DISSERTATION

DETECTION AND TRANSMISSION OF *RENIBACTERIUM SALMONINARUM* IN COLORADO INLAND TROUT

Submitted by

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ABSTRACT

DETECTION AND TRANSMISSION OF *RENIBACTERIUM SALMONINARUM* IN COLORADO INLAND TROUT

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD), is known to cause high mortality in both wild and cultured salmonids, causing concern for many of the salmonid populations. Bacterial kidney disease caused up to 80% mortality in cultured Pacific salmonids and 40% in Atlantic salmonids. Due to high mortality among salmonid species, the American Fisheries Society has defined *R. salmoninarum* as a regulated pathogen. Due to its regulated status, research efforts have focused on advancing fish health diagnostics and understanding the transmission of the bacteria. However, many of these studies focus on Pacific northwest salmonids and the understanding of *R. salmoninarum* dynamics is not well known among inland salmonids.

Aquaculture propagation of Greenback Cutthroat Trout (*Oncorhynchus clarkii*) is a necessary component of their management. Since their protection under the Endangered Species Act, broodstock of Greenback Cutthroat Trout have been established at Colorado Parks and Wildlife (CPW) hatcheries to allow more rapid reintroduction through stocking. In 2017, hatcheries rearing isolated strains of the Greenback Cutthroat Trout contributed 1.5 million eggs during the spawning season. However, one major constraint to maintaining spawning production of the Greenback is the spread of disease within a facility. Increased contact rates between fish in raceways may influence the transmission of a pathogen. To ensure fish health and promote best

practices in fish culture, fish health inspections have served as a critical step in identifying prohibitive and regulated pathogens entering or exiting the hatchery systems.

Various diagnostic methods have been established to detect *R. salmoninarum* in salmonids. Culturing the bacteria is the most accurate and reliable assay for detection; however, it is a slow process and not suited for rapid assessment. Other methods used to detect *R. salmoninarum* include Direct Fluorescent Antibody Tests (DFAT), Polymerase Chain Reaction (PCR), and Enzyme-Linked Immunosorbent Assays (ELISA) and are typically performed using lethally collected kidney tissue. Currently, kidney tissues are used to screen for the presence of the bacteria using DFAT as the initial test and PCR as a confirmatory test, following the American Fisheries Fish (AFS) Health Blue Book protocol. The protocol was developed using highly susceptible Pacific northwest salmonids and it is unknown if the protocol is appropriate for testing inland salmonids which may be less susceptible. In addition, the current protocol requires sacrificing fish, which is undesirable in situations with valuable and sometimes irreplaceable broodstocks. Therefore, I examined the efficacy of the current AFS detection protocol and compared it to other potential approaches (Chapter 2). I also assessed several non-lethal approaches to detecting the bacteria (Chapter 1 and 2).

In chapter 1, I compared non-lethal sampling methods with standardized lethal kidney tissue sampling that is used to detect *R. salmoninarum* infections in salmonids. I collected anal, buccal, and mucus swabs (non-lethal qPCR) and kidney tissue samples (lethal DFAT) from 72 adult Brook Trout (*Salvelinus fontinalis*) reared at the Colorado Parks and Wildlife Pitkin Brood Unit and tested each sample to assess *R. salmoninarum* infections. Brook Trout were used as a model species for Cutthroat Trout because they are described as highly susceptible species. Standard kidney tissue detected *R. salmoninarum* 1.59 times more often than mucus swabs,

compared to 10.43 and 13.16 times more often than buccal or anal swabs, respectively, indicating mucus swabs were the most effective and may be a useful non-lethal method. My study highlights the potential of non-lethal mucus swabs to sample for *R. salmoninarum* and suggests future studies are needed to refine this technique for use in aquaculture facilities and wild populations of inland salmonids.

In chapter 2, I assessed the probability of detecting the bacteria in several tissues using standard diagnostic tests. I collected three lethal tissue (kidney, liver, and spleen) and three nonlethal serum (blood, ovarian fluid, and mucus swabs) samples from 781 adult Greenback Cutthroat Trout at the Colorado Parks and Wildlife Poudre Rearing Unit. All tissues were tested for *R. salmoninarum* via DFAT and qPCR. The overall prevalence (all tissue types) of *R. salmoninarum* among the fish was 22.7% with DFAT and 81.8% with qPCR. Kidney and liver tissues resulted in the greatest number of detections using either assay. To calculate the probability of infection among kidney and liver tissues and probability of detection between assays, I developed a hierarchical occupancy model. The liver had the highest probability of infection among all fish (0.69) and the probability of detection within the liver was highest with qPCR (0.79). DFAT produced a high probability of false negative detections (0.58). Thus, I suggest that testing a combination of both kidney and liver tissues with qPCR may yield a higher detection rate that better predicts the probability of infection when performing fish health inspections.

Management of *R. salmoninarum* is particularly difficult because the bacterium utilizes both vertical and horizontal transmission. Vertical transmission occurs when infected brood fish transmit the bacterium to their eggs and ultimate their progeny. Previous studies suggest the bacterium cannot be paternally transmitted due to limited success of bacterial entry into the egg

from the spermatozoa. Thus, vertical transmission is suggested to be maternal. Horizontal transmission occurs among individuals through the ingestion of contaminated fecal matter or through direct contact with infected fish or water. In previous studies, horizontal transmission has been suggested to contribute more toward infection persistence than vertical transmission in wild and hatchery fish populations. However, the relative importance of horizontal transmission in hatcheries, where flow-through systems may expose multiple fish lots, has received little attention. I conducted experiments to determine rates of vertical and horizontal transmission.

In chapter 3, I examined the potential for horizontal transmission among hatchery-reared brood fish at an *R. salmoninarum*-positive hatchery facility. Juvenile Cutthroat Trout were placed in sentinel cages near positive adult Rainbow Trout and Cutthroat Trout for three, 30-day periods during optimal temperatures for infection. After exposure, the caged Cutthroat Trout were euthanized, and kidney tissue was tested for *R. salmoninarum* with qPCR. Only one out of 360 potentially exposed fish tested positive. My data suggest that horizontal transmission may play a small role in maintaining infection in hatchery-reared inland trout. However, I also show that horizontal transmission can occur in a short time, an important consideration when moving fish both within a hatchery or from one unit to another.

In chapter 4, I assessed whether the bacterium is vertically transmitted in Cutthroat Trout from the Poudre Rearing Unit in Colorado and the rate of transmission from paternal and maternal brood fish. Adult brood fish were lethally tested for *R. salmoninarum* and stripped of gametes to create 32 families among four *R. salmoninarum* infection treatments (MNFN, MNFP, MPFN, MPFP; M: male, F: female, P: positive, N: negative). Progeny from each spawning treatment were sampled at 6- and 12-month post swim-up to test for the presence of *R. salmoninarum* with an enzyme-linked immunosorbent assay (ELISA) and quantitative

polymerase chain reaction (qPCR). My study indicates that vertical transmission occurs in inland Cutthroat trout and transmission is high when examined at the family level but is low within a family. These results suggest that hatcheries should limit vertical transmission through practices such as lethal culling, but also that adopting other methods such as testing eggs for *R. salmoninarum* should be considered in the future.

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I am with you and will watch over you wherever you go

Genesis 28:15

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CHAPTER 1

EVIDENCE FOR THE USE OF MUCUS SWABS TO DETECT *RENIBACTERIUM SALMONINARUM* IN BROOK TROUT

1.1 Introduction

Disease outbreaks disrupt fish production efforts by reducing the number of fish cultured, the number available for stocking into the wild or delivered for consumption, and the ability to move fish among hatcheries or from the hatchery to wild populations. To reduce disease outbreaks at aquaculture facilities, it is crucial to monitor fish health and to detect the presence of regulated, virulent pathogens. Many of the current American Fisheries Society Fish Health Blue Book (AFS-FHS 2016) protocols to detect pathogens require lethal sampling. However, lethal sampling may be undesirable with valuable or rare broodstocks and developing non-lethal diagnostic techniques that allow for consistent detection of pathogens is a high priority. Detection of fish pathogens often entails euthanizing a proportion of the target population to collect organ tissues from an adequate number of hosts and determine the pathogen prevalence within the population. Dependent upon the population or lot size in a rearing facility, a large proportion of fish may need to be tested to estimate the pathogen's prevalence with a high degree of confidence (Ossiander & Wedemeyer 1973; Richards et al. 2017).

The development of non-lethal techniques may reduce the need to euthanize fish and be especially valuable for assessing the presence or absence of a pathogen among populations of sensitive or valuable species that cannot be lethally sampled (Powell et al. 2005). Non-lethal

methods may also allow the testing of more individuals than would be possible with lethal methods, thereby increasing the likelihood that a pathogen is detected. Infection dynamics can also be studied using non-lethal methods through repeated testing over time (Cornwell et al. 2013). For instance, diagnosis of infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) in Rainbow Trout (*Oncorhynchus mykiss*) previously required euthanizing fish, but researchers demonstrated the ability to diagnose and track infection status over multiple testing periods using fin clips from the same host (Bowers et al. 2008; Cornwell et al. 2013). Furthermore, surveillance of *Aeromonas salmonicida* in hatchery stocks of Atlantic Salmon (*Salmo salar*) utilized non-lethal mucus swabs for early detection of *A. salmonicida* leading to proper treatment prior to stocking (Cipriano et al. 1996). The development of more non-lethal detection methods for regulated fish pathogens may substantially benefit surveillance and management in cultured and wild fish populations.

Renibacterium salmoninarum, the bacterial pathogen that causes bacterial kidney disease, is a concern in salmonid populations. The bacteria can cause significant pathological effects among infected fishes. However, more often it exists sub-clinically and present no clinical signs of disease, making it difficult to observe signs of illness (Evenden et al. 1993). Bacterial kidney disease may also cause high mortalities among salmonids at all life stages, albeit with varying susceptibility and overall *R. salmoninarum* prevalence among species (Mitchum et al. 1979). Inland salmonid populations appear to exhibit a higher resistance to disease caused by *R. salmoninarum* infections than many species in the Pacific Northwest, including Chinook Salmon (*Oncorhynchus tshawytscha*) and Coho Salmon (*Oncorhynchus kisutch*; Starliper et al. 1997; Jones et al. 2007; Elliott et al. 2014), and as such, many *R. salmoninarum* studies have been

focused on anadromous salmonid populations. Consequently, non-lethal sampling methods to detect *R. salmoninarum* have not been implemented for use in inland salmonids.

In this study, I evaluated Brook Trout (*Salvelinus fontinalis*) collected from the Colorado Parks and Wildlife (CPW) Pitkin Brood Unit (Pitkin, Colorado, USA) to address two primary objectives: (1) to determine if non-lethal and standard lethal sampling methods gave similar predictions of *R. salmoninarum* presence, and (2) to determine which non-lethal sampling method had the highest rate of predicting infection status when the infection status is known using standard lethal diagnostic techniques. Specifically, I collected and evaluated bacterial presence using kidney tissue and compared those results to bacterial presence using non-lethal anal, buccal, and mucus swabs from 72 adult brook trout.

1.2 Methods

1.2.1 *Fish and Tissue Collection*

I sampled 72 brook trout from the CPW Pitkin Brood Unit on 3 May 2017. Average spring water temperature where the fish were located on the unit was 5.3 °C. Fish were intentionally selected from a lot of adult brook trout with an ongoing *R. salmoninarum* infection. Size and sex of fish were not recorded because this was an opportunistic collection. Three non-lethal samples and one lethal sample were collected from each fish. Non-lethal samples included individual swabs of anal and buccal areas as well as swabbing of the lateral line on both sides of the fish for mucus collection. Swabs were collected by firmly running a 2 mm-diameter, sterile, cotton-tipped applicator along each of the three surfaces ten times, depositing each swab into individual, sterile, 4-mL collection tubes, and placing them on dry ice. Following swab collection, fish were euthanized through immersion in tricaine methanesulfonate (MS-222; Syndel) for 10 to 15 min. Lethal samples consisted of whole kidney tissue collected through an

abdominal incision and placed into sterile Whirl-Pak bags on dry ice for transport. All samples were maintained at −20 °C until processed.

1.2.2 *Laboratory Analysis*

Kidney tissue samples were prepared for direct fluorescent antibody test (DFAT) and single-round PCR analyses as part of CPW's fish health inspection protocol; qPCR is not used during CPW health inspections. In addition, current standard techniques (AFS-FHS 2016) state that DFAT can only be used on kidney tissue and not to evaluate non-lethal swabs. Extractions of DNA from each kidney sample (approximately 0.25 g) were completed using the Qiagen DNeasy Blood and Tissue Kit protocol (Hilden, Germany) with known positive and negative control tissue samples. Single-round PCR was used to determine the presence of *R. salmoninarum* DNA in kidney tissues, with Forward 5′-TTTGGGGTGGCTCCTCTTGCG-3′, PM14, and Reverse 5′-ATTGGGGATGGCGCATTATCG-3′, PM15 primers targeting the major soluble antigen gene (*msa*; P57 protein) for amplification, and visual confirmation of band formation of the 377 base pair product (Fetherman et al. 2020). Kidney tissues were prepared for DFAT by making tissue imprints from each fish on a 12-well slide. Slides were stained utilizing a *Fluorescein*-labeled, affinity purified polyclonal antibody to *R. salmoninarum* (KPL; Milford, MA, USA) with eriochrome black T counterstain (Pascho et al. 1991). Slides were examined at 500 times magnification with a fluorescein isothiocyanate (FITC) fluorescent lamp at a wavelength of 400 nm. Tissue imprints showing visible fluorescent cells were further examined at 1000 times magnification to confirm identification through cell morphology and size (AFS-FHS 2016).

Anal, buccal, and mucus swabs were prepared for analysis via high throughput, real-time qPCR. DNA extraction was similarly completed using Qiagen DNeasy Blood and Tissue Kit

protocols. Additionally, I followed the Qiagen protocol for Gram-positive bacterial swabs, and the DNA elution step was increased with 200 µL of AE buffer (Elliott et al. 2013). I established standard curves for quantification by creating ten-fold serial dilutions of *R. salmoninarum* from pure bacterial culture grown in KDM2 broth at 15 °C for 9 days. The positive controls ranged from 1.1×10^5 to 1.1×10 bacterial cells. The qPCR cut-off Cq value was determined to be 37.75 (Chapter 2), which is considered an acceptable value (Sandell & Jacobsen 2011). Quantitative PCR was performed using ABI StepOnePlus System (Applied Biosystems, Foster City, CA, USA) to detect the *msa* gene in a final volume of 25 µL of DNA template and 12.5 µL of TaqMan Gene Expression Master Mix with primer sets RS 1238 Forward, 5′-GTGACCAACACCCAGATATCCA-3′, and RS 1307 Reverse, 5′-TCGCCAGACCACCATTTACC-3′, and MGB probe 1262, 5′-CACCAGATGGAGCAAC-3′ (Chase et al. 2006).

1.2.3 *Data Analysis*

I used a generalized linear mixed model (GLMM) to analyze pathogen detection (Bolker et al. 2009). The presence of *R. salmoninarum* was treated as a continuous, binomially distributed response variable in a GLMM with a logit link, using the glmer function in the lme4 package in R to perform multiple pairwise comparisons (Bates et al. 2015).

Detections of *R. salmoninarum* can differ among assays (Sandell & Jacobson 2011), including the assays used in this study (PCR, qPCR, and DFAT). I first compared the ability of each assay to predict the presence of *R. salmoninarum* in my samples. I characterized a positive assay result as a binomial response and each diagnostic assay type as a predictor (PCR, qPCR, and DFAT). Individual fish were included as a random intercept term to account for the repeated, non-independent observations on each fish (three assay types per fish: n = 216 observations from

72 Brook Trout). I report these findings as probabilities of detection, odds ratio contrasts obtained from the emmeans package, standard error (SE), and *z*- and *p*-values ($\alpha = 0.05$) with a Tukey adjustment for small sample size.

I compared the utility of each tissue in predicting the presence of *R. salmoninarum*. As predictor variables, I included kidney tissue and anal, buccal, and mucus swabs, with individual fish included as a random effect. All four tissues were tested from each fish, resulting in 288 observations from 72 brook trout. I report the probability of detection, odds ratios, SE, and *z*- and *p*-values ($\alpha = 0.05$) for each tissue.

A GLMM was also used to evaluate which non-lethal sampling method(s) best predicted an infection when the kidney tissue was positive by DFAT. The presence of *R. salmoninarum* was the response variable, as determined by positive kidney tissues, and anal, buccal, and mucus swabs were included as predictors. Individual fish were a random intercept term to account for the repeated and non-independent observations on each fish (three tissues tested per fish; n = 141 observations from 47 *R. salmoninarum*-positive Brook Trout). Similar to the analyses above, odds ratios and estimated detection probabilities are reported and used to assess the capacity of a non-lethal tissue to predict a known positive infection status.

1.3 Results

1.3.1 *Assay Performance*

Among the 72 brook trout collected from the CPW Pitkin Brood Unit, 21 were positive with single-round PCR (PCR), 47 were positive with direct fluorescent antibody test (DFAT), and 50 were positive using quantitative PCR (qPCR). The overall estimated detection probabilities for PCR, DFAT, and qPCR were 0.24, 0.70, and 0.74, respectively. Odds ratios indicated that DFAT predicted the presence of *Renibacterium salmoninarum* 7.27 times more

often than PCR ($p < 0.01$), and qPCR 9.26 times more often than PCR ($p < 0.01$). No significant difference in detecting *R. salmoninarum* was observed between DFAT and qPCR ($p = 0.82$; Table 1.1). Given the low detection probability for PCR, and similarity in the diagnostic capabilities of DFAT and qPCR, PCR was dropped from further analyses. As such, subsequent subsections highlight results for the tissues tested rather than assay type used.

1.3.2 *Tissue Comparisons*

Among the 72-brook trout collected, 47 fish (65.3%) were determined positive for *R. salmoninarum* using DFAT on kidney tissue samples. Positive detections by qPCR of nonlethal swab sampling with anal, buccal, and mucus swabs were 9 (11.1%), 11 (15.3%), and 39 (54.2%), respectively. *Renibacterium salmoninarum* infections among kidney tissue had the highest detection probability (Figure 1.1a). Among the non-lethal tissues, I was more likely to detect the bacteria using mucus swabs. *Renibacterium salmoninarum* detection probabilities also suggest kidney tissues and mucus swabs are the best tissues to sample, especially compared to anal and buccal swabs (Figure 1.1a). Furthermore, kidney tissue and mucus swab detection results had low odds of differing from one another (Table 1.1), indicating that mucus swabs are at least as effective as kidney samples in detecting *R. salmoninarum*. Buccal or anal swabs had much higher odds of differing from kidney tissue detection of *R. salmoninarum*, suggesting they may not be an appropriate non-lethal method for detection of the bacteria (Table 1.1).

Of the 47 fish positive using DFAT analysis of kidney tissue, 5 anal (10.6%), 9 buccal (19.2%), and 28 mucus (57.5%) samples were considered positive for *R. salmoninarum* using qPCR. Mucus swabs were the best non-lethal sampling method for determining if a fish was positive for *R. salmoninarum* when kidney tissue was positive compared to anal or buccal swabs (11.34 or 5.70 higher odds, respectively; Table 1.1). Likewise, the probabilities of detecting *R.*

salmoninarum indicated that mucus swabs were more likely to detect the bacteria than anal or buccal swabs (Figure 1.1b). Interestingly, when the bacterium was not detected in kidney tissues by DFAT, mucus swabs detected *R. salmoninarum* in an additional 11 fish, anal swabs in an additional 2 fish, and buccal swabs in 1 additional fish.

1.4 Discussion

The utilization of non-lethal methods is not well-developed for determining the presence or absence of *R. salmoninarum* in cultured or wild salmonid populations and, particularly, in inland salmonids. Therefore, evaluating the performance of non-lethal sampling methods for detecting and predicting the presence of *R. salmoninarum* is essential for validating and advancing their use in inland salmonid populations. Overall, my results indicate using kidney tissues (DFAT) and mucus swabs (qPCR) to test for *R. salmoninarum* in Brook Trout offers the highest detection probabilities for the tissues tested and are equally effective. Mucus swabs were also the best non-lethal sampling method for detecting *R. salmoninarum* when the fish was positive for the bacteria by testing kidney tissues (DFAT).

Confidently detecting pathogens often requires sacrificing a large number of fish (Ossiander & Wedemeyer 1973; Richards et al. 2017), but this is undesirable, particularly with species of high conservation concern. Ovarian fluid from spawning adult female fish has been used to detect pathogens, such as *R. salmoninarum*, non-lethally; however, this is limited to fish that are mature, gravid, and female (Pascho et al. 2002). Most fish are not typically held until they reach spawning maturity, limiting the usefulness of testing ovarian fluid. In previous studies, Chinook Salmon as young as 6 months old have been known to be naturally and/or experimentally infected (McKibben & Pascho 1999; Mesa et al. 2000; Rhodes et al. 2006), and inland Rainbow Trout have tested positive for *R. salmoninarum* as soon as 11 days after swim-up

(Fetherman et al. 2020). Therefore, non-lethal sampling methods are needed for all age classes and maturity statuses. My study suggests using mucus swabs as a sampling method, coupled with a qPCR assay, could be useful as a screening tool for *R. salmoninarum*. My data also suggest that mucus swabs coupled with qPCR are at least as effective as the standard kidney test, although additional controlled research with more species and testing at various time points of infection and infection level is needed.

Interestingly, mucus swabs detected *R. salmoninarum* in 11 fish that were negative by kidney tissue testing. It is possible that some of the positive mucus detections in my study could reflect bacteria present in the water and represent an exposure or subclinical infection but not an internal infection (Elliott et al. 2015; Suzuki et al. 2017). Mucus is a primary defensive mechanism of fish and can be shed and replaced to prevent the colonization of bacterial pathogens and active infections (Benhamed et al. 2014). I did not observe any external signs of disease and was therefore unable to include an ordinal visual disease assessment in this study and relate signs of disease to a positive mucus swab. This is typical because *R. salmoninarum* causes a systemic, slow-progressing disease with varying clinical signs, which reduces the probability of visually observing signs of acute and sub-acute clinical signs (Sanders & Fryer 1980; Bullock & Herman 1988; AFS-FHS 2016). Therefore, further experiments are needed to understand the meaning of a positive mucus swab. Positive results may indicate (1) a future infection, following attachment to the underlying dermis tissues in the mucus layer; (2) a previous or active infection, and the mucus may be aiding in clearing the bacteria from the fish; or, (3) the bacteria are present in the fish's environment, but detection is not indicative of a previous or future infection. Despite uncertainty about the status of fish testing positive with mucus swabs, they may advantageously be used to determine if *R. salmoninarum* is present in the environment. In the

case of anadromous or other migrating salmonids (O'Bryne-Ring et al. 2003), mucus swabs could present a means for determining whether fish traveled through areas where *R. salmoninarum* was present. Additionally, mucus swabs could allow the initial screening of wild fish that are being collected to supplement hatchery broodstocks for conservation purposes, and those fish could remain in isolation to prevent active transmission of *R. salmoninarum* into a hatchery unit.

