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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*, *cd8 $\alpha$*  and *yp*) 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 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; PCMV; 3Rs; *mx*; interferon

## 1 *1. Introduction*

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

22

## 23 *2. Material and methods*

### 24 2.1. Experimental design

25 This study was carried out in strict accordance with the UK Animals (Scientific Procedures)  
26 Act 1986 (ASPA) under project licence PPL3965. The protocol was approved by the Marine Scotland  
27 Ethical Review Committee. All procedures were performed under MS222 anaesthesia, and all efforts  
28 were made to minimise suffering. Twenty PIT tagged Atlantic salmon *Salmo salar* were provided by  
29 Landcatch Natural Selection (Hendrix-Genetics), transported to the Aquarium Facility at Marine  
30 Scotland and divided equally into two circular 1 m<sup>3</sup> tanks. The fish were screened for common  
31 pathogens, viral hemorrhagic septicaemia virus, infectious pancreatic necrosis virus, infectious  
32 salmon anemia virus, infectious hematopoietic necrosis virus, and *Aeromonas salmonicida* using  
33 standard methods in the laboratory (Collet et al. 2015). They were kept under natural photoperiod  
34 (October), sea water salinity 37 ‰ and at 10 °C. They were fed once a day with pellets (EWOS). After  
35 a week of acclimation, all the fish were anaesthetised, weighed (average weight 1,109 ± 167 g) and  
36 injected intra-muscularly with 100 µl heart tissue homogenate from salmon originating from a  
37 clinical case of CMS on the north-west coast of Norway and diagnosed with a piscine myocarditis  
38 virus infection by real-time PCR and with typical histo-morphological heart changes (N=10, 1 tank)  
39 or 100 µl tissue homogenate from healthy salmon (N=10, 1 tank). The PMCV-positive homogenate  
40 was tested earlier to yield high levels of PMCV genome in heart tested by real-time PCR (Haugland  
41 et al. 2010). The sampling model was as described by Collet et al. (2015). Immediately before  
42 injection, a small blood sample (150 µl) was collected from the caudal vein (Pre-infection bleed).  
43 Subsequently, non-lethal blood samples were collected repeatedly at 4, 8, 12, 16, 28, 35, 42, 49, 56,  
44 63, 70, and 77 days post infection (dpi). At 77 dpi all the fish were killed by exposure to an overdose  
45 of anaesthetic and blood collected immediately. For all samples, the blood was centrifuged for 30  
46 sec at 10,000g to separate blood cells and plasma, and stored at -80°C until processed further. Heart  
47 tissue was dissected and half was stored in 750 µl RNAlater (Sigma) at -80°C for subsequent viral  
48 load analysis and the other half was fixed in 10% phosphate buffered formalin, embedded in paraffin

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

## 51 2.2. RNA extraction, cDNA synthesis and real-time PCR

52 RNA purification and cDNA synthesis from whole blood cells and heart tissue was carried  
53 out using a method described by Collet et al. (2015). Real-time PCR analysis was performed for the  
54 house keeping gene *ef1α*, PCMV using forward primer 5'-TTCCAAACAATTCGAGAAGCG-3', reverse  
55 primer 5'-ACCTGCCATTTTCCCCTCTT-3' and Taqman probe 5'-6FAM-CCGGGTAAAGTATTTGCGTC-3'.  
56 (amplicon 141 nt, accession number NC\_015639), and immune genes (*mx*, *cd8α* and *γip*), as  
57 described by Collet et al. (2015).

58

## 59 2.3. Histopathology analysis.

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

## 63 2.4 Statistical analysis

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

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

71

72

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 PCMV 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 PCMV 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 spongy 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 not 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

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

157

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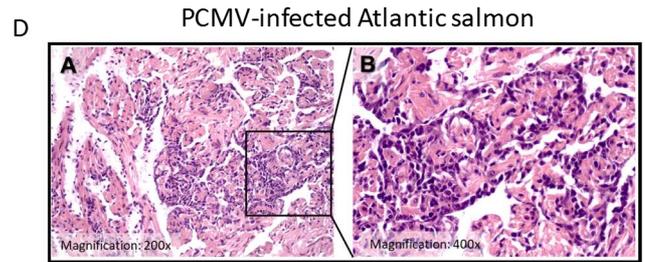
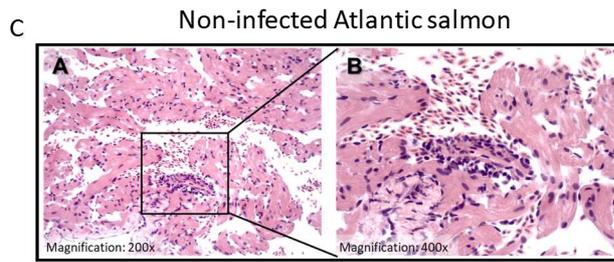
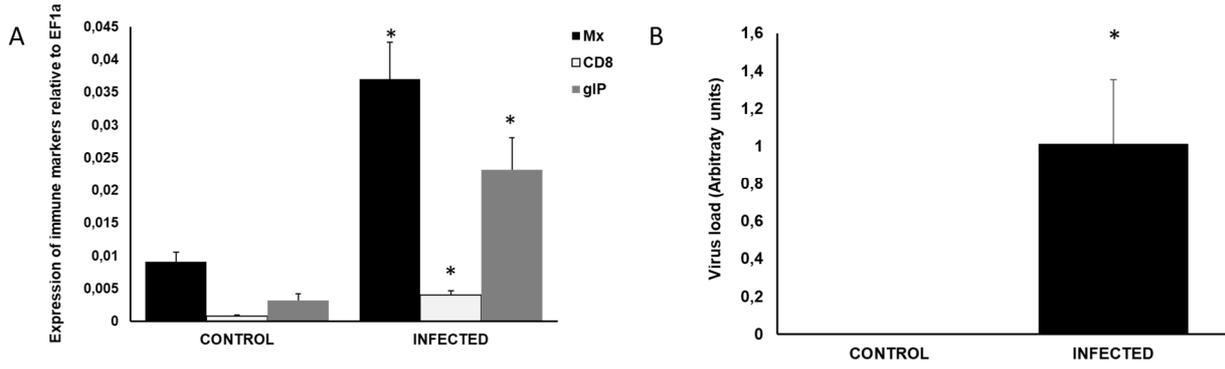
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231 **Figure legends**

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.

