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Pathogenicity of the bacterium New Zealand rickettsia-like organism (NZ-RLO2) in Chinook salmon *Oncorhynchus tshawytscha* smolt

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ABSTRACT: Farmed New Zealand Chinook salmon *Oncorhynchus tshawytscha* Walbaum have been found to be infected by rickettsia-like organisms (NZ-RLO). While these Gram-negative intra-cellular bacteria are closely related to *Piscirickettsia salmonis*, a significant pathogen for farmed salmon globally, the pathogenicity of NZ-RLO is unknown. The aim of the present study was to determine if one strain, NZ-RLO2, causes disease in Chinook salmon. Post-smolt salmon were inoculated with NZ-RLO2 by intraperitoneal injection at high, medium and low doses and observed for 30 d. All fish in the high and medium dosed groups died by the end of the study and 63% of the low dose group died within 30 d of inoculation. Necropsy revealed the fish inoculated with NZ-RLO2 had internal multifocal haemorrhages. The most consistent histological finding in fish inoculated with NZ-RLO2 was neutrophilic and necrotizing pancreatitis and steatitis with intra-cytoplasmic organisms often visible within areas of inflammation. Other histological lesions included multifocal hepatic necrosis, haematopoietic cell necrosis and splenic and renal lymphoid depletion. The presence of NZ-RLO2 within the inoculated fish was confirmed by replication in cell culture and qPCR. The results suggest NZ-RLO2 can cause disease in Chinook salmon and therefore could be a significant pathogen in farmed Chinook salmon.

KEY WORDS: Chinook · Rickettsia-like · *Piscirickettsia salmonis* · Histopathology · qPCR · Culture

1. INTRODUCTION

Chinook, or king salmon *Oncorhynchus tshawytscha* have been farmed in New Zealand since the 1980s after first being introduced from North America in the late 1800s (McDowall 1994). Chinook salmon are currently the only salmonid species farmed in New Zealand. As the majority of farmed salmon worldwide are Atlantic salmon *Salmo salar*, there has been little published research on disease in Chinook salmon under commercial conditions.

New Zealand Chinook salmon are considered to be relatively free of disease (Anderson 1996), with several major salmonid pathogens such as infectious salmon anaemia virus, viral haemorrhagic septicaemia virus, infectious haematopoietic necrosis virus, salmonid alphavirus and *Renibacterium salmoninarum* exotic to New Zealand (Diggles 2016). However, since 2012 higher than expected mortalities have occurred at some farmed sites (Norman et al. 2013). As these have occurred in the summer months they have been termed 'summer mortalities'. In 2015 during a summer mortality event, an investigation was carried

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out which led to the identification of 2 rickettsia-like organisms (RLOs) in farmed Chinook salmon that were subsequently named NZ-RLO1 and NZ-RLO2 (Brosnahan et al. 2017, Gias et al. 2018). These organisms are closely related to *Piscirickettsia salmonis*, a major cause of disease in salmon farms throughout the world, particularly in Chile where it is the most common infectious disease in farmed salmonids (Rozas & Enriquez 2014, Price et al. 2017). The pathogenicity of RLOs is dependent on the strain, host and the environment (House et al. 1999). The pathogenicity of *P. salmonis* and some closely related RLO strains have been evaluated in Atlantic salmon (House et al. 1999, Garćces et al. 1991, Smith et al. 2004, Morrison et al. 2016); however, the pathogenicity of the newly identified NZ-RLOs have not yet been evaluated in any fish species, including Chinook salmon.

The aim of the present study was to investigate the pathogenicity of NZ-RLO2 in Chinook salmon. We hypothesised that inoculating salmon with this bacterium would result in disease, supporting the notion that NZ-RL02 contributed to the summer mortality events in farmed Chinook salmon.

2. MATERIALS AND METHODS

2.1. Ethics statement

All experimental procedures involving the use of live fish were approved by the AgResearch Grasslands Animal Ethics Committee (Approval number AEC14122) under the New Zealand Animal Welfare Act 1999. Experiments were conducted in a PC2+ transitional containment facility at the Ministry for Primary Industries, Animal Health Laboratory (AHL), Wallaceville, New Zealand.

2.2. NZ-RLO2 isolate origin

Initial identification and isolation of the 2 NZ-RLO strains (NZ-RLO1 and NZ-RLO2) was carried out in an enhanced PC3 laboratory from which viable bacterial isolates are not permited to be removed. In order to determine if the bacteria caused mortality by *in vivo* methods, it was necessary to re-isolate these strains in a lower containment laboratory which was successful for NZ-RLO2 only. NZ-RLO2 was recovered from a skin ulcer of a moribund farmed Chinook salmon of harvest size (~3 kg) originating from the South Island of New Zealand as previously described (Gias et al. 2018).

The NZ-RLO2 isolate was cultured in a monolayer of epithelioma papulosum cyprini (EPC; ECACC-93120820) cell line grown in Hank's minimal essential medium (MEM; Gibco, Life Technologies) supplemented with 10% foetal bovine serum (FBS; HyClone) (MEM + 10% FBS) and passage number 3 was used for inoculation. The bacteria were incubated for 7 d at 15°C until approximately 85% cytopathic effect (CPE) was observed. The remaining monolayer was lifted by cell scraping and a titration was performed to determine the tissue culture infectious dose in 50% of the cells inoculated ($TCID_{50}$) following the Spearman-Karber method (Spearman 1908, Kärber 1931). The concentration of the inoculum administered to fish in the high dose group was calculated to be 8×10^5 $TCID_{50}$ per 50 µl. This was then diluted 1/10 and 1/100 with MEM + 10% FBS to obtain the inoculums administered to the fish in the medium (8×10^4 $TCID_{50}$ per 50 µl) and low (8×10^3 $TCID_{50}$ per 50 µl) doses.

2.3. Fish used in trial and tank setup

Chinook salmon smolt (n = 128) were obtained from a commercial freshwater hatchery and conditioned from freshwater to seawater immediately prior to the study. These fish were approximately 185 d post-hatch and had an average (±SD) size of 87 ± 18 g. To confirm the fish were naïve to NZ-RLOs, smolt (n = 20) were randomly selected and euthanized by iso-eugenol (AQUI-S) at a rate of 175 mg l^{-1} × 20 min. Fish were necropsied and the kidney, liver and spleen were aseptically removed and screened for the presence of NZ-RLOs using a generic quantitative PCR (qPCR) (Corbeil et al. 2003). These fish were also examined for any other pathogens using histology.

