Investigating the involvement of a *Midichloria* -like organism (MLO) in red mark syndrome in rainbow trout *Oncorhynchus mykiss*

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Justification: Red mark syndrome is a disease of economic concern that impacts on the marketability of rainbow trout stocks. The disease is still of unknown cause meaning that treatment is still symptomatic. Insight in the aetiology will increase treatment options and the welfare of the fish.

Key words: Red mark syndrome; *Oncorhynchus mykiss*; *Midichloria* -like organism; disease correlation, epidemiology, immunohistochemistry, quantitative PCR.

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Abstract

Red mark syndrome (RMS) is a skin condition in Rainbow trout *Oncorhynchus mykiss* that has been reported worldwide but was first seen in the United Kingdom (UK) in 2003. The current study was conducted to examine if there was an association between a *Midichloria*-like organism (MLO) and RMS using a statistically appropriate sample set, whilst determining if there is a lack of association with *Flavobacterium psychrophilum* implicated in disease in previous studies. Fish in this study were obtained from three sites positive for RMS in the UK and United States (US), and three sites in the UK and the Netherlands that had no previous history of this condition. Samples taken from RMS-affected sites were found to show typical RMS pathology. Analysis of the major organs of affected fish by quantitative polymerase chain reaction (qPCR) demonstrated a significantly higher presence of the MLO in the RMS-affected tissues. Although most of the tissues were positive for the MLO, the highest correlation was seen in the skin, whilst the tissues from the unaffected fish were all negative. Thus, a strong positive correlation was found between the MLO and RMS-affected fish, whilst no association was found between the RMS-affected fish and *F. psychrophilum* other than superficial presence in the skin. The use of immunohistochemistry showed positive staining of what was considered to be MLO-related antigens in the internal organs of most RMS-affected fish. Attempts were made to culture the MLO, but no MLO was isolated.

1. Introduction

Red mark syndrome (RMS) is a skin condition affecting rainbow trout (*Oncorhynchus mykiss*) in freshwater. Although the disease does not result in significant mortalities, it does cause considerable economic losses to the trout industry due to the downgrading of the fish at the time of harvest. RMS first appeared in the United Kingdom (UK) in late 2003 and has since spread to over more than 50% of trout farms in the UK (Verner-Jeffreys *et al.*, 2006). It has also now been reported in many European counties, and further afield as far as Iran and Chile (Sandoval *et al.*, 2016; Sasani *et al.*, 2016). A similar skin condition to RMS, known as strawberry disease (SD), has been present in rainbow trout in the United States (US) since the mid-1940s (Davis, 1946). The initial outbreak of RMS was linked to an egg importation from the US to the UK (Verner-Jeffreys *et al.*, 2006). Red mark syndrome is characterised by the clinical presentation of lesions that appear as slightly raised and well demarcated. The lesions are often located across or below the lateral line and can appear haemorrhagic with scales missing from the centre of the lesion. Acute inflammation with the presence of neutrophils can be observed around the area of scale loss together with high protein oedema (Ferguson, 2006; Oidtmann, 2013).

The aetiology of RMS has not been definitively identified to date, but the cause is believed to be bacterial in nature (Ferguson *et al.*, 2006; Verner-Jeffreys *et al.*, 2008). Ferguson *et al*. (2006) studied a small number of SD and RMS affected fish from the US and UK, respectively and suggested an association between RMS in Scottish fish and *Flavobacterium psychrophilum* using a polymerase chain reaction (PCR). Lloyd *et al.* (2008) later found a strong correlation between SD lesions and the presence of a Rickettsia-like organism (RLO) 16S rRNA sequence. Samples of RMS from UK gave similar results to those obtained for SD samples from the US when examined by quantitative PCR (qPCR) (based on the RLO 16S rRNA sequence) and immunohistochemistry (IHC) (Metselaar *et al.*, 2010). It is therefore,

64 considered that RMS and SD are the same condition in different geographic locations
65 (Metselaar *et al.*, 2010; Oidtmann *et al.*, 2013) and therefore only refer to the condition as RMS
66 in the present study. More recently a *Midichloria* -like organism (MLO), belonging to the
67 family Midichloriaceae within the Order Rickettsiales, has been associated with RMS (Cafiso
68 *et al.*, 2015) and we will therefore subsequently refer to RLO as an MLO.
69 The exact involvement of the MLO and *F. psychrophilum* in the pathology of RMS is
70 still unclear. The aim of the current study was to investigate this relationship further. In order
71 to have a clearer understanding of their role in RMS, 30 fish were sampled from RMS-affected
72 fish from sites in the US and UK with a history of the condition (positive farms) and compared
73 with 30 fish from unaffected farms that have never experienced RMS outbreaks and which
74 were located on water systems free of RMS-affected farms (negative farms) in the Netherlands
75 and UK. To confirm the disease status of the fish, tissues were examined for the presence of
76 RMS-related pathology. Primers for the MLO (Lloyd *et al.*, 2008; Lloyd *et al.*, 2011; Montagna
77 *et al.*, 2013) were used to investigate the association between the MLO and RMS in the samples
78 from the two sets of farms. As the qPCR allows the quantity of MLO DNA present in the tissue
79 to be determined, this can be correlated to the disease status of the animal, adding to the
80 evidence that the MLO is the causative agent of RMS. Immunohistochemistry with anti-
81 *Piscirickettsia salmonis* monoclonal antibodies (MAbs), shown to cross-react with RMS-
82 affected tissue (Metselaar *et al.*, 2010), and anti-*F. psychrophilum* polyclonal antibodies (PAb)
83 (Faruk *et al.*, 2002) were used to determine if there was an association of these pathogens with
84 the pathology. Several agars known to support the growth of intracellular bacteria were also
85 tried to establish if it was possible to culture any other bacterial agents from the fish. Standard
86 bacteriology was used to ascertain if *F. psychrophilum* was present, with predominant yellow-
87 pigmented bacteria recovered from the fish identified through conventional 16s partial rRNA

gene sequencing. Finally, a serological analysis was performed to evaluate the fish's antibody response to *F. psychrophilum* and MLO by ELISA.

