Ministry for Primary Industries
Manatū Ahu Matua

Salmon Mortality Investigation

REW-1017 Pelorus Sound

MPI Technical Paper 2013/19

By Richard Norman (Response Manager, MPI)
Cara Brosnahan (MPI)
Jeannine Fischer (MPI)
Jamie Frazer (MPI)
Colin Johnston (Brightwater Consulting Ltd)
Brian Jones (MPI)
Suzanne Keeling (MPI)
Anjali Pande (MPI)
David Pulford (MPI)
Tom Rawdon (MPI)
Rissa Williams (MPI)

ISBN No: 978-0-478-41497-4 (online)
ISSN No: 2253-3923 (online)

July 2013

New Zealand Government

Growing and Protecting New Zealand

Disclaimer

Every effort has been made to ensure the information in this document is accurate.

MPI does not accept any responsibility or liability whatsoever for any error of fact, omission, interpretation or opinion that may be present, however it may have occurred.

Requests for further copies should be directed to:

Publications Logistics Officer
Ministry for Primary Industries
PO Box 2526
WELLINGTON 6140

Email: brand@mpi.govt.nz
Telephone: 0800 00 83 33
Facsimile: 04-894 0300

This publication is also available on the Ministry for Primary Industries website at
http://www.mpi.govt.nz/news-resources/publications.aspx

Contents

Page

1 Executive Summary

On 1 March 2012, the New Zealand King Salmon Company (NZKS) notified MPI of a significant mortality event occurring in Chinook salmon (*Oncorhynchus tshawytscha*) at their sea farm in Waihinau Bay, outer Pelorus Sound.

Fish were sampled from the site at the time of notification (1/3/12 - 14 fish), and an additional two times (20/3/12 – 15 fish; 15/5/12 – 38 fish), for disease testing. As part of MPI's Animal Health Laboratory (AHL) standard procedures, a comprehensive diagnostic differential list specific for pathogens of king salmon was compiled. This list was later expanded to include other pathogens known to cause disease in salmonids. Testing performed at AHL ruled out a number of enzootic and exotic pathogens.

The following tests were conducted:

- *Aeromonas salmonicida* PCR
- *Yersinia ruckeri* PCR
- *Renibacterium salmoninarum* PCR
- General aquatic bacterial culture including Vibrio species
- Viral haemorrhagic septicaemia virus PCR
- Infectious hematopoietic necrosis virus PCR
- Infectious salmon anaemia virus PCR
- Mycoplasma PCR
- Iridovirus PCR
- Totivirus PCR
- Reovirus PCR
- Cytopathic viruses
- Histopathology.

All PCR tests produced negative results. General aquatic bacterial culture yielded no significant growth. Histopathology was consistently suggestive of an inflammatory process in the heart, liver and muscle. The findings were still not directly suggestive of infection with any specific OIE notifiable disease[1], however as frank clinical disease for many of the notifiable diseases has not been recorded for Chinook salmon, this conclusion is based on examination of available scientific literature and extrapolation from clinical presentation in Atlantic salmon (*Salmo salar*).

During the course of the investigation, it became public knowledge that a king salmon mortality event had occurred in Pelorus Sound and there was media speculation regarding the potential involvement of Infectious Salmon Anaemia virus (ISAV). ISAV has not been reported in the scientific literature to cause disease in Chinook salmon and nor did the gross pathology or histopathology of the affected fish suggest ISAV as a potential disease. However, as a risk reduction measure, a decision was made to seek laboratory analysis to rule out ISAV. ISAV belongs to Orthomyxoviridae - a family of viruses that are notable for rapid mutation and cross-species infection. During the initial stage of the MPI investigation, molecular capability for ISAV was not available at AHL. Subsequently, AHL established ISAV molecular capability using OIE-published assays and samples tested at AHL were negative.

1 New Zealand has international obligations to provide immediate notification to the World Organisations for Animal Health (OIE) when diseases on the notifiable organism schedule are detected.