While mucus swabs show promise for detecting *R. salmoninarum*, they may not be well-suited for testing all fish species or during certain life history periods. For example, the skin of anadromous salmonids and Brown Trout (*Salmo trutta*) is known to thicken during their spawning migration, which reduces the amount of mucus secreted (Pickering 1977; Pottinger et al. 1984). When mucus is not being replaced during spawning, mucus may not be indicative of the internal infection status of the fish. Anal and buccal swabs may also be affected by the timing of sampling, and this may be why I determined that anal and buccal swabs were not effective ways to sample for *R. salmoninarum*. For example, buccal swab effectiveness may be dependent on fish feeding and ingesting bacteria. Therefore, if fish are not feeding, then results from buccal swabs would be negative despite potential exposure. Fish ingesting *R. salmoninarum* could potentially lead to an infection through horizontal transmission (Balfry et al. 1996; Hamel 2005; Elliott et al. 2015). Similarly, timing issues are possible with anal swab effectiveness and may have led to my inference that they are not effective. Positive anal swabs may be dependent on the fish actively shedding the bacteria (McKibben & Pascho 1999), and a negative test may be misleading relative to the internal infection status of the fish. Clearly, more studies are needed to address potential issues regarding the timing of non-lethal sampling versus the level of infection,

but I feel that mucus testing offers the most promising avenue for non-lethal testing among inland salmonids.

My study suggests that single-round PCR, using the specified primers (Fetherman et al. 2020), has a lower probability of detecting *R. salmoninarum* than standard lethal methods (DFAT and qPCR) used by the AFS-FHS (2016). My conclusions are limited by the opportunistic study design that conducted assays at different time points, and a lack of additional tissue prevented rerunning samples with qPCR. The discrepancy between single-round PCR and qPCR may be related to the sensitivity and specificity of each assay (Faisal & Eissa 2009; Elliott et al. 2013; Elliott et al. 2015; Fetherman et al. 2020). For instance, nested PCR shows lower diagnostic sensitivity and specificity probabilities from qPCR when testing kidney tissues for *R. salmoninarum* (Elliott et al. 2013). However, the sensitivity and specificity for single-round PCR is not well known for *R. salmoninarum* in kidney tissues, as there has only been one study using this method (Fetherman et al. 2020). Future studies would benefit from using the same assays on the same tissues. This would allow us to optimize assay conditions and understand the reliability of these assays to detect *R. salmoninarum*, especially when comparing non-lethal to lethal sampling methods. I also recognize that due to my study design, the sample size is relatively small, and my analyses could be influenced by the low number of known positive samples.

My study offers a first step in utilizing non-lethal methods to detect *R. salmoninarum* in inland trout. Non-lethal sampling methods could be valuable in determining the presence of *R. salmoninarum* in populations of rare and vulnerable species and in aquaculture facilities where there may not be enough fish available for lethal testing. Therefore, the addition of non-lethal sampling techniques could allow for multiple testing, the monitoring of infections, and,

potentially, the fate of infections within populations, allowing for a more nuanced understanding of the consequences of *R. salmoninarum* infections.

Table 1.1. Results are based on three separate logistic regression models in which the presence of *Renibacterium salmoninarum* was treated as a binomial response variable. Individual fish were treated as random intercept terms. Model 1 (Assay Performance) accounts for 216 observations from 72 Brook Trout, model 2 (Tissue Comparisons) accounts for 288 observations from 72 Brook Trout, and model 3 (Comparisons when Kidney Tissue is Positive) accounts for 141 observations from 47 Brook Trout. Pairwise contrasts are given for each of the models, including odds ratios, standard error (SE), and *z*- and *p*-values ($\alpha = 0.05$) for each contrast.

Model	Contrasts	Odds Ratio	SE	*z*-Value	*p*-Value
Assay Performance	DFAT/PCR	7.27	3.35	4.30	<0.01
	DFAT/qPCR	0.78	0.32	-0.60	0.82
	qPCR/PCR	9.26	4.43	4.65	<0.01
Tissue Comparisons	Kidney/Anal	13.16	5.71	5.94	<0.01
	Kidney/Buccal	10.43	4.28	5.71	<0.01
	Kidney/Mucus	1.59	0.54	1.36	0.53
	Mucus/Anal	8.27	3.54	4.94	<0.01
	Mucus/Buccal	6.55	2.65	4.65	<0.01
Comparisons when Kidney Tissue is Positive	Mucus/Anal	11.34	6.32	4.36	<0.01
	Mucus/Buccal	5.70	2.70	3.67	<0.01
	Buccal/Anal	1.99	1.20	1.14	0.49

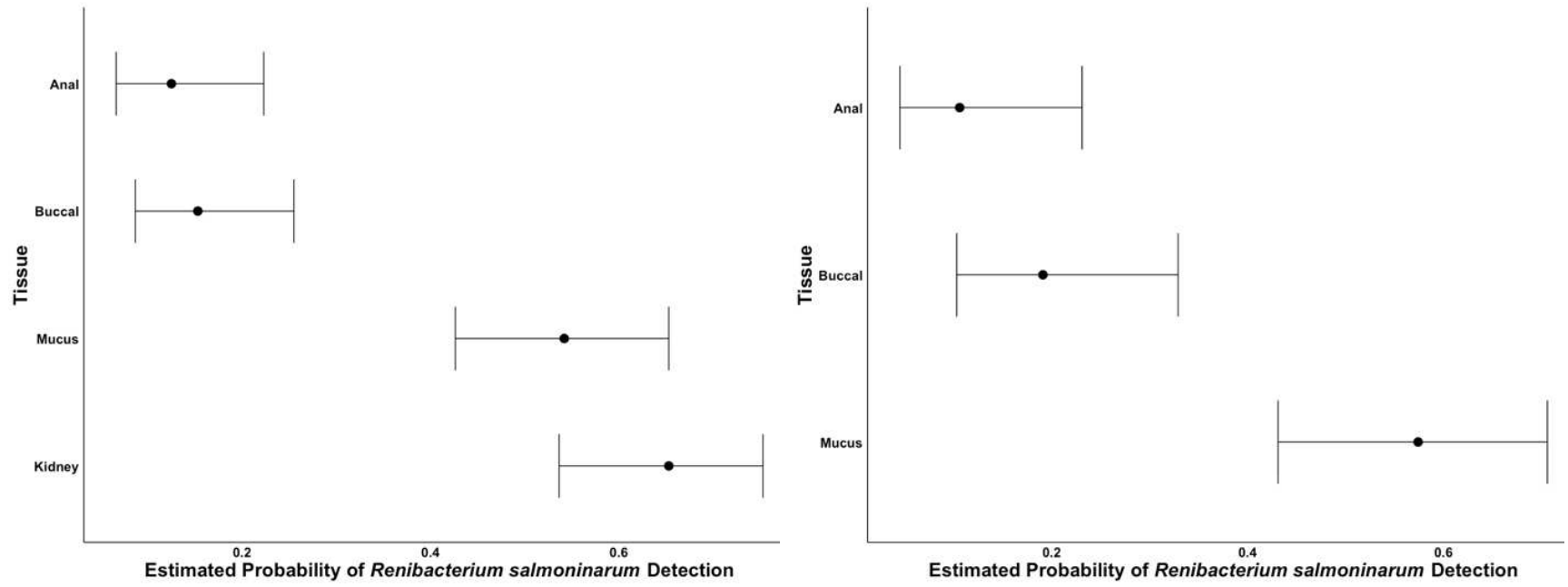

Figure 1.1. Model-based detection probability estimates (95% confidence interval bars) for *Renibacterium salmoninarum*; (**a**) using anal, buccal and mucus swabs, or kidney tissues, and (**b**) using anal, buccal, or mucus swabs when samples are known to be positive by testing kidney tissues with DFAT.

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CHAPTER 2

ESTIMATING *RENIBACTERIUM SALMONINARUM* INFECTION STATUS IN GREENBACK CUTTHROAT TROUT AMONG MULTIPLE TISSUES AND ASSAYS WITH VARYING DETECTION PROBABILITIES

2.1 Introduction

Propagation of fish species is often necessary to improve reintroduction, establishment, conservation, and management success in natural populations. Rearing fish in hatcheries often reduces natural mortality during vulnerable life stages and may increase abundance of wild fish following stocking (Støttrup & Sparrevohn 2007). Salmonid species reared in aquaculture facilities for conservation purposes include Atlantic Salmon (*Salmo salar*) and Brook Trout (*Salvalinus fontinalus*) in the eastern US and mid-Atlantic regions (Venture 2008; Hindar et al. 2011), lineages of the Cutthroat Trout (*Onchorhynchus clarkii*) in the Rocky Mountains (Metcalf et al. 2007; Heller 2021), and steelhead, Chinook Salmon (*O. tshawytscha*), Coho Salmon (*O. kisutch*), Pink Salmon (*O. gorbuscha*), and Sockeye Salmon (*O. nerka*) in the Pacific northwest (Eldridge & Killebrew 2007; Kostow 2009; Kline & Flagg 2014). Hatchery stocking programs have been successful in maintaining genetic diversity of Chinook Salmon in the North Fork Stillaguamish River (Washington, USA; Eldridge & Killebrew 2007), reestablishing an almost extirpated lineage of the Snake River Sockeye Salmon in Redfish Lake (Idaho, USA; Kline & Flagg 2014), and increasing a valuable population of the Bonneville Cutthroat Trout in Bear Lake (Idaho, USA; Heller 2021).

Although hatchery supplementation appears to be a viable option for aquatic species conservation, rearing conditions often involve high densities, exacerbating stress that can propagate disease epidemics (Fagerlund et al. 1981) and reduce the success of a conservation program. Common hatchery practices such as feeding, cleaning, and fish handling, have been shown to increase the prevalence of pathogen infections in hatchery-reared Chinook Salmon (VanderKooi & Maule 1999; Larson et al. 2020). In addition, high fish densities and stressful rearing environments can increase pathogen transmission rates. One such pathogen of concern is *Renibacterium salmoninarum*, the bacterium causing bacterial kidney disease in salmonids.

Renibacterium salmoninarum can be transmitted both horizontally and vertically within the hatchery environment. Horizontal transmission can occur through the ingestion of infected fecal matter (Balfry et al. 1996) or exposure to *R. salmoninarum*-contaminated water (Evenden et al. 1993). Vertical transmission occurs from the maternal fish to progeny through an intra-ovum infection (Evelyn 1984). Both vertical and horizontal transmission can contribute to increased prevalence of the bacteria in hatchery-reared salmonids. Prevalence of *R. salmoninarum* in farmed Pacific salmon has been as high as 80% despite low vertical transmission rates, suggesting both vertical and horizontal transmission contribute to infection within aquaculture facilities (Balfry et al. 1996). Disease caused by successful infection of *R. salmoninarum* can further increase transmission events and cause high mortalities in cultured fish (Pascho et al. 2002). The concerns of overt disease, high mortality rates, and increased transmission of the bacteria of *R. salmoninarum* has led many states to adopt disease management plans within hatchery facilities.

The American Fisheries Society (AFS) lists *R. salmoninarum* as a regulated pathogen among wild and cultured fish throughout the United States. Detection of regulated pathogens restricts

stocking or transfer of fish from infected hatchery units and can lead to lethal culling of fish to limit transmission (AFS-FHS 2016). Methods currently used to detect *R. salmoninarum* vary in their specificity, sensitivity, and reliability and there is no standard with 100% detection (Elliott et al. 2013; Elliott et al. 2015; Jaramillo et al. 2017). The AFS Fish Health Section has established methods for initial presumptive detection in fish tissues followed by a confirmatory test (Chase et al. 2006; Elliott et al. 2013). Presumptive testing includes direct fluorescent antibody tests (DFAT), membrane-filtration FAT, and enzyme-linked immunosorbent assays (ELISA; Pascho et al. 1991; AFS-FHS 2016). Various polymerase chain reaction assays (PCR) such as quantitative (qPCR) or nested (nPCR), can be used to confirm a presumptive positive (Chase & Pascho 1998; Chase et al. 2006; Elliott et al. 2013). Successful culture of *R. salmoninarum* on selective kidney disease medium followed by a biochemical test is the absolute confirmatory test. However, due to the slow growing nature of the bacteria in cultures (6-12 weeks; Gudmundsdóttir et al. 1991), this method may be unsuitable for rapid detection of the bacteria in fish health inspections. Therefore, AFS guidelines for screening tissues for *R. salmoninarum* are often followed.

Many state and federal agencies use DFAT as a presumptive diagnostic assay and qPCR as a confirmatory assay. DFAT detects the bacterial surface-associated 57 kDa protein using fluorescence (p57; Bullock 1980). Estimated diagnostic sensitivity (true positive detection rate) and specificity (true negative detection rate) of DFAT from the optimized assay are 0.76 and 0.85, respectively (Elliott et al. 2013). These estimates were determined based on the inoculation of Chinook Salmon with a virulent strain of *R. salmoninarum* known to produce large amounts of surface-associated p57 (GL–64 isolate from Lake Michigan, USA; O'Farrell et al. 2000; Elliott et al. 2013). Use of qPCR as a confirmatory diagnostic assay has increased due to its high

throughput and accuracy and it is a very sensitive and efficient technique for detecting and quantifying genomic DNA in tissue samples (Kubista et al. 2006; Bustin et al 2009). A qPCR assay has been developed and optimized for the detection and quantification of the major soluble antigen (*msa)* gene coding for p57 of *R. salmoninarum* from fish tissues (Chase et al. 2006) and has a diagnostic specificity of 1.0 and diagnostic sensitivity of 0.25 (GL-64 isolate; Elliott et al. 2013).

Current guidelines for tissue collection to test for the presence of *R. salmoninarum* specify using kidney homogenates as a lethal sampling method and ovarian fluid as a non-lethal sampling method (AFS-FHS 2016). However, ovarian fluid is limited to testing only adult spawning females. *Renibacterium salmoninarum* has been detected in other tissues including liver and spleen tissues, blood samples, fin clips, gill clips, intestinal lavage, and mucus swabs, but detection in these tissues can vary by fish species (Pascho & Mulcahy 1987; Bruno et al. 2007; Elliott et al. 2015; Gudmundsdóttir et al. 2017; Richards et al. 2017; Riepe et al. 2021). Although tissue testing and assay development have occurred for many years (Pascho et al. 2002), a method to test for *R. salmoninarum* that is 100% reliable has not been identified. Because there is no completely reliable testing method for *R. salmoninarum*, it is imperative to understand the efficacy and tradeoffs associated with specific tissues and assays for various species of susceptible fish.

Cutthroat Trout are an *R. salmoninarum*-sensitive species of conservation concern in Colorado, and many hatcheries produce Cutthroat Trout for stocking and re-establishing wild populations. Due to their susceptibility to *R. salmoninarum*, annual fish health inspections are completed at hatcheries and prior to stocking for detection of regulated pathogens. My goal was to determine which tissues to test, based on their probability of being infected, and which assay

resulted in the highest probability of detecting the bacteria in any given tissue or serum sample. I sampled adult spawning Cutthroat Trout from the Colorado Parks and Wildlife (CPW) Poudre Rearing Unit to evaluate two primary objectives: 1) compare the infection probability of *R. salmoninarum* in tissues and serum samples to determine which sample would provide the highest probability of detecting the bacteria if present and 2) evaluate the detection probability of DFAT and qPCR to determine which assay should be used when diagnosing *R. salmoninarum* infections in hatchery-reared populations.

2.2 Methods

2.2.1 *Tissue Collection*

I sampled 781 adult Greenback Cutthroat Trout (*Oncorhynchus clarkii*) at the CPW Poudre Rearing Unit during the 2019 spawning season to test for the presence of *R. salmoninarum*. I collected three serum samples (mucus, ovarian fluid, and blood) and refer to these as non-lethal samples. I also collected three internal tissue samples (spleen, liver, and kidney) and refer to these as lethal samples. All tissues were collected from individual three-year-old female and two-year-old male brood fish. I collected non-lethal mucus samples by swabbing both sides of each fish with sterile cotton swabs (Riepe et al. 2021). After collecting mucus, all fish were weighed (g), measured (mm), and euthanized using tricaine methanesulfonate (MS-222; Syndel). Individual pairs of female and male fish were stripped of gametes and spawned together in sterile bowls; however, ovarian fluid was collected with sterile syringes from the spawning bowls prior to adding milt. The artificial spawning was part of another experiment to determine the rate of vertical transmission of *R. salmoninarum* in Cutthroat Trout (Chapter 4). Whole blood was then collected through venipuncture of the caudal vein (non-lethal). Although fish were euthanized prior to collecting the three non-lethal samples,

mucus, ovarian fluid, and blood can be collected without having to euthanize the fish. Lethal spleen, liver, and kidney whole tissues were removed through an abdominal incision and placed in sterile whirl-pak-bags. Tissue and serum samples were labelled so they could be associated with individual fish. All samples were placed on dry ice until arrival at the laboratory (6 hours after collection) and then held at -20°C until processed.

2.2.2 *Laboratory Analysis*

All lethal tissue and non-lethal serum samples were analyzed for the presence of *R. salmoninarum* using two different assays, DFAT and qPCR. Samples were prepared for DFAT by making two tissue imprints or serum smears with sterile cotton swabs on individual wells located on a 12-well slide (6 individual fish per slide). A separate control slide with a smear of bacterial culture was also prepared with each set of slides to ensure that the reagents were working properly. I used a *Fluorescein isothiocyanate*-conjugate (FITC), affinity purified polyclonal antibody to *R. salmoninarum* (KPL; Milford, MA, USA) to stain the slides, followed by an eriochrome black T counterstain (Sigma-Aldrich®; Pascho et al. 1991). I examined all slides under 500 times magnification using a FITC fluorescent lamp with a wavelength of 400 nm. Samples exhibiting visible fluorescent cells were further examined at 1000 times magnification and were confirmed as *R. salmoninarum* by cell morphology and cell size (AFS-FHS 2016).

Replicate tissue and serum samples were used to screen for *R. salmoninarum* with qPCR. For DNA extraction, tissue and serum samples were thawed and homogenized. Serum samples were homogenized by vortexing in 2 mL microcentrifuge tubes. Mucus swabs were incubated in 1X PBS for three hours then homogenized by vortexing. Tissue samples were homogenized manually with a sterile rolling pin while remaining in the Whirl-Pak-Bags. Two replicates of

approximately 0.25 g of tissue or 200 µL of serum were prepared for DNA extractions. Tissue, blood, and ovarian fluid DNA extractions were completed using a Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany). The manufacture's protocol for Gram-positive bacterial swabs was used for DNA extractions on mucus. I changed the AE buffer in the DNA extraction protocols to 200 µL to increase DNA concentration in the final samples (Elliott et al. 2013).

I used an ABI StepOnePlus System (Applied Biosystems, Foster City, CA, USA) for all qPCR assays to detect the *msa* gene of *R. salmoninarum*. Each assay resulted in final volume of 5 µL of extracted DNA template and 12.5 µL of TaqMan Gene Expression Master Mix with predetermined primer sets RS 1238 Forward, 5'-GTGACCAACACCCAGATATCCA-3', RS 1307 Reverse, 5'-TCGCCAGACCACCATTTACC-3', and MGB probe 1262, 5'-CACCAGATGGAGCAAC-3' for a total of 25 µL for each PCR reaction (Chase et al. 2006).

Known positive controls, obtained from Rainbow Trout at the CPW Bellvue Fish Research Hatchery, were used with each qPCR run. The isolate for the positive controls was identified by the CPW Aquatic Animal Health Laboratory, confirmed with single-round PCR and qPCR, and stored as a stock culture in phosphate buffered solution (PBS) at −80°C until use. Positive controls were used to produce a standard curve for the absolute quantification of *R. salmoninarum* in each sample. I rehydrated the stock culture to inoculate kidney disease medium broth (KDM) and reinoculated the bacteria five times in new media over a total of 45 days. Stocks with 1 mL of the cultured bacteria were prepared with an optical density value of 0.081. Eight, ten-fold serial dilutions were prepared from the stock to quantify the number of bacteria in each dilution. I followed the protocols for membrane-filtration fluorescent antibody test (MFAT) to quantify the number of cells present in each dilution (Elliott & Barila 1987; Elliott & McKibben 1997). Bacterial cells in KDM broth were centrifuged at 5000 x *g* for 20 min at 4°C.

The pellet was re-suspended in 1X PBS-peptone. Syringes containing serial dilutions were fitted with Whatman® pop-top filter holders, 13 mm, 0.2 μm polycarbonate filters, and 13 mm, 5.0 μm nylon membrane filters, and samples were forced through the filter. Filters were rinsed with PBS plus Triton and incubated with 100 μL of *Fluorescein*-labeled, affinity purified polyclonal antibody to *R. salmoninarum* produced in goat for one hour; rinsed again and counterstained with eriochrome black T; and rinsed a final time with 1X PBS. Following rinsing, the polycarbonate filter was placed onto a glass microscope slide to air dry, and a glass coverslip was mounted with DABCO-glycerol medium. Filters on the slides were examined at 1000 times magnification to quantify the number of bacterial cells present in each dilution when there was an observable amount to count. I was able to quantify cells from the MFAT standards with the 10^{-6} dilution. All other dilutions had too many cells to count. Ten replicate 10^{-6} dilutions were created to count the number of bacterial cells within the dilution. The final five-point curve was generated by plotting five-$\log_{10}$ bacterial concentrations from 58 ten-fold serial dilutions against the Cq output values from qPCR to determine slope, the y-intercept which corresponds to the Cq cut-off value, and amplification efficiency (Bustin et al. 2009; Life Technologies 2012). The absolute number of *R. salmoninarum* bacterial cells in each qPCR sample was quantified based on the standard curve.

2.2.3 *Statistical Analysis*

To determine relationships between the number of bacteria among tissues in individual fish, I used a two-dimensional correlogram with associated r^2 values for each relationship. I also sought to determine if more detections may occur in the two most infected tissues, kidney, and liver, due to a higher number of bacteria in one tissue over the other. Differences between the

number of bacteria present in the tissues were determines by performing a Welch's two sample t-test in program R (version 4.1.0) with a significance (α) set at 0.05.

2.2.4 *Occupancy Model*

Multi-state occupancy modeling used to estimate *R. salmoninarum* infection probability in tissues and detection probabilities among assays. We explicitly define the probability of *R. salmoninarum* infection as the probability of occupancy. I used this modeling approach because of imperfect detection of *R. salmoninarum* with DFAT and qPCR and wanted to account for the possibility of false negatives (i.e., not detecting the pathogen when it is actually present). I focused on kidney and liver tissue for the modeling because kidney is the standard tissue recommended by AFS-Fish Health Blue Book for *R. salmoninarum* evaluation, and the data indicated that liver had the highest raw detections of *R. salmoninarum*. The sampling unit was an individual fish, and the kidney and liver tissues were considered "locations" within each fish (Nichols et al. 2008). The replicate testing on each tissue represented two surveys for each assay (DFAT or qPCR). DFAT and qPCR assays were treated as two, independent models. The model also assumed that there were not false positives, and I make this assumption based on the high specificity of both assays (Elliott et al. 2013).

