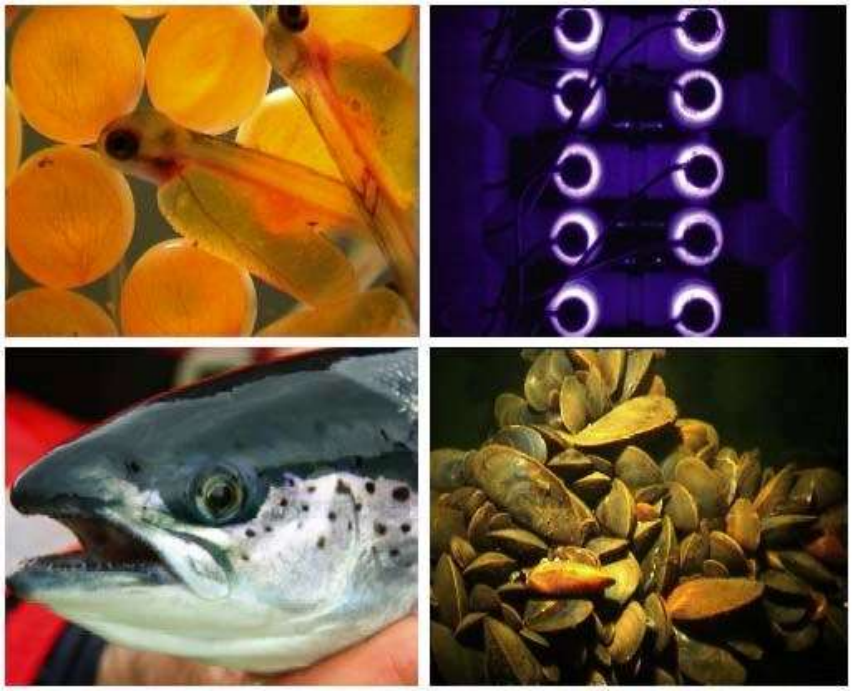
SARF094/SARF094B - Development of Management Strategies for Red Mark Syndrome (RMS)



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AND PREPARED BY

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Project Final Report Form

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Project Details

SARF Project ID Code: 094 & 094b	
Project Title: Development of Management Strategies for Red Mark Syndrome (RMS)	
Project: Start date 7/01/13	**End date** 31/12/16
Name(s) and address(s) of contractor organisation(s): University of Stirling	
Contractor's Project Manager: Professor Alexandra Adams	
SARF Project Manager: Richard Slaski	
Total SARF Project costs £25,000 + £37,000 = £62,000 (Actual costs £49225 + £75,000)	
Total **approved** project expenditure £62,000	Total **actual** project expenditure £124,225
Total ***approved** staff input 0.94 years	Total ***actual** staff input 1.5 years
Is there any Intellectual Property arising from this project which is suitable for commercial exploitation (*This question requires a YES/NO answer only. All other details of any Intellectual Property must be included under the Scientific Report or in an accompanying Annex*). .....YES NO ☐ ☒	
**Staff years of direct science effort*	

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Scientific objectives

List the scientific objectives as set out in the contract. If necessary these can be expressed in abbreviated form. Indicate where amendments have been agreed with the SARF Project Manager, giving the date of amendment.

Project 094 **Development of Management Strategies for Red Mark Syndrome**

1. Provide an overview of current red mark syndrome (RMS) research, summarising the state of knowledge up to the point of publication of SARF057, but particularly highlighting progress since then.
2. Suggest further work that could be done to overcome remaining knowledge gaps.
3. Design and implement experimental procedures to test hypotheses identified in Objective 2. This included:
   3.1. Attempt to identify and culture the aetiological agents of RMS
   3.2. Optimise methods to detect RLO DNA in fish tissue and eggs, and perform PCR on tissues and eggs.
   3.3. Vaccination with an existing commercial *Piscirickettsia salmonis* vaccine.
   3.4. On farm investigation of effective treatment and management practices.
4. Prepare a final report. *Preparation of the final report was deferred until completion of 094b.*

Project 094b Red Mark Syndrome – a longitudinal farm study

1. Screen rainbow trout populations over time for presence of RLOs.
2. Screen discrete populations of fish to investigate any differences in stock susceptibility to RMS.
3. Discussion of possibilities of performing a wider scale vaccination. *Decision taken not to go ahead due to unavailability of suitable contols.*
4. Attempt culture of the RLO
5. Prepare a final report for 094 and 094b.

Milestones

List the milestones. It is the **responsibility of the contractor** to check fully that **all** milestones have been met and to provide a detailed explanation if this has not proved possible.

Milestone		Target Date	Milestone Met	
Number 094	**Title**		**In Full**	**On Time**
1	Overview of red mark syndrome (RMS) research	311213	Yes	No
2	Suggest further research	310613	Yes	Yes
3.1	Attempt to identify and culture the aetiological agents of RMS	311213	Yes	Yes
3.2	Optimise methods to detect RLO DNA in fish tissue and eggs	310612	Yes	Yes
3.3	Vaccination with a *Piscirickettsia salmonis* vaccine and field challenge (initiated 310713) Added as an extra milestone	310714	Yes	Yes
3.4	On farm investigation of treatment and management practices. *On-going in other projects.	**310612***	Partially	Yes

094b				
1	Screen rainbow trout populations over time for presence of RLOs and record farm conditions	01/01/16	Yes	No
2	Screen discrete populations of fish to investigate any differences in stock susceptibility to RMS.	01/03/16	Yes	No
3	Discuss possibilities of performing a wider scale vaccination and perform if agreed.	01/09/15	Yes	Yes
4	Attempt culture of RLO	01/06/16	Yes	Yes
5	Prepare a final report of 094 and 094b	01/06/16	Yes	No

If any milestones have not been met please give an explanation below.
On farm investigation of treatment and management practices will continue through other projects (CEFAS).

Declaration

I declare that the information I have given in this form and in any associated documentation is correct to the best of my knowledge and belief.

Name: Alexandra Adams	Date: 9th June 2017
Position held: Professor	

Executive Summary

The executive summary must not exceed 2 sides in total of A4 (minimum font size 10) and should be understandable to the intelligent non-specialist. It should cover the main objectives, methods and research results, together with any other significant events and options for new work (the box below will expand to accommodate the Summary).

ABSTRACT (*This project report covers both projects 094 and 094b*).

Red mark syndrome (RMS) is a skin condition that affects rainbow trout (*Oncorhynchus mykiss*). It was first reported in the UK in 2003[1] and quickly spread to over 50% of trout farms in the UK [2,3]. RMS appears to be identical to strawberry disease (SD) in rainbow trout in the USA[4], which has been present there since the mid 1940's. RMS results in significant economic losses to the trout industry due to downgrading of the fish product [5]. Two pathogens have been reported to be associated with the disease; a rickettsia-like organism (RLO) [6-10] and *Flavobacterium psychrophilum* [3,11,12], although the later has not been identified in all cases[1,5,9,13,14]. The RLO has been recently been visualised by Transmission Electron Microscopy (EM)[15] and the 16S rDNA sequence of the RLO bacterium associated with RMS/SD lesions has been shown to belong to the family Midichloriaceae, within the order Rickettsiales[10].

This project, hosted at University of Stirling, was in partnership with CEFAS, Marine Science Scotland (MSS), Fish Vet Group (FVG), Dawn Fresh Fisheries (DFF), Selcoth Fisheries (SF) and Pharmaq (now Zoetis). It has been very successful in bringing the different sectors together to try to tackle the RMS problem in rainbow trout in the UK.

The overall aim of the project was to develop management strategies for RMS. The specific objectives were to:

1. Provide an overview of RMS research and suggest further research.
2. To learn more about the RMS by:
   -attempting to identify and culture the aetiological agents of RMS.
   -develop methods to detect RLO DNA in fish tissue and eggs.
   -determine if a commercial *Piscirickettsia salmonis* vaccine protects against RMS.
   -undertake on farm investigation of effective treatment and management practices.
3. Conduct a longitudinal farm study of RMS, including:
   -screening rainbow trout populations for the presence of RLO.
   -screening discrete populations of fish to investigate any differences in stock susceptibility to RMS.
4. Prepare a final report

New molecular methodologies (PCR) were developed to assist in the detection of RLOs in fish and eggs, and valuable information was gained on RMS in the field. Attempts were made to try to culture the causative agent of RMS on a variety of agar types and identification of any colonies was performed using different staining methods. Selected samples were sent for 16S rRNA sequencing. Very few bacterial colonies were observed on any of the media used and it was not possible to obtain sufficient amounts of material for a conclusive identification. Subtractive and non-subtractive libraries were prepared to determine if RLO or viral genes were present in RMS fish. The results indicated that RLO genes were present in RMS-infected fish. Although all of the egg samples were negative by PCR, eggs cannot be discounted as being positive for the RLO due the difficulty in extracting the DNA (even with 6 eggs per pool) and presence of PCR inhibitors. Further assay optimisation and validation is required if the PCR is to be used on fish eggs. PCR analysis of tissue samples appeared to be reproducible and positive PCR results were obtained from skin and spleen samples.