Fresh samples plus previously collected samples were sent to an OIE reference laboratory for ISAV- the Norwegian Veterinary Institute (NVI) - for ISAV testing. NVI was not able to detect ISAV in any of the samples using the standard procedures recommended in the OIE Manual of Diagnostic Tests for Aquatic Animals. Both NVI and AHL laboratories obtained negative results for ISAV from samples using OIE recommended methods. The case definition for confirmation of ISAV was never met, and given two independent sets of negative results, it is our opinion that ISAV need not be considered further as a causative agent in this case.

No cause for the mortality event was identified by the investigation, however in retrospect sampling was only carried out after the peak mortality. Further investigation to identify the cause of this annual mortality increase, and whether it is related to the external ulcers, heart pathology and suspected intracellular parasites is recommended in the future.

2 Background

2.1 NOTIFICATION

On 1 March 2012, the New Zealand King Salmon Company (NZKS) notified MPI of a significant mortality event occurring in Chinook salmon at their sea farm in Waihinau Bay, outer Pelorus Sound.

2.2 INVESTIGATION OBJECTIVES

An epidemiological and laboratory investigation was carried out to:

- Determine whether the mortality event in Chinook salmon reported from Waihinau Bay, Pelorus Sound was associated with an infectious agent,
- Rule out OIE listed diseases which can cause Chinook salmon mortality.

2.3 INVESTIGATION PLAN

In order to fulfil the investigation objectives, information was sought from NZKS about the Waihinau Bay farm, including environmental monitoring, production data and their operational procedures. This was analysed to identify any factors or events that may have contributed to the mortality event and identify any potential biosecurity risks, should an infectious agent be identified later. As well as collecting information on the farm, a sampling plan was developed to support diagnostic testing.

2.4 DESCRIPTION OF THE MORTALITY EVENT AT WAIHINAU BAY

Mortality in March was higher than typically expected at all cages across the Waihinau Bay salmon farm site.

Within the affected site, two cages had higher mortality than all other cages. Initial observations also included fish with skin lesions, reduced feed intake, and exhibiting lethargy.

The mortality appeared to peak in mid-March, but still remained higher than expected as moribund fish continued to drop out of the population. By the beginning of May, the farm reported that mortality rates had reduced to normal.

2.5 MORTALITY ASSESSMENT OF ALL NZKS SITES

Cumulative monthly mortality was compared across NZKS sites. Waihinau Bay appeared significantly higher compared with other NZKS sites in February and March 2012.

2.6 DESCRIPTION OF FARM AND OPERATION

Oncorhynchus tshawytscha, commonly known as Chinook salmon or king salmon, are produced in a vertically integrated system. Broodstock are selected from sea farms and collected from the wild, based on their desirable traits (e.g. fast growth rate). Broodstock are taken back to one of three hatcheries, where they are artificially stripped (eggs and milt removed), eggs fertilised and hatched, then fish are grown to smolt, whereupon they are transferred to sea cages to be grown to market size. The entire production cycle from hatching to harvest takes about 15 – 18 months.

NZKS have seven marine farm leases, five of which are currently operating, and own three hatcheries (Waiau, Takaka and Tentburn).

Waihinau Bay – the affected site – is located in the outer reaches of the Pelorus Sound. There are two other sites approved for fin fish aquaculture in Pelorus Sound: Crail Bay and Forsythe Bay. These other two sites were empty of fish at the time of the mortality event. The other operating sites are located in the Tory Channel, Queen Charlotte Sound, and therefore remote from Waihinau Bay.

2.7 ENVIRONMENTAL AND HUSBANDRY FACTORS

Dissolved oxygen and water temperatures are routinely monitored by the farm, but no obvious changes were observed by the farm operator that would account for the increased mortality (e.g., higher than usual water temperature, decreased dissolved oxygen, algal blooms). No recent husbandry activities such as grading or changes to feed were evident.

2.8 CONTACT TRACING

All fish being grown out at Crail Bay & Forsythe Bay and subsequently Waihinau Bay had originated from the Takaka hatchery, which also supplied smolt to the other NZKS sea pen sites - Te Pangu, Clay Point and Ruakaka. No unusual mortality was reported from the other three sites supplied by the Takaka hatchery.