The data consisted of detection histories from both assays. The detection histories were produced using the results of the two surveys for each tissue type, kidney ($K_{j=1}$; $K_{j=2}$) or liver ($L_{j=1}$; $L_{j=2}$), resulting in 16 possible detection histories (Table 2.1). For instance, the following detection history indicates that an individual fish tested negative on both surveys in each tissue (with two observations each for kidney and liver)

$$d = [K_1\ K_2\ L_1\ L_2] = [0\ 0\ 0\ 0]$$

The sixteen possible detection histories resulted in four possible states of infection for each assay; state 1: {K– L–}, state 2: {K+ L–}, state 3: {K– L+}, and state 4: {K+ L+} where K = kidney tissue and L = liver tissue (Table 2.1). When testing the kidney and liver tissues, *R. salmoninarum* may be present in the tissue (true positive = 1), not present (true negative = 0), or present but not detected (false negative = 0). Due to the possibility of false negative results, there may be multiple possible true states of infection for any detection history with a zero, ultimately affecting the classification of the observed infection state. In my detection history example above, the individual would be classified in observed state 1{K– L–}, which could correctly represent a true state of infection of {K– L–} if both tissues were truly negative. However, that individual could also be positive in one or both kidney surveys (a false negative for kidney) and in one or both liver surveys (a false negative for liver) and the true infection state could be {K+ L–}, {K– L+}, or {K+ L+}. The model is static because fish were euthanized, making multiple measurements over time impossible. Therefore, I model the probability that the observed state represents the true state of infection.

Prior to running the multi-state occupancy models, I used a multinomial logistic regression to evaluate if fish length, weight, and/or sex affected the state or observation process in kidney and liver tissues (Kéry & Schaub 2011). Only individual effect models were included because length, weight, and sex were known to be correlated since female fish were larger than male fish. No covariates were determined significant (Table 2.2); therefore, I did not use these covariates in the occupancy modeling.

The static multi-state occupancy model was fit in a Bayesian hierarchical framework (Figure 2.1) and was used to calculate bacterial occupancy (Ψ_k) among each state of infection

(k) for each fish (i) and detection probability in kidney (p_2) and liver tissues (p_3) for each assay (DFAT and qPCR; calculated independently) among two surveys (j).

$$\left[p_2, p_3, z_i, \beta_{\Psi,1}, \beta_{\Psi,2}, \beta_{\Psi,3}, \beta_{\Psi,4} \middle| y_{i,j}\right] \propto$$

$$\prod_{i=1}^{781} \prod_{j=1}^{2} \left[y_{i,j} \middle| p_2, p_3, z_i\right] \left[z_i \middle| \beta_{\Psi,1}, \beta_{\Psi,2}, \beta_{\Psi,3}, \beta_{\Psi,4}\right] [p_2]\ [p_3] \left[\beta_{\Psi,1}\right] \left[\beta_{\Psi,2}\right] \left[\beta_{\Psi,3}\right] \left[\beta_{\Psi,4}\right]$$

The observed dataset ($y_{i,j}$) was derived from 16 detection histories of 781 fish (i) with two surveys per fish (j) and consisted of the four infection states described above (Table 2.1). MacKenzie and Royle (2005) suggest when building an occupancy model, the optimal number of surveys needed for each tissue (i.e., site or location) is a function of known detection probabilities for the method of detection. The optimized DFAT and qPCR assays have diagnostic specificities of 0.85 and 1.0 (Elliott et al. 2013), respectively, and therefore two surveys on each tissue with each assay was determined optimal for my study. The model was split into two process models: the state process model and the observation process model (McKenzie et al. 2013) that both affect the outcome of infection status in individual fish. The state process model describes that the tissues representing the states of infection (k = 1, 2, 3, or 4) are either infected with *R. salmoninarum* (with probability Ψ_k) or not infected (with probability $1 - \Psi_k$) in each fish and z_i represents the true unknown latent state of infection for each fish derived from each Ψ_k (Figure 2.2). The observation process model describes the presence or absence of *R. salmoninarum* among each state of infection ($p_{i,j}$). These process models are described in detail below.

2.2.5 *State Process Model*

The state process model describes the probability of infection (Ψ_k) in a fish for each state (k) and the true latent state of infected fish (z_i). The latent state is calculated because of the

possibility of false negative results. z_i can only be equal to 1, 2, 3 or 4 indicating each true state of infection and is modeled as a categorical random variable from $\mathbf{\Phi}$:

$$z_i \sim \text{categorical}(\mathbf{\Phi})$$

$$\mathbf{\Phi} = [\, \Psi_1\ \Psi_2\ \Psi_3\ \Psi_4\,]$$

Probabilities of infection are calculated for each of the four states: Ψ_1 is the probability of infection for state 1, Ψ_2 is the probability of infection for state 2, Ψ_3 is the probability of infection for state 3, and Ψ_4 is the probability of infection for state 4. I assigned a Dirichlet prior (Kéry & Schaub 2011) for the probabilities of infection. To constrain the four probabilities, the hyperparameters ($\beta_{\Psi,k}$) within the Dirichlet prior were sampled from a non-informative gamma distribution (gamma(1,1)). This Dirichlet prior is the most commonly used prior distribution for categorical variables (Huang 2005) and allows me to represent the marginal probabilities such that Ψ_k sum to one. For instance, the probability of infection for kidney tissues is equivalent to $\Pr(\Psi^{Kidney}) = \Psi_2 + \Psi_4$, the probability of infection for liver tissues is equivalent to $\Pr(\Psi^{Liver}) = \Psi_3 + \Psi_4$, and the probability of infection within either tissues is $\Pr(\Psi^{Kidney} + \Psi^{Liver}) = \Psi_2 + \Psi_3 + \Psi_4$.

2.2.6 *Observation Process Model*

The observation process model describes the presence or absence of *R. salmoninarum* among each state of infection. In the observation process model, the observation process ($\boldsymbol{P}_{i,j}$) is linked to the observed data for each assay based on the repeated surveys (j) with each tissue.

$$y_{i,j} = \text{Categorical}(\boldsymbol{P}_{i,j}, z_i)$$

I define two detection probabilities (p_2, p_3) with each assay model. Specifically, I am most interested in understanding the detection probability for each assay on kidney (p_2) and liver tissues (p_3). Thus, I formulated an observation matrix for the parameters p_2 and p_3, and arranged

them where each row indicates the true state of infection, and each column indicates the observed state of infection. Therefore, the observation matrix is:

$$\boldsymbol{P_i} = \begin{array}{c} \\ \mathbf{1} \\ \mathbf{2} \\ \mathbf{3} \\ \mathbf{4} \end{array} \begin{array}{c} \begin{array}{cccc} \mathbf{1} & \mathbf{2} & \mathbf{3} & \mathbf{4} \end{array} \\ \begin{bmatrix} 1 & 0 & 0 & 0 \\ 1-p_2 & p_2 & 0 & 0 \\ 1-p_3 & 0 & p_3 & 0 \\ (1-p_2)*(1-p_3) & p_2*(1-p_3) & (1-p_2)*p_3 & p_2*p_3 \end{bmatrix} \end{array}$$

If the true infection state is state 1 {K– L–}, it is impossible to observe infection states 2, 3, or 4 because the fish is not infected. Therefore, the probability must be 1. When the true state of infection is state 2 {K+ L–} (second row of $\boldsymbol{P_i}$), the fish could be observed in either state 1 or state 2. As such, the probability I detect the fish in state 1 given the true state of 2, a false negative from the kidney tissue, is $Pr(1-p_2)$, and in state 2 given the true state of 2, a true positive, is $Pr(p_2)$. Similarly, when the true state of infection is state 3 {K–L+} (third row of $\boldsymbol{P_i}$), the fish could be observed in either state 1 or state 3. Therefore the probability I detect the fish in state 1 given the true state of 3 is $Pr(1-p_3)$ and in state 3 is $Pr(p_3)$, and state 2 or 4 cannot be observed. Finally, if the true state of infection is state 4 (row four in $\boldsymbol{P_i}$) it is possible to observe fish in all four states and the probabilities are defined in $\boldsymbol{P_i}$ matrix. The parameters p_2 and p_3 are defined as the probability of detection for kidney (state 2 {K+ L–}) or liver (state 3 {K– L+}), respectively, and can be derived from $\boldsymbol{P_i}$. I used non-informative priors for p_2 and p_3 drawn from a uniform distribution between 0 and 1.

2.2.7 *Model Implementation*

Posterior probability distributions of the model parameters ($p_2, p_3, \beta_{\Psi,2}, \beta_{\Psi,2}, \beta_{\Psi,3}, \beta_{\Psi,4}$) were estimated with separate models for each assay using a Monte Carlo-Markov chain (MCMC) algorithm in program JAGS (version 4.3.0) within program R with the library rjags.

Both assay models were fit for each state of infection using 2,500 MCMC iterations for three chains with a thinning interval of two, and a burn in value of 500 for each chain. Model convergence was determined based on the Gelman-Ruben statistic (Rhat) comparing within-chain variance to between-chain variance and was considered acceptable when the Rhat value was less than 1.1 (Gelman & Ruben 1992). The models were developed by fitting the model with simulated data to known parameters and recovering the parameters from the model. Recovering the parameters indicates no evidence for a lack of fit.

The model calculates the probability of detection from what is observed given the truth. However, for most situations I felt it would be more useful to evaluate the probability of an observed state being true or not (Figure 2.3). For instance, if I observe a fish in state 1, the true state could be any of the four states due to false negative results with kidney or liver tissues (states 2-4) or because the true state is state 1. I used the probability of detections from $\boldsymbol{P}_{i,j}$ for each of the DFAT and qPCR models (Figure 2.4) and transformed the values to determine the probability of the true state of infection given what was observed. The new probabilities are derived by taking each value in a row of the matrix $\boldsymbol{P_i}$ (true value) and dividing them by the sum of their column values (observed state) from $\boldsymbol{P_i}$. For instance, if I observe state 2 and want to know the probability that the possible true state of infection is state 2 then, from $\boldsymbol{P_i}$, I can divide the row value, p_2, by the sum of the second column, $p_2 + (p_2 * (1 - p_3))$. To determine the probability that the possible true state of infection is state 4 when I observe state 2, from $\boldsymbol{P_i}$ I divide the row value of $p_2 * (1 - p_3)$ by the sum of the second column, $p_2 + (p_2 * (1 - p_3))$. These quotients are the probabilities are the focus of the discussion below.

2.2.8 *Conditional Probability*

Using the probability of infection for kidney (Ψ_2) and liver (Ψ_3; Table 2.1) tissues from the posterior distribution, I computed the conditional probability of detecting *R. salmoninarum* in *K* number of surveys with a specific tissue and assay type given the tissue is infected. Conditional probability of detection was modeled as a function of the number of surveys for DFAT or qPCR assays on each tissue. I modified equation 1 from Chaudhary et al. (2020) where the numerator is the sum of the estimated *R. salmoninarum* occupancy in a tissue and the estimated probability of detection in *K* surveys for either DFAT or qPCR, given a tissue is infected. The denominator is the estimated probability neither kidney nor liver tissue is infected with *R. salmoninarum* (state 1). For example, when estimating the conditional probability of detection from liver tissue ($\hat{p}^{LK}$), I can compute $\hat{p}^{LK}$ where $\widehat{\Psi}^{\text{state 3}}$ and $\widehat{\Psi}^{\text{state 4}}$ is the marginal probability of occupancy for liver as:

$$\hat{p}^{LK} = \frac{(\widehat{\Psi}^{\text{state 3}} + \widehat{\Psi}^{\text{state 4}})}{1 - \widehat{\Psi}^{\text{state 1}}}$$

2.3 Results

2.3.1 *Tissue Collection Summary*

I evaluated the *R. salmoninarum* infection status of 781 Greenback Cutthroat Trout (392 2-year-old males and 389 3-year-old females) during the 2019 spawning season at the CPW Poudre Rearing Unit. I collected 770 mucus swabs, 781 blood samples, 314 ovarian fluid samples, 781 spleen samples, 776 liver samples, and 778 kidney samples from the 781 fish evaluated. Some samples were not included in the evaluation due to mishandling of the tissues and the potential for tissue contamination. Average weights of male fish were 242.7 $\pm$ 22.5 g (range 124 – 358 g), and average weights of female fish were 515.82 $\pm$ 135.86 g (range 157 –

1,089 g). The multinomial regression indicated no significant differences in *R. salmoninarum* detections between male and female fish for any tissue or assay (Table 2.1). Therefore, I did not include sex any further in my analysis.

2.3.2 *qPCR Standard Curve*

The original stock of bacterial cells for the development of the standard curve was equivalent to 4.4×10^7 cells/mL^{-1}. The total PCR product (200 µL) from the serial dilutions ranged from 2.2×10^5 to 2.2×10^1 bacterial cells/mL^{-1}. Since 5 µL of each standard per qPCR reaction was used, the final five-point standard curve generated a linear dynamic range from 1.1×10^5 to 1.1×10 bacterial cells/mL^{-1}. The standard curve was linear ($R^2 = 0.94$) indicating that the number of cells was correlated with the Cq value (Figure 2.5). The slope of the standard curve was -3.38, which corresponds to a qPCR amplification efficiency of 97.6% (MIQE standards; Bustin et al. 2009). I estimated the maximum Cq value from the intercept (i.e., the theoretical limit of detection) and used that estimate as the positive detection cutoff of 37.75, corresponding to an analytical sensitivity of 1.1 bacterial cells/mL^{-1}. The Cq value of 37.75 is an acceptable maximum Cq value to consider tissues positive for *R. salmoninarum* using qPCR (Sandell & Jacobson 2011).

2.3.3 *Tissue and Assay Data*

The number of *R. salmoninarum* detections with DFAT were lower than detections with qPCR overall. *Renibacterium salmoninarum* was detected in 177 fish (22.7%; 92 males: 85 females) using DFAT from any tissue or serum sample. Using the Cq cutoff value of 37.75, 639 fish tested positive with qPCR (81.8%; 311 males: 328 females). Only 45 kidney tissues (5.8% of all fish tested), 58 liver tissues (7.4%), and 45 spleen tissues (5.8%) tested positive by DFAT (Figure 4). More tissues were positive with qPCR. Specifically, 256 kidney tissues (32.7%), 447

liver tissues (57.2%), and 120 spleen tissues (15.4% were positive with qPCR; Figure 2.6). Detections with either assay in multiple tissues from the same fish was low. Among the 256 kidney tissues and 447 liver tissues positive by qPCR, only 151 fish were positive for *R. salmoninarum* in both tissues. Similarly, both tissues were positive in only 8 fish tested with DFAT among the 45 positive kidney and 58 positive liver tissues.

Renibacterium salmoninarum was detected in all three non-lethal serums, with positive detections from 258 fish (33.0%) with mucus swabs, 76 fish (9.7%) with ovarian fluid, and 14 fish (1.8%) with blood samples (Figure 2.6). Using ovarian fluid, I had 76 detections (19.5% of all females tested) with qPCR and 8 (2.1%) detections with DFAT. Average bacteria among the 8 fish positive by DFAT was 5.5×10^5 bacteria/mL^{-1} and two had less than 26 bacteria cells present in the ovarian fluid. I detected 76 positive female fish with qPCR and the average bacterial cell count was 1.4×10^4 bacteria/mL^{-1} but 73 of the fish had detections with lower than 26 bacterial cells present. Differences in the bacterial counts between positive samples potentially indicates lower sensitivity with DFAT than with qPCR. Using blood to detect *R. salmoninarum* also resulted in low detections (Figure 2.6). Interestingly, I did have more detections with DFAT (32 fish; 4.1%) than with qPCR (14; 1.8%) when testing blood indicating some intra-assay variability.

I examined linear relationships between tissues that had infections and bacterial counts greater than zero with qPCR (Figure 2.7). Strong positive relationships ($r^2 = 0.84$) were observed between kidney and liver bacterial quantities. However, the number of bacteria in kidney and liver tissues were not significantly different from one another when both tissues were positive in the same individual fish (t-statistic: -1.16, p-value: 0.248) with median levels of bacteria for kidney tissues of 2.0 bacteria/mL^{-1} and 2.9 bacteria/mL^{-1} for liver tissues. Weak

associations ($r^2 < 0.50$) were observed among mucus, liver, and kidney tissues indicating that an increase in bacteria in mucus swabs did not necessarily indicate an increase in bacteria in either kidney or liver tissues. Although relationships were observed among other tissue and serum comparisons, sample size was small because it was rare that these samples were both positive in the same fish, and therefore, no conclusions were drawn from these comparisons.

2.3.4 *State Process Model*

Both assays were modeled independently to estimate the probability of infection for each of the four states. When analyzed using DFAT, the probability of infection for state 1 (both tissues negative; Ψ_1) was the highest (0.83; CI:0.78–0.85; Table 2.2) indicating that most of the fish (681 (z_1)) were not infected (Figure 2.8). Probabilities of infection for state 2 (Ψ_2; kidney positive and liver negative) and state 3 (Ψ_3; kidney negative and liver positive) were <0.01 (CI: 0.001–0.03) and 0.10 (CI: 0.06–0.16), respectively, resulting in an estimated 6 fish in state 2 (z_2) 80 fish in state 3 (z_3; Figure 2.8). Marginal credible intervals for the probability of infection for kidney and liver tissues did not overlap, further suggesting higher probability of infection with liver tissues. The Dirichlet prior allowed me to constrain the probability of infection (Ψ_k) among each state such that I could calculate the marginal probability of infection for both tissues $\Pr(\Psi^{kidney} + \Psi^{liver}) = \Psi_2 + \Psi_3 + \Psi_4$. Thus, the probability of infection based on DFAT resulted in 0.17 (118 fish) that were infected with *R. salmoninarum*.

Higher probabilities of infection were calculated from the qPCR model compared to the DFAT model. The probability of infection (Ψ_3) was highest when analyzing liver tissue (0.43; CI: 0.38–0.43; Table 2.2) and resulted in an estimated 331 fish in state 3 (z_3). The estimated probability of infection when analyzing kidney tissues was lower than that of liver at 0.10 (CI: 0.08–0.13) and resulted in an estimated 80 fish in state 2 (z_2; Figure 2.8). Marginal credible

intervals from qPCR suggest that probability of infections in liver tissues are significantly greater than kidney since they do not overlap. Using Dirichlet distribution I estimated that the marginal probability of infection for liver tissue ($\Pr(\Psi^{liver}) = \Psi_3 + \Psi_4$) was 0.69 and the estimated true number of fish in states 2 and 4 equaled 528. The marginal probability of infection for kidney tissues ($\Pr(\Psi^{kidney}) = \Psi_2 + \Psi_4$) was 0.36 and the estimated true number of infected fish in those states equaled 276. The total probability of infection ($\Pr(\Psi^{kidney} + \Psi^{liver}) = \Psi_2 + \Psi_3 + \Psi_4$) was 0.79 and resulted in an estimate of 608 infected fish. Overall, the probability of infection was greatest when analyzing liver tissues regardless of assay, and infection probabilities were significantly lower with DFAT than with qPCR.

There were differences between the number of fish observed in a state and the estimated true number of fish (z_i) in that same state (Figure 2.8). Specifically, with both assays, the model indicated that fewer fish were uninfected (state 1 {K– L–}) when compared to the number observed (Figure 2.8). Conversely, the model indicated that more fish were infected than observed, especially in states 3 {K– L+} and 4 {K+ L+}, suggesting the possibilities of false negative assay results.

2.3.5 *Observation Process Model*

Detection probability of *R. salmoninarum* varied between assay type. The probability of detection was low for DFAT analyses in both kidney and liver tissues. The DFAT detection probability was 0.61 for kidney tissue (p_2; CI: 0.39–0.65) and 0.68 for liver tissue (p_3; CI: 0.58–0.81) and overlapping credible intervals suggested no differences in detection probability between the two tissues. Detection probabilities indicated a high potential for false negative results when using DFAT (Figure 2.9). For instance, when the observations indicated state 1 {K– L–}, the probability of this being the true state was 0.42 while the sum of all other true states of

infection was 0.58 (Figure 2.9a). When the observed state was {K+ L−}, i.e., state 2, the probability of that being the true state was 0.61, with a probability of 0.39 for being misclassified in state 4 (Figure 2.9b). The results were similar when the observed state was {K− L+}, i.e., state 3, with a slightly higher probability of the observed state being the true state 0.68 (Figure 2.99c). Overall, the detection probability when using qPCR was higher for comparing the observed to the estimated true state than that seen with DFAT. The qPCR detection probability was 0.72 for kidney tissue (p_2; CI: 0.70–0.74) and 0.79 for liver tissue (p_3; CI: 0.77–0.81) and non-overlapping credible intervals suggested that liver tissue had a higher detection probability than kidney tissue. Detection probability using qPCR indicated a high potential for false negative results (Figure 2.9). For instance, when my observations indicated state 1 {K− L−}, the probability of this being the true state was 0.56 and the sum of all other true states of infection was 0.44 (Figure 2.9a). This suggests a high probability of falsely classifying a fish as negative for *R. salmoninarum* based on testing with qPCR, but it is better than DFAT. When the observed state was {K+ L−}, i.e., state 2, the probability of that being the true state was 0.72, with a probability of 0.28 for being misclassified in state 4 (Figure 2.9b). The results were similar when the observed state was {K− L+}, i.e., state 3, with a slightly higher probability of the observed state being the true state 0.79 (Figure 2.9c). For both DFAT and qPCR, if fish in state 2 or 3 were misclassified in state 4, they would still be considered infected during a fish health inspection. Differences were detected with kidney tissues with either assay but were not detected between liver tissues.

2.3.6 *Conditional Probability of Detection*

The conditional probability of detecting ($\hat{\mathrm{p}}^{\mathrm{t}K}$) *R. salmoninarum* with DFAT or qPCR was highest in liver tissues for both assays (Figure 2.10). The $\hat{\mathrm{p}}^{\mathrm{t}K}$ also increased more rapidly in liver

tissues for both assays as the number of surveys increased, suggesting liver tissue is the best tissue to sample for *R. salmoninarum* if only one tissue can be sampled. Also, $\hat{p}^{tK}$ for kidney tissue in either assay never increased above 0.50 regardless of the number of surveys completed. Liver $\hat{p}^{tK}$ reached greater than 0.80 when five surveys were completed with DFAT, and three surveys completed with qPCR, further suggesting testing liver tissues with qPCR is the best combination for *R. salmoninarum* detection.

2.4 Discussion

The goal for fish health diagnostics is to use sampling methods and diagnostic assays that have the capacity to detect and measure low numbers of pathogens present in fish tissues. *Renibacterium salmoninarum* detection within various fish tissues and assays remains variable, despite decades of research (Pascho et al. 2002; Elliott et al. 2013). Currently there is no protocol or tissue that results in detection certainty (i.e. a gold standard) for detection of *R. salmoninarum*. Current standards to test salmonids in hatchery facilities and wild populations include DFAT as a presumptive testing method and a PCR (nested or quantitative) assay as a confirmatory method. The practical simplicity, sensitivity, and specificity of DFAT coupled with the high analytical and diagnostic specificity of PCR provides a useful method for screening for and confirming *R. salmoninarum* presence in tissues. However, the estimated detection probabilities for the two assays suggest that it might be possible to miss infections with lower number of bacteria present in Cutthroat Trout reared in Colorado.