It had been shown previously that Piscirickettsia-*like* antigens were present in tissue samples from fish presenting with RMS. Thus, a vaccine containing antigens to *Piscirickettsia salmonis* (also an RLO) may confer a degree of cross protection to fish. Therefore fish on two sites were vaccinated with a commercial *P. salmonis* vaccine and then exposed to a natural RMS challenge on the farms. The results of both vaccination trials indicated that vaccination with a commercial *P. salmonis* vaccine (Alphaject Micro 2) does lead to a significant reduction in RMS. This, however, may represent a positive effect of the adjuvant included in the vaccine as Alphaject 2-2, which does not contain *P. salmonis* antigens, but also led to a reduction in RMS. This may provide one possible control strategy for the future.

There were still many unanswered questions when this project was due to terminate and therefore an extension was granted (project 094b) to perform a longitudinal study. This project (094b and an associated MSc project) therefore enabled further screening for RMS to be performed in 2015 and 2016 using the knowledge and methods previously developed in project 094. The results indicated that RLOs were first detected by RLO PCR in July 2015 in skin and spleen samples of rainbow trout and the incidence increased in liver, spleen and skin samples of fish over the following months. The RLOs could be detected in fish with

lesions, especially in skin samples until October. Lower numbers of RLO positive samples were detected in samples collected in 2016 versus 2015 (also later, August 2016), coinciding with a reduced incidence of RMS. This may be related to the introduction of new diets on the farm or to other factors. In addition, there appeared to be differences in RMS susceptibility between fish from different genetic sources, although direct comparisons could not be made as the different groups entered the farm at different time points.

Although RLOs have been identified in RMS affected fish using molecular methods, the organism has still not been cultured. This should be pursued further so that an RMS specific vaccine can be developed. In addition the presence of other pathogens (as either co-infections or carrier states) on progression and spread of the disease are currently not known; this is very important. Fish genetic background is also a critical factor and a direct comparison between populations is therefore required.

An overview of current RMS research, summarising the state of knowledge up to the point of publication of SARF057 (a previous RMS project), but particularly highlighting progress since then is presented in Annex 1. This will be submitted for publication.

1. OBJECTIVES AS SET OUT IN THE CONTRACT (SEE ABOVE)

2. EXTENT TO WHICH THE OBJECTIVES SET OUT IN THE CONTRACT HAVE BEEN MET

All the objectives set out in the project were met although clearly further research is required on this important disease.

3. MATERIALS AND METHODS

3.1. Culture and identification of the aetiological agents of RMS

Attempts were made to try to culture the causative agent of RMS on a variety of agar types and identification of any colonies was performed using different staining methods and selected isolates were sent for 16S rRNA sequencing. In addition, next generation sequencing (NGS) using subtractive libraries was performed (CEFAS).

The spleen and liver samples were homogenised in 3 ml phosphate buffered saline (PBS) (0.02 M phosphate, 0.15 M NaCl, pH 7.2) using the plunger of a sterile syringe (BD, USA) and sieved through a 100 µm filter (Fisher Scientific, UK) to form a homogenate. The homogenate from each tissue was plated out onto three different media in duplicate and then incubated at 6-8 °C and 15 °C. The plates were incubated for at least three weeks and then checked for bacterial growth.

Different media were selected on the basis of their properties to grow organisms with similarities to RLO. These included Austral-TSFe media which is a novel media suitable for the isolation of *P. salmonis* [16]*;* Cysteine heart agar (Difco™ Cysteine Heart Agar, BD diagnostics, USA), that has been used to grow *P. salmonis, Francisella tularensis* and other intracellular bacteria; and Campylobacter Blood-Free selective agar base (Thermo Scientific, UK) that has been applied to culture fastidious and microaerophilic pathogens like *Campylobacter jejuni, Campylobacter coli* and *Campylobacter laridis*.

In addition, as many rickettsias require an invertebrate vector to reproduce [17,18], wax moth larvae (*Galleria mellonella*) were injected with RMS infected tissue as a novel model to culture the bacterium.

Injection of wax moth (*Galleria mellonella*) larvae and collection of haemolymph: Wax moth larvae (UK waxworms Ltd, UK) were left for 1 d after arrival at 15 °C to acclimatize. Fish samples (homogenates as described above) were then injected into the larvae. Ten microliters of each tissue homogenate was injected into the left, last pro-leg of three larvae using a Hamilton syringe (Hamilton, Switzerland) (Fig. 1). Before use, the syringe was sterilised by rinsing four times with 20 % bleach (Jeyes, UK), 70 % ethanol (Fisher Chemicals, UK) and distilled water (DW). The negative controls consisted of one group of three larvae that was injected with only PBS and additional group which was not injected. Between every inoculation the needle was rinsed three times using sterile PBS. The three larvae of each group were stored together in a Petri dish at 15 °C and were checked regularly for mortalities.

The first batch of haemolymph was collected 7 d post injection (PI). From each group of 3 larvae, only one was used to collect haemolymph. The last two segments (Figure 1) of the larvae were removed using a pair of sterile scissors and the larvae squeezed gently. The haemolymph was collected into an individual 2.0 ml cryotube (Alpha laboratories, UK) which was then frozen on dry ice

and stored in a – 70 °C freezer. Fourteen days PI a second batch of haemolymph was collected using the method described above.

Figure 1. Wax moth larvae of approximately 20 mm in length (Galleria mellonella). Bold arrow indicates the left last pro-leg which is the injection site. Narrow arrow indicate last two segments (www.bbb-tech.com).

3.1.1 Sample collection

Samples were collected for bacteriology, DNA (for PCR), and electron microscopy from three fish farms, including Tervine and, Braevalich Farms, Loch Awe, Argyll and Selcoth Fisheries (SF), Moffat, Dumfriesshire. Skin, spleen and blood samples were streaked on to agar. In the longitudinal study only fish from SF were sampled. Thirty fish were samples per time point.

3.1.2 Staining

Staining of any bacterial isolates cultured was performed using Gram, Giemsa, Pinkerton's, Macchiavello's, Rapid Romanowsky, Gimenez, and Castaneda stains.

3.1.3 Next Generation Sequencing

Subtractive and non-subtractive libraries were prepared to determine if RLO or viral genes were present in RMS infected fish.

3.2. Optimisation of methods to detect RLO DNA in fish tissue and eggs

3.2.1 Optimisation for nested PCR for eggs

Eggs were collected from a site with active RMS and from a site which had never recorded RMS. Eggs collected from rainbow trout (4-9 lb) were placed dry into individual sterile universal containers and shipped overnight to Marine Scotland Science, Aberdeen, in a polystyrene box with ice packs. The eggs were surface disinfected by immersion in 1% Buffodine and rinsed in sterile saline, drained and stored at -20°C in 95% ethanol.

3.2.2 Method validation at MSS

The 16S rRNA nested PCR method was validated with regards to extraction, amplification using ATPase primers.

For extraction of DNA, eggs were homogenised at 25 Hz in a Tissue Lyser (Qiagen) using a 7 mm stainless steel bead (Qiagen) and shaking for 4 min in 180 µl lysis buffer (20 mM Tris HCl pH 8.0, 20 mM EDTA, 200 mM NaCl, 4% SDS). After addition of 25 µl Proteinase K, the homogenate was

incubated overnight at 56°C. Subsequent steps followed the manufacturer's instructions from the DNeasy Blood and Tissue Kit (Qiagen). The DNA was finally eluted in 100 ul Buffer AE.

The 16S rRNA nested PCR was carried out in a 50 µl reaction volume, using 2 U Biotaq (Bioline), 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 100 pmol of each primer (see Table 1). For amplification, 5 µl template and 1 µl template was used for the first and second round PCR respectively.