2. Material and methods

2.1 Fish sampling

Sixty fish (ten per farm) were randomly selected from six unrelated rainbow trout farms (details of each site are withheld for confidentiality). Sample size was based on OIE recommendations for detection of disease prevalence of >10% with 95% confidence (OIE - Aquatic Animal Health Code, 2015). Tissue samples from skin (affected as well as unaffected skin), heart, liver, gill, kidney and spleen were collected for histology. Four farms in the UK were sampled two farms that had never experienced an outbreak of RMS and were not located on water systems with farms with a history RMS outbreaks (Farms 1 and 2) and two farms with on-going clinical RMS (Farms 4 and 5). In addition, samples were collected from one farm in the USA (Farm 6), where fish were exhibiting clinical signs, and, from a single farm (Farm 3) in the Netherlands, regarded as RMS-free. Bacteriological samples were taken from the skin lesions or unaffected skin where appropriate. Tissue samples were also collected from lesions, unaffected skin (opposite side of the body, distal to the lesion), heart, liver, gill, kidney and spleen and stored in 95 % ethanol (Farm 1-6) and formalin (Farms 1, 2, 4 and 5). Serum was also collected from the fish sampled in the UK for serology.

2.2 Histology

Tissues from the farms sampled in UK were processed for histology using standard protocols and five µm sections were stained with haematoxylin and eosin (H&E) and examined for the presence of RMS-associated pathology as described by Ferguson *et al.* (2006) and Oidtmann *et al.* (2013).

2.3 Immunohistochemistry

Additional five µm wax tissue sections were placed on glass slides and incubated overnight at 60°C. Tissues were dewaxed and encircled with a wax pen. Slide were quenched for endogenous peroxidase activity following the procedures detailed in Metselaar *et al.* (2010). All sections were washed in 0.1M phosphate-buffered saline (PBS) and non-specific binding sites were blocked using a 1/10 dilution of goat serum for 30 min at 22°C. To investigate the presence *F. psychrophilum*, a cocktail of 2 rabbit anti *F. psychrophilum* sera (Faruk *et al*., 2002) were used at a 1/5000 dilution. The slides were incubated with the PAbs for 1 h at 22^{o}C before capture with anti-rabbit IgG-HRP diluted 1/250 in PBS and visualised with a VIP chromogen kit with a methyl green counterstain.

The presence of the MLO was investigated using an anti- *P. salmonis* MAb (Metselaar *et al.*, 2010) at 10 µg/ml incubated for 1 h at 22°C. This was captured with an anti-mouse IgG-biotin conjugate, diluted 1/200 in PBS and streptavidin-HRP diluted 1/200 in PBS for 30 min at RT. This reaction was developed as described above. Negative controls were incubated with either an isotype-matched MAb (anti-white spot virus Mab 4), normal rabbit serum (1/5000) or PBS where appropriate. Negative tissue was obtained from the samples taken from negative farms (Farm 1 and 2) and unaffected skin on RMS-affected fish.

2.4 Bacteriology

Bacteriology samples were collected from 10 fish from all of the UK farms (Farms 1, 2, 4 and 5). Swabs were taken from the rainbow trout at the site of the lesion (on negative fish in a similar location mid flank) and cultured on tryptic soya agar (TSA); Veggietone Agar (MVA) (Ngo *et al.,* 2017); and Austral-TSFe (Yanez *et al.,* 2012). Skin from unaffected fish was sampled from a similar position as that of the lesions. The agar plates were incubated at 10˚C for 9 days, and the colony morphology described. Yellow pigmented colonies from the MVA plates, which resembled *Flavobacterium* sp., were selected and sub-cultured onto fresh MVA.

Gram staining was performed on all passaged bacteria and all Gram-negative rods, again resembling *Flavobacterium* sp., were sub-cultured onto fresh MVA plates. Small white colonies were selected from the Austral-TSFe media.

2.5 Sequencing of the bacteria isolates

In order to confirm the identity of the bacterial isolates conventional 16s rRNA gene sequencing was performed on fresh cultures of selected isolates (see Table 1 for details). The DNA was extracted using Proteinase-K and amplified using KBiosciences standard protocols. The resulting PCR products were sent to GATC-Biotech Ltd. for Sanger sequencing. The forward and reverse sequences were aligned using SeqMan® software and compared against the EMBL/GenBank sequence databases using the BLAST algorithm (http://www.ncbi.nlm.nih.gow/BLAST).

2.6 Quantitative PCR for MLO

In order to investigate levels of the MLO in the fish tissues qPCR was performed. DNA was extracted from all seven tissues (affected skin, unaffected skin, heart, liver, gill, kidney and spleen). Tissues were homogenised 1:10 in G2 buffer (Qiagen, UK) and digested with 6 milli-Anson units of proteinase K at 55°C for 3 h. DNA was then extracted from 200 l of homogenized tissue using an EZ1 DNA tissue kit in an EZ1 extraction robot (Qiagen). DNA was eluted in 50 l of elution buffer, according to the manufacturer's instructions. Insulin growth factor (IGF-1) was used to normalise the MLO copy numbers and a standard (2.5×10^6 to 250 copies) was made from liver tissue from a specific pathogen free (SPF) fish. For the MLO standard, a 149 bp segment of the MLO was cloned using a PGMT-easy plasmid kit (Promega). The SPF fish DNA (used as the IGF-1 standard) was added to MLO standard at 375 ng/reaction.

The qPCR was performed using the primers (Table 1) and protocol as described the method of Lloyd *et al.* (2011) with modifications as following: 2 min at 50˚C, 10 min at 95˚C, 40 cycles of 95˚C for 15 sec, 55˚C for 30 sec and 60˚C for 30 sec. These were used to represent

the MLO (Cafiso et al., 2015). The Cq values were measured with the Applied Biosystems StepOne Plus® and downloaded into the StepOne® software and normalised against IGF-1. Negative DNA extractions and non-template were included as controls. MLO copy number was plotted against the corresponding Cq value and standard curves were generated by linear regression of plotted points. The amplification efficiency was assessed using the calculation E = −1 + 10(−1/slope). Efficiency was found to be 98%. The results were calculated as an average from three replicates and normalised against the IGF1 according to Lloyd et al., (2011). If only one of three replicates showed amplification, the result was considered to be insignificant or too near to the detection level to be considered positive. Only Cq values below <35 were included in this study.

2.7 ELISA

Fish serum was collected from four farms (Farms 1, 2, 4 and 5) and analysed by ELISA to determine the host antibody response to *F. psychrophilum* and the MLO. The MLO associated with RMS has not yet been isolated therefore it was postulated that, as the anti-*P. salmonis* MAbs reacted with RMS-affected tissue, *P. salmonis* could be used as the capture antigen. Capture antigens comprised of *F. psychrophilum* (type strain NCIMB 1947^{T}) and three related *Flavobacterium* spp. (*F. hydatis* ARF07; *Flavobacterium* sp. BGARF; *Chryseobacterium piscium* MOF25P) and *P. salmonis (*ATCC VR 1369^{T}). ELISA reactions were optimised according to standard protocols (Cobo Labarca *et al.*, 2015). Negative wells incubated with PBS were used to calculate the background and the threshold of the ELISA was taken as three times the background absorbance value obtained with PBS.