The remaining fish were divided between 18 individual 100 l tanks containing aerated artificial seawater, 33 ppm (salt) (AquaOne), at 15 ± 1°C. Fish were provided with a 12 h light:12 h dark photoperiod. Each tank possessed independent mechanical sponge filtration, chemical filtration and biological filtration and were held at a stocking density of approximately 5 kg m^{-3} (6 fish $tank^{-1}$). Fish were acclimated to these tank conditions for 2 wk before being inoculated with NZ-RLO2. Throughout the acclimation and experiment, fish were fed once per day (Skretting; 51% protein, 21% lipid, 3 mm pellets) with any uneaten food being removed from each tank daily.

2.4. Challenge with NZ-RLO2

The number of fish used in each of the treatments was based on sample size calculations. These were performed based on conservative predictions that were lower than the mortality of reported overseas strains of RLO (Birkbeck et al. 2004, Morrison et al. 2016, Valenzuela-Miranda & Gallardo-Escárate 2016). These predictions were that fish infected with a high, medium and low dose of NZ-RLO2 would have mortalities of at least 70, 50 and 30% respectively by the end of the 30 d trial. The predicted values balanced the need to reduce the number of animals used and to ensure suitable power to detect statistically significant differences between the inoculated groups when compared to the controls. However, these numbers did not allow for a statistically significant difference to be detected between the dosed groups.

All fish were sedated using iso-eugenol (AQUI-S) at 25 mg l^{-1} and tagged (VI alpha tags, Northwest marine tech) while under sedation. All injections were administered as an intraperitoneal (i.p.) injection with 23 gauge × 5 mm needles (Eurovet). All fish, control and challenged, were kept at a density of 6 $tank^{-1}$. Control fish (n = 60) were subdivided evenly into 2 groups (i.p. and no-i.p. control fish) with the i.p. control fish receiving a 0.1 ml i.p. injection of cell culture media (MEM + 10% FBS). Based on sample size calculations, the number of tanks $dose^{-1}$ were 1, 2 and 5 for the high, medium and low dosed group respectively with fish receiving a 0.1 ml i.p. injection of the required dose of NZ-RLO2.

During the experiment, fish were checked at least 3 times d^{-1} with mortalities being removed immediately. Fish showing overt signs of disease such as darkening of skin, loss of equilibrium or not responding to stimuli were euthanized using iso-eugenol (AQUI-S). Euthanized fish and mortalities were processed for diagnostic testing including necropsy, blood smears, histology, cell culture and molecular tests. All fish were euthanized at the end of the experiment at 30 d post infection (dpi) and processed as above.

2.5. Pathology

2.5.1. Necropsy and gross pathology

Following euthanasia or death, each fish was measured (fork length), weighed and visually assessed for any abnormalities. A blood sample was taken from each fish by caudal venous puncture. A blood smear was created and stained with modified Giemsa stain (Sigma) for 30 min prior to microscopic examination for the presence of NZ-RLO2.

2.5.2. Histopathological examination

Samples were taken from the gills, skin/skeletal muscle, heart, liver, pyloric caeca, spleen, anterior and mid-kidney, brain and mid-intestine from all fish and fixed in 10% buffered formalin for at least 1d. Samples were then transferred to 70% ethanol prior to being embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E) for histological examination.

2.6. Recovery of NZ-RLO2 from fish tissue in cell culture

A representative number of fish were sampled for cell culture from each dose; high (n = 6), medium (n = 8), low (n = 27) and control groups (n = 6). Kidney, spleen and liver were aseptically removed from each fish and combined into a sterile vial (~500 mg in total). These samples were diluted 1/10 in MEM + 10% FBS with the addition of penicillin (100 µg ml^{-1}), homogenised then diluted further to 1/100 and 1/1000 in MEM + 10% FBS. An aliquot (100 µl) of each dilution was inoculated into separate wells of a 24 well plate seeded with a monolayer of EPC. Following adsorption for 30 min at room temperature, 1 ml of MEM + 10% FBS was carefully added to each well and cultures were incubated at 15°C for 14 d. Cultures were observed under light microscopy for the presence of CPE. Cultures displaying no CPE after 14 d were then passaged once by transferring 100 µl of the first culture into new wells of a 24 well plate seeded with EPC cells at the same cell rate and re-incubated for a total of 28 d.

DNA was extracted from 2 samples at each dose displaying CPE; high, medium and low, to confirm replication of NZ-RLO2 in the cell culture. For each of these samples, an aliquot of 200 µl of the culture showing CPE after 14 d (P_1) and from the initial homogenate (P_0) was taken. DNA was then extracted and subjected to NZ-RLO2 qPCR as described below. If the CPE was due to replication of NZ-RLO2 a decrease in the cycle threshold (C_T) value was expected in the P_1 samples compared to that in the P_0 samples.

2.7. Molecular tests

2.7.1. DNA extraction from tissues

Fish sampled on arrival. Following euthanasia, ~200 mg of the kidney, liver and spleen was aseptically removed from each fish and combined into a MagNA lyser green bead tube (Roche). Phosphate buffered saline (PBS) was then added (500 µl) and the tissue was homogenised in the MagNA lyser (Roche) at 6500 rpm for 30 s. A subsample of this homogenate (80 µl) was then used for DNA extraction using the QIAamp mini kit (Qiagen) as per the manufacturer's protocol.

Experimental fish. Following euthanasia, individual samples of approximately 20 mg of each kidney, liver, digestive tract (mid gut), skin ulcer (when present) and 10 mg of spleen were aseptically collected from each fish. DNA was extracted from each tissue sample separately on the QIAcube high throughput automated extraction machine using the QIAamp HT kit as per the manufacturer's protocol (Qiagen).

All fish. Extracted DNA was assessed for suitability in qPCR by performing an internal control 18S rRNA qPCR following the manufacturer's protocol (Life technologies; Ribosomal 18S rRNA Endogenous Control).

2.7.2. Generic NZ-RLO qPCR to detect NZ-RLO in fish on arrival to the facility

The presence of NZ-RLO DNA in these fish was evaluated using a previously published qPCR targeting the 23S rRNA gene of *Piscirickettsia salmonis* (Corbeil et al. 2003). Per reaction, ~150 ng genomic DNA (i.e. 2 µl) was used and all samples were tested in duplicate. DNA extracted from a pure cell culture of NZ-RLO2 was run as a positive control and molecular grade water was used as a no template control to assess environmental contamination with each qPCR.

2.7.3. Specific qPCR to detect NZ-RLO2 in experimental fish

DNA extracted from samples of kidney, liver, spleen and digestive tract from all 108 experimental fish were analysed by qPCR for the presence of NZ-RLO2 (n = 432) including skin ulcers where present (n = 12).

