Takle, H., Afanasyev, S., Nilsen, P., Rode, M., Jørgensen, S.M., 2012. Comparison of Atlantic salmon individuals with different outcomes of cardiomyopathy syndrome (CMS). BMC Genomics 13, 205. https://doi.org/10.1186/1471-2164-13-205

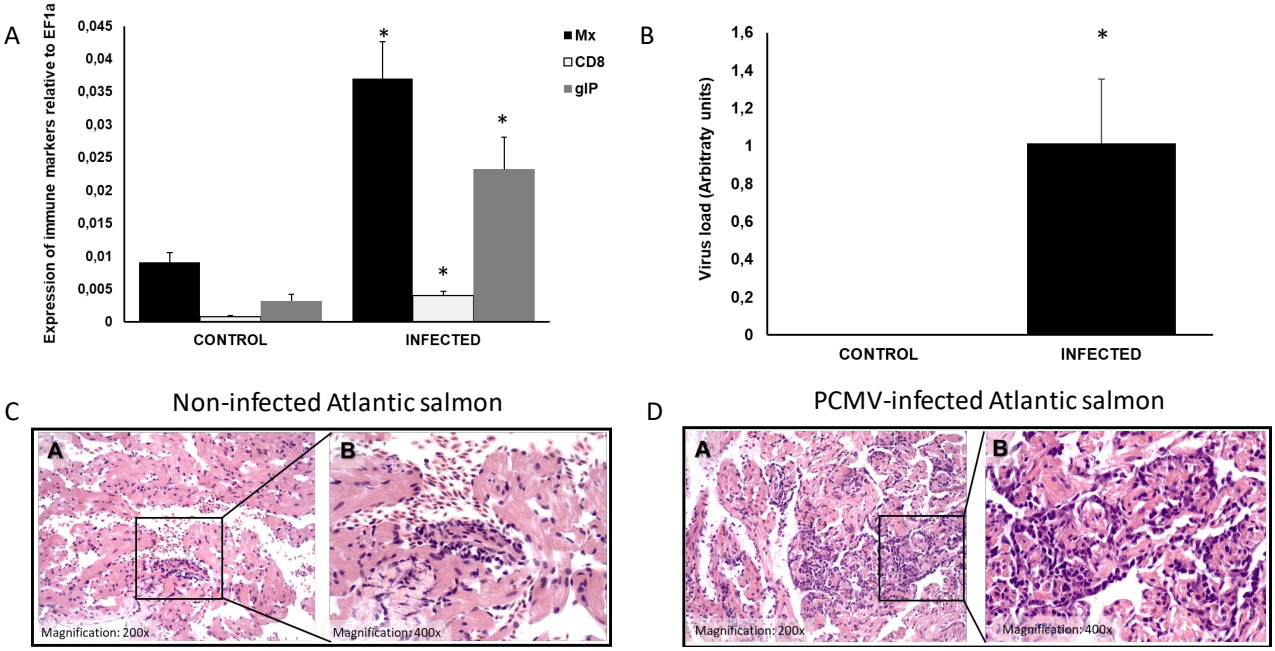
21. Urquhart, K., Collins, C., Monte, M., Sokolowska, J., Secombes, C., Collet, B., 2016. Individual measurement of gene expression in blood cells from Rainbow trout Oncorhynchus mykiss (Walbaum). J. Exp. Appl. Anim. Sci. 2, 1. https://doi.org/10.20454/jeaas.2016.1077

Figure legends

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

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

Figure 1


A
Expression of immune markers relative to EF1a
0,045
0,04
0,035
0,03
0,025
0,02
0,015
0,01
0,005
0
Mx
CD8
gIP
*
*
*
CONTROL
INFECTED
B
Virus load (Arbitraty units)
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
*
CONTROL
INFECTED
C
Non-infected Atlantic salmon
A
B
Magnification: 200x
Magnification: 400x
D
PCMV-infected Atlantic salmon
A
B
Magnification: 200x
Magnification: 400x