Table 1. First and Second round PCR primers used in the RLO 16S rRNA nested PCR

Primer	Reference	Sequence (5' to 3')
First round forward primer	Eubacterial 16S 24F	GCA GGC YTA ACA CAT GCA AGT CGA
First round reverse primer	Eubacterial 16S 518R	CGT ATT ACC GCG GCT GCT GG
Second round forward primer	RLO specific RLO1 F (Lloyd *et al*. 2008)	ATC GCT ACA AGA CGA GCC CAT GCA
Second round reverse primer	RLO specific RLO2 R (Lloyd *et al*. 2008)	TAT TAC CGC GGC TGC TGG CA

Thermocycling conditions were as follows:

1st round

94°C	4 min	
94°C	40 s	30 cycles
55°C	40 s	
72°C	60 s	
72°C	5min	

2nd round

94°C	4 min	
94°C	40 s	30 cycles
60°C	40 s	
72°C	60 s	
72°C	5 min	

During assay optimisation, PCR for rainbow trout ATPase was carried out on DNA extracted from 1, 2 and 3 eggs in order to determine if amplification was inhibited by higher numbers of eggs. The efficiency of extraction between Chelex and the adapted QIAgen method was also compared. In addition, 1 µl purified RLO DNA at dilutions up to 1/16 was used to spike egg samples prior to homogenisation and DNA extraction, in order to confirm that RLO DNA could be detected in egg material. The effect of annealing temperatures (50°C - 62°C) and $MgCl_2$ concentrations (1.0 - 3.0 mM) on the yield and specificity of the RLO amplification were assessed. The identity of PCR products was confirmed by sequencing.

3.2.2.1 DNA extraction from trout eggs

A modified DNeasy Blood & Tissue Kit method (Qiagen) was used based on extraction of DNA from animal tissues (Spin Column Protocol). One egg was added to 180 ul of extraction buffer and homogenised using a Bead Beater (30 s x 3 with 2 metal beads) and transferred into a 1.5 ml homogenisation tube. Then 20 µl of Proteinase K solution (Qiagen kit 600 mAU/ml solution) was added and incubated at 56^{O}C overnight. Buffer AL (200 µl) was then added to the sample and vortexed. Next 200 µl of 100% ethanol was added and the sample vortexed before adding onto a DNeasy Mini spin column that was placed into a 2 ml collection tube. The column was incubated for 5 min then centrifuged at 13,000 g for 1 min. The DNeasy Mini spin column was then placed into new collection tube, 250 µl Buffer AW1 x 2 added (incubated for 5 min first aliquot), and centrifuged for 1 min at 13,000 g. The centrifugation was repeated with 250 µl wash Buffer AW2 x 2 for 3 min at 20,000 g and the empty column centrifuged for a further minute to dry the membrane. The column was then placed in an open 1.5 ml micro-centrifuge tube and placed on heating block at 55°C for 5 min to ensure all ethanol had evaporated from the column prior to elution with 30 µl EB buffer (5 mM Tris pH 8.5) preheated at 55°C directly onto the DNeasy membrane. The sample was then incubated at room temperature for 1 min and centrifuged for 1 min at 18,000 g to elute.

3.2.2.2 ATPase PCR

An ATPase PCR was run to validate the method, using 1X ReddyMix Master mix (Thermo Scientific AB-0575/DC/LD/A), 1 µl DNA template, 0.8 µM ATPaseF, 0.8 µM ATPaseR in a 25 ul volume. The PCR was run as follows:- 95°C 1 min, 50°C 1 min, 72°C 1 min (35 cycles), and 72°C 5 min.

3.2.2.3 RLO PCR

A 16S rRNA nested PCR was performed to detect the RLO using first round universal 16S primers primers 20F and 1500R at 0.2 uM in a 25 ul volume of 1X ReddyMix Master mix and 1 µl DNA template. The second round PCR included specific primers RLO1 and RLO2 at 0.2 uM using the same PCR master mix and 1 ul of the PCR product from the first round PCR as template. The PCR cycling conditions were as follows:

1st round primers – 20F & 1500R

95°C	5 min	
95°C	30 s	35 cycles
45°C	90 s	
72°C	120 s	
72°C	5 min	

2nd round primers – RLO1 & RLO2

95°C	5 min	
95°C	30 s	35 cycles
57°C	30 s	
72°C	20 s	
72°C	5 min	

3.3 Vaccination

Fish on two sites were vaccinated with a commercial *P. salmonis* vaccine and then exposed to a natural RMS challenge on the farms (Figure 2). The vaccine used was ALPHA JECT Micro 2®, an oil-adjuvanted IP injectable vaccine which is licensed for use in Chile in Atlantic salmon, Pacific salmon and Rainbow trout for the reduction in mortality from Salmon Rickettsial Syndrome (SRS) and Infectious Pancreatic Necrosis (IPN). It contained antigenic components from both IPNV as well as *P. salmonis.* The timeline of vaccination is shown in Table 1.

Figure 2: Vaccination of Rainbow trout with commercial vaccines

3.1 Vaccination studies

3.1.1 *Study one*

Rainbow trout (130,000) were vaccinated in July 2013 at Site A with ALPHA JECT Micro 2®. To account for any protection which might be conveyed from the IPNV component in this vaccine, an additional 115,000 fish were vaccinated with ALPHA JECT 2-2® a bi-valent vaccine containing *Aeromonas salmonicida* and IPNV antigens in an oil-adjuvanted emulsion. This was administered as a 0.05 ml dose. All fish were also vaccinated by immersion and oral boost for ERM. The efficacy of the vaccine was determined by comparing vaccinated fish with 115,000 unvaccinated fish, based on the severity of RMS lesions as described in Section 3.1.3.

3.1.2 *Study two*

A total of 100,000 Rainbow trout were vaccinated in October 2013 at Site B with ALPHA JECT Micro 2®. To account for any protection which might be conveyed from the IPNV component in this vaccine, 100,000 additional fish were vaccinated with ALPHA JECT 2-2® a bi-valent vaccine containing *A. salmonicida* and IPNV antigens in an oil-adjuvanted emulsion. This was administered as a 0.05 ml dose. All fish were vaccinated by immersion and were given an oral boost for ERM.

Table 1: Timeline for vaccination of Rainbow trout with commercial vaccines

	2013						2014				
	July	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**	**Jan**	**Feb**	**March**	**April**	**May**
Study 1	130,000 fish Vaccinated with AJ Micro 2		Fish moved to Challenge site				First visual assessment		Second visual assessment	Third visual assessment	Forth visual assessment
Study 1	115,000 fish Vaccinated with AJ 2-2 (0.05 ml dose)		Fish moved to Challenge site				First visual assessment		Second visual assessment	Third visual assessment	Forth visual assessment
Study 1	Unvaccinated controls		Fish moved to Challenge site				First visual assessment		Second visual assessment	Third visual assessment	Forth visual assessment
	July	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**				**Jun**	**July**
Study 2				100,000 Fish Vaccinated with AJ Micro 2						First visual assessment	Second visual assessment
Study 2				115,000 vaccinated with AJ Micro 2						First visual assessment	Second visual assessment

3.1.3 Grading (assessment) for RMS following vaccination and natural RMS challenge

Fish were graded according to the lesions observed, as shown in Figure 3 below.

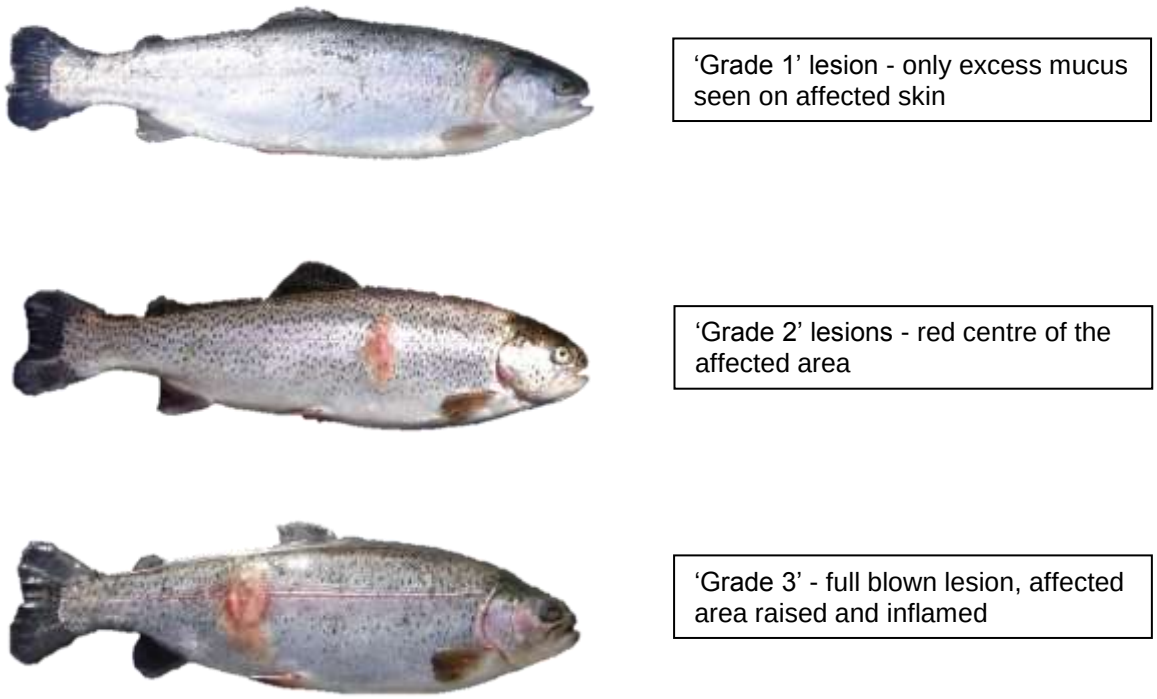

Figure 3: Grading (assessment) of RMS affected fish

3.1.4 Serology

Blood samples were also collected when the fish were graded and serum was prepared from these. Serum samples from each time point were pooled (pools of 5 samples x 4 per time point) and then analysed by antibody capture ELISA to determine if a specific antibody response to *P. salmonis* had been elicited following vaccination.