The NZ-RLO2 qPCR was performed as previously described (Gias et al. 2018). Briefly, each reaction consisted of 2 µl of DNA (~150 ng genomic DNA), 10 µl 2× Bio-rad SsoAdvanced Universal Probes Supermix, 0.5 µM of each primer, 0.2 µM of probe and water to a final volume of 20 µl. The cycling conditions were as follows: initial denaturation at 95°C for 2 min, 50 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s.

2.8. Statistical analysis

Statistical analysis was performed in R v.3.5.2 (R Core Team 2015). The weight and lengths of fish were compared using a 1-way ANOVA to examine differences between the control and dosed fish. A generalised linear model (GLM) with weight or lengths of fish as the response variable and treatment type (dosed and control) as the explanatory variable was fitted to the data with a Gaussian distribution (R package 'multcomp'; Hothorn et al. 2008).

Cumulative mortality rates of each dosed fish group were compared to both control groups in a GLM with a binomial error distribution. The 2 control groups were also compared using this model. Specific pair-wise differences between the groups were tested using Tukey contrasts and p-values were adjusted using the Benjamini & Hochberg method (R package 'multcomp'; Hothorn et al. 2008). This analysis was carried out to compare mortalities between the control and dosed groups from 1 to 22 dpi and 1 to 30 dpi respectively. For the low dose group, analyses were also carried out to compare mortalities to the control groups from 23 to 30 dpi.

Two analyses of C_T values from the NZ-RLO2 qPCR were carried out: (1) C_T values detected from all organs (kidney, liver, spleen and digestive tract) were compared between the inoculated groups (high, medium and low); and (2) C_T values detected from all organs were compared within the inoculated groups. Analysis 1 was carried out to determine if the load of bacteria detected among the 3 inoculated groups differed significantly. Analysis 2 was carried out to determine if any organ within each dosed group had a significantly different load of bacteria. The response variable in the model was the C_T value, which was log transformed to meet the assumptions of normality. Analysis of specific pair-wise differences between groups was carried out as above.

The significance of the explanatory variables in all models was assessed using likelihood ratio tests; p-values < 0.05 were considered statistically significant.

3. RESULTS

3.1. Cumulative mortality

Death of fish injected with a high dose of NZ-RLO2 first occurred at 6 dpi and all fish had died by 8 dpi. Fish infected with a medium dose first died at 7 dpi and all fish had died by 23 dpi. The first deaths of fish infected with a low dose occurred at 11 dpi and reached 63% by the end of the 30 d trial. By the end of the trial, the cumulative mortalities of the fish in the control groups, no-i.p. and i.p. reached 17 and 10% respectively. At 23 dpi, a mechanical failure occurred in the laboratory air conditioning unit which resulted in a 2°C rise in water temperature for a 24 h period. A spike in mortality in all groups followed this temperature increase (Fig. 1).

The mortality of fish in the high dose group was significantly higher than the mortality of both control groups at 22 dpi (χ^2 = 11.64, $p < 0.01$) and 30 dpi (χ^2 = 7.98, p = 0.02). The Tukey contrast test showed these differences were significant between the high dose and both control groups ($p < 0.01$). Mortalities of fish in the medium dose group were significantly higher than in both control groups at 22 and 30 dpi respectively (χ^2 = 8.85, p = 0.01, χ^2 = 6.77, p = 0.03). The Tukey contrast test showed these differences were significant between the high dose and control groups ($p < 0.01$). The mortality rates in the low dose group were compared to the controls at 22 and 30 dpi as well as comparing the difference in low dose mortalities to the controls from the temperature spike onwards (23–30 dpi). At 22 dpi there were no significant differences (χ^2 = 1.11, p = 0.57) between the low dose group and the control groups. At 30 dpi the differences were not significant when using the Tukey test (p = 0.06). When comparing the low dose group to the controls from the temperature spike onwards (23–30 dpi) there was a significant difference (χ^2 = 8.3, p = 0.02) and the Tukey contrast test showed the differences observed were between the low dose and both control groups (p = 0.02).

There were no significant differences in cumulative mortalities between the 2 control groups (p = 1).

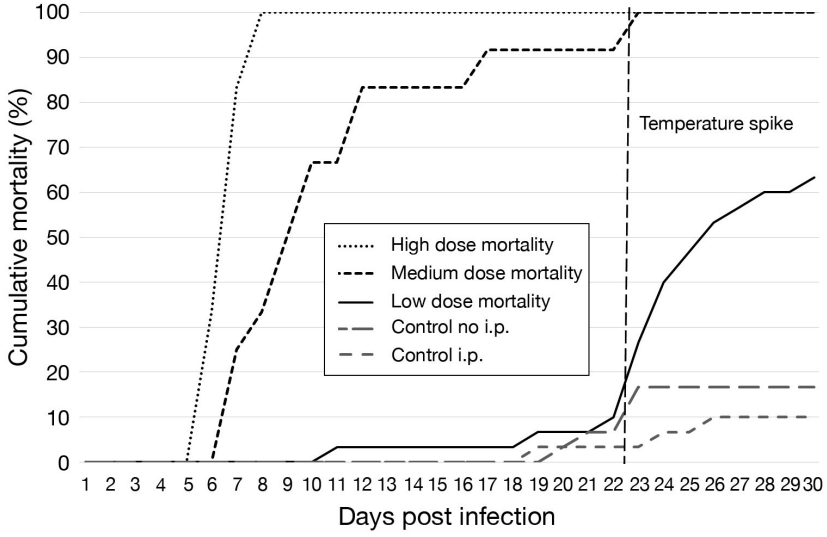

Fig. 1. Cumulative mortalities of Chinook salmon inoculated with high, medium and low dose of New Zealand rickettsia-like organism (NZ-RLO2) and control groups over 30 d. Vertical dashed line: day of temperature increase

3.2. Pathology

3.2.1. Gross pathology

There were no significant differences in average weight (χ^2 = 4.97, p = 0.29) and length (χ^2 = 7.47, p = 0.11) of fish between the experimental groups.

NZ-RLO2 was visible within the leucocytes obtained from the caudal vein (Fig. 2) in 2 fish from each of the high and medium dose groups and from 6 fish in the low dose group.

Necropsy examination of fish in the high dose group revealed 83% had petechial haemorrhage in the adipose tissue around the pyloric caeca (Fig. 3). Other common findings were pale liver (33%) and an increase of clear fluid in the coelomic cavity (33%). No abnormalities were observed in any other organs.