2.8 Statistical analysis

To examine which tissue had the highest PCR results, negative results below the limits of detection were replaced with a value of half the smallest value found elsewhere in the dataset. All data were then converted into base-10 logarithms. Since the resulting distributions would

not satisfy ANOVA assumptions (bimodality due to the negative test results), A Friedman test was then used to compare tissues, accounting for fish as a blocking factor. This test was then followed by post-hoc comparisons using the frdAllPairsExactTest function provided by the PMCMRplus package in R. In all tests, a significance level of P=0.05 was taken. An alternative, robust, approach using a chi-square test with post-hoc pairwise Fisher exact tests was used. This provided similar results and only the Friedman test results are presented here.

Of interest is the potential for clustering of test results amongst tissues. To investigate this, PCR results were converted to simple presence/absence and analysed with multiple correspondence analysis (MCA) in R using the FactoMineR and FactoExtra packages. Similar results were found using principal components analysis on the numerical results, due to the bimodality of the tissue-level data, and therefore the more robust MCA results are presented here.

To test for differences in isolate presentation between farm statuses, the ELISA data were analysed by fitting linear mixed-effect models using R (R Core Team, 2020) and the lme4 package, since initial analysis indicated significant farm-level variation for some isolates. Three models were fitted for each isolate. First, the null model. Second, a model containing farm only, as a random effect. Third, a model containing farm status as an additional fixed effect. These nested models were compared using likelihood ratio tests to determine the significance of farm and status for each isolate. No correction was made for multiple comparisons here, but additionally, a MANOVA was performed on all isolates, with farm ID nested within status.

3. Results

3.1 Clinical signs and histopathology

The fish from four farms sampled (Farms 1, 2, 4 and 5) from the UK were examined for gross pathology and histopathology (Table 2). There was a difference in the severity in the clinical signs between fish from the RMS-affected farms with fish from Farm 4 showing no signs of RMS in three fish and mild to moderate lesions in the remaining fish, while all ten fish from

Farm 5 had moderate to severe gross signs of RMS. There were signs of the skin swelling at the site of the lesion and with scale loss. A haemorrhagic dermatitis was evident, with the lesions often present around the lateral line. Histopathology typical of that previously reported for RMS was evident in the majority of fish from Farms 4 and 5 (Metselaar *et al.*, 2010). Skin showed multifocal lymphocytic intra-epithelial infiltration at the site of the lesion with evident acanthosis (with high mitotic numbers) and erosion and ulceration of the epithelium. The subcutis showed necrosis with fibro-angioblastic tissue and inflammatory reaction with heterophilic granulocytes and macrophages. There was very extensive necrosis of underlying musculature with a severe non-suppurative inflammatory response along the intermuscular septa. The spleen was hyperaemic with hyperplasia of the white pulp and perivascular histiocytic cell proliferation. The kidneys had moderate to severe diffuse histiocytic proliferation of interstitial haematopoietic tissue, with the liver showing no pathological changes. The gills showed fusion of the secondary lamellae in the distal areas of the primary lamellae, hyperplasia of goblet cells and interstitial oedema with heterophilic granulocytes. This may be unrelated and within normal limits and was classed incidental. Subepithelial multifocal non-suppurative infiltrates were present in the gill arch.

None of the tissues sampled from the RMS-negative farms had any pathology indicative of RMS. One fish did have mild focal myositis, but this was considered as an incidental finding and not related to RMS (Oidtmann, 2013).

3.2 Immunohistochemistry

A positive reaction was seen with the anti-*P. salmonis* MAb in most of the affected fish from Farms 4 and 5 (Table 3), with staining mostly seen in internal organs. The rainbow trout sampled from Farm 4 were in the final stage of the condition, as the infection had started to regress on this farm. One fish from one of the unaffected farms (Farm 1) had mild staining in its kidney and four fish from the other unaffected farm (Farm 2) also had mild staining in their

spleen, although this was much less severe than the reactions seen in the RMS-affected fish. Staining can be seen in Figure 1

The fish screened for *F. psychrophilum* showed a positive reaction in the skin and one of the internal organs of two of the RMS-affected fish. Positive staining was also seen in unaffected skin from one of these fish. There was a mild reaction within the skin, kidney and gill of three non-RMS-affected fish (Farms 1, 2 and 3). Staining can be seen in Figure 2.

The negative controls with normal rabbit serum or PBS were negative, while the *F. psychrophilum*-positive control tissue was positive. The isotype matched control was also negative.

3.3 Bacteriology

The TSA plates were discarded after nine days due to non-specific overgrowth by bacteria and/or fungus. No notable colonies were obtained on the Austral-TSFe agar. Yellow and yellow/white pigmented bacteria on the MVA plates were stained with Gram stain after 27 days of culture, and all gram-negative rods were sub-cultured onto fresh agar plates. In total 116 gram negative isolates were cultured from mucus, internal and external surfuces of skin from the RMS-affected or unaffected rainbow trout from the four fish farms (Table 4).

3.4 Sequencing of 16s rRNA gene

In general, it was found that the forward and reverse sequences resulted in >1000 bp. These were aligned and submitted to the NCBI website for identification, after which the best identification match was selected. Various *Flavobacterium* isolates were seen in both RMS-affected and unaffected groups of fish and although some bacteria were only found in either affected or non-affected fish, there was no discernible association with *F. psychrophilum* and RMS (Figure 3).

3.5 Quantification of the MLO in tissue of fish using a qPCR

Quantification of the MLO in the various tissues of fish from the unaffected farms (Farms 1-3) and the affected fish (Farms 4-6) by qPCR can be seen in Table 5 and Table 6 respectable and summarised in Figure 4. Four fish from the unaffected farms had amplification in one of the three replicates in the qPCR but were not considered to be positive for the MLO. With the affected farms almost, all fish showed amplification of the MLO in one or more of their tissues. The detection limit of the qPCR assay used in this study is estimated to be at least 10 copies.

3.6 ELISA using serum from RMS affected fish

Antibody titres against *P. salmonis* and four *F. psychrophilum* isolates were generally very low and ranged between 1/64 and 1/256 (Table 7).

3.7 Statistical analysis

Looking at differences in PCR data between tissues for diseased fish, the Friedman test indicated that significant differences were present amongst tissues ($\chi 26=54.2$; $P<0.001$). Pairwise post-hoc tests then showed there to be significant differences between higher levels in diseased skin and several other tissues: normal skin ($P=0.12$), heart ($P<0.001$), liver ($P<0.001$), kidney ($P=0.026$) and spleen ($P=0.014$). There were no other significant differences between any tissues.