4. RESULTS AND DISCUSSION

An overview of current RMS research, summarising the state of knowledge up to the point of publication of SARF057, but particularly highlighting progress since then is presented in Annex 1. This is currently in draft form with plans to publish the review.

4.1. Attempt to culture and identify the aetiological agents of RMS

Attempts were made to try to culture the causative agent of RMS on a variety of agar types and identification of any colonies was performed using different staining methods, and selected samples were sent for 16S rRNA sequencing. Very few bacterial colonies were observed on any of the media used and it was not possible to obtain sufficient amounts of material for a conclusive identification.

Subtractive and non-subtractive libraries were also prepared to determine if RLO or viral genes were present in RMS fish. This was done as adeno-virus had been proposed as another possible aetiological agent of RMS [19]. The results indicated that RLO genes were present in RMS-infected fish.

4.2. Optimise methods to detect RLO DNA in fish tissue and eggs and perform PCR on eggs

4.2.1 Results from initial method development at MSS:

Examination of products from ATPase PCR indicated that the highest DNA yield was obtained from a single egg using the adapted Qiagen method. The PCR method successfully amplified the RLO 16S rRNA gene from RMS-positive skin samples. In addition, RLO DNA could be detected at dilutions up to 1/16 in spiked egg material. However, screening of eggs from RMS-positive fish using the optimised PCR method produced non-specific amplification products.

Rainbow trout eggs from RMS-positive and RMS-negative (no history of RMS) sites were obtained, and used in the analysis at IoA. The extraction method was first validated using an ATPase PCR as shown in Figure 4. The amount of DNA obtained from eggs was found to be very low and ATPase was not always detected in some samples (Figure 5).

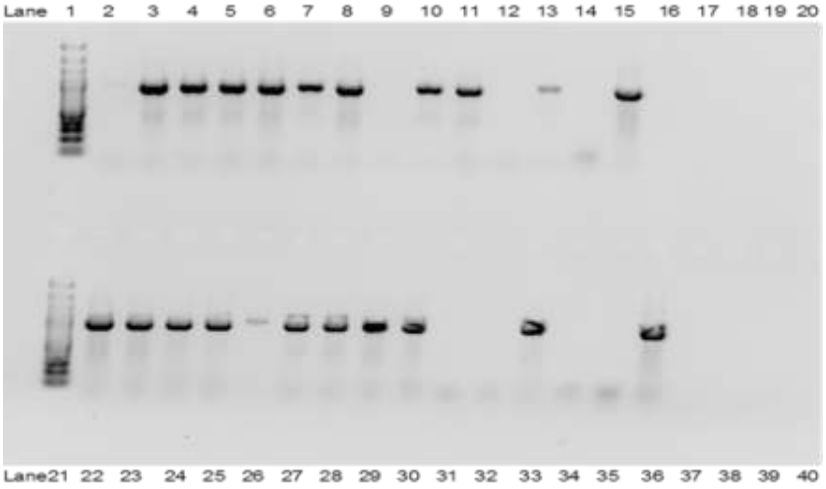

Figure 4: Agarose gel electrophoresis of ATPase PCR products from samples extracted from rainbow trout eggs. Lane 1 and 21: Molecular weight standard; Lanes 2-15 and 22-36: Individual fish egg samples.

Pooling of six eggs improved the yield and quality of the DNA obtained. Once the extraction method had been validated, the RLO PCR was performed on egg samples. Of the 32 egg samples collected from RMS-positive sites, DNA was extracted from 6 eggs from each sample and the RLO PCR performed. Although all of the egg samples tested negative by PCR, eggs cannot be discounted as being positive for the RLO due the difficulty in extracting the DNA (even with six eggs per pool) and presence of PCR inhibitors. Further assay optimisation and validation is required if the PCR is to be used on fish eggs. Conversely, PCR analysis of tissue samples appeared to be consistent *i.e.* sensitive and reproducible (see longitudinal study, Section 4).

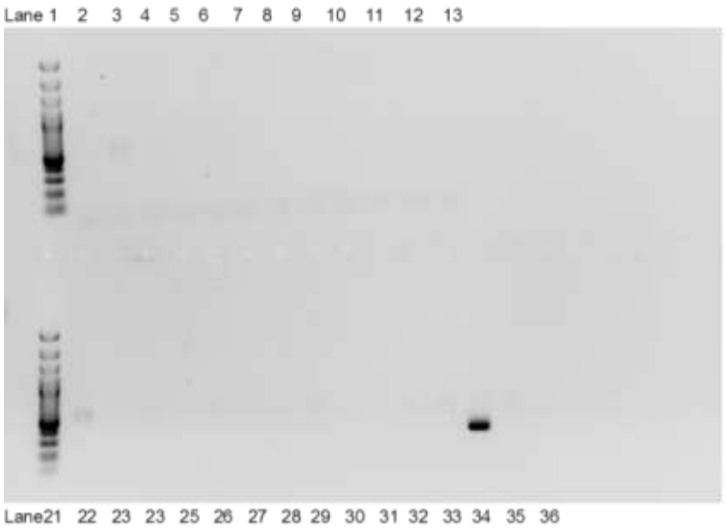

Figure 5: Agarose gel electrophoresis of RLO PCR products from samples extracted from rainbow trout eggs. Lanes 1, 21: Molecular weight standards; Lanes 2 – 13 and 22 - 33: Fish egg samples; Lane 34: RLO positive control.

4.3 Vaccination with an existing commercial *P. salmonis* vaccine

It had been shown previously [8,9] that Piscirickettsia-like antigens were present in tissue samples from fish presenting with RMS. An assumption was made that these antigens derive from a Rickettsia-Like Organism (RLO) which may be the causative agent of Red Mark Syndrome. Thus, a vaccine containing antigens to *Piscirickettsia salmonis* (also an RLO) may confer a degree of protection to fish against RMS. Therefore, fish on two sites were vaccinated with a commercial *P. salmonis* vaccine and then exposed to a natural RMS challenge on the farms. Fish were assessed for RMS over a time course, as shown in Table 2 and 3 and Figure 6.

The results of both vaccination trials (Tables 2 and 3) indicated that vaccination with a commercial *P. salmonis* vaccine (Alphaject Micro 2) did lead to a significant reduction in RMS (especially vaccination trial 2, Table 3), although it is likely that this could be further improved with a specific RMS vaccine. The protection given by the existing commercial vaccines may actually represent a positive effect of the adjuvant included in the vaccines as Alphaject 2-2, which does not contain *P. salmonis* antigens, also led to a reduction in RMS (Table 2).