When testing for regulated pathogens during a fish health assessment in Colorado, DFAT is used as the presumptive test and a PCR method is only used to confirm presence of the pathogen if DFAT is positive. Thus, if a fish is negative by DFAT it is never tested by qPCR. Therefore, if false negatives occur with DFAT, infected fish could be incorrectly diagnosed and cleared for

transportation to other facilities or stocked into the wild, potentially resulting in transmission of the pathogen into other hatchery or wild populations. In my study, 723 fish tested negative for *R. salmoninarum* by DFAT using kidney tissues and would have never been tested with qPCR. If my study was part of a standard fish health inspection, many 382 fish tested would be reported as a false negative results by DFAT and not confirmed with a follow-up qPCR test, leading to movement or stocking of infected fish throughout the state. Thus, I feel that it is important to consider detection probabilities for the two assays used to detect the presence of *R. salmoninarum* and make decisions regarding fish transportation from hatchery facilities.

2.4.1 *Assay Detection Probability*

The use of occupancy modeling has increased in disease ecology for estimating disease prevalence under the assumptions of laboratory assay uncertainty (McClintock et al. 2010; Lachish et al. 2012; Miller et al. 2012). Such models have been useful to successfully determine malaria parasite detection abilities with qPCR from blood samples of blue tits (Lachish et al. 2012) and detection probability of avian influenza virus in waterfowl populations (McClintuck et al. 2010). I used a similar modeling approach with multiple tissues that is conceptually analogous to a single-season multi-state occupancy model. This approach allows for the estimation of detection probabilities between DFAT and qPCR, allowing us to account for the probability of false negative results (i.e., not correctly detecting bacteria when it is present in the tissue). A high probability of false negative results occurred most often with DFAT compared to qPCR, validating that there is a higher detection probability for *R. salmoninarum* with qPCR. Although detection probability for qPCR was still lower than what is desired for an optimized assay (< 0.90), the low numbers of bacteria present in the fish I sampled could have influenced these results. Calculating detection probabilities with known infection intensities ranging from low to

high would result in better estimates of detection probability. The low detection probability when using DFAT was not surprising as I have seen similar patterns during routine fish health inspections and other studies within the state. In a statewide survey to determine the prevalence and distribution of *R. salmoninarum* in Colorado, DFAT and qPCR were used to screen kidney tissues in naturally infected Cutthroat Trout and Rainbow Trout populations. Positive detections with qPCR resulted in an estimated 23.5% of populations that were infected with *R. salmoninarum* whereas only 4.8% were infected based on DFAT. In addition, the two assays agreed on infection status in only one of the populations tested (Kowalski et al. 2022). In contrast, detection of *R. salmoninarum* with DFAT in kidney tissues was near 100% in Chinook Salmon (O'Farrell et al. 2000). The reason for the apparent lack of detection with DFAT in my study is not currently known. However, one possible explanation could be related to the specific strain of *R. salmoninarum* I detected in Colorado among inland trout.

Virulent strains of *R. salmoninarum* include detectable p57 associated proteins on the bacterial cell surface (Bruno 1988; Daly & Stevenson 1989). In contrast, attenuated virulent strains often show little to no detectable p57 (Bruno 1988; O'Farrell & Strom 1999; Senson & Stevenson 1999; O'Farrell et al. 2000; Elliott et al. 2013). The presence of the p57 protein is also the detectable protein for which the DFAT assay has been optimized, with a high specificity. Thus, without the presence of the surface protein, the *Fluorescein*-labeled antibody to *R. salmoninarum* used in the DFAT assay cannot bind to the bacteria, resulting in a lack of or poor fluorescence. O'Farrell et al. (2000) noted tissues extracted from fish injected with an attenuated virulent strain had poor fluorescence compared to fish infected with a virulent strain. In another study (Appendix I), I also showed that the strain found in at least one Colorado hatchery has the potential to be an attenuated virulent strain. Thus, the poor diagnostic performance I observed

with DFAT may be due to the low specificity to the un-identified strain detected in the fish tissues tested in this study.

2.4.2 *Probability of Tissue Infection*

Kidney tissue is the most widely used tissue when testing for the presence of *R. salmoninarum*. The AFS Fish Health Blue Book recommends sampling kidney tissues and was developed based on infection patterns in highly *R. salmoninarum*–susceptible salmonids (AFS-FHS 2016). This is likely because the salmonids are the principal reservoirs of infection and mortalities have been as high as 80% with Pacific salmon and 40% with Atlantic salmon (Evenden et al. 1993; Weins 2011). Internal clinical signs of disease have been observed in kidney tissues and include kidneys that are grey, swollen with fluids, and have white nodular lesions (AFS-FHS 2016). Not only are clinical signs of disease seen in kidney tissues, but kidney tissues are the primary site for bacterial uptake for many aquatic pathogens including *R. salmoninarum* and *Aeromonas salmonicida* (Ferguson & McCarthy 1978; Ferguson et al. 1982). Despite high prevalence of *R. salmoninarum* infections among inland trout of Colorado, we rarely see signs of clinical disease in kidney tissues (Kowalski et al. 2022). However, other haemopoietic tissues may harbor the bacteria in inland Cutthroat Trout.

In contrast to other studies, my study revealed a high probability of detecting infections in liver tissues compared to kidney tissues. Although kidney tissue is the most important organ for clearing bacteria from the blood through passive uptake and phagocytosis by reticuloendothelial cells (Dos Santos et al. 2001; Ferguson et al. 1982), the liver tissue may be acting as an essential part of the innate immune response. The innate immune response of teleost fishes typically occurs in the head kidney, spleen, and mucosal sites (Causey et al. 2018). Although not completely known, the liver may be serving a secondary role in supporting host

defenses, such that the kidney acts as the first defense by phagocytosis then the liver removes the foreign materials from the circulation of blood with activation of the innate immune response (Causey et al. 2018). Therefore, I suspect I am detecting a higher number of fish infected with *R. salmoninarum* using liver tissues because the liver is acting as a site to further destroy the bacteria before it is completely cleared from the fish. I would suggest attempting to culture the bacteria from liver tissues suspected to have a high intensity bacterial infection to determine if the bacteria is viable. If culturing the bacteria from the liver was unsuccessful despite a relatively high intensity of bacteria detected using qPCR, the liver may be acting as part of an immune response to rid the infection versus a source of active infection.

2.4.3 *Conditional Probability of Detection*

One challenge is understanding how many times to test a single tissue to ensure a high probability of detection (Chaudhary et al. 2020). When comparing liver and kidney tissue, liver tissue always had the highest conditional probability of detection with both DFAT and qPCR. In addition, as the number of surveys increase, the conditional probability of detection for liver is always higher than kidney tissues and exceeds 80% when the tissue is tested at least three times with qPCR or five times with DFAT. Typically, only one survey is completed in a standard fish health inspection using kidney tissue and a DFAT test. Therefore, the conditional probability of detection, given the kidney tissue is infected, is only 21%. In comparison, the conditional detection probability using DFAT on liver tissue is 32%. Both conditional probabilities are significantly lower than expected for most standard diagnostic methods. Therefore, I recommend collecting both tissues and testing as one homogenate sample multiple times, which will increase the probability of detection. If that cannot be completed, I suggest collecting and testing liver tissue with qPCR.

2.4.4 *Non-lethal Detection*

The development and use of non-lethal methods to detect aquatic pathogens have been increasing, especially among populations of conservation concern. The AFS Fish Health Blue Book recommends collecting non-lethal ovarian fluid from adult spawning fish for detection of *R. salmoninarum* (AFS-FHS 2016). I did not detect *R. salmoninarum* in the ovarian fluid of many female fish in which other tissues were positive, especially when using DFAT. Others have shown that using DFAT with ovarian fluid is not reliable when infection intensity is low in the sample (Armstrong 1989; Elliott & McKibben 1997). My results also show that when infection intensity was high in an ovarian fluid sample by qPCR, more ovarian samples were also positive by DFAT. Detections with DFAT decreased when the number of bacteria in the ovarian fluid was less than 26 bacterial cells/mL^{-1} by qPCR. Thus, I suggest that when using ovarian fluid as a non-lethal sampling method to screen for *R. salmoninarum*, using qPCR over DFAT may be best since it can detect low level infections. I did notice higher detections with mucus swabs than other non-lethal serums collected, suggesting that this could be a better screening method for both sexes; however, I need to determine what a positive detection means relative to an internal infection before implementing this method as a fish health diagnostic method. For instance, a positive mucus swab may represent a pre-, active, or post-infection of the fish. Alternatively, the mucus could simply be acting as a potential barrier and indicator of the bacteria in the environment (Riepe et al. 2021). Lastly, low detections in whole blood were observed and may be due to PCR-inhibitory components that may occur in blood samples (Abu Al-Soud & Radstrom 2001; Elliott et al. 2015). For further studies, I suggest testing blood plasma to limit the potential for any inhibitory effects.

2.5 Conclusion

In this study, I tested three non-lethal serum (blood, ovarian fluid, mucus swabs) and three lethal tissues (spleen, liver, kidney) with DFAT and qPCR. DFAT is typically used as a presumptive diagnostic testing method and qPCR as a confirmatory method in fish health inspections. When the detection probability of the presumptive method is low, high rates of false negative results occur and the potential for stocking unknown infected fish into the wild can arise. Compared to qPCR, I found that testing tissues with DFAT results in low detection for *R. salmoninarum.* Fish should be tested with the more sensitive, specific, and reliable qPCR test if it is believed that infections are present in the hatchery or wild population. I also suggest multiple testing of lethal liver tissues or testing combined homogenate sample of kidney and liver tissues for detecting *R. salmoninarum* in Cutthroat Trout. Although I had high detections with mucus swabs and low detections with ovarian fluid, the objective of the testing may aid in determining which non-lethal test to use. If the goal is to limit vertical transmission on a hatchery unit or within a wild population, testing ovarian fluid may be beneficial. If this method is used in inland salmonids, I suggest testing samples with qPCR rather than DFAT. Mucus swabs may be used swabs for *R. salmoninarum* detection in instances where knowing the presence or absence of the bacteria in the fish or the environment is needed to determine if fish should be transported since detection may or may not represent an active infection (Riepe et al. 2021). My results contrast with the suggested methods of testing kidney tissues or ovarian fluid with DFAT for presumptive testing. Therefore, determining detection probability of assays with different fish species, bacterial strains, and tissue types may be useful for setting regulations based on optimal detections of *R. salmoninarum.*

Table 2.1. Possible detection history for each state of infection from a combination of *Renibacterium salmoninarum* detection or non-detection status in the kidney or liver tissues. Each detection history consists of two surveys (S) for each tissue (kidney and liver). Probability of infection (Ψ) for each assay is listed for each possible state of infection.

Detection History						
Kidney		Liver				
S1	S2	S1	S2	Infection State	Ψ (DFAT)	Ψ (PCR)
0	0	0	0	State 1 {K–L–}	0.83	0.21
0	1	0	0	State 2 {K+L–}	<0.01	0.10
1	0	0	0	State 2 {K+L–}		
1	1	0	0	State 2 {K+L–}		
0	0	1	0	State 3 {K–L+}	0.10	0.43
0	0	0	1	State 3 {K–L+}		
0	0	1	1	State 3 {K–L+}		
1	0	1	0	State 4 {K+L+}	0.06	0.26
1	0	0	1	State 4 {K+L+}		
0	1	0	1	State 4 {K+L+}		
0	1	1	0	State 4 {K+L+}		
1	1	1	1	State 4 {K+L+}		
1	1	1	0	State 4 {K+L+}		
1	1	0	1	State 4 {K+L+}		
1	0	1	1	State 4 {K+L+}		
0	1	1	1	State 4 {K+L+}		

Table 2.2. Multinomial logistic regression results comparing covariates affecting *Renibacterium salmoninarum* presence detected by either DFAT or qPCR for each state of infection represented by z-test statistic and p-value (significance $\alpha = 0.05$).

Model	Covariate	State of Infection	Z	p-value
qPCR	Sex	State 1 {K– L–}	0.38	0.71
		State 2 {K+ L–}	-0.38	0.71
		State 3 {K– L+}	0.99	0.32
		State 4 {K+ L+}	0.21	0.83
	Length	State 1 {K– L–}	0.50	0.61
		State 2 {K+ L–}	-0.50	0.61
		State 3 {K– L+}	-1.22	0.22
		State 4 {K+ L+}	-0.48	0.63
	Weight	State 1 {K– L–}	1.13	0.26
		State 2 {K+ L–}	-1.13	0.26
		State 3 {K– L+}	-0.82	0.41
		State 4 {K+ L+}	-1.11	0.27
DFAT	Sex	State 1 {K– L–}	1.26	0.21
		State 2 {K+ L–}	-1.26	0.21
		State 3 {K– L+}	-0.26	0.79
		State 4 {K+ L+}	-0.73	0.46
	Length	State 1 {K– L–}	-1.24	0.21
		State 2 {K+ L–}	1.24	0.21
		State 3 {K– L+}	-0.66	0.51
		State 4 {K+ L+}	0.15	0.88
	Weight	State 1 {K– L–}	-1.23	0.22
		State 2 {K+ L–}	1.23	0.22
		State 3 {K– L+}	-0.40	0.69
		State 4 {K+ L+}	0.08	0.93

$$y_{i,j} \sim \text{categorical}(\boldsymbol{P}_{i,j} * z_i)$$

$$z_i \sim \text{categorical}(\boldsymbol{\phi})$$

$$\boldsymbol{\phi} = [\Psi_1, \Psi_2, \Psi_3, \Psi_4]$$

$$\boldsymbol{P}_{i,j} = \begin{bmatrix} 1 & 0 & 0 & 0 \\ 1-p_2 & p_2 & 0 & 0 \\ 1-p_3 & 0 & p_3 & 0 \\ (1-p_2)*(1-p_3) & p_2*(1-p_3) & (1-p_2)*p_3 & p_2*p_3 \end{bmatrix}$$

$$\beta_{\Psi,k} \sim \text{gamma}(1,1) \quad k = 1,2,3, \text{or } 4$$

$$\Psi_1 = \frac{\beta_{\Psi,1}}{\sum_{i=1}^{4} \beta_{\Psi,k}}$$

$$\Psi_2 = \frac{\beta_{\Psi,2}}{\sum_{i=1}^{4} \beta_{\Psi,k}}$$

$$\Psi_3 = \frac{\beta_{\Psi,3}}{\sum_{i=1}^{4} \beta_{\Psi,k}}$$

$$\Psi_4 = \frac{\beta_{\Psi,4}}{\sum_{i=1}^{4} \beta_{\Psi,k}}$$

$$p_2 \sim \text{uniform}(0,1)$$

$$p_3 \sim \text{uniform}(0,1)$$

Figure 2.1. Model statement for the static multi-state occupancy model in a Bayesian hierarchical framework.

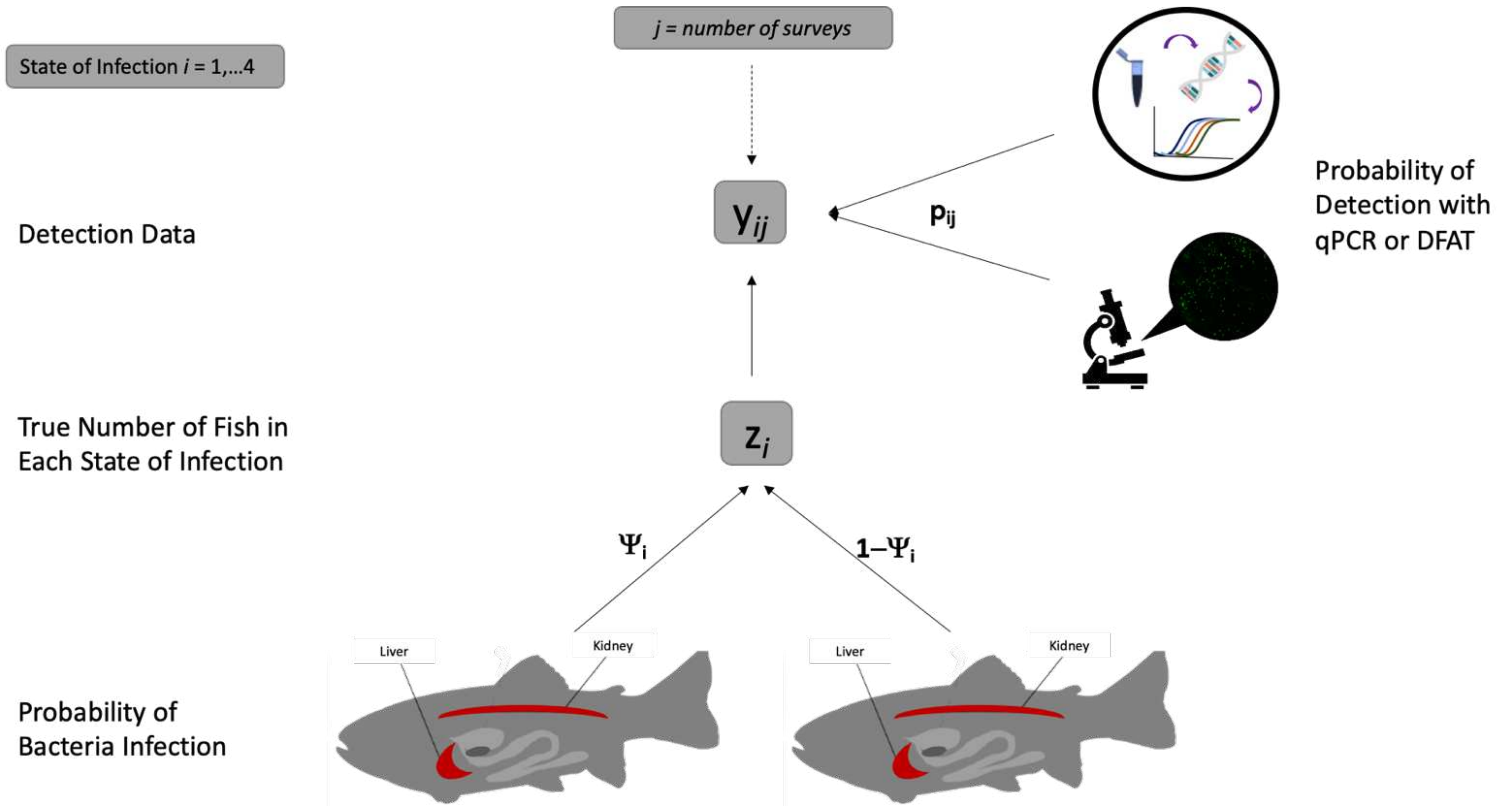

Figure 2.2. Conceptual diagram of hierarchical occupancy model where Ψ_i is the probability of infection for each state of infection, z_i is the true occupancy within each state of infection, p_{ij} is the probability of detecting *Renibacterium salmoninarum* from either DFAT or qPCR (modeled independently) for the state of infection i during survey j, and y_{ij} is the detection data (1, 2, 3, or 4) for each state of infection i during survey j.

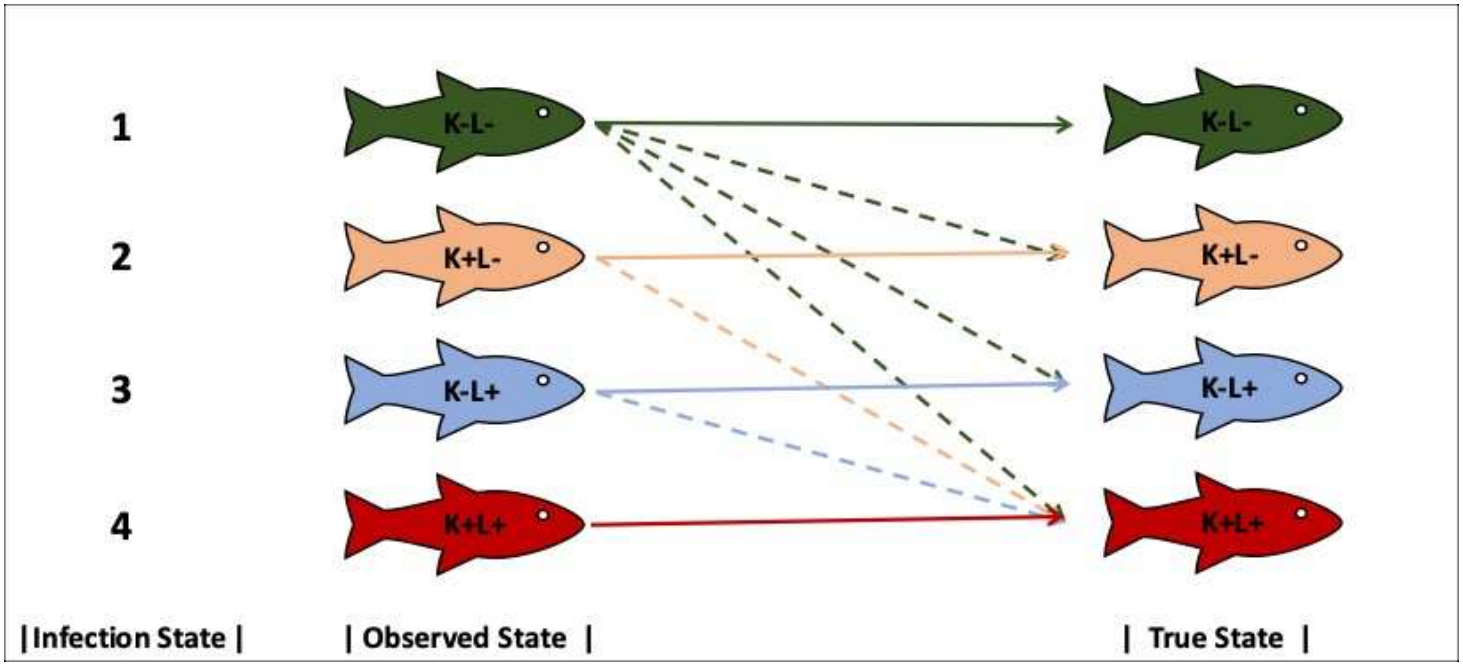

Figure 2.3. Conceptual diagram of observed and true infection states and possible combinations of how a true infection state may be observed with DFAT or qPCR (indicated by colored lines; K: Kidney, L: Liver, -: negative detection, +: positive detection). Solid lines indicate the probability the observed fish is in the true state and dashed lines indicate the probability of the observed fish in a different true state.

		Observed State			
		1	**2**	**3**	**4**
True State	**1**	1	0	0	0
	2	0.46	0.54	0	0
	3	0.64	0	0.36	0
	4	0.29	0.35	0.17	0.19

		Observed State			
		1	**2**	**3**	**4**
True State	**1**	1	0	0	0
	2	0.27	0.73	0	0
	3	0.39	0	0.61	0
	4	0.11	0.28	0.16	0.45

Figure 2.4. Values represent the probability of observing an infection state given the probability of the true state of infection for each of the assay (DFAT top, qPCR bottom).