Multiple correspondence analysis was used to investigate patterns of PCR positive results amongst fish and tissues. No strong signal here was in evidence, with the first two of seven dimensions accounting for 25.2 and 22.8% of variance, respectively. There is some suggestion that liver, heart, and gill may contribute towards one dimension preferentially, and kidney, skin and spleen to another. (Fig. 5)

With the ELISA data, MANOVA indicated significant contributions across isolates of both farm status ($F5,32 = 6.88$; $P<0.001$; Pillai's Trace = 0.52) and farm ID nested within status ($F10,66=3.36$; $P=0.001$; Pillai's Trace=0.67). The linear mixed-effect models found farm ID to

be significant for two isolates (NCIMB fp and BG ARF) but in no case was farm status significant (Table 8). Effect sizes were small and only a small proportion of deviance explained by the models. Normality plots were checked for the residuals of each model and found to be satisfactory.

4. Discussion

The current study appears to suggest a degree of involvement of MLO's with RMS, however, no MLO-like bacteria were described in any of the H&E stained tissues sampled from RMS-affected fish in the publications to date. The MLO's have been seen in fresh spleen smears of affected rainbow trout in Italy using Giemsa staining (Galeotti, *et al.,* 2011) and by TEM with the presence of intracytoplasmic microorganisms resembling Rickettsiales observed within macrophages, fibroblasts and erythrocytes of rainbow trout. The microorganisms observed were oval/short rod in shape (400–800 nm in length and 100–200 nm in width) (Galeotti *et al.,* 2016). In the histology, although all fish from Farm 5 displayed classical gross signs of RMS, not all the fish from Farm 4 (an RMS-affected farm) exhibited typical histopathological signs. This was not unexpected as the RMS lesions had started to resolve on this farm and many of the lesions on these fish were in an advanced stage of healing, with three fish having no signs of the disease when sampled. It was noted that when RMS lesions were more advanced and showing signs of healing that lesions were often covered with a protective film of clear mucus. There was also no sign of clinical bacterial cold-water disease, caused by *F. psychrophlilum*, in these fish.

There was a clear difference in the degree of staining in the IHC with the anti-*P. salmonis* MAb between the affected and the non-affected groups, with a strong association seen between the affected tissue and the staining, thought to represent the presence of the MLO (Metselaar *et al.,* 2010). There was mild staining in the kidney of one RMS-unaffected fish and the spleen of another, but this staining was much weaker than that seen with affected fish. The

anti-*F. psychrophilum* PAbs used reacted minimally with a few of the samples, which were from both affected and unaffected fish. It has been suggested that a particular strain of *F. psychrophilum* may be involved in RMS and may differ from other strains involved in RTFS (Ferguson *et al.*, 2006), although authors did not specify or report any differences. It is known that these PAbs react with many different *F. psychrophilum* isolates, but not with all *F. psychrophilum* isolates (Faruk *et al.*, 2002), and therefore a cocktail of the two sera made by Faruk *et al.* (2002) was used. It is possible that for some *F. psychrophilum* isolates, if present, may not have been recognised by these antisera, however this study found no association with *F. psychrophilum* in RMS-affected tissue using the described techniques.

The MLO associated with RMS has recently been allocated to the family Midichloriaceae, within the order Rickettsiales (Cafiso *et al.*, 2015) which requires host cells to support growth. No bacterial growth was noted on the Austral-TSFe agar. We have previously tried to culture the MLO in fish cell lines unsuccessfully (data not presented). The authors believe that the Austral-TSFe agar can support intracellular organisms such as the MLO, but we cannot be certain of this.

The original qPCR assay reported by Lloyd *et al.*, (2011) was able to detect 100 copies of the MLO. The detection limit of the qPCR assay used in this study is estimated to be at least 10 copies in the presence of background rainbow trout DNA. The results of the qPCR showed that a single MLO was present for every 10,000 host cells. The previous studies by by Lloyd *et al.*, (2011) showed much higher copy numbers, believed to be unusually high and the current ratio does support the lack of visualisation. In the original study by Lloyd *et al.* (2011) related to the development of the qPCR, there was a significant difference in copy number across the range of RMS lesion severity with the qPCR. A significant difference was seen in the results of the qPCR here, with highly significant differences in the presence of the MLO detected in the affected fish between the different tissues. In the MCA numbers of data were small. It

should be noted that there is no test of significance presented here for this approach. The approach shown here suggests that there might be clustering amongst the skin, kidney and spleen where there was no significant difference with the affected skin, compared to liver and heart ($P<0.001$). The former tissues are often target for bacterial infections and thus can form the basis of future, more targeted attempts for isolation.

Serology is regularly used in both clinical and veterinary medicine to indicate previous exposure to pathogens by the detection of specific antibodies in the serum of animals (Yuce *et al.*, 2001; Fournier & Raoult, 2003). Serology can be useful when it is not possible to isolate the pathogen by traditional methods or when rapid tests to identify the pathogen have not yet been developed (Adams & Thompson, 2006). Sero-conversion can be used to confirm that certain pathogens are related to the symptoms seen. The preferred method to examine this is by ELISA, but this requires purified pathogen to coat the ELISA plate, which was not possible for RMS. As such *P. salmonis*, based on potential cross binding, was used to establish if there was a difference between samples obtained from the two sets of farms. Antibody titres obtained against *Flavobacterium* isolates and *P. salmonis* were low and did not appear to demonstrate any differences in antibody responses between RMS-affected and non-affected sites. The MANOVA does not treat Farm ID as a random factor, only as a fixed one, as a limitation of the approach used, however it is useful as an indication that some differences exist within the dataset. The apparent differences are between farm status; however, this appears to be due to differences in individual farms and the small number of farms sampled.

5. Conclusion

In conclusion, the results of this study strongly indicate that the MLO are involved in RMS rather than *F. psychrophilum*. Analysis, of a statistically appropriate sample set, appears to confirm a positive correlation between the MLO's and RMS-affected tissue, mainly in the skin, further supported by the results of IHC tests. It was not possible to confirm whether the inferred

presence of MLO had a primary or secondary role in RMS. However, it may be important to note that the finding of MLO's was a consistent feature even when sampling RMS-affected fish from several discrete geographical areas. The failure to date to directly culture any MLO's warrants further investigation. No association was found between the RMS-affected fish and *F. psychrophilum* other than superficial presence in the skin.