Table 2 Results of RMS Grading at Braevallich-2014

GROUP	16/01/14			06/03/14			10/04/14			01/05/14		
	Score			Score			Score			Score		
	0	1	2	0	1	2	0	1	2	0	1	2
Alphaject Micro 2 (Trial)	97.4 341	2.6 9	0	98.9[a] 346	1.1[a] 4	0	99.4[a] 348	0.6[a] 2	0	98.8[a] 346	0.6[a] 2	0.6 2
Alphaject 2-2 (Injected control)	100 0	0	0	100[a] 0	0[a]	0	99.4[a] 348	0.6[a] 2	0	100[a] 0	0[a]	0
Control (Unvaccinated)	95.1 333	4.6 16	0.3 1	97.7[b] 342	2.3[b] 8	0	98.0[b] 343	2.0[b] 7	0	93.4[b] 327	4.6[b] 16	2.0 7

GROUP	12/06/14				01/07/14				
	Score				Score				
	0	1	2	3	0	1	2	3	1R
Alphaject Micro 2 (Trial)	85.7[a] 300	10.6[a] 37	2.9[a] 10	0.8[a] 3	60.0[a] 210	34.0[a] 119	5.7[a] 20	0.3[a] 1	0[a]
Alphaject 2-2 (Injected control)	97.4[a] 341	1.7[a] 6	0.6[a] 2	0.3[a] 1	69.1[a] 242	28.0[a] 98	2.3[a] 8	0.6[a] 2	0[a]
Control (Unvaccinated)	67.4[b] 236	25.4[b] 89	5.7[b] 20	1.4[b] 5	58.0[b] 203	22.0[b] 77	6.0[b] 21	1.7[b] 6	12.3[b] 43

A total of 350 fish scored from each cage for signs of RMS. The results are represented as percentages of fish sampled. Actual fish numbers in blue. Different letters indicate significant differences at P<0.001

Table 3 Results of RMS Grading at Trossachs Trout (10th June 2014)

GROUP	SCORE			
	0	1	2	3
Vaccinated	95.7* 335	2.9* 10	1.4* 5	0* 0
Control (Unvaccinated)	69.7 244	20 70	8.3 29	2.3 8

A total of 350 fish were examined from each cage for signs of RMS and scored. *P<0.001

The results are represented as percentages of fish sampled. Actual fish numbers in blue.

Figure 6 On-site assessment of rainbow trout for Red Mark Syndrome following vaccination with a commercial *Piscirickettsia salmonis* vaccine

Serum samples from each group at the various time points were analysed by antibody capture ELISA to determine if *P. salmonis* specific IgM had been produced following vaccination. Antibodies were detected in the samples collected in November 2013 from fish vaccinated with Alphaject Micro 2 in two out of the four pooled samples measured (OD 0.316 and 0.308 at 1/128 serum dilution). Lower levels of antibodies were also detected in the serum from fish vaccinated with Alphaject 2-2 in one of the four pools of serum samples (OD 0.108 at 1/128 serum dilution).

4.4. On farm investigation of effective treatment and management practices

A previous study led by CEFAS, in co-operation with MSS, collected information on risk factors for the development of RMS. It also collected information about management procedures and treatments effective in controlling RMS. During this project the CEFAS FHI re-visited several RMS affected farms in England and Wales and they will continue to provide updated information about effective treatment methods. Substances or management procedures that emerged as effective in treating RMS or preventing its occurrence will be studied further in farm based trials in on-going projects. Farmers were asked to dedicate tanks or raceways (units) to the study and follow management and treatment procedures as defined by the study requirements.

4.5 Longitudinal study (including MSc Project[20])

The extension of project 094 enabled further screening for RMS to be performed using the knowledge and methods developed in the SARF RMS project. Culture of the RLO was also attempted in a new model, the wax moth larvae (*Galleria mellonella).*

Screening was performed from April to October in 2015 and 2016 to determine the incidence of RLOs in liver, spleen and skin samples, to investigate when the RLOs are present in the fish and to determine the relationship between entry of the bacterium and appearance of lesions. By collecting farm records, the trend between the water temperature and the appearance of RMS lesions on the farm could be plotted (Figure 7). The first RMS lesions started to appear in August 2015 after the temperature had dropped having reached a maximum of 11.5 °C in July. The incidence of new lesions

increased to reach a maximum between November and March 2015 after which no new lesions were seen.

In 2015, sampling started in April, and the first RLO PCR positive results were detected in July in spleen and skin samples. The later were from apparently “healthy” skin tissues, suggesting the presence of RLO within the skin before lesions develop. The RLOs were then detected in skin, liver and spleen samples from fish with lesions in (August to October sampling). In October fish without lesions were positive for RLO in spleen and liver but negative in the skin for those fish tested.

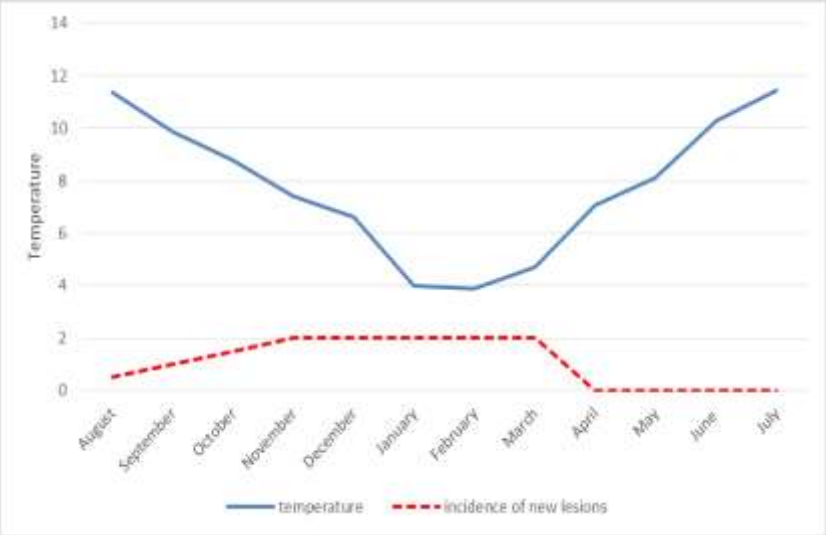

Figure 7. Monthly average water temperature in °C (blue) and the incidence of new RMS lesions (red) present on the farm in 2015.

There appeared to be lower numbers of RLO positive samples detected in 2016 versus 2015, accompanied by reduced incidence of RMS on the farm in 2016. This may be related to the introduction of new fish diets or to other factors. In 2016, all samples (spleen, liver and skin) collected from January - July tested negative for the presence of RLO by PCR (Table 4). The first positive results were noted in August in 6/30 skin samples and 1/30 spleen samples. Positive results were also recorded in September (8/30 skin samples and 1/30 liver samples) and October (5/30 skin samples), as shown in Table 4.

Additional attempts to isolate the organism on specialised culture media from these tissues were unsuccessful. Most probably this was due to the media lacking the exact nutritional requirements for the RLO. Culture of the RLO in wax moth larvae appeared to show potential since RLO positive PCR results were shown in larvae haemolymph after one and two weeks of incubation with suspected RMS infected tissues (MSc project). This was recently repeated by IoA staff with an improved disinfection protocol for the syringe to avoid sample carry over. Although the results were negative, this method should still be pursued as neither tissue collection nor larvae were optimal in the trials.

Table 4

Tissue samples testing positive by RLO PCR in 2016

Number of RLO PCR positive samples			
Month (2016)	**Spleen**	**Liver**	**Skin**
January	0/30	0/30	0/30
March	0/30	0/30	0/30
April	0/30	0/30	0/30
May	0/30	0/30	0/30
June	0/30	0/30	0/30
July	0/30	0/30	0/30
August	1/30	0/30	6/30
September	0/30	1/30	8/30
October	0/30	0/30	5/30

Three different genetic populations of fish were periodically on the farm and although it appeared that that there may be differences in RMS susceptibility between fish from different genetic sources, direct comparisons could not be made as the different groups entered and left the farm at different time points.

MAIN IMPLICATIONS OF THE FINDINGS

- This project based at University of Stirling, in partnership with CEFAS, MSS, FVG, DFF, SF and Pharmaq (now Zoetis) has been very successful in bringing the different sectors together to try to tackle the RMS problem in rainbow trout.
- New methodologies were developed to assist in the detection of RLOs in fish and eggs, although the assay for eggs needs further optimisation.
- Valuable information has been gained on RMS in the field, with some limited protection provided through vaccination with commercial vaccines. This may provide one possible control strategy for the future.
- The results from a longitudinal farm study indicated that RLOs were first detected by PCR in July in 2015 (skin and spleen) and August in 2016 (skin and spleen) in rainbow trout. The RLOs could be detected in fish with lesions, especially in skin samples, until October in the fish sampled.
- Lower numbers of RLO positive samples were detected in 2016 versus 2015 and this coincided with a reduced incidence of RMS. This may be related to the introduction of new diets on the farm (new Skretting diets had been used) or to other factors. Introduction of new feeds may therefore represent another possible control strategy for the future.
- It appeared that there may be differences in RMS susceptibility between fish from different genetic sources, although direct comparisons could not be made as the different groups entered and left the farm at different time points.