Similarly to fish in the high dose group, petechial haemorrhage in the adipose tissue around the pyloric caeca was the most frequent lesion seen in fish necropsied following exposure to a medium dose of NZ-RLO2 (86%). Additionally, splenomegaly (33%) and pale liver were observed (17%). Ascites was seen in one fish necropsied (8%). No abnormalities were detected in any other organs.

In the low dose group, petechial haemorrhage in the adipose tissue around the pyloric caeca was observed in 47% fish necropsied. Of this 47%, 74% had died during the study with the remainder being euthanized at 30 d. Fish exposed to a

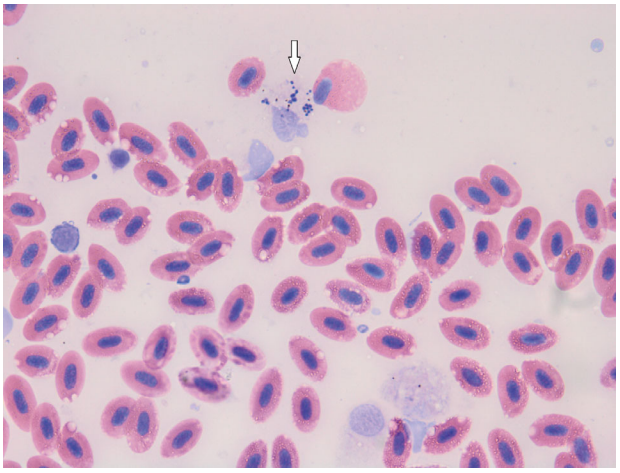

Fig. 2. Giemsa-stained Chinook salmon blood smear showing New Zealand rickettsia-like organism (NZ-RLO2) within a leucocyte (arrow)

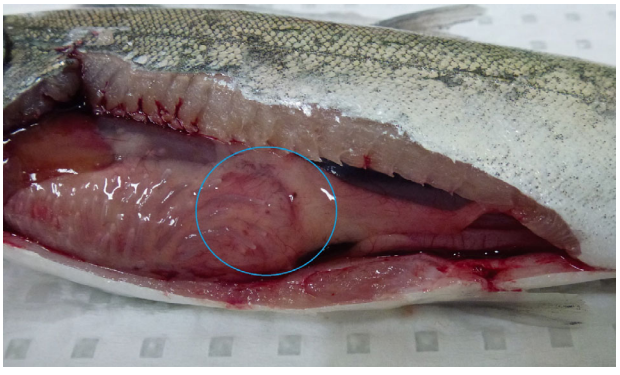

Fig. 3. A common gross finding in Chinook salmon inoculated with New Zealand rickettsia-like organism (NZ-RLO2): petechial haemorrhage in the internal body fat (circle)

low dose of NZ-RLO2 were the only group where skin lesions were observed. This finding was observed in 40% of fish from 23 dpi onwards. Of these fish, 33% were fish that died during the study and 67% were euthanized at 30 d. Skin lesions presented as ulcers approximately 2–3 mm diameter in 9 fish and as lesions with scale loss not extending into the musculature in 3 fish (Figs. 4 & 5). Splenomegaly was observed in 10% of fish, 2 fish that died during the study and one that was euthanized at the end of the study. Pale liver was observed in 7% from fish that died during the study. No abnormalities were detected in any other organs.

Of the control fish that died during the study, 5 had a fluid-filled stomach, 1 had reddening in the hind gut and 2 had no abnormalities detected. Necropsy examination of the control fish that were euthanized at the end of the study did not reveal any significant gross abnormalities.

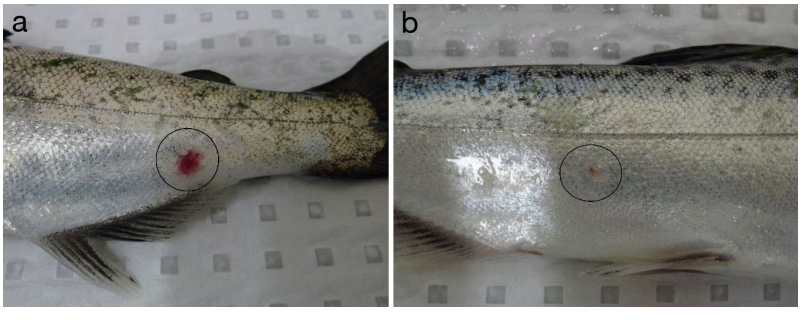

Fig. 4. Examples of skin ulcers (black circles) in Chinook salmon inoculated with a low dose of New Zealand rickettsia-like organism (NZ-RLO2). (a) Reddened ulcer extending into the musculature; (b) ulcer not extending into the musculature. All samples were positive for NZ-RLO2 by qPCR

3.2.2. Histopathology

The most frequent histological lesion was the presence of necrosis and moderate to severe neutrophilic inflammation of the exocrine pancreas and peri-pancreatic adipose tissue. These lesions were observed in 100, 92 and 37% of fish in the high, medium and low dose groups respectively (Fig. 6a). NZ-RLO2 was frequently visible as 0.8 to 1.2 µm basophilic cocci as pairs or clusters within infiltrating inflammatory cells (Fig. 7a–c).

Examination of the liver of fish inoculated with NZ-RLO2 revealed multifocal areas of hepatocellular necrosis (Fig. 7d). These foci appeared as hepatocytes showing nuclear pyknosis and karyorrhexis. The foci of necrosis were associated with mild to moderate predominantly mononuclear infiltrates. Liver necrosis was seen in 83, 67 and 54% of fish in the high, medium and low dose groups respectively. Intracytoplasmic pairs or clusters of rounded basophilic single cells organisms, interpreted as NZ-RLO2, were present associated with the foci of hepatocellular necrosis (Fig. 7e). Examination of the kidneys revealed moderate diffuse individual cell karyorrhexis and pyknosis with apparent depletion of haematopoietic cells. There was occasional or complete loss of tubular epithelium in areas. Renal necrosis was seen in 100, 67 and 33% of fish in the high, medium and low dose respectively. The spleen showed a depletion of both lymphatic and haematopoietic cells which was observed in 100, 83 and 57% of fish in