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Table 1. Overview of primers in polymerase chain reactions

Name	Sequence (5'- 3')	Reference
RLO qPCR probe	(6FAM) CCC AGA TAA CCG CCT TCG CCT CCG (BHQ1)	Lloyd *et al.* (2011)
RLO qPCRf	GGC TCA ACC CAA GAA CTG CTT	Lloyd *et al.* (2011)
RLO qPCRr	GTG CAA CAG CGT CAG TGA CT	Lloyd *et al.* (2011)
IGF-1 qPCR probe	(6FAM)CCG TGG TAT TGT GGA CGA GTG CTG C(BHQ1)	Kelley *et al.* (2004)
IGF-1 qPCRf	CAG TTC ACG GCG GTC ACA T	Kelley *et al.* (2004)
IGF-1 qPCRr	CCG TAG CTC GCA ACT CTG G	Kelley *et al.* (2004)

483 Table 2. Histopathology of *Oncorhynchus mykiss* from red mark syndrome (RMS)-affected and
484 non-affected farms

Farm	Fish	RMS Status	Histopathology
1	1-10	-	No pathology in all but 1 fish; mild focal mysositis (not in *stratum compactum*). Considered an incidental finding and not RMS
2	1-10	-	No pathology
4	1	+	RMS confirmed
	2		Possibly incipient RMS
	3-4		Moderate/Marked RMS involving the musculature
	5		Moderate RMS with loss of epidermis
	6-7		Mild RMS
	8-10		No pathology
5	1-3,5,8-9	+	Marked RMS
	4,6		Moderate RMS
	7		Mild/Moderate RMS
	10		Marked RMS with pericarditis

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Table 3. Immunohistochemistry reaction in red mark syndrome-affected and unaffected *Oncorhynchus mykiss* tissue using an anti-*Piscirickettsia salmonis* MAb 23.

Farm	Fish	RMS Status	Non affected skin	Affected skin	Kidney	Spleen	Liver	Heart
1	1-10	-	-	-	1x +/-	-	-	-
2	1-10	-	-	-	-	-	-	-
4	1-10	+	-	1x +	3x +	2x +-	6x +-	1x +-
5	1-10	+	-	-	1x +-	1 x +	5x +	1x +

490 Table 4. The number of yellow, Gram negative bacteria isolated from *Oncorhynchus mykiss*
491 sampled from the farms in the UK and the location of their isolation on the fish.

Farm	RMS status	Mucus	Exterior	Interior	Total
1	-	(9)	(10)	(7)	(26)
2	-	(12)	(11)	(4)	(27)
4	+	(5)	(16)	(9)	(30)
5	+	(12)	(13)	(7)	(33)

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Table 5 Quantitative polymerase chain reaction results (average of 3 replicates) for RMS unaffected Farm 1-3. Relative MLO presence is presented as the MLO copy number divided by the IGF-1 copy number to normalise the results.

	no.	Affected skin	Non affected skin	Heart	Liver	Gill	Kidney	Spleen
Farm 1 (RMS negative)	1	-	0.17**	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Farm 2 (RMS negative)	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-	-	-	3.35* $x10^{-6}$	3.97* $x10^{-7}$	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-
	8	-	-	-	-	-	-	-
	9	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-
Farm 3 (RMS negative)	1	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-
	3	-	-		-	-	-	-
	4	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-
	7	-	-	-	1.79* $x10^{-6}$	-	-	-
	8	-	-	-	5.94* $x10^{-6}$	-	-	2.46* $x10^{-6}$
	9	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-

***Not significant; one of three replicates amplified**
**** Outlier; one out 3 replicates significantly higher**
- No amplification of the *Midichloria* -like organism

Table 6 Quantitative polymerase chain reaction results (average of 3 replicates) for RMS-affected Farm 4-6. Relative MLO presence is calculated by dividing the MLO copy numbers by the IGF-1 copy number to normalise the results**.**

	no	Affected skin	Non affected skin	Heart	Liver	Gill	Kidney	Spleen
Farm 4 (RMS positive)	1	3.29×10^{-5}	-	-	-	-	-	-
	2	-	-	-	$9.61*\times 10^{-7}$	-	$1.96*\times 10^{-6}$	1.26×10^{-6}
	3	1.87×10^{-3}	-	-	-	-	-	$1.78*\times 10^{-6}$
	4	1.58×10^{-4}	9.35×10^{-6}	$1.26*\times 10^{-6}$	-	$7.22*\times 10^{-7}$	9.13×10^{-7}	2.21×10^{-6}
	5	3.14×10^{-5}	6.98×10^{-5}	-	-	-	1.52×10^{-6}	-
	6	6.56×10^{-4}	1.17×10^{-5}	-	-	-	9.01×10^{-7}	$1.42*\times 10^{-6}$
	7	1.55×10^{-4}	-	4.62×10^{-6}	6.65×10^{-6}	3.69×10^{-4}	$1.21* 10^{-6}$	-
	8	5.53×10^{-6}	-	-	$3.59*\times 10^{-6}$	-	-	-
	9	-	-	-	-	$6.26*\times 10^{-7}$	-	-
	10	$1.34*\times 10^{-5}$	$3.41*\times 10^{-5}$	1.42×10^{-6}	1.20×10^{-6}	$1.55*\times 10^{-6}$	-	-
Farm 5 (RMS positive)	1	8.38×10^{-4}	2.51×10^{-5}	5.03×10^{-6}	-	1.40×10^{-4}	8.57×10^{-6}	1.30**
	2	0.68**	-	$4.00*\times 10^{-6}$	$2.75*\times 10^{-6}$	1.52×10^{-6}	9.38×10^{-7}	1.63**
	3	6.07×10^{-7}	-	$8.40*\times 10^{-9}$	-	-	5.56×10^{-9}	-
	4	1.37×10^{-6}	1.44×10^{-4}	$1.36*\times 10^{-6}$	$8.80*\times 10^{-7}$	9.40×10^{-7}	3.15×10^{-6}	1.79×10^{-6}
	5	4.71×10^{-4}	5.96×10^{-5}	-	-	$3.26*\times 10^{-7}$	$2.93*\times 10^{-7}$	-
	6	1.22×10^{-4}	$1.18*\times 10^{-5}$	2.55×10^{-6}	-	$1.15*\times 10^{-6}$	3.68×10^{-6}	2.71×10^{-6}
	7	-	-	-	-	1.24×10^{-6}	-	$4.60*\times 10^{-6}$
	8	-	6.26×10^{-5}	-	-	-	-	-
	9	7.35×10^{-6}	-	-	-	8.40×10^{-7}	-	-
	10	1.64×10^{-4}	1.38×10^{-5}	$1.11*\times 10^{-6}$	$5.20*\times 10^{-7}$	$5.07*\times 10^{-7}$	$4.14*\times 10^{-7}$	$4.70*\times 10^{-3}$
Farm 6 (RMS positive)	1	7.41×10^{-4}	1.81×10^{-5}	-	-	$5.43*\times 10^{-6}$	-	5.92×10^{-6}
	2	3.93×10^{-4}	-	-	-	-	7.86×10^{-6}	1.49×10^{-3}
	3	1.86**	2.89×10^{-5}	-	4.55×10^{-6}	-	1.10×10^{-5}	7.39×10^{-6}
	4	1.06×10^{-3}	-	-	-	$1.76*\times 10^{-6}$	-	1.55×10^{-6}
	5	2.28×10^{-4}	-	-	$2.92*\times 10^{-6}$	39.23**	1.9×10^{-6}	3.72×10^{-6}
	6	3.83×10^{-5}	$3.3* \times 10^{-5}$	-	-	-	-	-
	7	1.78×10^{-4}	$1.57*\times 10^{-5}$	-	-	3.93×10^{-6}	1.02×10^{-6}	1.17×10^{-5}
	8	1.67×10^{-4}	4.51×10^{-4}	$3.83*\times 10^{-5}$	5.25×10^{-6}	1.19×10^{-5}	3.69×10^{-5}	7.11×10^{-5}
	9	4.69×10^{-5}	1.25×10^{-4}	-	-	$1.00*\times 10^{-5}$	$3.22*\times 10^{-6}$	2.09×10^{-6}
	10	1.20×10^{-4}	$2.04*\times 10^{-4}$	1.23×10^{-5}	-	-	1.26×10^{-5}	7.64×10^{-6}