POSSIBLE FUTURE WORK; AND ANY ACTION RESULTING FROM THE RESEARCH (*e.g.* IP, KNOWLEDGE TRANSFER)

There are still many unanswered questions and although RLOs have been identified in RMS affected fish using molecular methods, cultured organisms have so far still not been identified. This should be pursued further so that a specific RMS vaccine can be developed. In addition the presence of other pathogens (as either co-infections or carrier states) on progression and spread of the disease are currently not known; this is very important. Fish genetic background is also a critical factor and a direct comparison of populations is required.

References to published material

This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project (the box below will expand).

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ANNEX 1

Red Mark Syndrome - 10 years on *(draft)*

Abstract

Red mark syndrome (RMS) is a skin condition affecting rainbow trout (*Oncorhynchus mykiss*), appearing in the United Kingdom (UK) in late 2003 (Verner-Jeffreys, Algoet, Feist, Bateman, Peeler & Branson 2006). Since then, RMS has quickly spread to over 50% of trout farms in the UK (Adam 2009; Noguera 2008). RMS appears to be identical to strawberry disease seen in rainbow trout in the United States of America (USA), which has been present there since the mid 1940's (Metselaar, Thompson, Gratacap, Kik, LaPatra, Lloyd, Call, Smith & Adams 2010). RMS results in significant economic losses in the trout industry due to downgrading of the fish product (Verner-Jeffreys, Pond, Peeler, Rimmer, Oidtmann, Way, Mewett, Jeffrey, Bateman, Reese & Feist 2008). Ten years since the original outbreak of RMS in the UK, the cause of the disease is still unclear. Two pathogens have been associated to disease; a rickettsia-like organism (RLO) (Lloyd, LaPatra, Snekvik, St-Hilaire, Cain & Call 2008; Lloyd, LaPatra, Snekvik, Cain & Call 2011; Metselaar 2010; Metselaar 2013; Cafiso, Sassera, Serra, Bandi, McCarthy & Bazzocchi 2015) and *Flavobacterium psychrophilum* (Ferguson, Girons, Rizgalla, LaPatra, Branson, MacKenzie, Davies, Collins, Diab. & Crumlish 2006; Noguera 2008; Wallis, Dalsgaard, Hopewell & Kardos 2009). The 16S rDNA sequence of the RLO bacterium associated with RMS/SD lesions was included in a phylogenetic study, and was shown to belong to the recently described family Midichloriaceae, within the order Rickettsiales (Montagna *et al*. 2013). *F. psychrophilum* has not been identified in all studies carried out (Verner-Jeffreys *et al.* 2006; Verner-Jeffreys *et al.* 2008; Bruno, Crumlish, LaPatra, Noguera & Verner-Jeffreys 2007; Schmidt-Posthaus, Bergmann, Knusel, Heistinger & Licek 2009; Metselaar 2013) and the RLO has still to be isolated or visualised by histology (Metselaar 2013). Both pathogens have however been identified consistently enough to warrant further research. Other possible aetiological agents of RMS include an adeno-like virus, however, no recent work has been done on this pathogen in relation to RMS (Fleury, Vuillaume & Sochon 1985). Skin conditions, especially those of unknown aetiology, are getting more common and are a true challenge to the trout industry.

Red mark syndrome, Strawberry disease and other skin conditions in trout

Red mark syndrome (RMS) first appeared at the end of 2003 at a single Scottish farm (Girons Ingles 2005). Since then, RMS spread around Scotland and the rest of the United Kingdom (UK) to over 50% of the trout farms. The initial outbreak was possibly linked to a single batch of imported eggs from the United States (US) and spread could be linked back to the original outbreak (Girons Ingles 2005; Verner-Jeffreys *et al*. 2006, Algoet, Feist, Bateman, Peeler & Branson 2006). Based on the outbreak pattern, it has been suggested that there have been further imports of the disease in subsequent years (Adam 2009).

A similar condition to RMS was first seen in the US by Davis (1946) and referred to as strawberry disease (SD). A case study was published in 1965 (Ericson) where several studies on the disease were described. The initial outbreak was linked to one specific batch of eggs (Nevada Department of Wildlife 1974). Research has shown a strong correlation between SD lesions from the US and the presence of DNA with a Rickettsia-like organism (RLO) 16S rRNA sequence (Lloyd *et al.* 2008). Immunohistochemistry (IHC) was used to screen RMS samples using a panel of anti-*P. salmonis* antibodies. The staining observed in the RMS samples was similar to the staining obtained in tissue from SD-affected samples using the same panel of antibodies. A polymerase chain reaction (PCR) using RLO-specific primers was also performed on samples from RMS affected fish from the UK, and SD affected fish from the US. Both sets of samples were positive for the RLO 16S rRNA sequence. These findings suggest that the same aetiological agent may be responsible for RMS in the UK and SD in the US. SD and RMS are now believed to be the same condition (Metselaar 2010; Oidtmann, Verner-Jeffreys, Pond, Noguera, Bruno, LaPatra, St-Hilaire, Schubinger, Snekvik, Crumlish, Green, Metselaar, Hughes, Rodger Schmidt-Posthaus, Galeotti & Feist 2012).

Warm water strawberry disease (WWSD), another skin condition in trout, has a different pathology and temperature range when compared to RMS. Therefore, it is believed that WWSD is not the same condition as RMS based on epidemiological and pathological characteristics (Ferguson, Girons, Rizgalla, LaPatra, Branson, MacKenzie, Davies, Collins, Diab & Crumlish 2006; Verner-Jeffreys *et al.* 2008; Oidtmann *et al.* 2012). Rash, a skin pathology seen only in the US, might resemble that of WWSD and also differs from RMS (Oidtmann *et al.* 2012).

A more recent disease seen in the UK, called puffy skin disease (PSD), is still of unknown aetiology. A case definition for this disease was made based on epidemiology, clinical signs and presentation, gross pathology, normal and electron microscopy. This skin disease, unlike RMS, does affect the appetite and overall health status, even causing mortality (Maddocks, Nolan, Feist, Crumlish, Richards & Williams 2014).

RMS in other countries

The condition is also a problem in continental Europe, with reports of RMS outbreaks coming from Switzerland, Austria, Japan, France, Finland, Germany, Spain, Serbia, Italy and Chile (Bruno *et al.* 2007; Ferguson *et al.* 2006; Licek, Schmidt-Posthaus & Heistinger 2008; Radosavljevic, Jeremic, Cirkovic & Milosevic 2009; Schmidt-Posthaus, Bergmann, Knusel, Heistinger & Licek 2009; Galeotti, Giavenni, Volpatti, Beraldo & Feist 2011; Sandoval, Montoya, Ifante, Ilardi, Battaglia & McLoughlin 2013).

Histology and case definition

RMS presents as raised erythematous multifocal lesions with scale loss in the centre. Most commonly the lesions are located on the flanks, but can be seen all over the body of the animal. RMS has only been confidently identified in rainbow trout (*Oncorhynchus mykiss*). Lesions similar to RMS have been seen in brown trout, wild caught rainbow trout, sea-going rainbow trout, cutthroat trout, white fish and sea bream (Bruno *et al.* 2007; Metselaar, Thompson & Adams 2011; Oman 1990; Zarza & Padros 2007), in none of these cases RMS could be confirmed by histology. Histologically, the dermis and hypodermis at the site of the lesions appears inflamed, together with the muscle and adipose tissue. The epidermis is however not always involved (Noguera 2008). An infiltration of high numbers of inflammatory cells such as lymphocytes, heterophils and macrophages is seen, which macroscopically causes the lesions to appear raised. Loss of scales is common at the lesion site, which is most likely due to the large infiltration of neutrophils and oedema around the scale pockets. Ulceration has been described in the condition (Verner-Jeffreys *et al.* 2006), however, it has been thought that this is due to secondary infections (Ferguson *et al.* 2006; Oidtmann *et. al*. 2012). In very early lesions, the necrotic and inflammatory foci are seen in the deeper layers of the skin. It was therefore concluded that the causative agent is present in the fish instead of entering the body

through the site of the lesion. It is thought that the resulting external lesions are caused by a hypersensitivity reaction of the host (Ferguson *et al.* 2006; Noguera 2008; Verner-Jeffreys *et al.* 2006). This reaction could possibly be triggered by factors such as water quality, temperature and/or stress inducing procedures like grading and handling; the exact trigger, however, still needs to be elucidated. Concurrent myocarditis has also been reported (Ferguson *et al.* 2006) and 20% of the affected fish showed cardiac pathology. Acute necrotising myocarditis was detected in smaller fish (Noguera 2008).