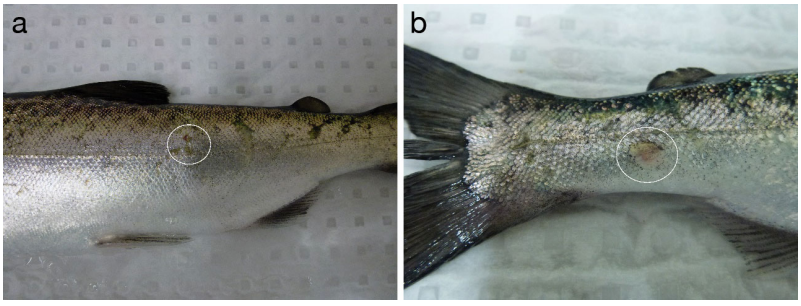

Fig. 5. Examples of scale loss and reddening (white circles) in Chinook salmon inoculated with a low dose of New Zealand rickettsia-like organism (NZ-RLO2). (a) Ulcer not extending into the musculature; (b) skin ulcers extending into the musculature. Both samples were negative for NZ-RLO2 by qPCR

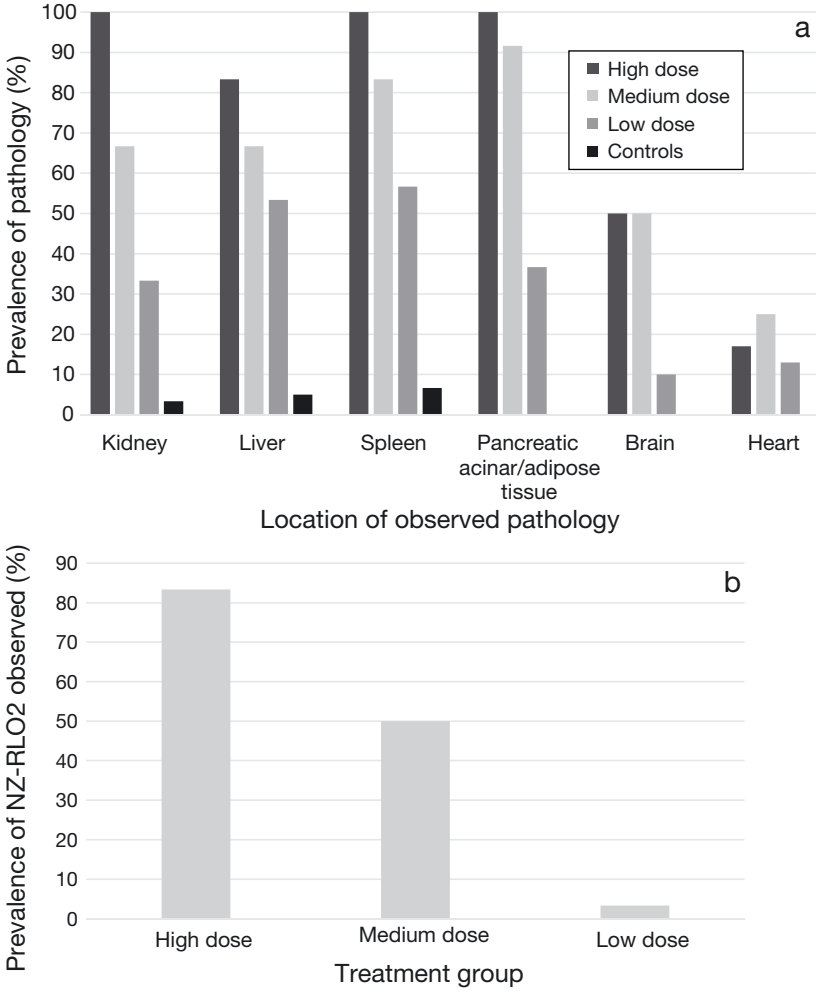

Fig. 6. (a) Prevalence of pathology (%) observed within the tissues of Chinook salmon inoculated with New Zealand rickettsia-like organism (NZ-RLO2) in all treatment groups; (b) percent prevalence of NZ-RLO2 observed within any tissue under histopathology in all dosed groups

the high, medium and low dose group respectively. Pericarditis that was associated with the presence of visible NZ-RLO2 bacteria was observed in 17, 25 and 13% of fish in the high, medium and low groups respectively.

On examination of the brain, congestion in the meningeal blood vessels was observed in 50% of the fish for both high and medium doses and 10% in the low dose. NZ-RLO2 bacteria were not observed in these affected areas.

In the skeletal muscle of inoculated fish, areas of inflammation were often observed underneath the stratum compactum, between the red and the white layers of muscle and within the red layer of muscle sometimes tracking down vessels. Thickening of artery walls with associated inflammation (Fig. 7f) was observed in 33, 50 and 23% of fish exposed to high, medium and low dose, respectively. Inflammation was also noted occasionally in control fish (12%). NZ-RLO2 bacteria were visible in at least one of the tissues in 83, 50 and 3% of fish in the high, medium and low dose group respectively (Fig. 6b).

Significant histological lesions were not observed in sections of the gill and intestine from any of the fish inoculated with NZ-RLO2. No significant lesions were observed in the control groups analysed or the fish initially assessed for general health.

3.3. Recovery of NZ-RLO2 from tissue in cell culture

In cell culture, NZ-RLO2 was recovered from all high dosed fish, 6 of the 8 medium dosed fish and 10 of the 27 low dosed fish tested by the observation of CPE after 14 d incubation (Table 1). None of the 6 control fish tested exhibited CPE in cell culture after 28 d incubation. The presence of replicating NZ-RLO2 in cell culture was confirmed by qPCR with the P_1 material resulting in C_T values ranging from 21.31 to 24.59, equating to $\geq 10^4$ $TCID_{50}$, an increase from the original P_0 material which gave C_T values ranging from 31.90 to 39.03, equating to $\leq 10^2$ $TCID_{50}$ (Table 2).