Link to NZKS biosecurity protocol:
http://www.kingsalmon.co.nz/KingSalmonEvidence_documentation/2430009%20Mark%20Preece%20-%20Operations%20Annexsure%203%20-%20v1.pdf

2.9 ROUTINE DISEASE SURVEILLANCE

2.9.1 Salmon testing scheme – export to Australia

Salmon that are exported from New Zealand to Australia undergo testing for health surveillance as per the 'MAF standard for health surveillance in approved establishments for export of salmon for human consumption to Australia" implemented on 1 February 2000.

Participation in this standard is voluntary; however, participation is required for approval to produce salmon for export to Australia under MPI export certification.

Waihinau Bay and Forsyth Bay has been involved in salmon export testing scheme since 1998.

Salmon at this site get tested:
- Once on the farm as 1 year old fish
- Once at the factory after harvest.

At these times, the fish undergo:
- Bacteriology (exclusion of *Aeromonas salmonicida*, *Renibacterium salmoninarum* and *Yersinia ruckeri* and identification of any significant dominant bacteria)
- Virology (exclusion of cytopathic viruses)

- Protozoans (exclusion of *Myxobolus cerebralis*).

All results to date for this testing has been negative.

2.9.2 On farm health monitoring

At the time of the incident, NZKS were contracting a private veterinarian, based in Australia, for fish health support.

2.10 CASE MANAGEMENT

In order to manage any further notifications of mortality events in salmonids during the investigation, a planning document was developed, including a case definition for report cases, to guide which cases would meet the threshold for investigation.

No other sites or farms notified MPI of mortality events meeting the report case definition for the duration of the response.

Planning and development of case definitions also took place, to help provide some framework to refer to when describing the syndrome, both for the purposes of the investigation, and to refer to when describing to domestic and international stakeholders. .

3 Diagnostic Findings

3.1 SUMMARY OF DIAGNOSTIC TESTING CONDUCTED AT AHL ON ACCESSION W12_00272

Pathogen/Test	Sample type	Results	Comments
Aeromonas salmonicida PCR	Kidney Lesion	NEGATIVE	Direct and enrichment samples tested
Yersinia ruckeri PCR	Kidney Lesion	NEGATIVE	
Renibacterium salmoninarum PCR	Kidney	NEGATIVE	
General bacterial pathogens including *Vibrio* species	Kidney Lesion	Light mixed growth was recovered from most samples, however there was no consistent colony morphology between the same sample types or for all samples indicating that there was no significant bacteriology associated with the salmon mortalities.*	LJ slopes were discontinued due to histopathology results showing no sign of Mycobacterium sp.
Viral haemorrhagic septicaemia virus PCR	Kidney Lesion	NEGATIVE	
Infectious hematopoietic necrosis virus PCR	Kidney Lesion	NEGATIVE	
Infectious salmon anaemia PCR	VTM homogenates of kidney and spleen (PO and P1)	NEGATIVE	
Cytopathic viruses	VTM homogenates of kidney and spleen	NEGATIVE	

VTM – viral transport media

*As bacteriology samples received were swabs, there was concern about the survival of *Vibrio* species, so subsequent collection was of actual tissue.

Peer review of histopathology was carried out by Brightwater Consulting Ltd. and agreed with findings above. At this point there was no histopathological evidence for a wide list of aetiologies, including:

- salmonid herpesvirus
- infectious pancreatic necrosis virus
- infectious haematopoietic necrosis virus
- viral haemorrhagic septicaemia virus
- infectious salmon anaemia virus
- erythrocyte inclusion body syndrome
- pancreas disease
- ichthyophonus (or other fungus) infection
- neoplasia