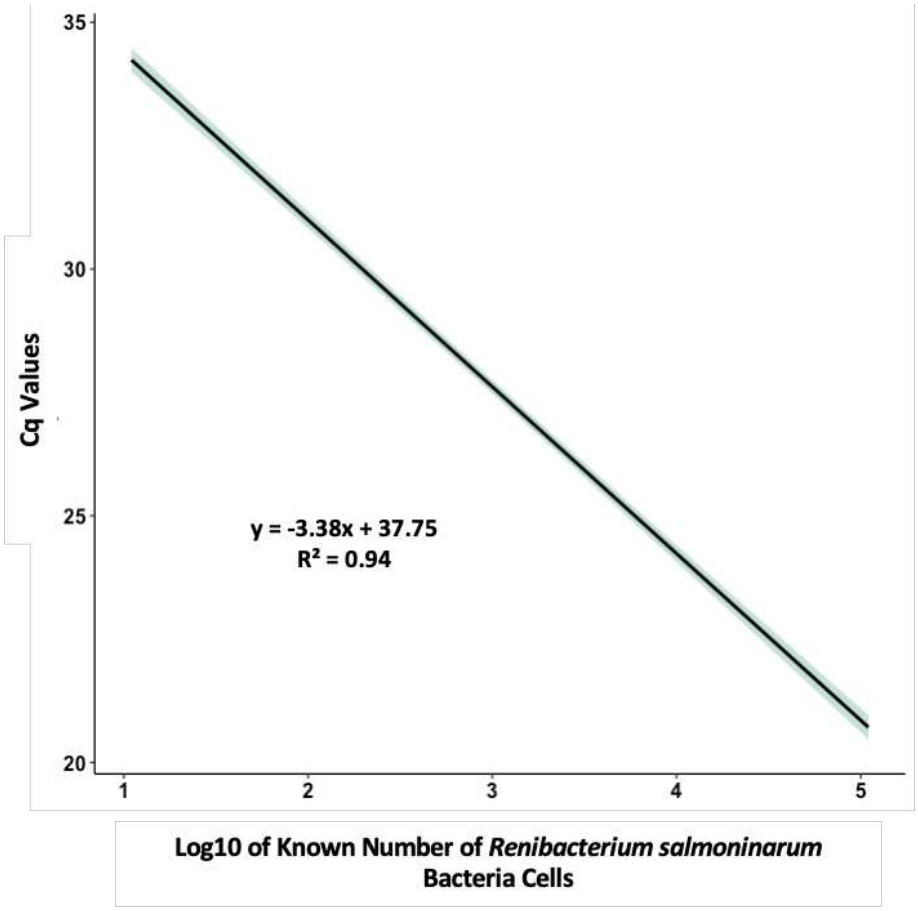

Figure 2.5. Standard curve generated from 58, five-$\log_{10}$ serial dilutions of *R. salmoninarum* with associated confidence intervals. Cq values are plotted as a function of five-$\log_{10}$ of the known number of bacterial cells that were quantified by membrane-filtration fluorescent antibody test from pure culture of *R. salmoninarum* collected from Rainbow Trout at the Colorado Parks and Wildlife Bellvue Fish Research Hatchery.

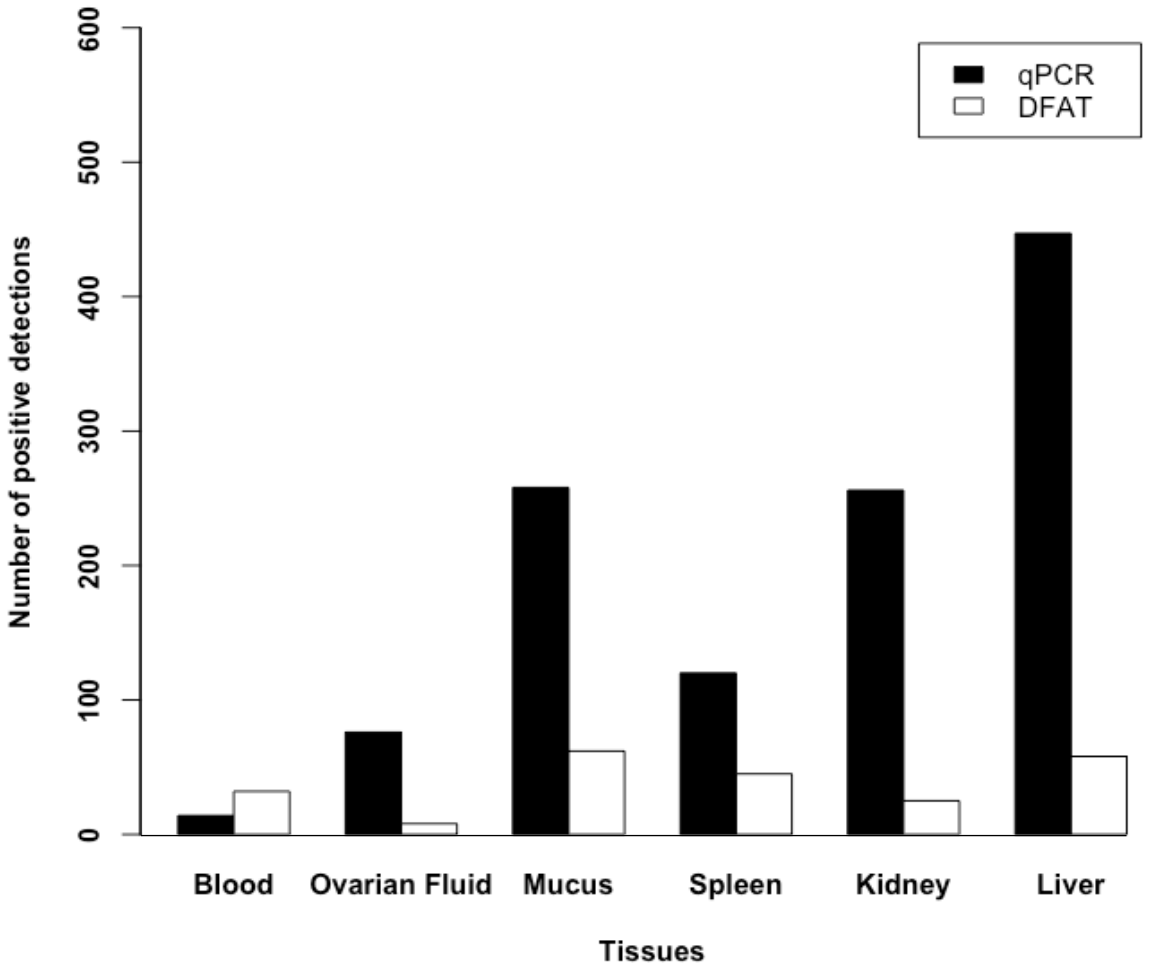

Figure 2.6. Number of positive *R. salmoninarum* detections from six tissues (non-lethal serum samples: blood, ovarian fluid, mucus; lethal tissues samples: spleen, kidney, liver) using qPCR (black bars) and DFAT assays (white bars).

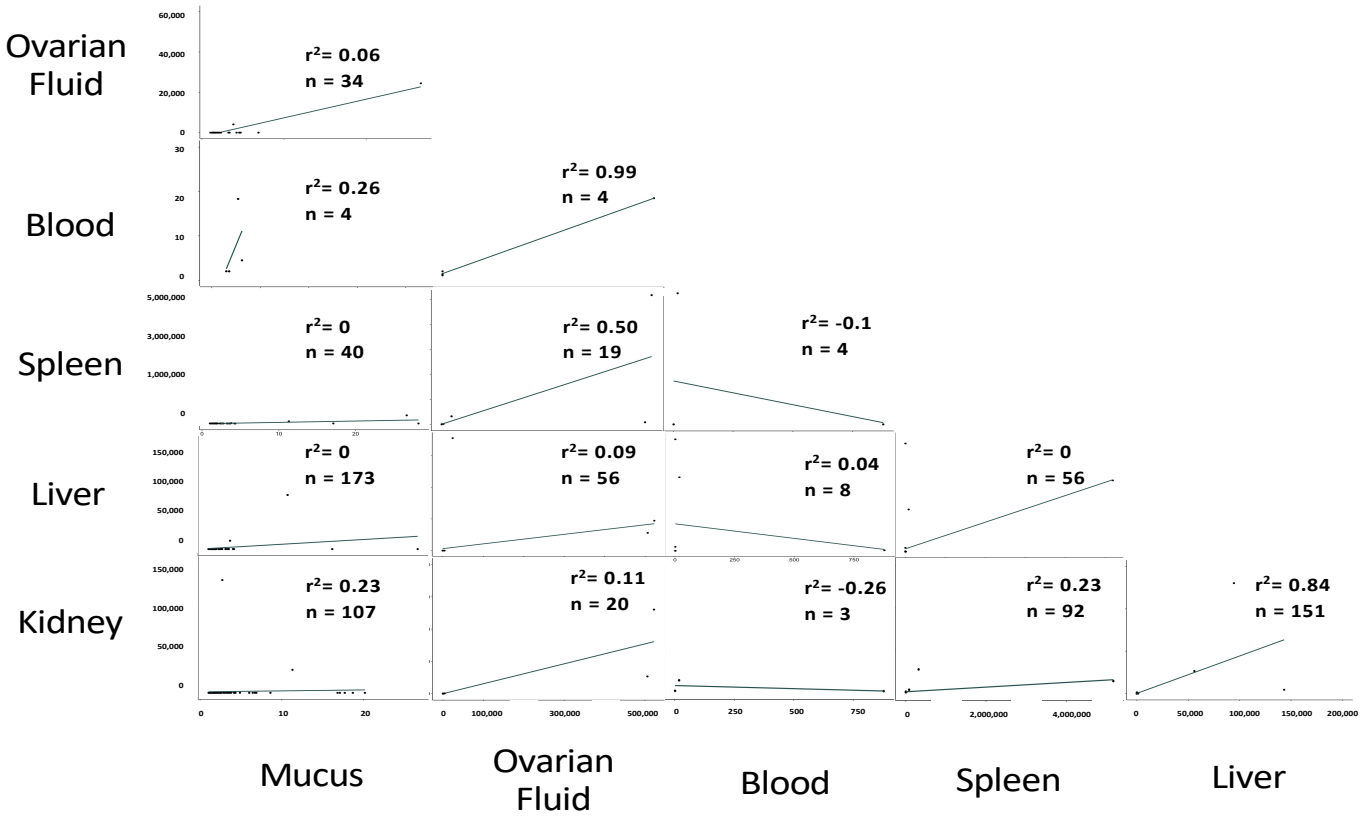

Figure 2.7. Correlations of quantities of bacterial cells present in each tissue from qPCR reactions. Each panel shows the linear relationship between a pair of tissues when both tissues being compared are positive for the detection of *R. salmoninarum* within the same fish. The association (r^2) and the number of tissues (n) is represented for each association.

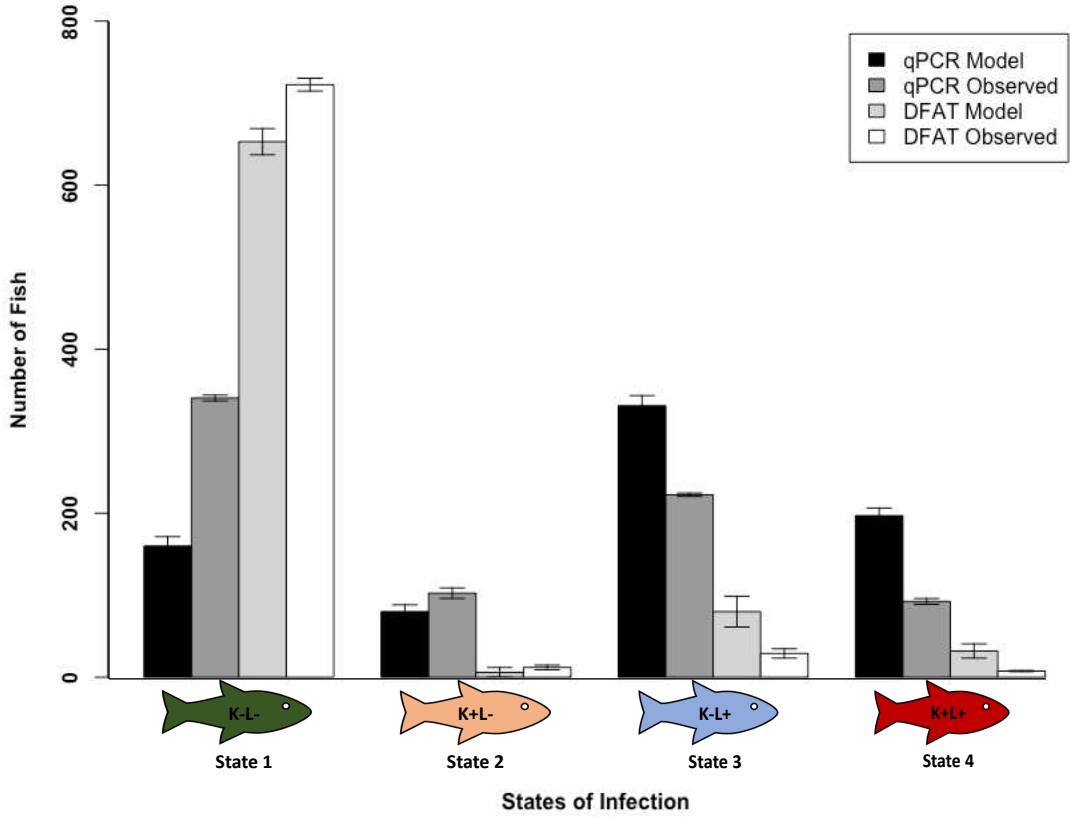

Figure 2.8. The number of fish observed and the number of fish estimated from the qPCR or DFAT state process model for each state of infection.

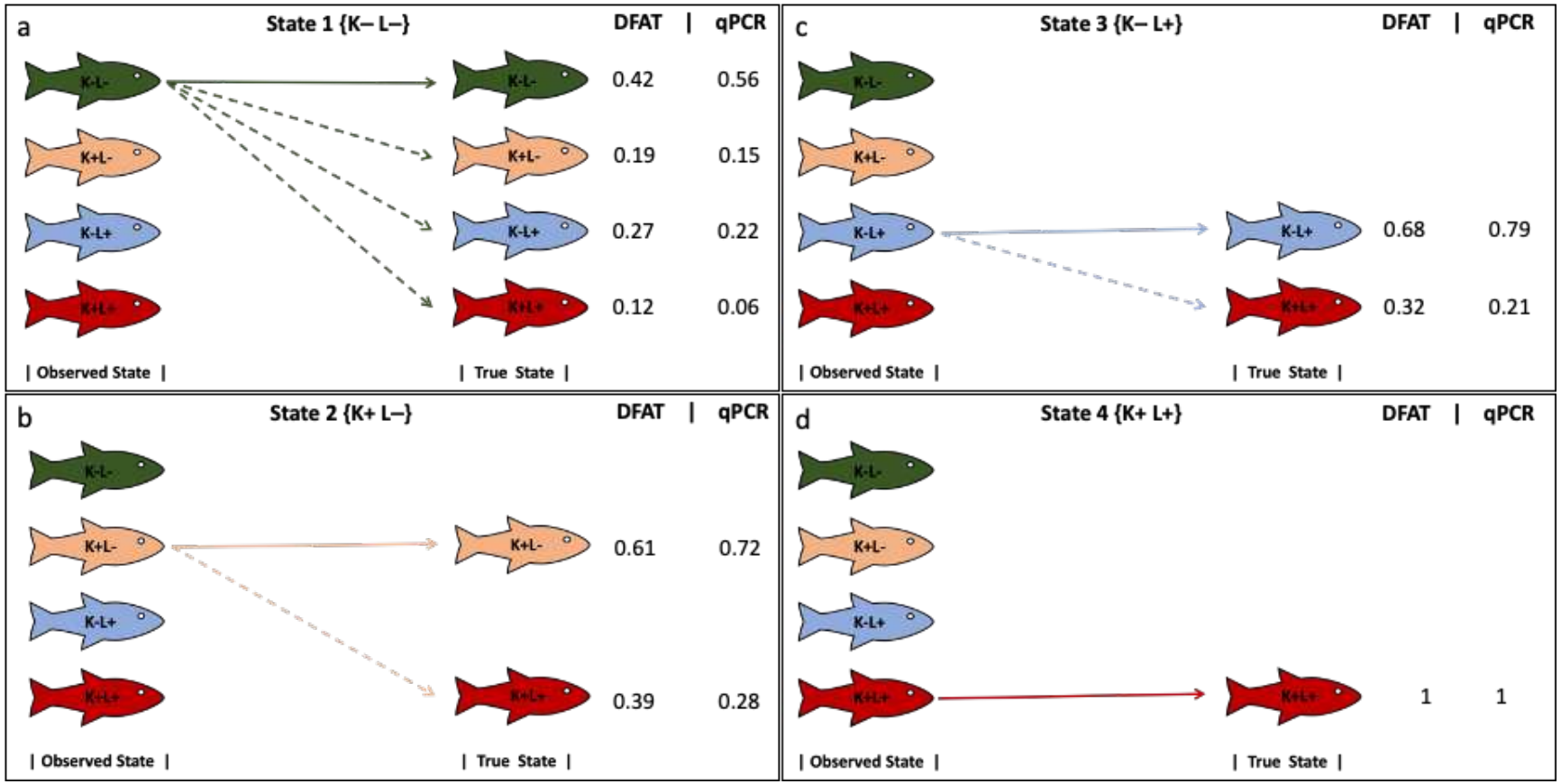

Figure 2.9. Each panel represents the probability that the observed state of infection represents the same or different true state of infection. Solid lines represent the probability of observing a fish in the true state of infection. Dashed lines indicate the probability of observing a state of infection different from the true infection state due to false negative results with DFAT and/or qPCR (a: State 1 {K–L–}, b: State 2 {K+L–}, c: State 3 {K–L+}, d: State 4 {K+L+}).

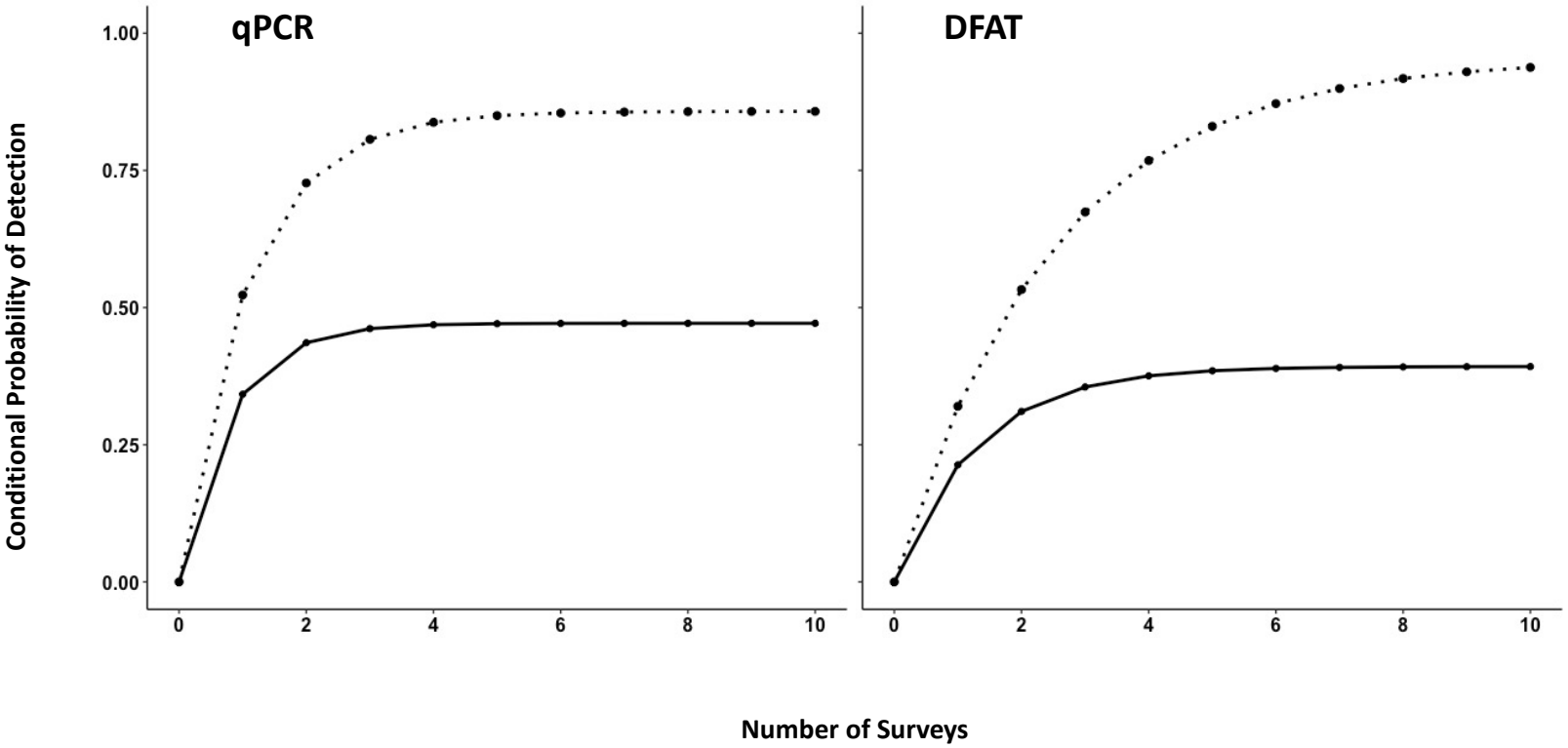

Figure 2.10. Conditional probability of detecting *Renibacterium salmoninarum* in liver (dotted line) or kidney tissue (solid line) given the tissue is infected, as a function of the number of surveys conducted with each assay (DFAT or qPCR).

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CHAPTER 3

HORIZONTAL TRANSMISSION OF *RENIBACTERIUM SALMONINARUM* IN A FLOW THROUGH HATCHERY SYSTEM

3.1 Introduction

Flow-through fish culture systems have been widely used for rearing salmonids for stocking and broodstock maintenance (Stickney 2016). In a flow-through system, surface water from a nearby source is diverted by gravity or underground pipes from the inflow to hatch houses or nurse basins housing younger age classes of fish. The effluent water then enters raceways used for growing fish to larger sizes and maintaining brood fish and leaves the system to reenter the surface water or to irrigate pastures or crops. To support high fish densities, the effluent from each location may contain self-cleaning features such as internal standpipes and settling ponds (Stickney 2016). Settling ponds are used to collect wastewater, remove suspended solids (i.e., unused feed, fecal matter, pathogens, etc.), and filter water before it leaves the unit. These methods do not always effectively remove all solids from the system and, consequently, organic material can build up as the water flows through the facility (Larmoyeux & Piper 1973). Organic material such as fecal matter can travel up to 200 meters from the source and throughout flow-through hatchery systems (Gowen 1987). Suboptimal water quality from the buildup of these solids may create favorable conditions for horizontal transmission of pathogens, adversely affecting fish health both on and below the hatchery.

Bacterial kidney disease (BKD), caused by the bacterium *Renibacterium salmoninarum*, is one of the most harmful diseases of salmonids worldwide, decimating fish in both wild and

aquaculture populations (Pascho et al. 2002). Chronic disease can cause external lesions, ascites, exophthalmos, hemorrhaging in the abdomen, and granulomatous lesions in the kidney tissue or other hematopoietic tissues (AFS-FHS 2016). Mortality from BKD has been observed as high as 80% in Pacific salmon (*Oncorhynchus* spp.; Evenden et al. 1993). However, fish more often exhibit subclinical infections, with low infection intensities, than overt disease (Delghandi et al. 2020). This can be attributed to the ability of the bacteria to evade host defense mechanisms by invading and multiplying in host macrophages and remaining in a dormant state (Young & Chapman 1978). Fish in a subclinical infection state may be carriers of the bacteria, able to spread *R. salmoninarum* to uninfected fish.