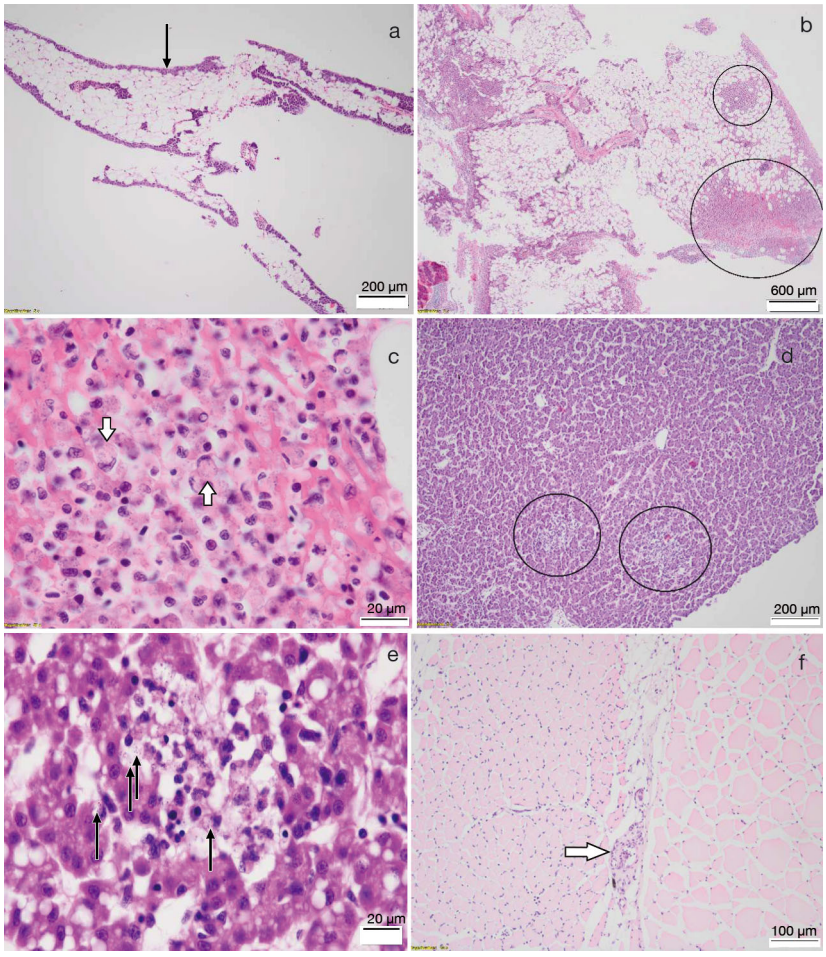

Fig. 7. (a) Histology section stained with H&E displaying normal pancreatic tissue from a control intraperitoneal (i.p.) injection. Chinook salmon with no inflammation (arrows); (b) severe pancreatitis and steatitis from a fish inoculated with New Zealand rickettsia-like organism (NZ-RLO2) with large areas of inflammation (circles); (c) pancreatic tissue with inflammatory cells full of intracellular RLO (basophilic cocci) from a fish infected with NZ-RLO2 (arrows); (d) liver tissue from a fish infected with NZ-RLO2. The liver is displaying multi-focal necrosis (circles); (e) prominent intracellular RLO (basophilic cocci) (arrows) within the areas of focal necrosis in the liver; (f) skin tissue showing thickening of artery walls with associated inflammation (arrow)

3.4. Evaluation of tissues using qPCR

All DNA recovered from tissue samples were shown to be appropriate for PCR by amplification in the internal control 18S rRNA PCR.

Tissue samples from all 20 fish analysed at the beginning of the experiment showed no amplification in the generic NZ-RLO qPCR.

Amplification by qPCR revealed all tissue samples tested from all fish in the high dose group had NZ-RLO2 present. NZ-RLO2 DNA was amplified from at least one tissue from 92% of fish in the medium dose group. In the low dose group, NZ-RLO2 DNA was amplified from at least one tissue of 43% of fish. Of the 12 skin ulcers tested, NZ-RLO2 DNA was amplified from 7 of these using the NZ-RLO2 qPCR (Table 1). The NZ-RLO2 qPCR did not amplify DNA from any tissues from any of the 60 control fish.

There was a significant difference in the C_T values from the NZ-RLO2 qPCR when comparing all organs between each of the dosed groups; high, medium and low (χ^2 = 16.9, $p < 0.01$). Differences were significant between the high and medium dose groups compared to the low dose (both $p < 0.01$). There was no significant difference seen between the medium and high dose groups ($p = 0.34$).

There was no significant difference between the C_T values from the NZ-RLO2 qPCR between any of the organs within each dosed group; high, medium or low (χ^2 = 2.94, $p = 0.4$, $\chi^2 = 2.03$, $p = 0.57$, χ^2 = 3.66, $p = 0.3$).

4. DISCUSSION

Elevated mortalities of Chinook salmon at farmed sites in the Marlborough Sounds have occurred since 2012 (Norman et al. 2013) and 2 strains of NZ-RLO have been identified from fish during these summer mortality events (Brosnahan et al. 2017, 2019). However, the exact relationship between NZ-RLOs and mortalities remain unknown. This study was performed to determine the pathogenicity of NZ-RLO2 in Chinook salmon smolt, and demonstrated that NZ-RLO2 can cause death of inoculated salmon. The mortalities induced by NZ-RLO2 were not unexpected as this bacteria was originally isolated from moribund fish. However, it was considered possible that NZ-RLO2 could either be of low pathogenicity or a secondary pathogen. The results of the present study confirm that NZ-RLO2 can cause death in salmon, and therefore suggest this organism could contribute to summer mortalities.

Table 1. New Zealand rickettsia-like organism (NZ-RLO2) quantitative PCR (qPCR) cycle threshold (C_T) values of all individual Chinook salmon tissues in all treatments; high dose (n = 6), medium dose (n = 12) and low dose (n = 30). **Boldface**: positive growth in cell culture; *italics*: negative growth in cell culture; plain text: sample not tested for cell culture; N: negative qPCR result; na: no lesion present