E

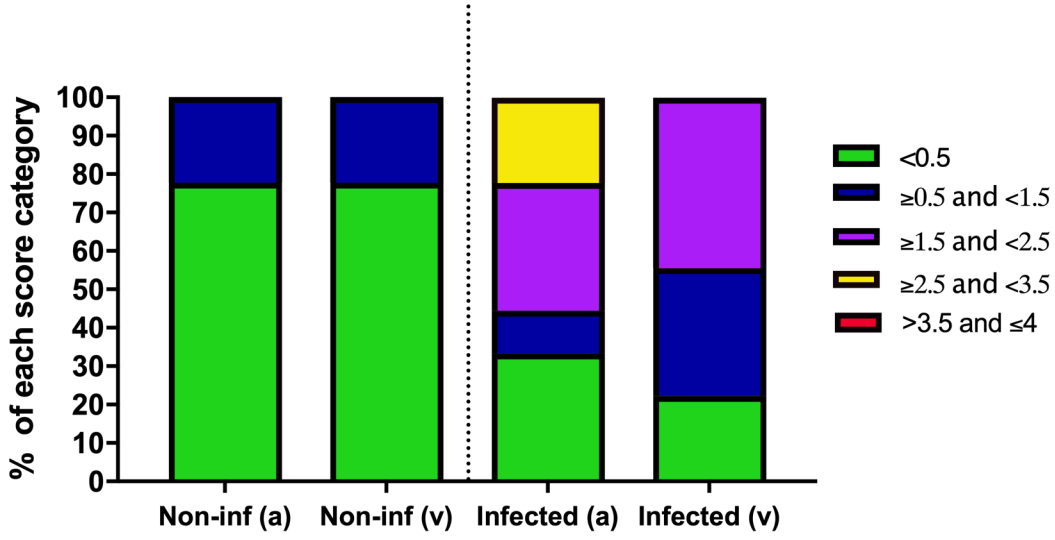
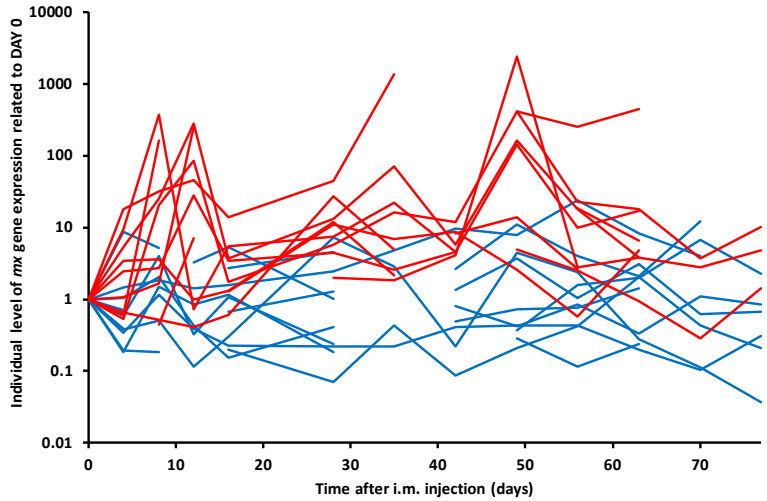

% of each score category
100
90
80
70
60
50
40
30
20
10
0
Non-inf (a)
Non-inf (v)
Infected (a)
Infected (v)
<0.5
≥0.5 and <1.5
≥1.5 and <2.5
≥2.5 and <3.5
>3.5 and ≤4

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

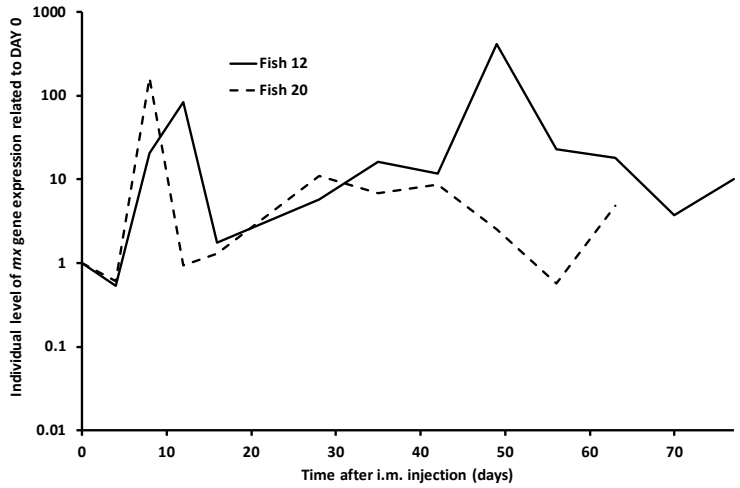

Individual level of mx gene expression related to DAY 0
1000
100
10
1
0.1
0.01
Fish 12
Fish 20
0
10
20
30
40
50
60
70
Time after i.m. injection (days)

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