Transmission of *R. salmoninarum* to uninfected fish can occur via vertical and/or horizontal transmission. Vertical transmission is primarily maternal, originating in the ovarian tissues, resulting in an intra-ovum infection (Evelyn et al. 1986). Horizontal transmission can occur through the ingestion of contaminated fecal matter or through entryways on the fish including external lesions or the gills (Evelyn 1988; Evenden et al. 1993). Aquaculture facilities provide more opportunities for horizontal transmission than in wild populations due to increased contact with infected fish, high fish densities, and stressful rearing environments (VanderKooi & Maule 1999; Larson et al. 2020). Stressors include crowding, increased fish handling, poor water quality, and low nutritional value of available diets. Both high and low water temperatures can influence the severity of BKD and intensity of *R. salmoninarum* in infected fish, depending on the species (Sanders et al. 1978; Purcell et al. 2016; Plumb 2018). Temperatures can also affect survival of the bacteria in the environment, and suitable temperatures for viability between 10–18°C allow the bacteria to remain in the water for up to 21 days (Pascho et al. 2002; Balfry et al. 1996). Many hatchery facilities in Colorado rear *R. salmoninarum*-susceptible species such as

Rainbow Trout (*Oncorhynchus mykiss*) and Cutthroat Trout (*Oncorhynchus clarkii*) between 8–16°C, which overlaps with the optimal range for the *R. salmoninarum* persistence.

Renibacterium salmoninarum was detected at the Colorado Parks and Wildlife (CPW) Poudre Rearing Unit (PRU) during annual fish health inspections conducted between 2015 and 2019 in the broodstocks of Rainbow Trout and Cutthroat Trout and the source of infection was unknown. The objective of my study was to monitor the potential transfer of *R. salmoninarum* from infected brood fish to juvenile Cutthroat Trout at the PRU during optimal water temperatures for horizontal transmission. Specifically, I measured *R. salmoninarum* prevalence in Cutthroat Trout located near *R. salmoninarum* infected broodstock, in settling ponds, in the influent source, and in the hatchery effluent, which reenters the river after use and could potentially influence transmission to wild salmonid populations.

3.2 Study Site

The CPW PRU is a flow-through hatchery producing and stocking over fifty thousand Rainbow Trout and Cutthroat Trout annually and maintains broodstocks of both species for egg production. The PRU is located about 50 miles west of Fort Collins, CO and is adjacent to the Cache la Poudre River. The PRU obtains surface water from the river through an inflow pond prior to entering the hatchery and does not include any biosafety mechanisms to decontaminate water prior to entering the facility (Figure 1.3). From the pond, the influent enters an underground pipe, is mixed with well water, and directed to raceways containing *R. salmoninarum*–negative catchable fish reared on the unit. These catchable fish are located in separate raceways above the broodstock used in this study. The water from the first raceways is directed to the north and south broodstock raceways. The north raceway contained two and three-year old Cutthroat Trout (1,000 fish) that were previously estimated to exhibit an *R.*

salmoninarum infection prevalence of 79% (Chapter 2). The south raceway contained three-year-old Rainbow Trout (50 fish) that had an estimated *R. salmoninarum* prevalence of 80% (CPW Aquatic Animal Health Lab *unpublished data*).

I evaluated the potential for horizontal transmission of *R. salmoninarum* through the water to naïve Cutthroat Trout at six locations. I deployed one cage in the water inflow pond to test if the surface water supply could be a source of *R. salmoninarum.* Two cages were deployed in each of the north and south raceways to determine if horizontal transmission can occur between the two infected species and near infected fish. In the south raceway, cages were located in the same section as the Rainbow Trout. Cages in the north raceway were set approximately 200 ft downstream of the infected Cutthroat Trout to determine if proximity to infected fish could influence successful transmission. The north raceway drains into settling ponds #1 and #2 in succession. The south raceway drains directly into settling pond #2, from which all effluent exits the hatchery back into the Cache la Poudre River. Two cages were placed in the settling ponds to determine if they were acting as pathogen removal before entering the effluent. The last cage location was below the settling ponds and in the hatchery effluent entering the Cache la Poudre River to determine if *R. salmoninarum* was leaving the hatchery and entering the river, potentially exposing fish in the river to the bacteria.

3.3 Methods

In 2019, I deployed a total of 360, 12-month-old Cutthroat Trout collected from the PRU Isolation Building that is fed with well water and not connected to any surface water. Twenty fish from the PRU Isolation Building were collected and tested lethally for the presence of *R. salmoninarum* before the start of the experiment to ensure the sentinel fish were not currently infected. At each of the six locations described above (Figure 3.1), two cages containing ten fish

each were set in a single deployment (120 fish/deployment) and were deployed over three, 30-day time periods (D1: May 15– June 16, D2: June 16 – July 14, D3: July 14 – August 13, D# = deployment period). Deployment dates were set using historical records of water temperatures at the PRU that were consistent with the optimal water temperatures for *R. salmoninarum* to persist (10–18°C; Pascho et al. 2002). Cages were fabricated using 6-inch schedule 40, PVC pipes fitted with screw top lids. A 6" by 8" hole was cut into the side of the pipe and fitted with a 0.5 inch wire mesh screen to ensure fish were exposed to the surrounding water but would not escape. Fish were fed through the wire mesh with Rangen size three fish feed, once per day. Any mortalities that occurred during the experiment were removed and cause of death was not determined because fish tissues were typically decayed. All fish were weighed (g) and measured (mm) prior to deployment. After 30 days, fish were collected from the cages and a new set of 120 fish were deployed. Fish remaining in each cage after 30 days were weighed, measured, euthanized using tricaine methanesulfonate (MS-222; Western Chemicals), and frozen on dry-ice for transportation to the laboratory.

Tissues were collected from each fish to determine the rate of horizontal transmission of *R. salmoninarum* and the bacterial intensity among infected fish. Fish were thawed at the laboratory, and liver, spleen, and kidney tissues were collected through an abdominal incision and placed together in a sterile Whirl-Pak-Bag in which they were homogenized using sterile rolling pins. Approximately 0.25 g of tissue was collected from the bag for DNA extractions. Extractions were completed using a DNeasy Blood and Tissue Kit (Qiagen, Inc.; Hilden, Germany) with the addition of an extra elution step of 200 µL AE buffer to increase DNA concentration (Elliott et al. 2013). Quantitative PCR (qPCR) was used to detect and quantify the presence of *R. salmoninarum* targeting the major soluble antigen (*msa*) gene with predetermined

primer and probe sets; RS 1238 Forward, 5'-GTGACCAACACCCAGATATCCA-3', RS 1307 Reverse, 5'-TCGCCAGACCACCATTTACC-3', and MGB probe 1262, 5'-CACCAGATGGAGCAAC-3'. Each PCR reaction resulted in a final volume of 25 μL with 5 μL of DNA, 12.5 μL TaqMan Gene Expression Master Mix, 2.375 μL of dH_2O, and a total of 5.125 μL of the primers and probe (Chase et al. 2006). Positive detections in tissues were determined based on a maximum Cq value of 37.75, and a standard curve was used to quantify the number of bacteria present in infected tissues from a positive qPCR result (Chapter 2).

Fish weights were recorded at the start and end of each 30-day deployment. A one-way analysis of variance (ANOVA) was used to determine if the change in weight (ending weight – starting weight) affected the successful infection of *R. salmoninarum* in the caged fish. A post hoc Tukey's Honest Significant Difference (HSD) test was used to determine differences in the change in weights between deployments when the ANOVA p-value was less than 0.05.

Temperatures were recorded from one cage at each location using a thermochron high resolution iButton programmed to log temperatures every 255 minutes (roughly five times per day) to determine if deployment dates were within the optimal range for survival of *R. salmoninarum* in water. An ANOVA was used to determine if there were differences in daily average temperatures across the three deployments. A second one-way ANOVA was used to determine if temperature differed among the six locations within a deployment period. If differences were detected, a post-hoc Tukey's HSD test was employed to determine significant differences in mean temperatures among locations. This was completed for all three deployments. All analyses were performed in RStudio with the aov function and Tukey's HSD function in the R library agricolae and significance (α) was set at 0.05.

3.4 Results

3.4.1 *Fish Collection and Tissue Analysis*

No fish collected from the cages tested positive for *R. salmoninarum* during the first deployment (Table 3.1). The mortality rate during the first deployment was 9.1% (11 fish), with the highest mortality of 25% (5 fish) occurring in the cages near the infected Rainbow Trout in the south raceway. One fish tested positive for *R. salmoninarum* near the infected Rainbow Trout (south raceway) during the second deployment, with an average intensity of 6.5 $\pm$ 1.4 cells/mL^{-1} in the PCR sample (Table 3.1). Four fish (3.3%) died during the second deployment, with the highest mortality of 10% (2 fish) occurring in the south raceway. In the third deployment, no fish tested positive for *R. salmoninarum*. Mortality rates were highest in the third deployment, with a rate of 13.3% (16 fish) and the highest mortality rate of 65% (13 fish) in the south raceway (Table 3.1).

Fish lost weight in all deployments (D1: -1.13 $\pm$ 3.23 g, D2: -0.92 $\pm$ 3.65 g, D3: -1.92 $\pm$ 5.56 g). There was a difference in the change in weight among the three deployments (ANOVA $F_{2,326}$; 22.6, p-value < 0.001), with fish in deployment 3 losing significantly more weight than those in deployments 1 and 2 (TSD D2-D1: p-value = 0.90, D3-D1: p-value < 0.001, D3-D2: p-value < 0.001). Since the greatest change in weight was not observed in deployment 2, it was unlikely that weight loss facilitated the infection observed during that deployment. The caged fish in the south raceway during deployment 2 had an average weight change of -1.09 g (CI: -2.98 – 0.81 g) and the one infected fish had an average weight change of -2.30 g, suggesting that infection did not result in higher weight loss compared to uninfected fish.

3.4.2 *Temperature*

Water temperatures across the three deployments ranged from below the optimal *R. salmoninarum* temperature range (1.8°C) to above the optimal range (21.5°C; Figure 3.2). Average daily water temperatures were significantly different among the three deployments ($F_{3,18}$; 36.6, $p < 0.05$: Figure 3.2). The first deployment occurred from May 15 – June 16, 2019, and average water temperatures were 7.3 $\pm$ 1.9 °C (range: 1.75 – 12.6°C) across all locations (Figure 3.3). Only one day out of 30 was in the optimal range for *R. salmoninarum* persistence in the environment (10–18°C; Balfry et al. 1996). The second deployment occurred from June 16 – July 14, 2019. Average temperature was 10.7 $\pm$ 2.1°C (range: 5.4 – 16.1°C), with 19 days in the optimal range of 10–18°C. The third deployment was from July 14 – August 13, 2019. Average water temperature was 14.4 $\pm$ 1.94°C (range: 10.5–21.5°C), with all 30 days within the optimal range. Average daily water temperatures varied among locations within a deployment (Figure 3.4; Tables 3.2-3.4). Of the two deployment locations in closest proximity to infected fish (north and south raceways), the south raceway where the one infected fish was observed during the second deployment, was generally warmer and had more days within the optimal range of 10-18°C than the north raceway within each of the deployment periods (Figures 3.3 and 3.4).

3.5 Discussion

The goal of my study was to determine the rate of horizontal transmission of *R. salmoninarum* in a flow-through hatchery system. I detected the bacteria in one sentinel fish deployed near the infected Rainbow Trout in the south raceway, indicating that horizontal transmission is low but can occur near infected fish and within 30 days. Horizontal transmission in my study was lower than what was expected and inconsistent with previous research (Balfry et al. 1996). The three-year old Cutthroat Trout had an estimated *R. salmoninarum* infection

prevalence of 79% and the Rainbow trout had an estimated prevalence of 80% (Chapter 2). Additionally, only 1-21% of individual progeny originating from infected Cutthroat Trout held on the unit were also infected through vertical transmission (Chapter 4). Therefore, I expected higher horizontal transmission and prevalence of *R. salmoninarum* in the sentinel fish. Despite the low transmission rate, this finding is useful for *R. salmoninarum* management in Colorado because before this study, it was unclear if horizontal transmission could occur on flow-through hatcheries.

Transfer of *R. salmoninarum* through the fecal-oral route from infected fish has been suggested as the most likely way for horizontal transmission to occur, however direct contact with infected fish is also presumed to promote transmission (Balfry et al. 1996). The lack of direct contact with infected fish could have limited successful transmission in my study. The design of the cages only allowed the fish minimal exposure to fecal matter and no direct exposure to infected fish. The success of directly transmitted diseases often involves high contacts that drive the spread of the pathogens between infected and susceptible individuals. Previous studies have suggested that the high density of fish in a hatchery create favorable conditions for pathogen transmission (Pulkkinen et al. 2010). In addition, injuries and fin deformities often occur when fish densities are high in hatcheries (Noble et al. 2011). Open injuries create entryways for pathogens to leave the infected fish and enter uninfected fish. Broodstock at the PRU often have visible lesions and eroded fins from high densities and/or the tools used to clean the raceways. *Renibacterium salmoninarum* has been known to enter susceptible fish through open lesions, gills, and the eyes (Delghandi et al. 2020). Therefore, if the broodstock were actively shedding the bacteria, direct contact with the infected Rainbow Trout and Cutthroat Trout may have increased the rate of horizontal transmission in the sentinel fish.

The low level or lack of transmission may have also been due to timing, where the brood fish may not have been actively shedding bacteria into the water to promote transmission. An estimated 80% of the Rainbow Trout in this study were infected, but previous fish health assessments did not determine bacterial levels within the fish. A low infection intensity in the brood fish would lead to low shedding rates of bacteria in fecal matter (McKibben & Pascho 1999). Therefore, it is possible that more sentinel fish could have been infected had the number of bacteria in the brood fish been higher. Water temperature is also an important factor that may influence the shedding rate of the fish because it has been suggested to regulate the replication of the bacteria and the immune response of the broodfish (Purcell et al. 2016; Rozas-Serri et al. 2020). Higher temperatures (>11°C) tend to increase the shedding of bacteria from infected fish (Purcell et al. 2016). Lower temperatures have been speculated to slow the growth and replication of *R. salmoninarum* in the infected fish and promote metabolic dormancy of the bacteria (Hirvelå-Koski 2005). This dormancy is likely to lead to little or no shedding from the infected fish. This complex relationship between shedding of the bacteria and water temperatures have been observed in wild populations of spawning Pacific salmonids. In the Pacific Northwest, water temperatures vary throughout the spawning runs, but tolerable temperatures range from 3.3°C–13.3°C in the spring-summer and 10.6°C–19.4°C during the fall run (Bell 1986; Bjornn & Reisere 1991; Spence et al. 1996). Consequently, the fall run temperatures overlap with the optimal temperature for *R. salmoninarum* to persist in the water and, coupled with high fish density and high virulence, increased horizontal transmission of *R. salmoninarum* contributes to high prevalence in the Pacific salmon during this time (Rhodes & Mimeault 2019).

It is also important to understand the potential for variable transmission between virulent and attenuated virulent strains of *R. salmoninarum*, which may differ in their properties needed

for successful transmission. Multiple strains of *R. salmoninarum* have been described and isolated from the Pacific northwest, but recent tests suggest that the strain seen in at least one Colorado hatchery is an attenuated virulent strain, which could have affected transmission during my study (Appendix I). Thus, strain identification could be used to understand the risk of infection after *R. salmoninarum* has been detected on a hatchery unit.

3.6 Conclusion

Implementing biosecurity measures, frequent fish health diagnostics, and eliminating infected fish may help decrease the prevalence of infections on a hatchery. Although infection studies such as this provide opportunities to evaluate the potential transmission risk to fish on the unit when other infected fish are present, they cannot address the full complexity of infection dynamics that exist at a flow-through hatchery. As such, future studies should focus on horizontal transmission throughout the year, especially during the spawning season(s) when infected ovarian fluid may be expelled into the water, the viability of *R. salmoninarum* in the water, and how bacterial intensity of brood fish relates to bacterial shedding and transmission. Lastly, there is still not a gold standard diagnostic test for detecting *R salmoninarum* with a high level of certainty in either the fish or the water source. My results suggest that horizontal transmission from infected to naïve fish can occur relatively quickly (within 30 days), and despite negative results from recent fish health assessments, moving fish around could have the unintended consequence of spreading the bacteria to other fish on a unit, between units, or to wild fish populations. Reducing fish transfers within or between units could prevent the spread *R. salmoninarum* from units where the bacteria has become established but has not yet been detected.

Table 3.1. Percentage of fish mortality (from 20 total fish at each location) and infections of *Renibacterium salmoninarum* (Rsal) detected in fish at the end of each deployment from each location across the Poudre Rearing Unit. Quantities of *R. salmoninarum* are included when the bacteria were detected with qPCR.

Deployment	Location	Mortality	Rsal	Rsal Quant cells/mL $^{-1}$
1	Water Inflow	5.0 (1)	0	0
1	North Raceway	15.0 (3)	0	0
1	South Raceway	25.0 (5)	0	0
1	Settling Pond #1	5.0 (1)	0	0
1	Settling Pond #2	5.0 (1)	0	0
1	Effluent	0	0	0
2	Water Inflow	5.0 (1)	0	0
2	North Raceway	0	0	0
2	South Raceway	10.0 (2)	5.6 (1)	6.5
2	Settling Pond #1	5.0 (1)	0	0
2	Settling Pond #2	0	0	0
2	Effluent	0	0	0
3	Water Inflow	0	0	0
3	North Raceway	10.0 (2)	0	0
3	South Raceway	65.0 (13)	0	0
3	Settling Pond #1	0	0	0
3	Settling Pond #2	5.0 (1)	0	0
3	Effluent	0	0	0

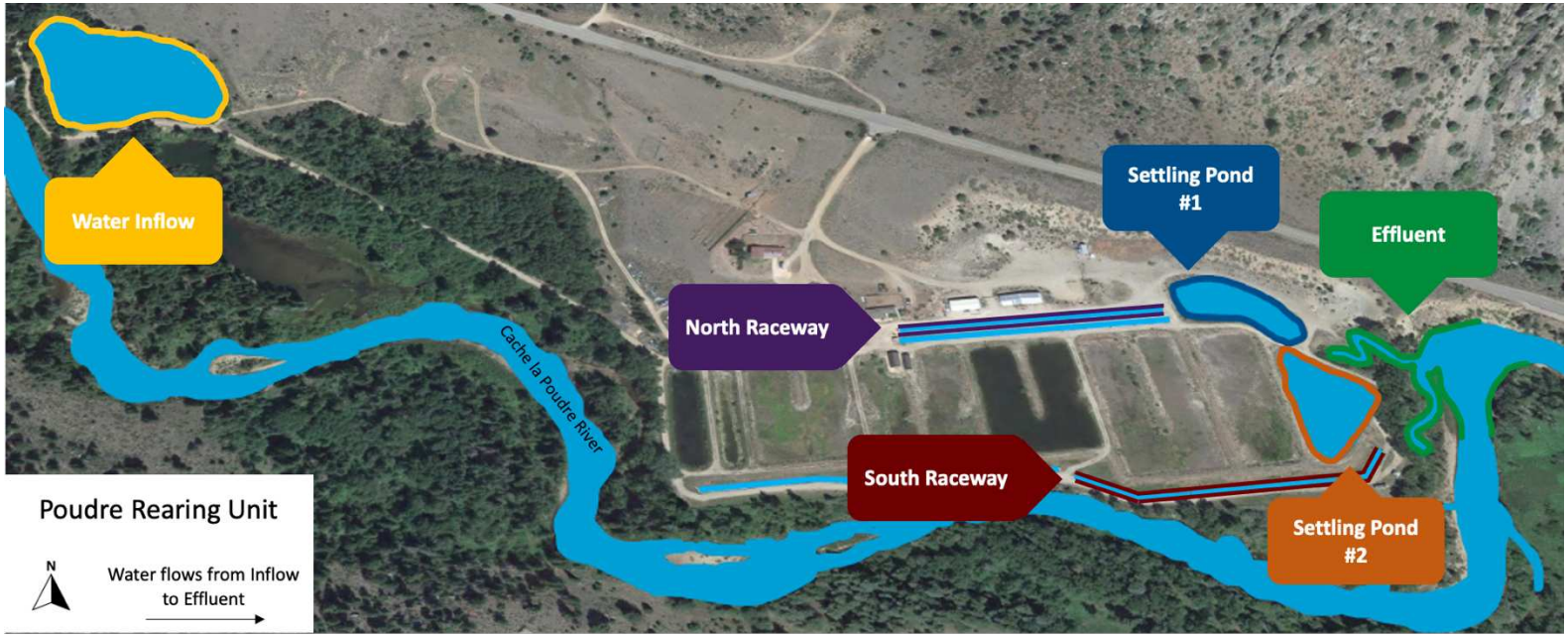

Figure 3.1. Map of the flow-through Poudre Rearing Unit rendered from Google Earth Search Engine with highlighted locations for deployments of cages. The water flows from the pond above the hatchery (water inflow) and is directed to the north and south raceway. The north raceway flows into settling pond #1 which flows into settling pond #2. Water from the south raceway flow into settling pond #2 which then empties in the effluent mixing back with the Cache la Poudre River. *Renibacterium salmoninarum* infected Cutthroat Trout are located in the north raceway and infected Rainbow Trout are located in the south raceway.

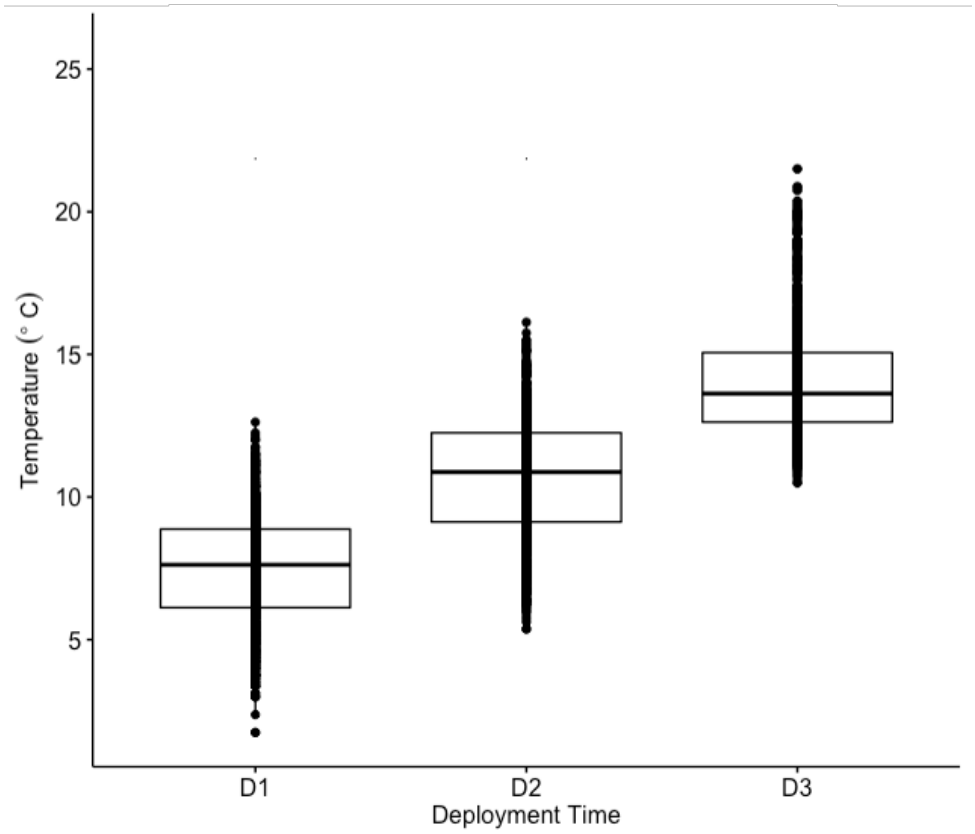

Figure 3.2. Average temperatures between three deployment periods. D1: deployment 1 from May 15 – June 16, 2019, D2: Deployment 2 from June 16 – July 14, D3: Deployment 3 from July 14 – August 13.