Dose	Mortality (Mo) or survivor (S)	C_T value				
		Kidney	Liver	Spleen	Digestive tract	Skin ulcer
High	Mo	**26.37**	**28.54**	**26.71**	29.01	na
	Mo	**33.14**	**29.1**	**30.08**	27.81	na
	Mo	**28.63**	**27.59**	**27.26**	26.31	na
	Mo	**28.34**	**29.26**	**29.1**	28.02	na
	Mo	**25.99**	**28.26**	**24.54**	24.31	na
	Mo	**37.87**	**37.65**	**35.69**	41.43	na
Medium	Mo	**34.85**	**32.51**	**32.55**	38.36	na
	Mo	29.18	30.24	29.88	30.08	na
	Mo	**31.41**	**28.99**	**30.23**	30.12	na
	Mo	**34.67**	**31.54**	**32.73**	30.44	na
	Mo	**30.7**	**31.24**	**28.43**	30.41	na
	Mo	45	39.07	38.74	45	na
	Mo	**26.41**	**27.57**	**27.28**	23.89	na
	Mo	29.34	29.38	N	32.71	na
	Mo	N	38.6	N	N	na
	Mo	*N*	*N*	*N*	N	na
	Mo	**36.51**	**N**	**42.98**	N	na
	Mo	*40.76*	*N*	*44.92*	N	na
Low	Mo	N	N	N	N	na
	Mo	*N*	*N*	*N*	N	na
	Mo	*N*	*N*	*N*	N	N
	Mo	*N*	*N*	*N*	N	na
	Mo	**N**	**N**	**35.81**	33.65	32.67
	Mo	*N*	*N*	*N*	39.15	na
	Mo	*N*	*N*	*N*	N	na
	Mo	**N**	**N**	**N**	N	na
	Mo	*N*	*N*	*N*	N	na
	Mo	*39.59*	*N*	*N*	N	na
	Mo	**N**	**N**	**N**	39.76	na
	Mo	*40.62*	*N*	*N*	N	na
	Mo	**N**	**N**	**38.61**	42.25	31.02
	Mo	*N*	*N*	*N*	N	na
	Mo	*42.96*	*43.8*	*40.01*	N	33.25
	Mo	*N*	*N*	*N*	N	N
	S	**37.51**	**41.97**	**40.39**	38.33	38.74
	S	**35.05**	**N**	**N**	N	na
	S	**40.98**	**37.25**	**34.93**	32.85	na
	S	44.04	N	N	N	27.82
	S	*N*	*N*	*37.64*	N	N
	S	*38.39*	*N*	*N*	N	N
	S	*N*	*N*	*N*	N	na
	S	*N*	*N*	*N*	N	na
	S	**N**	**N**	**N**	N	41.65
	S	*N*	*N*	*N*	N	na
	S	**N**	**N**	**N**	N	N
	S	*N*	*N*	*N*	N	na
	S	40.98	N	43.79	N	na
	S	**N**	**40.76**	**40.24**	N	26.7

While the gross lesions found on Chinook salmon during summer mortalities have not been fully defined, a significant proportion of fish from these mortality events have been observed to have reddening within the abdominal fat and skin ulcers. In the present study, fish infected with NZ-RLO2 were observed to have similar reddening and skin ulcers. While many different disease processes can result in these gross lesions, it is possible that NZ-RLO2 was the cause of these lesions observed during the summer mortality event. Additionally, petechial haemorrhage within the abdominal adipose tissue has been described in Atlantic salmon that were experimentally infected with Tasmanian-RLO (Morrison et al. 2016). This suggests this lesion frequently develops due to RLO infection in salmon. However, gross lesions described from Atlantic salmon infected with *Piscirickettsia salmonis*, such as the presence of creamy coloured circular nodules in the liver and swollen kidneys (Fryer & Mauel 1997, Birrell et al. 2003, Rozas & Enriquez 2014), were not observed in the present study nor have we observed them during summer mortality events.

It is possible that infection with NZ-RLO2 caused skin ulcers in the infected fish. Skin ulcers were only observed in fish in the low dose group from 23 dpi onwards following a temperature spike. This suggests that the infection has to be present for a longer time, or an increase in water temperature needs to occur for this clinical sign to develop. Perhaps the longer NZ-RLO2 is present in the fish, the more it is likely to replicate and shed into the water column via faeces or mucus, leading to infection via the skin. The temperature increase may have led to increased replication of NZ-RLO2 but also may have impacted the mucosal barriers of the skin (Jensen et al. 2015), leading to an increased likelihood of infection by bacteria.

NZ-RLO2 was not detected by qPCR in all ulcers. It is therefore possible that these ulcers developed due to secondary infection by a different organism in a fish stressed by NZ-RLO2 and increased temperature. Alternatively, NZ-RLO2

Table 2. New Zealand rickettsia-like organism (NZ-RLO2) quantitative PCR (qPCR) cycle threshold (C_T) values and equivalent tissue culture infectious dose in 50% of the cells inoculated ($TCID_{50}$) of cell cultures with cytopathic effects (CPEs) to confirm growth of NZ-RLO2. P_0: original tissue homogenate; P_1: growth in cell culture material after 14 d incubation

Dose	P_0 C_T	$TCID_{50}$ equivalent	P_1 C_T	$TCID_{50}$ equivalent
High	32.36	10^2	21.40	10^5
High	31.90	10^2	22.02	10^5
Medium	35.51	10^1	21.31	10^5
Medium	39.03	10^1	21.875	10^5
Low	36.96	10^1	23.14	10^5
Low	37.12	10^1	24.59	10^4

could have directly caused the ulcers, but the bacteria was missed within the ulcer due to sampling for DNA extraction, as the entire ulcer was not assessed. In the future, homogenisation of all of the skin ulcer with a sub-sample for DNA extraction may help to eliminate false negative results. Whether or not NZ-RLO2 can cause skin ulcers is important, and the presence of NZ-RLO2 on the skin of fish suggests likely horizontal transmission from close contact with infected fish or from NZ-RLO2 in the water column. Such transmission has been shown to be important for the spread of *P. salmonis* and *P. salmonis*-like organisms in other fish species (Cvitanich et al. 1991, Fryer & Mauel 1997, Smith et al. 1999, Chen et al. 2000). Immersion or cohabitation studies would be valuable to confirm this route of transmission for NZ-RLO2.

Histology of fish from summer mortality events have predominantly revealed focal areas of necrosis in the liver and depletion of haemopoetic tissue in both the kidney and spleen. Similar lesions were observed in fish inoculated with NZ-RLO2 in the present study. However, in the fish deliberately infected by NZ-RLO2, neutrophilic inflammation and necrosis of the exocrine pancreas and peri-pancreatic adipose tissue was most consistently seen. This lesion has not been previously reported in fish from summer mortality events. The differences in the histological lesions could suggest that NZ-RLO2 does not cause summer mortality. Alternatively, the differences in the lesions could simply be due to the use of i.p. injection to experimentally inoculate the fish in the present study. NZ-RLO2 bacteria were observed in the lesions of many of the fish from the high or medium dose groups. This is in contrast to fish from summer mortality events which RLOs are not typically observed. The lack of visible RLOs in the fish from summer mortality events could suggest a different cause of disease. However, as RLOs were rarely seen in fish in the low dose groups, this could be due to the artificially high infectious dose of RLOs given to the fish in the experimental conditions.

In the present study, pairs or clusters of NZ-RLO2 were visible within the inflammatory cells within tissues that showed evidence of necrosis in fish from the high and medium dose groups. The presence of RLOs in association within the lesions adds evidence that these bacteria were causative of the lesions rather than being present incidentally in the tissues.