***Not significant; one of three replicates amplified**

**** Outlier; one out 3 replicates significantly higher**

- No amplification of the *Midichloria* -like organism

Table 7. Enzyme-linked immunosorbent assay to measure the antibody titre ($-\log_{2+}1$) of with serum from red mark syndrome (RMS) negative (Farm 1 & 2) and RMS-positive fish (Farm 4 & 5) to different *Flavobacterium* spp. and *Piscirickettsia salmonis.*

		***Flavobacterium* sp.**				***P. salmonis***
Farm	RMS Status	NCIMB Fp^T	ARF07	MOF25	BGARF	
1	-	5.8 ± 1.4	8.1 ± 1.2	7.0 ± 1.2	7.9 ± 0.7	7.7 ± 0.8
2	-	7.5 ± 1.0	7.1 ± 0.6	7.6 ± 1.2	7.3 ± 0.5	7.4 ± 0.5
4	+	7.3 ± 2.4	6.9 ± 2.4	7.1 ± 2.3	6.9 ± 2.4	7.0 ± 2.4
5	+	8.0 ± 0.5	7.0± 0.5	7.5 ± 0.5	7.0 ± 0.5	7.8 ± 0.4

Results represent the mean of 10 duplicate samples per farm ±SD

Table 8. Linear mixed-effect models for isolate ELISA results, accounting for farm status and farm ID. Coefficients are illustrated from the full models. Likelihood ratio tests compare the nested models.

Isolate	Model	Coefficient [b]	SE [b]	t_{36} [b]	(Δ) Deviance	P
NCIMB fp	Intercept	6.90			126.61	
	+ Farm [a]		0.60		−9.69	0.001 **
	+ Status (affected)[a]	0.70	0.67	1.04	−0.96	0.33
ARF 7	Intercept	7.55			109.27	
	+ Farm		0.15		−1.39	0.24
	+ Status (affected)	−0.55	0.32	−1.73	−2.23	0.14
PS	Intercept	7.55			89.60	
	+ Farm		0.14		−0.24	0.62
	+ Status (affected)	−0.10	0.27	−0.37	−0.14	0.71
MCF 25p	Intercept	7.55			113.49	
	+ Farm		0.28		−0.94	0.33
	+ Status (affected)	−0.15	0.41	−0.36	−0.13	0.72
BG ARF	Intercept	7.7			100.8	
	+ Farm		0.15		−3.86	0.050 *
	+ Status (affected)	−0.70	0.28	−2.48	−3.71	0.054

[a] All contrasts use one degree of freedom.

[b] Derived from the third model in each set.