3.2 SUMMARY OF DIAGNOSTIC TESTING CONDUCTED AT AHL ON ACCESSION W12_00369

Pathogen/Test	Sample type	Results	Comment
Aeromonas salmonicida PCR	Kidney Lesion	NEGATIVE	Fish 8-15
Mycoplasma PCR	Kidney, spleen, heart	NEGATIVE	Fish 8-15
General bacterial pathogens including *Vibrio* species	Kidney Lesion	NEGATIVE	Fish 1-15
Viral haemorrhagic septicaemia virus PCR	Kidney Spleen	NEGATIVE	Fish 8-15
Infectious hematopoietic necrosis virus PCR	Kidney Spleen	NEGATIVE	Fish 8-15
Totivirus PCR	Heart	NEGATIVE	Fish 8-15
Reovirus PCR	Heart	NEGATIVE	Fish 8-15
Iridovirus PCR - Megalocytivirus - Ranavirus	Kidney, spleen, heart	NEGATIVE	Fish 8-15
Infectious salmon anaemia PCR	VTM homogenates, P1, heart, kidney, spleen, liver	NEGATIVE	Fish 8-15
Cytopathic viruses	Kidney Spleen	NEGATIVE	Fish 8-15
Electron Microscopy	Heart	no viral particles evident	Fish 1-7

3.3 SUMMARY OF DIAGNOSTIC TESTING CONDUCTED AT AHL ON ACCESSION W12-00699

Pathogen/Test	Sample type	Result
Infectious salmon anaemia virus PCR	Heart gills	NEGATIVE
Cytopathic viruses	Kidney/spleen heart	NEGATIVE

3.4 SUMMARY OF DIAGNOSTIC TESTING CONDUCTED AT AHL ON W12_00700

Pathogen/Test	Sample type	Result
Infectious salmon anaemia virus PCR	Heart gills	NEGATIVE
Cytopathic viruses	Kidney/spleen heart	NEGATIVE

4 Exclusion of Infectious Salmon Anaemia Virus

4.1 ANIMAL HEALTH LABORATORY, WALLACEVILLE

AHL implemented the OIE recommended real time PCR assays.
http://www.oie.int/fileadmin/Home/eng/Health_standards/aahm/2010/2.3.05_ISA%20.pdf

This involved establishing methods for extracting RNA from tissues, designing synthetic positive controls (plasmid with target sequence insert), implementing and optimising assays using AHL equipment and procedures.

A high throughput tissue lyser was purchased to enable processing of tissues for optimal RNA recovery. Once tissues were homogenised using the tissue lyser, the samples were then further processed using the automated X-tractor gene robot as per manufacturer's protocol to recover RNA.

OIE assays implemented target genomic segment 7, genomic segment 7 (European isolates) and genomic segment 8. Assays targeting genomic segment 7 (not European) and genomic segment 8 were used to test samples.

As newer real time PCR enzyme chemistry has become available since the publication of the OIE recommended assays, the assays were validated for use at AHL with enzymes recommended for use with AHL equipment. However, a comparison was made between published primer and probe concentrations and primer and probe concentrations recommended for use with the 'newer' enzyme chemistry. Results indicated comparable levels of detection using a synthetic positive control (linearised plasmid with target sequence insert), indicating that the assay was suitably optimised for use.

AHL routinely uses an upper and lower concentration of DNA/RNA when testing for aquatic animal pathogens, so this approach was adopted for ISAV testing. Each sample was tested using 1 and 4 µL RNA in a 10 µL reaction. The reasoning behind this is to enable detection of low levels of infection as well as clinical cases. AHL has proven this approach when testing for Ostreid herpesvirus.

Extracted RNA was also tested for PCR suitability using 18S and/or elongation factor α, both housekeeping genes.

Once assays were in place, selected samples from W12_00272, W12_00369, W12_00699 and W12_000700 were tested using the real time PCRs for ISAV. **All samples tested negative for ISAV.**

AHL retained histopathology samples, samples for electron microscopy, and tissues at -80°C Gross pathology and histopathology observations indicated no evidence of pathogenic ISAV. Further, heart tissue were examined using an electron microscope and no evidence of viral particles were identified.

At no time were the OIE criteria for confirmation of infection fulfilled.

The Norwegian OIE Reference Laboratory was engaged to test samples.