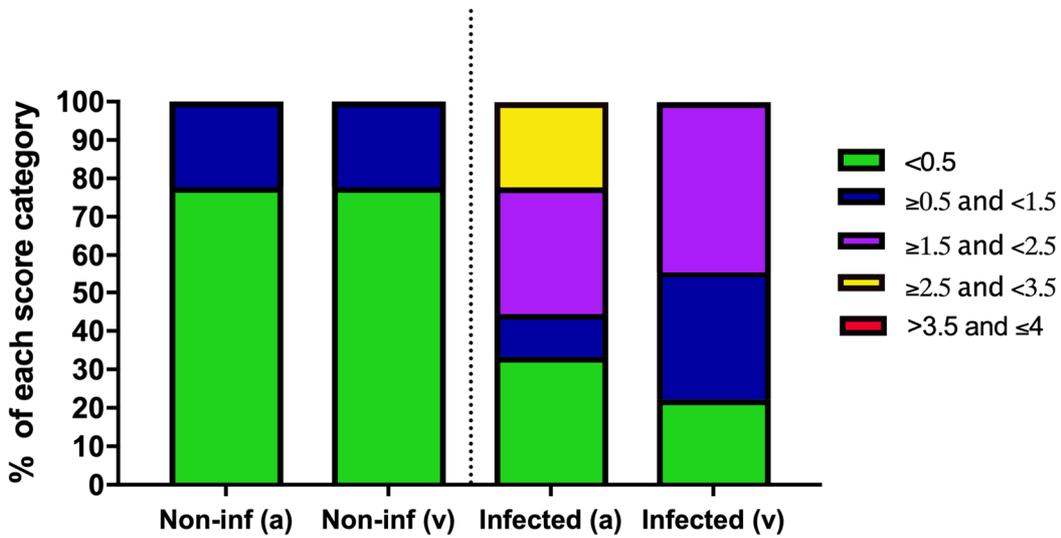
250 **Figure 2. Individual mx gene expression level in the cells of blood collected from non-infected and**  
251 **PCMV-infected Atlantic salmon over a 77-day infection gene expression.** Data represent the  
252 individual fold change relative to pre-infection (day 0) (1 line = 1 animal) in n=10 (blue 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



255

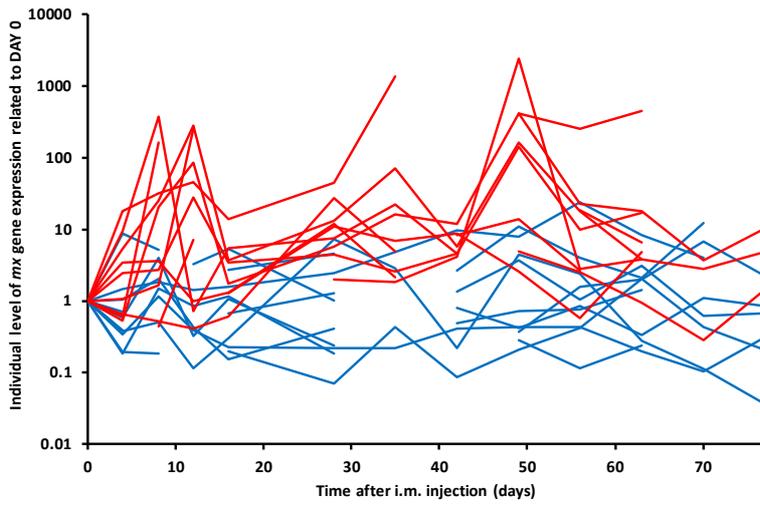
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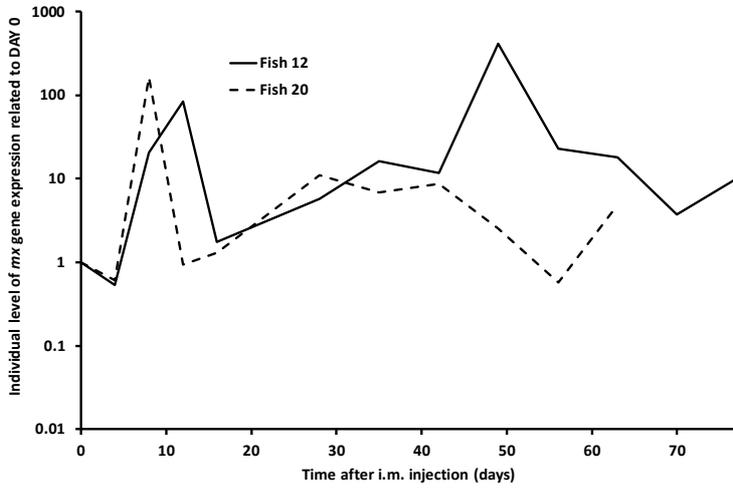
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258

259 **Figure 2**  
260 **A**



261 **B**  
262



263  
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265  
266  
267

Table 1. Scoring histopathological examination in heart

<b>Fish ID</b>	<b>Atrium</b>	<b>Ventricle</b>	<b>Epicard</b>
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

268  
269  
270  
271  
272

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