d

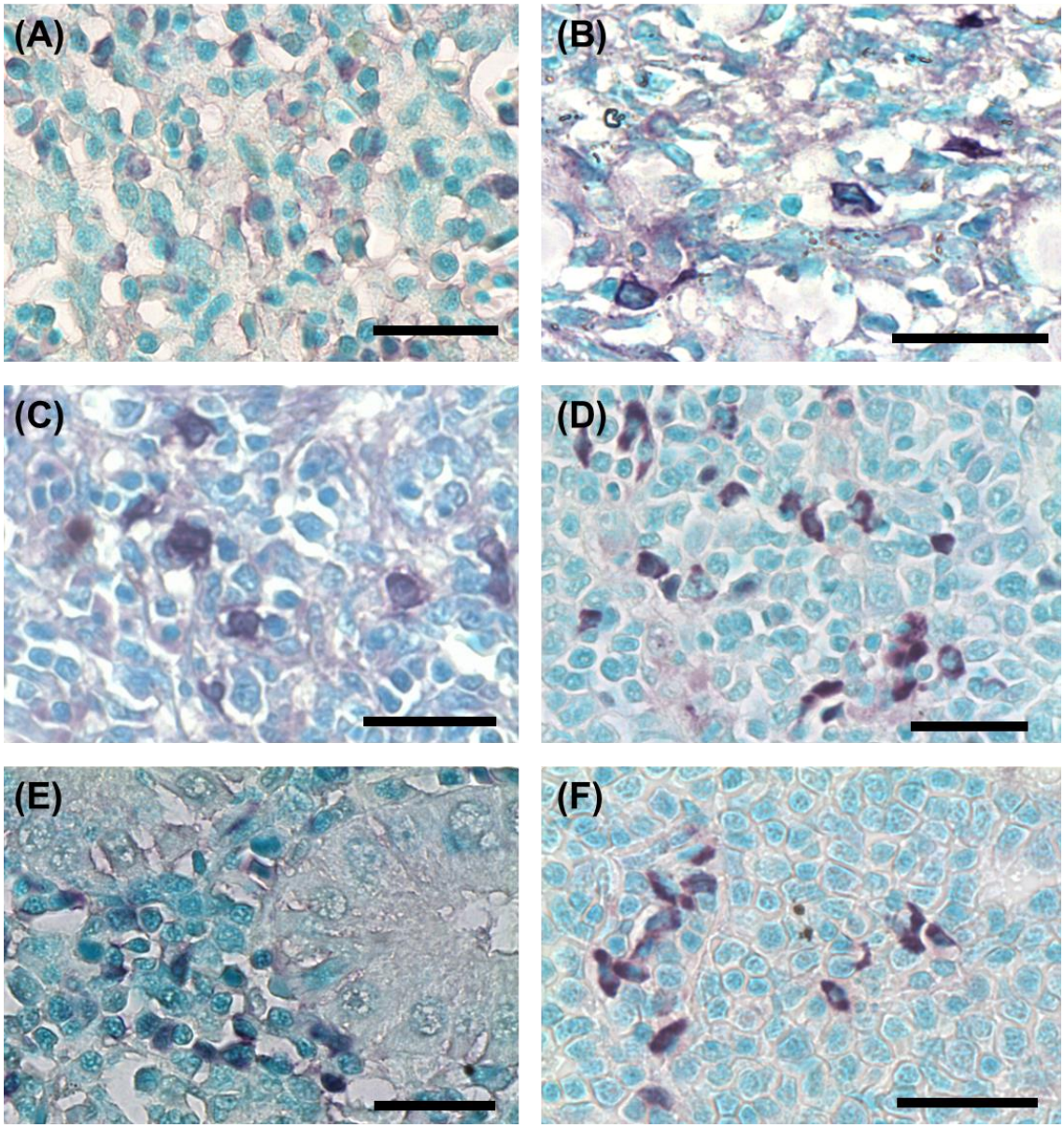

Figure 1 Reaction of rabbit anti-*P. salmonis* serum in immunohistochemistry of tissues affected with red mark syndrome (RMS) from the United Kingdom (UK) and strawberry disease (SD) in United States of America (USA). (A) Skin RMS UK; (B) Skin SD USA; (C) Spleen RMS UK; (D) Spleen SD USA; (E) Kidney RMS UK; (F) Kidney SD USA. (Magnification 400x, Bar is 25 μm)

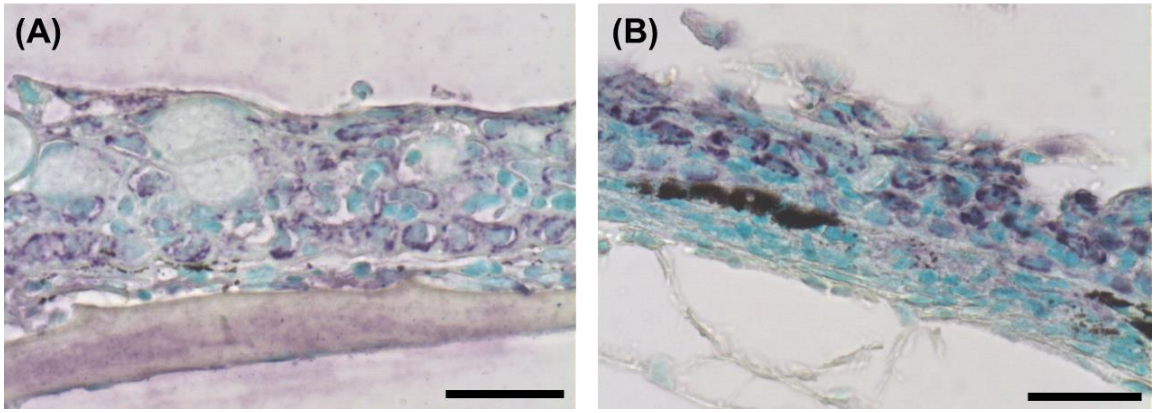

Figure 2 Reaction of rabbit anti-*F. psychrophilum*. (A) positive staining seen in the epidermis, around the lesion in affected skin and (B) normal skin. No staining was seen in the dermis or muscle layer. (magnification 400x, Bar is 25 µm)

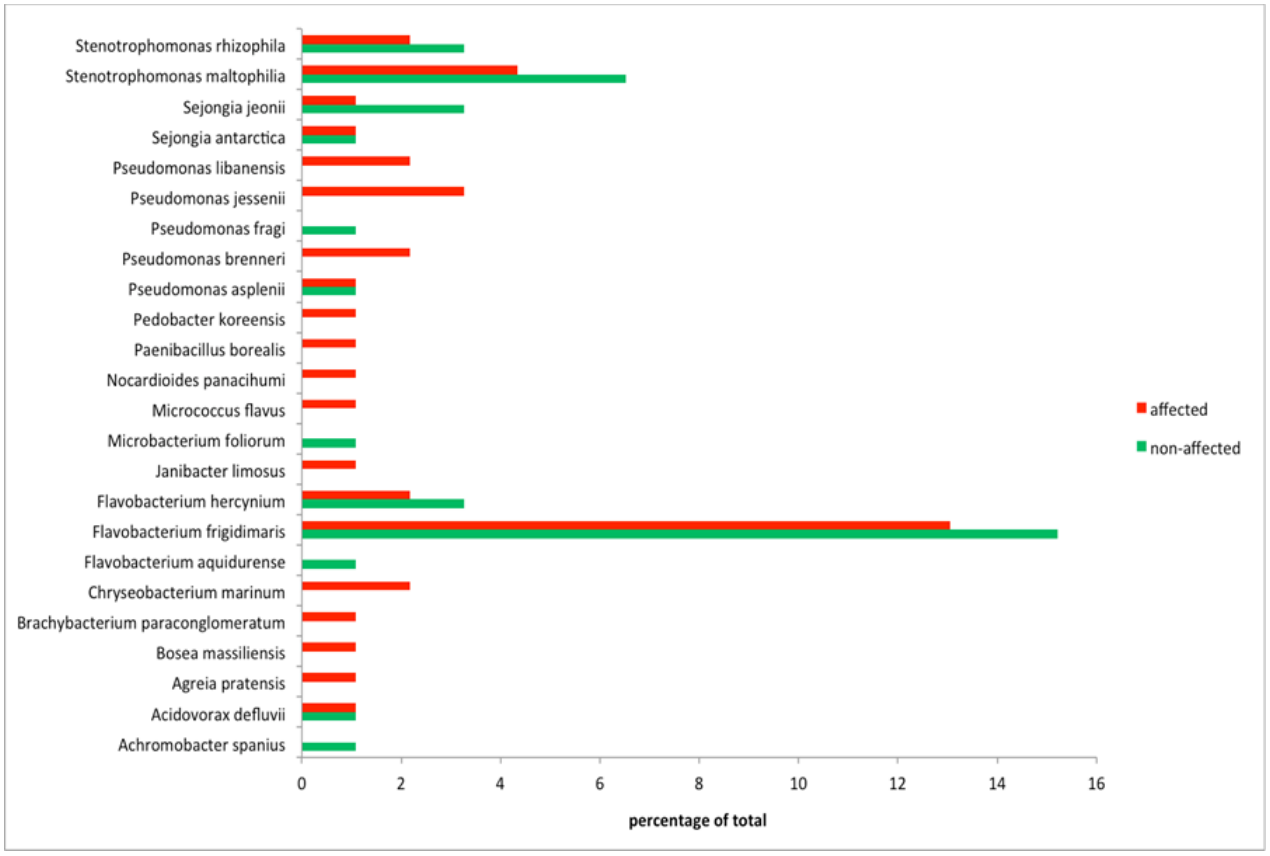

Figure 3. Identification using 16s rRNA gene sequencing of isolates cultures from red mark syndrome (RMS)-affected (red) and unaffected (green) *Oncorhynchus mykiss*, although some bacteria are only found in either affected or non-affected with no strong association apparent with *Flavobacterium psychrophilum*.