Interestingly, no RLOs were observed within sections of the brain or heart. This is in contract to infections by RLOs in other fish species in which the brain and heart consistently show histological evidence of infection, often including the presence of visible organisms (Skarmeta et al. 2000). The brain and heart are sites that *P. salmonis* and Tasmanian-RLO have been observed from tissue smears, immunohistochemistry or histology (Cvitanich et al. 1991, McCarthy et al. 2005, Morrison et al. 2016). In the present study, salmon infected with NZ-RLO2 were found to have pericarditis but NZ-RLO2 organisms were rarely observed in association with this lesion. In contrast, changes in the brain tended to be less consistent in the experimentally infected fish and NZ-RLO2 bacteria were not detected in sections of the brain. It is possible that evaluation of these organs by molecular testing, immunohistochemistry or *in situ* hybridization may allow more frequent detection of NZ-RLO2 in these organs. However, brain pathology has been reportedly linked to later stages of disease progression, i.e. >35 dpi (Rozas-Serri et al. 2017), which may account for not observing this in a 30 d trial.

In the present study, histology of the skin and muscle revealed inflammation in a proportion of fish infected with NZ-RLO2. However, as a proportion of sections from control fish showed similar inflammation, it cannot be confirmed that the dermatitis and myositis observed in the fish was due to NZ-RLO2. The fish in this study had been handled as part of the experimental manipulation and it is possible that the histological changes could have been due to handling of the fish.

Intra-cytoplasmic organisms were visible in blood smears from fish experimentally infected with NZ-RLO2. This suggests that NZ-RLO2 is able to spread systemically in the body through the blood. Such dissemination in the body has been well documented in the literature for RLOs in Atlantic salmon and other finfish (Birrell et al. 2003, Morrison et al. 2016, Marcos-López et al. 2017, Rozas-Serri et al. 2017).

NZ-RLO2 was most observed in fish from the high dose group and lowest in fish from the low dose group. In fish infected with a low dose of NZ-RLO2, these organisms were not abundant and if visualisation of organisms is required in these lower infections, either serial sections of tissue or the use of immunohistochemistry or *in situ* hybridisation may be necessary to visualize the NZ-RLO2. The focal nature of the organism within the tissue could also help when understanding the cell culture results in comparison with the qPCR results. In the high and medium dosed fish, organs with lower C_T values all resulted in a positive cell culture result; however, in the low dosed fish, 2 of the 27 samples were cell-culture negative but qPCR positive and 3 of the 27 samples were cell-culture positive but qPCR negative.

The qPCR testing gave an indication of the infection level in the tissues tested. The number of copies of NZ-RLO2 detected in the fish from summer mortality events are similar to the copy numbers detected in fish from the low and medium dosed fish infected in the laboratory with NZ-RLO2 (Ministry for Primary Industries unpubl. data). This comparison further suggests NZ-RLO2 involvement in the summer mortalities. Other diagnostic avenues carried out during the summer mortalities did not reveal other pathogen involvement (Brosnahan et al. 2017).

The results of our study suggest a dose-dependent relationship between NZ-RLO2 and pathogenicity in Chinook salmon and that many fish are likely to survive a low level infection under typical environmental conditions. Statistical analysis carried out on cumulative mortalities gave further confidence to this hypothesis as there was no significant difference in the mortalities when comparing the low dose mortalities with the control fish prior to the unexpected temperature spike.

Interestingly, there was no significant increase in the mortality rate of fish in the low dose group compared to the control group until the water temperature was accidently increased. After the transient 2°C increase in water temperature, the mortality rates of fish in the low dose group were significantly higher than the rates in the controls. This suggests that Chinook salmon could frequently be infected with NZ-RLO2, but this infection does not cause significant mortality at low water temperatures. It is possible, then, that the mortality events that are associated with NZ-RLO2 only develop in summer due to an interaction between increased water temperature and increased pathogenicity of the organism. Increased water temperature is a well-established risk factor for many diseases of aquatic animals and increased water temperature has been associated with outbreaks of disease due to *P. salmonis* (Branson & Nieto Diaz-Munoz 1991, Gaggero et al. 1995, Stene et al. 2014, Rees et al. 2014). Furthermore, NZ-RLO were detected at a higher prevalence in the summer months (Brosnahan et al. 2019). During the summer mortality events, sea temperatures were consistently above 17°C (New Zealand King Salmon unpubl. data) and out of the optimal temperature range for Chinook salmon, which is reported to be between 12 and 17°C. While a combination of NZ-RLO2 infection and warmer waters is an attractive hypothesis to explain the development of summer mortalities in New Zealand Chinook salmon, further controlled studies assessing temperature, as well as other possible stress factors, would need to be carried out to confirm the relationship between environmental stressors and mortality in NZ-RLO2 infected fish.

There were no significant differences in concentration of NZ-RLO2 as assessed by qPCR, between the 4 organs collected for analysis from the inoculated fish. This suggests that the NZ-RLO2 bacteria are able to survive and replicate in multiple organs within the body. This observation is also useful when planning survey strategies to detect this organism in infected fish. A widespread distribution of the organism in the body has also been reported for *P. salmonis* and Tasmanian-RLO (Almendras et al. 2000, Morrison et al. 2016).

Inoculation by i.p. injection was used in the present study to allow the dose of bacteria administered to be controlled. As this is not the natural route of infection, immersion or co-habitation trials should be performed to study the pathogenicity and resulting disease manifestations when infection occurs by this more likely natural transmission route.

The fish used during this study were post-smolt. Fish of this age were used because studies of other RLOs suggest that NZ-RLO2 is likely to be rapidly inactivated in freshwater (Morrison et al. 2016, Fryer & Mauel 1997). If NZ-RLO2 cannot live in freshwater, fish are most likely to be first exposed, and subsequently infected, as they enter seawater at the post-smolt stage. Additionally, smoltification is a particularly stressful event in the production cycle of salmon due to the dramatic physiological and anatomical changes making this age group more susceptible to any further environmental changes and disease outbreaks (Roberts & Pearson 2005). Therefore, using fish that were stressed by smoltification at the time of inoculation would be expected to maximize the chances of inducing disease and mortalities due to inoculation by the bacteria. By carrying out the

experiment in saltwater, the likelihood that additional infections from shedding and re-infection would occur was also maximized. However, exposure of fish of different ages to this pathogen would also be interesting to see if fish of different ages vary in their susceptibility to NZ-RLO2 induced disease.

In conclusion, this study has demonstrated that NZ-RLO2 can cause mortalities in Chinook salmon smolt when administered by i.p. injection in a laboratory environment. This bacterium was recoverable in pure culture from infected fish at all infection doses, fulfilling Koch's postulates for NZ-RLO2. The mortality rate due to inoculation was dose dependent with lesions within the pancreas and liver developed most consistently in infected fish.

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