Both RMS and SD have limited epidermal involvement and extensive lymphocytic infiltration into the sub-dermal layer and are both described as full thickness dermatitis (Verner-Jeffreys *et al.* 2008). There are however some differences in the pathology reported between the two conditions. Heterophils can be seen within established RMS lesions in connective tissues, between epidermis and dermis, and between dermis and subcutis. Pathology is not exclusively cutaneous in RMS; changes are also noted in the liver, kidney and spleen and additional pathologies include exophthalmia, myocarditis and skeletal deformities (Lloyd *et al.* 2008). It is not clear if the differences between the two syndromes are caused by genetic changes affecting immunity or different presentations caused by a different aetiopathogenesis (Lloyd *et al.* 2011).

RMS is only seen in water temperatures below 15˚C. Therefore RMS is also known as cold water strawberry disease or gravel rash (Girons Ingles 2005; Oidtmann *et al.* 2012). RMS is occasionally reported in warmer water, but because of the long latency period, it is unclear if RMS actually develops at higher temperatures or just manifests at this temperature. RMS has been seen in the marine environment. In the authors' experience, this has always been shortly after transfer and therefore most likely originates from fresh water.

RMS lesions can be graded on the basis of their severity. An early lesion can be described as Grade 1 and are identified as small spots of increased mucus production. In Grade 2 lesions, the characteristic redness appears in the centre of the lesion. Lesions described as Grade 3 have more extensive erythema and scale loss in the centre of the lesion (Metselaar 2010). The lesions do not affect the appetite or growth of the fish, but the effect on the appearance of the fish can result in economic losses (Ferguson *et al.* 2006; Noguera 2008). Mortality is usually not associated with RMS, despite the high number of fish that can be affected. Morbidity is normally between 5 and 50%, but has been described as high as 90% (Noguera 2008).

Verner-Jeffreys *et al.* (2008) artificially transmitted RMS through cohabitation infections, repeating the results from an earlier American study with SD (Ericson 1965). That same study showed that the clinical signs of the disease improved with the use of antibiotics (oxolinic acid, oxytetracycline and florfenicol), suggesting a bacterial agent as the contributing factor of the disease. In a later study RMS like lesions also appeared on cohabitant fish, but the results were inconclusive (Metselaar 2013).

Epidemiology

An epidemiological study showed that once RMS was observed on a farm, 10 to 60% of fish developed clinical signs of RMS within 2 to 3 weeks (Verner-Jeffreys *et al.* 2006). The same study observed a rolling infection, with the first affected fish already recovering while other fish were just starting to develop clinical signs. No reoccurrence of disease outbreak was observed in the same batch once it had recovered. Treatment was possible with broad-spectrum antibiotics such as oxolinic acid, oxytetracycline and florfenicol. Disinfectants like chloramine T and 10% neutral buffered formalin (NBF) were able to clear up the condition before harvest, but were less effective. RMS can be transmitted to naïve fish by indirect contact, which together with the response to antibiotics, suggests that RMS may be caused by a bacterial agent (Verner-Jeffreys *et al.* 2008). Another epidemiological study investigating several risk factors discovered no difference in the mean water temperature between RMS affected and control farms (Adam 2009). The same study also showed that there might have been two separate introductions of the condition into the country, one in 2003 and another in 2006, when there was a steep increase in the number of newly infected farms. Potential risk factors for infection included all mechanical methods of handling fish. It is therefore advised that high-risk farms keep mechanical handling to a minimum and implement “old-fashioned good husbandry”. Many factors that induce stress did not appear to have an elevated risk, but hand-netting and handling seem to be protective. The number of suppliers was also important, if the farm sought fish from more than four suppliers this was also found to increase the risk of fish developing RMS. The number of deliveries or connections through the river network did not seem important. Movement of fish or ova onto the site appeared to be the most important route of introduction of the condition. This is supported by the observation that large-scale production sites have a high likelihood of becoming infected (Adam 2009).

Aetiology

The cause of the condition has not yet been established. Since the condition appears to respond to antibiotics and the condition is shown to be transmissible to naive fish via partial cohabitation, this strongly suggests the involvement of an infectious agent, possibly a bacterial agent.

In 1985, an adeno-like virus was isolated from rainbow trout with RMS-like symptoms. Under transmitting electron microscope (TEM), virus particles were visible with an icosahedral symmetry with 6 capsomeres on each side giving a total of 252 capsomeres. No envelope was seen and the total diameter was about 65 nm, consistent with an adeno-like virus. Parasitic and fungal investigations were negative and haematology results within normal limits. At a later stage of the condition *Flexibacter columnaris* and *Flavobacterium sp*. could be isolated, but could not be associated with the onset of the condition and were therefore not thought to be the primary cause of the infection (Fleury, Vuillaume & Sochon 1985). The adenovirus was again identified by Oman in 1990, but despite TEM studies, could not be consistently found in later studies (Girons Ingles 2005; Verner-Jeffreys *et al.* 2008). Adeno viruses have been described in fish and other animals, but has not been linked to other any disease in trout (Benko, Elo, Ursu, Ahne, LaPatra, Thomson & Harrach 2002)

After an outbreak in 1993 in Spain, of what the author described as RMS in rainbow trout, an epidemiological and aetiological study was performed (Planas, Ortega, Muzquiz, Docando, Alonso, Sanz, Ramos & Arnal 1993). Two bacteria; *Pseudomonas* sp. and *Aeromonas hydrophila* were separately isolated in outbreaks a month apart and it was concluded that although not the primary cause of the outbreak these bacteria were associated with the condition. Attempts to culture any virus did not result in any cytopathogenic effect (CPE) in the cell cultures used.

More recently an association between RMS and *F. psychrophilum*, the causative agent of rainbow trout fry syndrome (RTFS) has been suggested (Ferguson *et al.* 2006). The bacterium was detected by a nested PCR in skin and heart lesions from two out of three formalin-fixed wax-embedded archived tissue samples from RMS-affected fish and pooled hearts (Crumlish, Diab, George & Ferguson 2007). The same association was not found in the SD tissues tested. Heart lesions of the type commonly seen in RTFS were also observed. The bacterium was not isolated using standard bacteriological techniques, but *Flavobacterium psychrophilum* requires a low nutrient medium making it difficult to culture. At Marine Scotland Science, Aberdeen, *F. psychrophilum* was

frequently found in RMS- affected fish, not only in the lesions, but also in the organs and the back of the eye (Noguera 2008). *Flavobacterium psychrophilum* isolates were found in brain and liver of fully recovered fish months after the peak. It was therefore suggested, together with the overall clinical presentation and characteristic histopathology, that RMS is a chronic, sub-lethal septic infection of *F. psychrophilum* and that the type strain may vary from the one causing RTFS (Noguera 2008). A further indication for the role of *F. psychrophilum* in RMS comes from an autogenous vaccine for *F. psychrophilum*, which was thought to protect against RMS as one study showed a lower prevalence in vaccinated groups compared to non-vaccinated groups (Wallis, Dalsgaard, Hopewell & Kardos 2009). The trialled vaccine is oil-based and there is a possibility that the oil- based adjuvant leads to protection. In the study no non-adjuvant control has been used and so warrants further investigation. Testing this hypothesis was part of the current Scottish Aquaculture Research forum (SARF) project. The results showed that the oil in the vaccine gives a significant boost to the immune system, and the prevalence of RMS appears lower in oil-vaccinated fish compared to non-vaccinated fish (data not published).

In addition to an epidemiology study (Verner-Jeffreys *et al.* 2006) efforts were made to isolate the causative agent of RMS, and a diverse range of bacteria were recovered from the lesions using standard methods. No agents could be related to the condition, including *F. psychrophilum.* No association could be found between possible agents and RMS in studies on 16S rRNA gene libraries using both RMS affected and naïve fish. Although *F. psychrophilum* was found in some of the RMS fish, no firm association was made between this bacterium and RMS (Verner-Jeffreys *et al.* 2008). During a later outbreak of RMS in Switzerland and Austria, Schmidt-Posthaus *et al.* (2009) tried to culture *F. psychrophilum* on Cytophaga agar plates, but no agents could be isolated related to the RMS outbreaks. In a comparative study between RMS affected and naïve fish no association was found between *F. psychrophilum* and RMS in either skin or other organs using PCR (Metselaar 2013). Therefore, there is still some debate as to the role of *F. psychrophilum* in RMS*.* At the European Association of Fish Pathologists (EAFP) workshop on RMS in Grado (2007) comments were made that *F. psychrophilum* can be found in 48% of the fish in Finland, without having any signs of RMS (Bruno *et al.* 2007). Clearly this bacterium can be found in some cases of RMS, but Koch's postulates have not yet been fulfilled. IHC using anti- *F. psychrophilum* polyclonal antibodies (PAbs) did show that the bacterium was present in RMS, but only in a few fish mostly on the skin. The anti-*F.*

psychrophilum PAbs used in this study do not detect all *F. psychrophilum* strains (Faruk, Campbell, Thompson, Rangdale & Richards 2002), and the MALDI-TOF-MS results showed that the majority of *Flavobacterium* spp were different strains that could not be detected by the PAbs. Antibodies that detect a wider range of *Flavobacterium* spp. would be useful for subsequent studies.