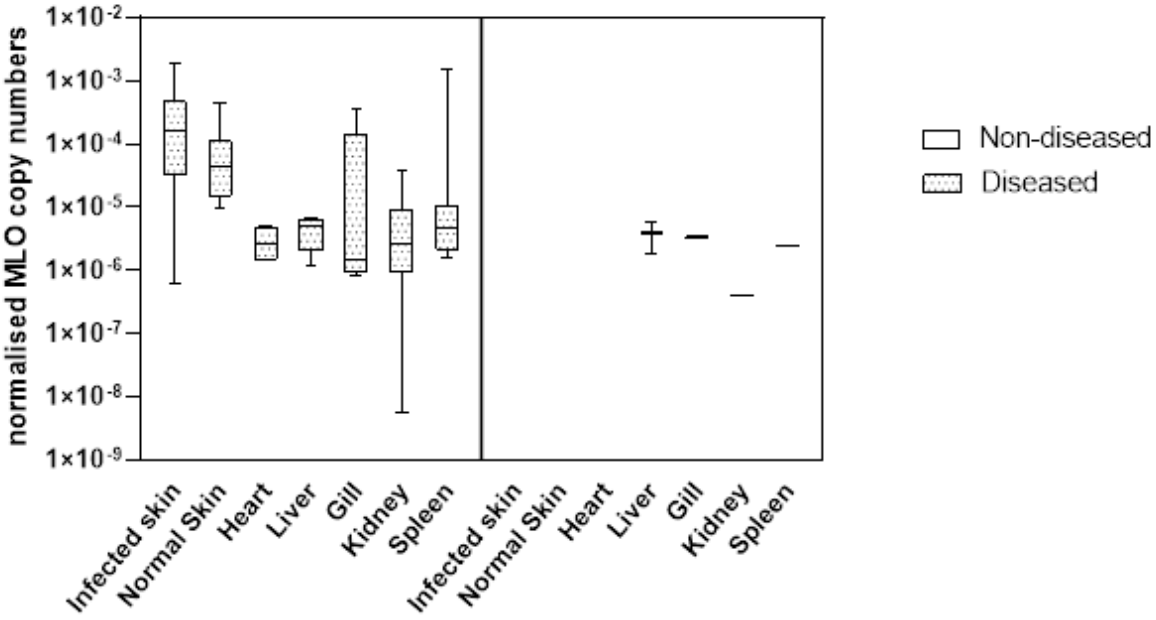

Figure 4 Boxplot of the normalised MLO copy numbers for seven different tissues in both affect (left) and unaffected farms (right) combined.

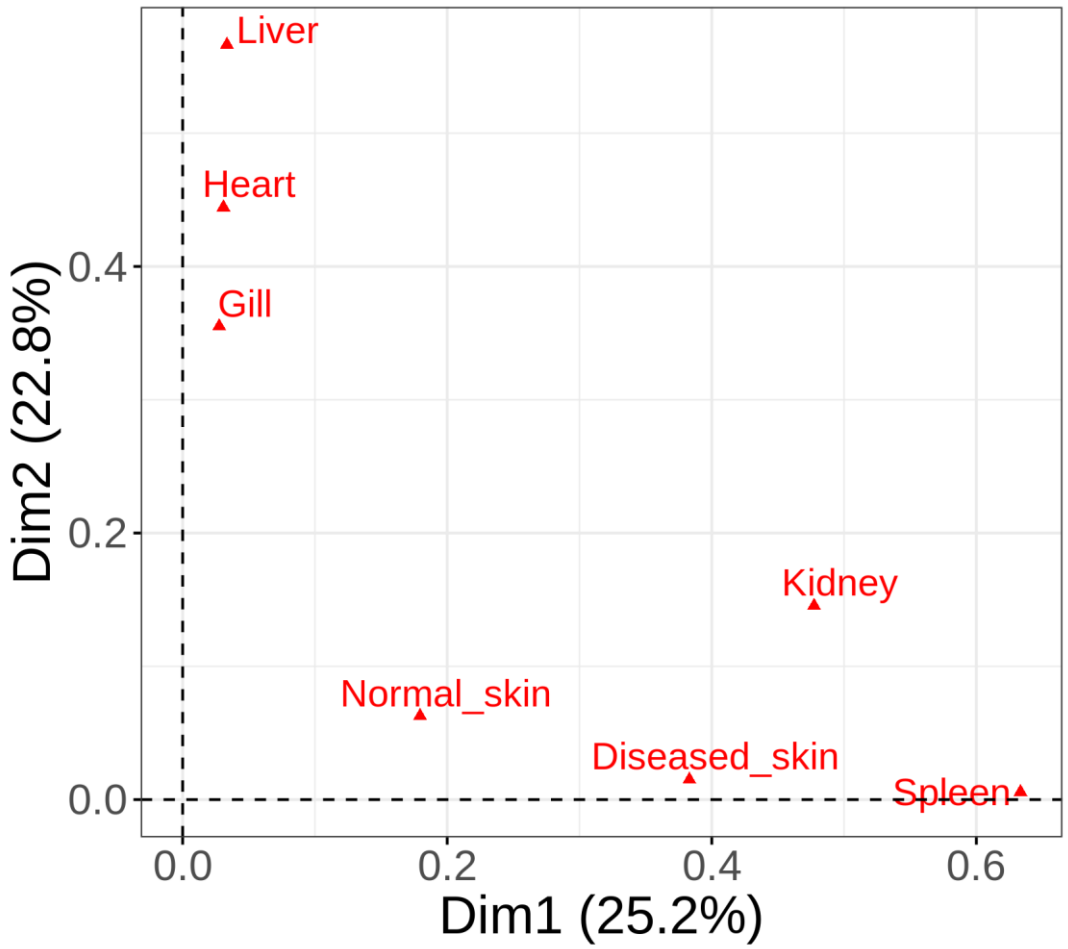

Figure 5. Multiple correspondence analysis on tissue-level PCR results for positive fish. Variable weightings for the first two dimensions are shown.