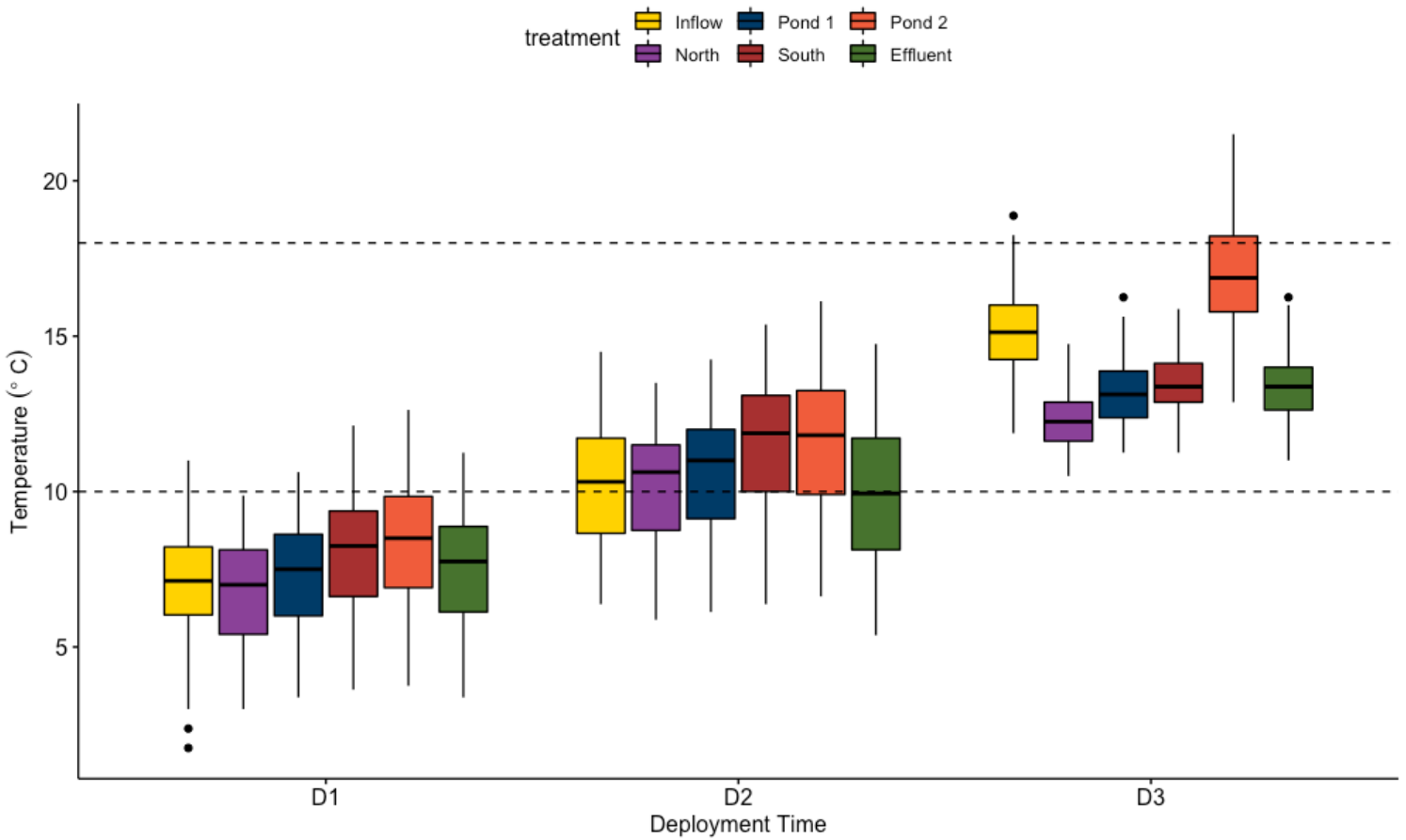

Figure 3.3. Average temperatures across three deployments from six locations on the Poudre Rearing Unit. Horizontal dotted lines represent minimum (10°C) and maximum (18°C) values *Renibacterium salmoninarum* is known to persist in water. D1: deployment 1 from May 15 – June 16, 2019, D2: Deployment 2 from June 16 – July 14, D3: Deployment 3 from July 14 – August 13.

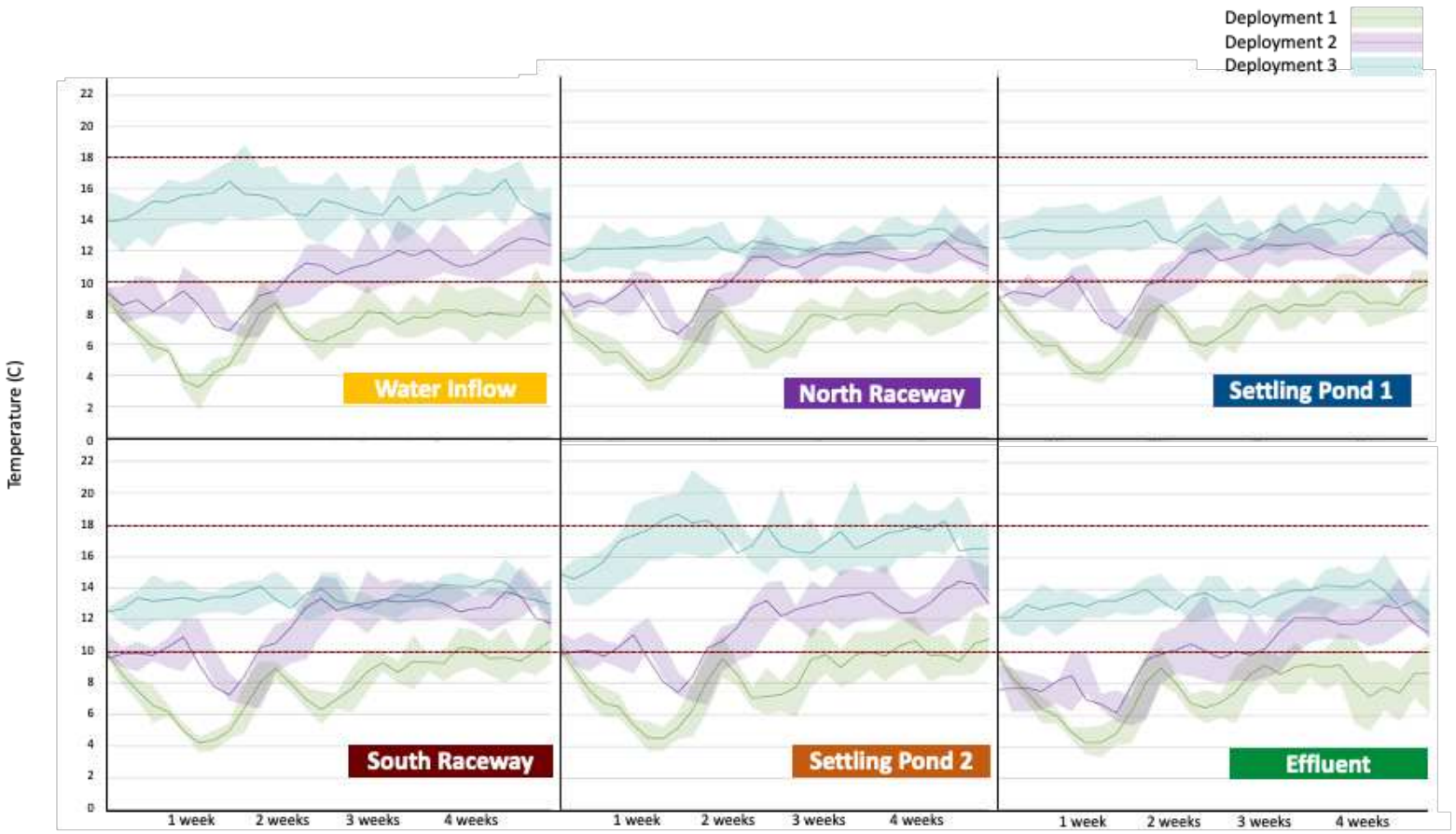

Figure 3.4. Average daily temperatures (°C) across three deployments from six locations located on the Poudre Rearing Unit. Red horizontal dotted lines represent minimum (10°C) and maximum (18°C) values *Renibacterium salmoninarum* is known to persist in water. Green lines = deployment 1, purple lines = deployment 1, blue lines = deployment 3.

Table 3.2. Pairwise comparisons of temperatures across six locations on the Poudre Rearing Unit during deployment 1 (May 15 – June 16, 2019). Differences in mean temperatures by location, 95% confidence intervals, and p-values are reported from a post-hoc Tukey's Honest Significant Difference test.

Location 1	Location 2	Difference	Conf Low	Conf High	P-value
Inflow	North Raceway	-0.19	-0.77	0.39	0.94
Inflow	South Raceway	0.99	0.41	1.57	<0.005
Inflow	Settling Pond #1	0.27	-0.32	0.85	0.78
Inflow	Settling Pond #2	1.32	0.74	1.90	<0.005
North Raceway	South Raceway	1.17	0.60	1.75	<0.005
North Raceway	Settling Pond #1	0.45	-0.13	1.03	0.23
North Raceway	Settling Pond #2	1.50	0.92	2.08	<0.005
Settling Pond #1	Settling Pond #2	1.05	0.47	1.63	<0.005
Settling Pond #1	South Raceway	0.72	0.14	1.30	<0.005
Settling Pond #2	South Raceway	-0.33	-0.91	0.251	0.59
Effluent	Inflow	-0.50	-1.08	0.83	0.14
Effluent	North Raceway	-0.68	-1.26	-0.10	0.01
Effluent	South Raceway	0.49	-0.09	1.07	0.15
Effluent	Settling Pond #1	-0.23	-0.81	0.35	0.87
Effluent	Settling Pond #2	0.82	0.24	1.40	<0.005

Table 3.3. Pairwise comparisons of temperatures across six locations on the Poudre Rearing Unit during deployment 2 (June 16 – July 14, 2019). Differences in mean temperatures by location, 95% confidence intervals, and p-values are reported from a post-hoc Tukey's Honest Significant Difference test.

Location 1	Location 2	Difference	Conf Low	Conf High	P-value
Inflow	North Raceway	-0.09	-0.72	0.55	0.99
Inflow	South Raceway	1.23	0.60	1.87	<0.005
Inflow	Settling Pond #1	0.39	-0.25	1.02	0.55
Inflow	Settling Pond #2	1.41	0.78	2.04	<0.005
North Raceway	South Raceway	1.32	0.68	1.95	<0.005
North Raceway	Settling Pond #1	0.47	-0.16	1.11	0.27
North Raceway	Settling Pond #2	1.50	0.86	2.13	<0.005
Settling Pond #1	Settling Pond #2	1.02	0.39	1.66	<0.005
Settling Pond #1	South Raceway	0.85	0.21	1.48	<0.005
Settling Pond #2	South Raceway	-0.18	-0.81	0.46	0.97
Effluent	Inflow	0.37	-0.27	1.00	0.56
Effluent	North Raceway	0.28	-0.35	0.92	0.80
Effluent	South Raceway	1.60	0.97	2.23	<0.005
Effluent	Settling Pond #1	0.75	0.12	1.39	<0.005
Effluent	Settling Pond #2	1.78	1.14	2.41	<0.005

Table 3.4. Pairwise comparisons of temperatures across six locations on the Poudre Rearing Unit during deployment 3 (July 14 – August 13, 2019). Differences in mean temperatures by location, 95% confidence intervals, and p-values are reported from a post-hoc Tukey's Honest Significant Difference test.

Location 1	Location 2	Difference	Conf Low	Conf. High	P-value
Inflow	North Raceway	-2.84	-3.21	-2.48	<0.05
Inflow	South Raceway	-1.64	-2.01	-1.28	<0.05
Inflow	Settling Pond #1	-1.91	-2.28	-1.54	<0.05
Inflow	Settling Pond #2	1.84	1.47	2.21	<0.05
North Raceway	South Raceway	1.20	0.83	1.57	<0.05
North Raceway	Settling Pond #1	0.94	0.57	1.31	<0.05
North Raceway	Settling Pond #2	4.68	4.32	5.05	<0.05
Settling Pond #1	Settling Pond #2	3.75	3.38	4.12	<0.05
Settling Pond #1	South Raceway	0.26	-0.11	0.63	0.33
Settling Pond #2	South Raceway	-3.49	-3.85	-3.12	<0.05
Effluent	Inflow	1.76	1.39	2.12	<0.05
Effluent	North Raceway	-1.09	-1.46	-0.72	<0.05
Effluent	South Raceway	0.11	-0.26	0.48	0.96
Effluent	Settling Pond #1	-0.15	-0.52	0.22	0.85
Effluent	Settling Pond #2	3.60	3.23	3.94	<0.05

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CHAPTER 4

VERTICAL TRANSMISSION OF *RENIBACTERIUM SALMONINARUM* IN COLORADO CUTTHROAT TROUT

4.1 Introduction

Understanding the transmission of pathogens among hosts is important to reduce losses in aquaculture facilities from disease outbreaks. Bacterial diseases are often one of the leading causes for high mortality events in hatchery facilities success (Meyer 1991; Pridgeon & Klesius 2011; Sudheesh et al. 2012). The number of pathogenic bacteria that are being isolated from fish reared in aquaculture facilities has been steadily increasing and is likely due to high transmission (Harvell et al. 1999). Bacterial coldwater disease, caused by *Flavobacterium psychrophilum*, can cause up to 90% mortality among reared salmonids (Nilsen et al. 2011; Barnes & Brown 2011). Sudden mortality events have been observed in Rainbow Trout (*Oncorhynchus mykiss*) due to *Pseudomonas fluorescens*, reaching 100% loss in some populations (Pękala-Safińska 2018).

Renibacterium salmoninarum is the bacteria responsible for bacterial kidney disease in salmonid species. Bacterial kidney disease has led to 80% mortality among reared Pacific salmonids and 40% in Atlantic salmonids (Evenden et al. 1993; Weins 2011). The bacteria can be transmitted through both vertical and horizontal transmission. Horizontal transmission occurs from direct contact with infected fish or water, or through the ingestion of contaminated fecal matter (Balfry et al. 1996). Vertical transmission typically occurs from the maternal fish to the progeny through an intra-ovum infection (Evelyn et al. 1986a). *Renibacterium salmoninarum* is primarily detected from hematopoietic tissues but has also been detected in ovarian fluid of adult

spawning fish, fertilized eggs, progeny, and milt (Brown et al. 1994; Larenas et al. 2003; Hamel 2005, Fetherman et al. 2020). Infections of progeny or eggs may occur early during egg development (oogenesis) or acquired from the infected surrounding ovarian fluids such that the pathogen passively enters through the micropyle of the egg (Potts & Rudy 1969; Evelyn et al. 1984a). Pseudo-vertical transmission, where the bacteria are in the water and enter the egg during the water hardening process of fertilization, may also be possible (Kumagai et al. 2000). *Renibacterium salmoninarum* has also been detected from spermatozoa but the significance of the presence of bacteria on the spermatozoa for successful vertical transmission is not clear (Daly & Stevenson 1989). Thus, vertical transmission is thought to be primarily driven by the female brood fish.

Currently there is no treatment to eliminate the bacteria from the environment or from an infected population. Therefore, efforts to limit transmission have been developed. Attempts to decrease prevalence and limit transmission have included depopulation of fish from an infected hatchery unit, lethal culling to reduce potentially infected eggs, and injections of erythromycin to reduce the probability of vertical transmission from adult broodstock to progeny (Fetherman et al. 2020). Depopulation and lethal culling of brood fish seem to be the most effective methods to reduce transmission. Lethal culling includes spawning brood fish and lethally sampling tissues to test for *R. salmoninarum*. If the brood fish test positive for an infection, subsequent eggs are discarded. However, this method resulted in a loss of approximately 2,800 Cutthroat Trout (*Onchorynchus clarkii*) broodstock from a Colorado hatchery between 2017 and 2019. Many hatcheries in Colorado maintain rare lineages of Cutthroat Trout and whirling disease resistant Rainbow Trout broodstock and produce millions of eggs for production around the state. Therefore, the lethal culling or depopulation to potentially eradicate *R. salmoninarum* may not be

appropriate in all hatcheries, especially in hatcheries that have rare or important brood stocks for conservation and management. Although erythromycin injections seemed promising, they did not stop all transmission from occurring (Fetherman et al. 2020) and may be a costly method to use among all infected fish in hatcheries.

Although there are methods to limit transmission, we have yet to understand the rate of vertical transmission among Cutthroat Trout. By estimating the rate of vertical transmission, we may be able to better evaluate which method(s) may be suitable to lower *R. salmoninarum* prevalence on a hatchery unit. My study aimed to estimate the rate of vertical transmission of *R. salmoninarum* in an infected broodstock. Specifically, I artificially spawned Cutthroat Trout at the Colorado Parks and Wildlife (CPW) Poudre Rearing Unit (PRU) and evaluated vertical transmission from infected broodstock to the progeny.

4.2 Methods

During the 2019 Cutthroat Trout spawn at the CPW PRU, I screened 3-year-old female and 2-year-old male broodstock for *R. salmoninarum*. Adult fish were initially swabbed for a non-lethal mucus sample (Riepe et al. 2021), weighed (g), measured (mm), and euthanized using an overdose of tricaine-methanesulfate (MS-222: Western Chemicals). One adult female and one adult male were stripped of gametes and spawned together in a clean, dry container (352 spawning pairs). Ovarian fluid was collected prior to the addition of milt with a sterile syringe and placed in a 2 mL centrifuge tube. I did not collect milt due to the risk of not having enough milt for fertilization. After spawning, blood was collected through intravenous puncture of the caudal vein and placed in a 2 mL microcentrifuge tube (Chapter 2). I also collected whole spleen, liver, and kidney tissues through an abdominal incision from each fish. Each sample was placed into individual Whirl-Pak-Bags and labeled with a unique identifier for individual fish. All

samples were frozen on dry ice for transportation to the laboratory where they were stored at -20°C until sample processing.

4.2.1 *Adult Tissue Testing*

Initial kidney tissue testing with quantitative polymerase chain reaction (qPCR) occurred within 72 hours of collection to determine infection status of broodstock to classify the eggs into appropriate treatments (discussed below). All other tissues were tested within six months of collection. I followed the AFS-Fish Health Blue Book (2016) recommended testing procedures for screening tissues for *R. salmoninarum* with qPCR. Tissues were homogenized in sterile Whirl-Pak-Bags with sterile rolling pins and serums vortexed in microcentrifuge tubes. To prepare mucus swabs, I incubated each swab in a 2 mL microcentrifuge tube for three hours with 1.5 mL of 1X phosphate buffered solution (PBS) at room temperature. Duplicate samples of tissues (25 mg) and serums (200 µL) from the homogenized sample were collected for DNA extractions. I followed the protocol for Qiagen DNeasy Blood and Tissue Kit (Hilden, Germany) for tissues, blood, and ovarian fluid and the Qiagen protocol for Gram-positive bacterial swabs for mucus samples with the addition of an extra elution step to increase DNA concentration in all samples (Elliott et al. 2013). I used 5 µL of extracted DNA for each qPCR reaction with 2.25 µL of forward primer (RS 1238 5'-GTGACCAACACCCAGATATCCA-3'), 2.25 µL of reverse primer (RS 1307 5'-TCGCCAGACCACCATTTACC-3'), and a probe with 3' MGBNFQ quencher (RS 1262, 5'-CACCAGATGGAGCAAC-3'). A TaqMan Gene Expression Master Mix was used at 1X concentration for a final volume of 12.25 µL per reaction. An Applied Biosystems Step One Plus system was used with an initial incubation time at 50°C for two minutes, 90°C for ten minutes, and 40 denaturing cycles at 95°C for 15 seconds followed by 60 seconds of annealing at 60°C. Samples below a Cq value of 37.75 were considered positive for

the presence of *R. salmoninarum.* Analysis of qPCR output was compared to a previously developed standard curve to calculate the absolute bacterial numbers from Cq values for each positive tissue sample (Chapter 2).

4.2.2 *Egg Fertilization and Transportation*

Immediately following milt extraction from the male brood fish, well water was added to the eggs and gently agitated for two minutes to induce egg fertilization. After fertilization, about half of the fertilized eggs from each pairing were subsampled and placed into individual egg incubation cups. Incubation cups were fabricated from 101.6 mm diameter schedule 40 PVC pipe that were 50.8 mm tall and fit with a 152.4 mm diameter and 125.4 mm lid with mesh screens to allow for water to flow through. Four incubation cups, labeled by family, were placed into a half-gallon water jugs, filled with 1,000 ppm iodine mixed with water for surface disinfection and water hardening of eggs for one hour. Following water hardening, each jug was rinsed with well water, filled to the top, and transported to the CPW Bellvue Fish Research Hatchery. Upon arrival each jug was rinsed, filled with 1,000 ppm ovadine for an additional surface disinfection of eggs for 10 minutes and rinsed again. Egg cups were placed into health stack incubator trays, with five egg cups per tray. Once eggs were eyed, unfertilized eggs were removed, and the egg cups were randomly assigned to one of 32, 75.7-L tanks based on family treatment assignment.

4.2.3 *Family Treatment Assignment*

After I screened kidney tissues from brood fish for *R. salmoninarum* with from qPCR, I assigned each family to a specific treatment based on *R. salmoninarum* infection status. Brood fish kidney tissues were determined positive when qPCR was positive. The number of families for each treatment resulted in 8 “control” MNFN (M: male, F: female, N: negative) families that

allowed me to evaluate if *R. salmoninarum* transmission was occurring through contact with the water source; 8 MNFP (P: positive) families to determine vertical transmission from female brood fish; 5 MPFN families to determine vertical transmission from male brood fish; and 11 MPFP families to determine vertical transmission from both male and female brood fish.

I tested all other adult tissues using the same qPCR methods described above. This resulted in a post-hoc reassignment of family treatments because some tissues indicated that brood fish were positive, contrary to the assignment with the initial kidney testing. A positive status was assigned only when at least one spleen, liver, or kidney tissue was considered positive and not when a non-lethal method alone was positive. Final treatment assignments resulted in 1 MNFN, 2 MNFP, 4 MPFN, and 25 MPFP families. All families were randomly assigned a tank number 1–32.