A strong association was convincingly demonstrated by Lloyd *et al.* (2008; 2011) between an RLO and SD. Although at that time it was unclear if SD and RMS were the same condition, it is now believed that they are (Metselaar 2010; Oidtmann *et al.* 2012). Lloyd used 16S rRNA gene sequencing with different universal primers compared to Verner-Jeffreys in 2008, to identify an RLO for which a nested PCR was developed (Lloyd 2008), and later improved (Lloyd 2011). Using this nested PCR further samples were tested, showing an association between the bacterium and the disease. RMS samples tested with the published nested PCR showed the same association with the RLO. This nested PCR was than transformed to a qPCR test and further research, using RMS affected fish and naïve fish showed none of the naïve fish to be positive for the RLO DNA, whereas all the RMS fish had RLO DNA present in the skin and internal organs. Unpublished work by Cefas, presented at the SARF meeting, independently found the same RLO using advanced subtraction next generation sequencing. It was also reported that joint work between MSS and an Italian group had confirmed the presence of the *Midichloria* species (Cafisio, 2015).

Although the above shows a strong correlation, the RLO has not been visualised in routine histology of RMS samples. *Rickettsia* spp. are intracellular organisms that are large enough to visualise using conventional light microscopy. Special stains used to highlight these intracellular organisms also failed to show the RLO in RMS affected fish. This is not uncommon as it was initially not possible to see the *Francisella* in histology (Zerihun, Feist, Bucke, Olsen, Tandstad, & Colquhoun 2011). Only recently has an RLO been seen in fresh spleen smears of RMS-affected fish (Galeotti *et al.* 2011). It is hypothesised that RMS results from a previous infection with the RLO (Metselaar 2013). The response seen in RMS does resemble an allergic reaction seen in other animals (Ferguson *et al.* 2006; Noguera 2008; Verner-Jeffreys *et al.* 2006), which would fit with the hypothesis. It could of course be possible that the RLO is a secondary infection as it was detected by IHC, PCR and qPCR when the lesions were already present on the fish. If it is a secondary infection it has however consistently been found in three different geographical areas without apparent contact. To further investigate the role of the RLO, a cohabitation study was set up to investigate early stages

of RMS. The overall results do strongly suggest that there is an involvement of the RLO, however as the number of infected fish in cohabitation was low, results were inconclusive and no distinction could be made if the RLO was a primary or secondary infection. Although little is known about the RLO, antibodies specific to *P. salmonis* were used in IHC as tools to detect what is thought to be the RLO in RMS–affected fish. The IHC had the same staining in all cases of RMS-affected tissue (100 % of samples), while only 5 internal organs from 22 RMS-unaffected fish weakly stained with these antibodies; therefore it was believed that the positive results obtained by IHC were genuine and that the RLO was associated with the RMS-affected fish. As no intact bacteria were seen in the IHC using the anti-*P. salmonis* antibodies, care should also be taken when interpreting the positive results of the IHC even though there is strong evidence that this represents direct binding of the anti-*P. salmonis* antibodies to common epitopes on an RLO present in the infected tissue section, as the reaction was seen as intracellular staining in mainly the RMS-affected fish, which showed a positive correlation with the detection of the RLO by qPCR (Metselaar 2013).

The RLO described here would be the first true rickettsia described in fish. Most Rickettsiae or RLOs described in fish to date refer to *Piscirickettsia salmonis* (Mohamed 1939; Ozel & Schwanzpfitzner 1975; Davies 1986; Chen, Tung, Chen, Tsai, Wang, Chen, Lin & Adams 1994; Comps, Raymond & Plassiart 1996; Chen, Wang, Tung, Thompson & Adams 2000; Yuksel, Thompson & Adams 2006). The studies all use IHC as a confirmation of the diagnosis. *P. salmonis* is a member of the gamma proteobacteria, whereas the true Rickettsia sp. are members of alpha proteobacteria (Fryer, Lannan, Giovannoni & Wood 1992). As there now is evidence suggesting that there is cross reactivity between the RLO and *P. salmonis,* any further positive IHC should be followed by DNA sequencing to differentiate between the two organisms. An association of the RLO with RMS is very likely, but a more complex pathology could be possible. Further investigations as to what role the RLO plays in RMS needs to be established (Botelho-Nevers & Raoult 2011). The severity of an infection with an RLO is dependent on several factors. RMS is noted to be multifactorial and certain farming practices and environmental factors make some stocks of fish more susceptible to the condition (Bruno *et al.* 2007; Botelho-Nevers & Raoult 2011).

Therapy

It is possible to treat RMS with antibiotics and disinfectants, helping to clear the fish of RMS prior to harvest, but there are major drawbacks with the use of these because of a required withdrawal period

and the additional cost of the treatment itself. The most common treatment is oxytetracycline, but other treatments used are Branzil (oxolininc acid), Florocol (florphenicol), chloramine T, Salt 3%, Skretting response diet, formalin or mild stress (e.g. grading) (Pond 2007; Verner-Jeffreys *et al.* 2008). Topical treatment with antibiotics is also occasionally applied (oxytetracycline) (author's experience).

However, left untreated, RMS will resolve without major marks or mortalities. There are some reports of systematic fallowing and disinfection of the ponds helping to eradicate the condition from the farm with good results (Adam 2010; Metselaar 2010). Moving fish to warmer water also results in more rapid recovery (Bruno *et al.* 2007).

Discussion

Red mark syndrome first appeared in the winter of 2003 on a Scottish farm and has since spread to over 50% of the rainbow trout farms in the UK (Noguera 2008). This spread was relatively quick and does highlight a potential issue. In an epidemiological study one of the risk factors that was mentioned was the number suppliers used by the farm. The farms positive for RMS often had more than four suppliers (Adam 2009). The biggest risk to biosecurity of any farm is always the importation of livestock, receiving and supplying livestock from multiple sources multiplies the risk many fold.

The current findings have no direct impact on the management of the condition. If RMS can be treated with antibiotics such as oxytetracycline, this is indicative that there is a bacterial involvement in the condition. Leaving the fish to recover over time is a method used often (Adam 2009; Adam 2010), especially if withdrawal periods make the use of antibiotics impractical, as seen in the UK unlike in the US. Another solution is prevention of contracting diseases. Fifty percent of farms not affected by RMS are mostly smaller, closed farms. Increasing biosecurity by importing only eggs or having farm based broad stock should be considered. A closed farm where no livestock is imported and movement of animals is restricted to move only from the brood stock to the hatchery on to the on-growers will be less likely to contract new diseases. A further challenge unique to aquaculture is the presence of wild stock swimming freely in the water supply. Although connections through the river network was not identified as an additional risk factor for RMS (Adam 2009) this concept of biosecurity should be expended to the point where a single company has control over all the farms on a single water supply to further reduce risk.

The current disease is and might remain of unknown aetiology. Working with diseases of unknown causality has several challenges especially with intracellular pathogens like RLO. In order to fulfil Koch's postulates or develop preventive measures such as a vaccines, it is necessary to isolate the causative agent. Not knowing the cause of the condition decreases the chances recovering any infectious agent from affected material. Although selective media can be carefully chosen for the suspected causative agent, it does not always guarantee growth of the bacterium. This was the case with *Francisella* which are also not always readily observed in histology (Colquhoun & Duodu 2011).

At the present time no pathogen has either been satisfactorily isolated, or visualised from any of the samples prepared from RMS or SD-affected rainbow trout. The exact role of *F. psychrophilum* remains unclear. Many researchers have failed to find an association between *F. psychrophilum* and RMS or SD-affected fish, but it has been found consistently enough to warrant further investigation. The two strongest candidates are still the RLO and *F. psychrophilum*, but possibly also the adeno-like virus. All other possible agents mentioned lack sufficient evidence to be associated to RMS. The results have provided more evidence about which bacteria are involved and more importantly which are not, but Koch's postulates have not been fulfilled so far.

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Iomairt na Gaidhealtachd 's nan Eilean

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Rivers & Fisheries Trusts of Scotland
Safeguarding Scotland's Rivers & Lochs
ASFB The Association of Salmon Fishery Boards