4.2 NORWEGIAN VETERINARY INSTITUTE, NORWAY

Samples from MPI accession W12-699/700 were collected from NZKS, Pelorus Sound, Marlborough on 14th-15th May 2012 and were dispatched to Norway on 17th May 2012 by World Courier. Samples dispatched included solid pieces of tissue in RNALater (an aqueous, nontoxic tissue storage reagent used to stabilize and protect the integrity of RNA in unfrozen tissue samples, minimizing the need to immediately process or freeze tissue samples in liquid nitrogen) from gill, spleen, heart, liver and kidney for 35 salmon fish. In addition, clarified homogenates of heart and spleen/kidney tissues in fish virus transport media (VTM) were also dispatched. The virus homogenates were in approximately 5 mL volumes and were snap frozen at -80°C for 30 minutes. All samples were transported in chilled box, filled with frozen gel pads, so VTM samples would slowly thaw over time as requested by NVI.

Samples were received at the Norwegian Veterinary Institute, Oslo on 21st May 2012.

All testing performed on heart, spleen and kidney samples returned negative results when nucleic acid extracts were tested on Seg 8 and Seg 7 real-time TaqMan tests (these were the same real time Taqman tests performed at AHL). This resulted in NVI requesting more samples from the earlier part of the investigation (samples from MPI accessions W12_272 and W12-369). The additional testing of samples from earlier time points closer to the peak of the mortality adds assurance. Depending on the circumstances a disease can progress at varying rates through a farm, and collecting samples at different time points may improve chances of detection. The second dispatch to NVI occurred on 24th May 2012 and included VTM samples from both W12_272 and W12_369 plus paraffin fixed tissues. The dispatched samples were received on 29th May 2012.

PCR testing was performed by NVI and their results were reported to AHL on 21/6/2012, one month after receiving the samples. During testing there was one sample that produced a non-negative result on one PCR (W12_369#1), but this result was not able to be reproduced when the test was repeated. This sample was also put into cell culture on ASK-2 cells but no ISAV was detected, as reported by NVI via email on 11 July 2012.

NVI also undertook histopathological analysis. Some evidence of intracellular parasites was apparent. NVI indicated that identification of these parasites may not be straightforward, would take time and significant resourcing. However, in order to understand their significance, identification and determination of prevalence is required.

5 Conclusions and recommendations from epidemiological investigation

The sudden increase mortality did not appear linked to recent movements of fish on to the site, as this had not occurred since November 2011. This was not considered typical of an infectious agent.

A longitudinal study, to monitor mortality and histological lesions across fish classes, pens and sites would be useful to try and identify what these predisposing factors are, including whether the heart pathology is related to mortality or not.

The epidemiology did not appear to point toward a typical infectious agent, but rather to a multi-factorial disease. Further work to understand how it is occurring is required before methods in which it can be prevented and/or controlled can be identified.

5.1 CONCLUSIONS

AHL conducted extensive investigation into the Chinook salmon mortality event using a combination of diagnostic techniques. The AHL investigation was not able to identify a specific causative agent.

The following tests were conducted:

- *Aeromonas salmonicida* PCR
- *Yersinia ruckeri* PCR
- *Renibacterium salmoninarum* PCR
- General aquatic bacterial culture including Vibrio species
- Viral haemorrhagic septicaemia virus PCR
- Infectious hematopoietic necrosis virus PCR
- Infectious salmon anaemia virus PCR
- Mycoplasma PCR
- Iridovirus PCR
- Totivirus PCR
- Reovirus PCR
- Cytopathic viruses
- Histopathology.

All PCR tests produced negative results.

5.2 NEXT GENERATION SEQUENCING

As standard diagnostic techniques had not yielded any obvious results, next generation sequencing (NGS) was employed. Two fish showing heart pathology that were collected during the third sampling on the 15th May were used as candidates for NGS. Next generation sequencing offers the potential to identify the presence of unculturable and/or unknown but important pathogens as well as providing information on the host organism (i.e. king salmon).

Initial data analyses from the Roche GS Junior of the heart tissues did not show any significant similarity with known pathogen sequences in Genbank. Extensive metagenomic analysis will take time and resourcing. Preliminary analysis shows the presence of immune response markers which could have been stimulated by the presence of a pathogen. However,

a range of other factors could also stimulate the presence of immune markers and without evidence of a pathogen the significance of the immune response markers is unclear. It is recommended that further samples closer to the peak mortality undergo NGS analysis using an alternative platform better suited to metagenomic analysis.