4.2.4 *Rearing Conditions*

After eggs hatched, I waited until 50% of the progeny were swim-up fry to start feeding BioOregon size #0 feed. After 30 days of initial feeding, I subsampled 100 fish to remain in each tank for the duration of the experiment; other fish were euthanized and removed from the experiment. Fish were maintained in 75.7-L flow-through tanks with 13.0 $\pm$ 0.1°C well water with a flow rate of 7.6 L/min. Every two weeks, all fish were weighed to determine average batch weight for each family. Once the average weight of individual fish in each family was equal to 0.4 g, I changed the feed to Rangen feed size #1. Feed was increased throughout the experiment based on the average weight of fish in each family every two weeks and the manufactures recommendation for a 3% maintenance diet. Cleaning occurred every two days and each family was assigned its own brush and suction tubing for cleaning to minimize the potential for cross contamination of the bacteria.

4.2.5 *Progeny Sampling*

The timing of sampling progeny was based on the day I subsampled 100 fish for the experiment (hereafter referred to as post swim-up). At 6 months post swim-up, 50 fish were selected and euthanized to sample tissues for *R. salmoninarum* testing. The remaining fish were kept in the experiment for an additional 6 months. Fish were weighed, measured, and euthanized with MS-222. An abdominal incision was made to collect spleen, liver, and kidney tissues which were pooled in one Whirl-Pak-Bag and uniquely labeled by individual fish and treatment. Samples were immediately placed on dry ice for transportation to the laboratory. After 12-months post swim-up, I sampled the remaining fish in each tank and collected the same tissues as I did at 6-month post swim-up. Any mortalities over the course of the experiment were noted, but these fish were not tested for the presence of *R. salmoninarum* because fish tissues were typically decomposed.

All tissue samples were tested for *R. salmoninarum* with qPCR. DNA extraction and qPCR analysis were followed as outlined above. Pooled spleen, liver, and kidney tissues from progeny were also screened with a double sandwich enzyme-linked immunosorbent assay (ELISA). Homogenized tissues were prepared to a 1:4 (w/v) dilution with PBS, 0.05% (v/v) Tween-20, and 0.01% (w/v) thimerosal. Following an established ELISA protocol (Pascho et al. 1991), I used an affinity purified *R. salmoninarum*-goat antibody as a coating antibody (KPL, Milford, MA, USA) and a horseradish-peroxidase (HRP) labeled *R. salmoninarum*-antibody as the conjugate (KPL, Milford, MA, USA). Each family was tested separately and replicates of the HRP conjugate, substrate-chromogen, cell culture water, and tissues from known negative Rainbow Trout tissues were plated as assay controls. Four *R. salmoninarum* positive control dilutions (BacTrace, KPL, Milford, MA, USA) were prepared at 1:100, 1:1000, 1:2000, and

1:5000. A UV-Vis microplate spectrophotometer with a monochromator-based absorbance was used at 405 nm to determine optical density values. I used a conservative threshold of greater than 0.10 to determine positive samples (Munson et al. 2010; Elliott et al. 2013; Kowalski et al. 2022). Like other studies, I set criteria to characterize antigen load levels (Elliott et al. 2013): low (OD: 0.100–0.199), intermediate (OD: 0.200–0.999), and high (OD: > 1.000). Sensitivity of the ELISA assay was between 2 and 20 ng of *R. salmoninarum* (Pascho & Mulcahy 1987).

4.2.6 *Statistical Analysis*

I compared if detection of *R. salmoninarum* differed among the four treatments using a Chi-square test for unbalanced designs from an analysis of variance (ANOVA). Then I evaluated if positive detections of *R. salmoninarum* among families were different among the three positive treatments (MNFP, MPFN, MPFP) as a function of assay type, age, weight, and length of progeny with a generalized linear mixed model (GLMM) with a logit link. The model was fit in the lme4 package using the glmer function in R version 4.1.0. Treatment and average weights or lengths among each family were treated as fixed effects, and the age of progeny when sampled, assay type, and individual family were treated as random effects. Weight and length were evaluated in separate models because they are known to be correlated. Models were compared using the Akaike Information Criteria (AICc) and coefficients were presented from the top model. The coefficients from the top model were used to calculate the proportion of positive families (p) for each assay and treatment combination using a logit link function:

$$p = \text{inv.logit}(\beta_0 + \beta_1 + \beta_2 \ldots \beta_N)$$

Lastly, I used Pearson's correlation test to determine if the number of bacteria present in the adult female brood fish tissues influenced the number of positive progeny within a family by either assay. Because the bacterium is known to be maternally transmitted, I only included the

number of bacteria in tissues from female brood fish in the correlation analysis. All tests were performed in R and significance was set at 0.05 (α).

4.3 Results

4.3.1 *Between Treatment Data*

Nineteen of the 32 families were positive for the detection of *R. salmoninarum* among the progeny. Within each treatment there were no families positive in the MNFN treatment, two of the families (100%) in the MNFP treatment, one of families (25%) in the MPFN treatment, and 16 families (64%) in the MPFP treatment. I detected a treatment level effect on positive *R. salmoninarum* detection between all the treatments (ANOVA: $F_{3,124} = 4.19$, p-value < 0.05). The MNFP treatment was the only treatment significantly different from the control (MNFP: p-value < 0.05, MPFN: p-value = 0.98; MPFP: p-value = 0.74). Age of the progeny tested among each tank did not seem to influence infection status (Table 4.1)

The AIC analysis indicated that positive detections were a function of treatment and average length of progeny and were influenced by the assay type (Table 4.2). The calculated probability of detection among treatments from the top model regression coefficients (Table 4.3) indicated lowest detections among the MPFN treatment and higher in the two treatments where females were positive (Figure 4.1). The two treatments with positive females resulted in the most detections, and detections overall indicated high within-treatment vertical transmission. Our results also indicate that males can contribute to vertical transmission.

4.3.2 *Within Treatment Data*

All 32 families started with 100 fish, but due to natural mortality or mishandling of fish or tissue samples, the number of fish tested was not equal across all families. The average number of 6- and 12-month post swim-up fish sampled was 46 $\pm$ 4 fish and 46 $\pm$ 3 fish,

respectfully. The average weights for all 6-month post swim-up fish were 15.42 $\pm$ 2.01 g and 76.43 $\pm$ 9.40 g for 12-month post swim-up. The average lengths for 6-month post swim-up fish were 118.53 $\pm$ 5.61 mm and 191.10 $\pm$ 47.0 mm for 12-month post swim-up fish. Length was included in the top model and had a effect on infection status (p-value < 0.05; Table 4.3). Weight was not included in the top model as an explanatory variable.

The progeny in the "control" treatment (MNFN) did not test positive for the detection of *R. salmoninarum* by either assay. Although only one tank may not indicate that the water source did not contain the bacteria, another experiment conducted a year prior to ours at the Bellvue Fish Research Hatchery and fed with the same water source, did have an adequate number of control tanks and those fish did not test positive by similar methods (Fetherman et al. 2020). Thus, I concluded any detectable infection in progeny among other families was a result of vertical transmission from infected brood fish.

I observed low-within family detections of *R. salmoninarum* in the progeny. The highest proportion of progeny infected within a single family was 21% by ELISA (Table 4.4). The average number of bacteria from qPCR among positive progeny were low. Eight of the families were noted as having low levels of detectable antigens with ELISA, six with intermediate levels, and one with high levels. A few of the families did have intermediate to high OD levels or high number of bacteria in progeny (Table 4.4), but I did not observe any signs of overt disease.

The number of bacteria in positive female brood fish affected the proportion of progeny positive in a family when kidney (kidney: t_{15}=2.13, p-value < 0.05), liver (liver: t_{15}=2.09, p-value < 0.05), spleen (spleen: t_{15}=2.07, p-value = 0.06), or ovarian fluid (t_{15}=2.07, p-value = 0.06) was positive. Positive detections in the adult female mucus or blood did not correlate with the

proportion of positive progeny (mucus: t_{15}=0.94, p-value = 0.36; blood: t_{15}=1.40, p-value = 0.18; Table 4.5).

4.4 Discussion

A better understanding of *R. salmoninarum* vertical transmission rates is needed to efficiently reduce infection among hatchery fish. Vertical transmission of *R. salmoninarum* has been demonstrated mostly among Pacific salmonids (Evelyn et al. 1986a,b), but there are limited studies that include inland salmonids. In this study, I evaluated the rate of vertical transmission from an inland Cutthroat Trout broodstock, the relationship between successful infection in progeny, and whether male or female brood fish contributed to an infection in progeny. Our study demonstrates high vertical transmission from the infected Cutthroat Trout broodstock to progeny. Our study also shows evidence for transmission of *R. salmoninarum* from the male brood fish to progeny, suggesting that vertical transmission does not only occur from female brood fish.

Many studies regarding vertical transmission of *R. salmoninarum* have focused on the number of individual infected eggs or progeny, and not the proportion of vertical transmission success from spawning adult pairs, thus reporting low vertical transmission rates (5-15%; Evelyn et al. 1984a,b; Evelyn et al. 1986b). I looked at the proportion of families positive and the results suggest high rates of vertical transmission occur from infected Cutthroat Trout brood fish to progeny. This is especially evident in the MNFP treatment where both tanks (100% of families) contained at least one infected progeny. Although I did observe low transmission within individual families, any level of vertical transmission is a concern for hatcheries rearing *R. salmoninarum*-infected broodstock because the bacteria may also be perpetuated in the population through horizontal transmission.

Renibacterium salmoninarum can be transmitted by both vertical and horizontal transmission, but whether vertical transmission occurs from male brood fish is debated (Klontz 1983; Evelyn et al. 1986a; Balfry et al. 1996). Daly and Stevenson (1989) note the presence of the bacteria on the tail region of the spermatozoa, but never the head region. Therefore, they suggest the bacteria may not enter the micropyle of the egg during fertilization since the tail, and attached bacteria, is lost upon contact with the egg. In our study, one family from a positive male brood fish and negative female brood fish resulted in an *R. salmoninarum* positive tank by ELISA. My model suggests positive male brood fish may account for 21-36% of transmission from spawning pairs in an infected broodstock. The optical density of the fish tissue sample was 0.10, suggesting a low-level infection (Meyers et al. 1993; Pascho et al. 1998; Faisal et al. 2009). This infection may indicate an initial recovery stage of the fish (Faisal et al. 2009). Nevertheless, our finding supports the supposition that male brood fish contribute to vertical transmission. Thus, it may still be important to test the male brood fish where infections are prevalent in the population to reduce any chance of vertical transmission.

This study was structured such that adult kidney tissues were initially tested with qPCR for treatment assignment, but after testing all other tissues, the assignments dramatically changed the number of families in each treatment. Initial treatment assignment had to occur within a 24-hour period upon brood fish tissue collection because there was not enough space to hold all 352 individual families that were made during spawn. I also did not have enough time to test all six tissues from the adults within a 24-hour period, thus I only chose to initially test the kidney tissue. Kidney tissues are the suggested tissues to test for the presence of *R. salmoninarum* (AFS-FHS 2016) and are used to test hatchery fish in Colorado. Thus, I anticipated low false negative results using kidney tissues coupled with the specific and sensitive qPCR assay (Elliott et al.

2013). However, results from another study conducted in conjunction with this one (Chapter 2) indicated the detection probability with qPCR on kidney tissues is only 0.72 resulting in the potential of 0.28 false negative results.

Initially, I assigned eight control families where *R. salmoninarum* was detected in either the male or female adult broodfish kidney tissues. After testing all other tissues, seven of the eight control families were re-assigned into positive treatments. Four of the six re-assigned tanks had successful vertical transmission occur, leading to infection in the progeny. This has important implications. To limit vertical transmission in Colorado, kidney tissues are tested from the adult broodfish in lethal culling practices and associated eggs are destroyed from the infected spawning pair. If the adults do not test positive for the bacteria, the coinciding eggs are eyed, hatched, and transported around the state for stocking efforts. If eggs are not discarded by identifying infected brood fish, we will increase the risk for disseminating the pathogen. My study suggests that it may be unwise to rely only on testing adult kidney tissue if the goal is to disrupt vertical transmission of *R. salmoninarum* by detecting it in the adult brood fish. Therefore, testing other tissues or using multiple tests on the kidney tissue (Chapter 2) should be considered.

Bacteria has been known to be localized in reproductive tissues leading to successful transmission and a high prevalence of infection among eggs or progeny (Brown et al. 1994; Long et al. 2012). In addition, other studies have observed high number of *R. salmoninarum* in progeny when ovarian fluid or other tissues from adult brood fish were infected with high numbers of bacteria in the adult brood fish (Evelyn et al. 1986a,b; Pascho et al. 1991). Similarly, in my study maternal infection intensity in internal tissues (liver, kidney, spleen) and ovarian fluid influenced the number of positive progeny in an individual family. Although the number of

bacteria in kidney tissues influenced the number of positive progeny, I recommend testing other tissues because I have previously found kidney tissues tested by qPCR can lead to high false negative results (Chapter 2). Thus, testing a combination of kidney, liver, spleen, and ovarian fluid will increase detection probabilities of *R. salmoninarum* among brood fish. It also appears that even when bacterial numbers in adult fish are low and ovarian fluid is negative, an infection can still be transmitted to the progeny, as evidenced by most of the MPFP families. This must be taken it consideration when using qPCR results to select for eggs free of an infection as ovarian fluid may not be a good measure of vertical transmission risk when infection intensity is low.

Variability in the number of *R. salmoninarum* detections by ELISA or qPCR occurred between treatments and within families. The double-sandwich antibody ELISA method detects the soluble antigen fractions of *R. salmoninarum* in the tissue samples and cannot distinguish between current or previous infection, whereas qPCR detects the genomic DNA in the sample, thus a current infection or presence of live bacteria (Pascho et al. 1998; Faisal et al. 2009; Elliott et al. 2013). Generally, detection of *R. salmoninarum* was highest among progeny when using the ELISA assay, with few fish that were also found to be positive by qPCR. There were also few instances where detections of the bacteria were only found by qPCR and not by ELISA. Faisal et al. (2009) describes similar infection patterns from naturally infected salmonid species, and the disagreement between the assays may reflect different stages of infection.

An initial stage of infection often results in low levels of bacteria within the fish tissues and is likely only detectable by qPCR (Faisal et al. 2009). Four families in my study were positive by only qPCR, indicating a low infection level as an infection was initially progressing. The next stage of infection may represent an infection that has progressed in the fish, resulting in an increased number of bacteria, and therefore detection by both ELISA and qPCR can occur.

Two of my families resulted in high number of fish positive by ELISA and a couple of those fish were also positive by qPCR. Although this type of infection pattern is suggestive of an active infection (Faisal et al. 2009), the lack of positive qPCR results may indicate an initial stage of recovery where the bacteria is rarely present in fish, but the detectable soluble antigen by ELISA remains in the tissues. When detections of *R. salmoninarum* only occur with ELISA, Faisal et al. (2009) suggest that this is indicative of an advanced stage of recovery in which we are detecting small traces of the bacteria or antigen that remain in the tissues. Across all positive detections, more progeny were positive by ELISA, and OD values were categorized as low to intermediate, which may indicate that most of the fish were already in that late stage of recovery. Lastly, the progeny in families that resulted in no infections may have been refractory from infection and therefore vertical transmission was unsuccessful or clearance of the infection occurred prior to sampling and qPCR or ELISA did not detect the bacteria or antigen.

4.5 Conclusion

Detection of *R. salmoninarum* in progeny is often difficult since there may be a lack of space or resources to rear the fish until they are able to be tested at 6 months old. Therefore, strategies to limit transmission have relied heavily on the development of vaccines or chemotherapy injections, depopulation, or lethal culling of female and/or male brood fish (Evelyn 1986a; Fetherman et al. 2020; Chapter 3). Lethal culling has been used in Colorado to limit transmission by testing kidney tissues from adult broodstock. Our results suggest that infections among adult fish may be missed when only testing kidney, thus not successfully preventing all vertical transmission. Therefore, I suggest testing a combination of kidney, liver, spleen, and ovarian fluid to assess whether the brood fish may transmit the bacteria to progeny. In addition, other studies have shown that testing a subsample of eggs for *R. salmoninarum* with

a specific, sensitive, and reliable method, such as qPCR or ELISA, may allow for decreased transmission without the high-volume loss of broodstock from lethal culling. Testing DNA extracted from eggs with qPCR has shown to detect as few as two bacterial cells and therefore may be a feasible alternative in future *R. salmoninarum* management in hatcheries (Gudmundsdóttir et al. 2000; Brown et al. 1994). I also suggest continued testing of males either by lethal methods or testing milt to further limit transmission. The potential rate of vertical transmission from males, especially in inland salmonid populations, should be studied further.

Table 4.1. Proportion of positive families (M: male, F: female, N: negative, P: positive) among treatments by assay type and age of progeny at the time of testing for *Renibacterium salmoninarum*. Total number of families positive listed in parenthesis.

Treatment	Assay	Age	Positive Families
MNFP	ELISA	6 Months	100% (2)
		12 Months	50% (1)
	qPCR	6 Months	50% (1)
		12 Months	50% (1)
MPFN	ELISA	6 Months	25% (1)
		12 Months	0
	qPCR	6 Months	0
		12 Months	0
MPFP	ELISA	6 Months	24% (6)
		12 Months	32% (8)
	qPCR	6 Months	12% (3)
		12 Months	12% (3)

Table 4.2. AICc model comparisons for proportion of positive detections and family, age, size, or detection assay components.

Model	AICc	ΔAICc	Wt
Treatment + Length + Assay	122.13	0	0.31
Treatment + Length + Family	123.42	1.29	0.16
Treatment + Length + Assay + Family	124.27	2.14	0.11
Treatment + Length + Assay:Age	124.37	2.24	0.10
Treatment + Weight + Assay	125.29	3.16	0.06
Length + Family	125.81	3.68	0.05
Treatment + Weight + Family	126.30	4.17	0.04
Treatment + Length + Assay:Age + Family	126.55	4.42	0.03
Treatment + Assay	127.22	5.09	0.02
Treatment + Assay + Weight + Family	127.37	5.24	0.02
Treatment + Weight + Assay:Age	127.54	5.41	0.02
Treatment + Family	128.50	6.50	0.01
Weight + Family	128.90	6.77	0.01
Treatment + Assay + Family	129.34	7.21	0.01
Treatment + Assay:Age	129.42	7.29	0.01
Treatment + Weight + Assay:Age + Family	127.46	7.52	0.01

Table 4.3. Top model regression coefficients, 95% confidence interval (CI), standard error (SE), z- and p-value for fixed effects and variance, standard deviation (SD), and associated regression coefficients for the random effect of ELISA and qPCR assays from the top model.

Type	Covariate	Coefficient	95% CI	SE	z-value	p-value
Fixed Effects	Treatment: MPFN	-3.26	-6.45 – -1.02	1.29	-2.53	< 0.05
	Treatment: MPFP	-1.97	-3.67 – -0.43	0.80	-2.46	< 0.05
	Treatment: MNFP	2.33	< 0.01 – 4.91	1.22	1.90	0.05
	Length	-0.01	-0.03 – < -0.01	0.01	-1.99	< 0.05
	Variance	**SD**	**Covariate**	**Coefficient**		
Random Effect	0.20	0.45	ELISA	0.30		
			qPCR	-0.28		

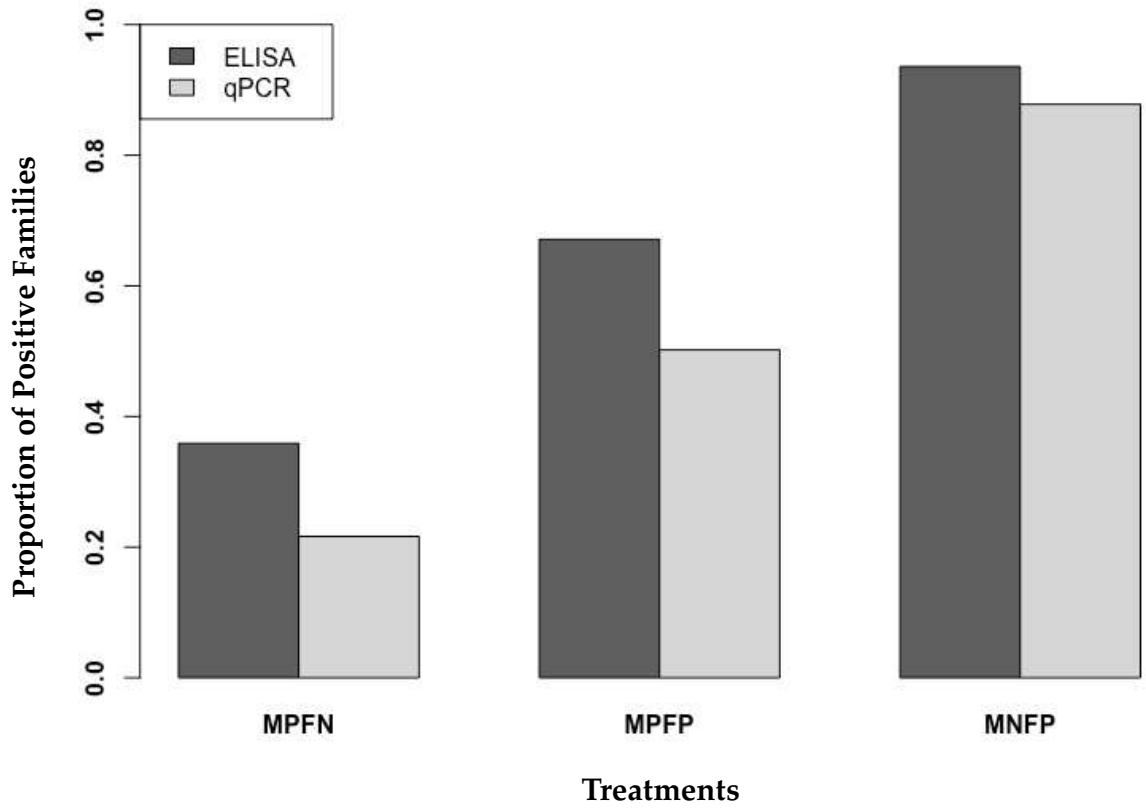

Figure 4.1. Model estimates of the proportion of families positive for the detection of *Renibacterium salmoninarum* by ELISA (dark) or qPCR (light) among the three positive treatments (M: male, F: female, N: negative, P: positive).

Table 4.4. Detection of *Renibacterium salmoninarum* among progeny (%) in each treatment and family by ELISA and qPCR. Number of infected progeny per tank are represented in the parenthesis. Optical density values (OD) are listed for assay measurements for ELISA from positive fish (low (OD: 0.100–0.199), intermediate (OD:0.200–0.999), and high (OD: > 1.000)) and the number of bacteria (bacteria/mL $^{-1}$) are listed from qPCR from positive fish. (M: male, F: female, N: negative, P: positive)

Treatment	Family	Assay	Positive Progeny	Assay Measurement	ELISA Level
MNFN	Tank 7	ELISA	0	–	–
		qPCR	0	–	–
MNFP	Tank 27	ELISA	9.4% (8)	0.32 ± 0.03	Intermediate
		qPCR	8.2% (7)	178.41 ± 443.57	–
	Tank 28	ELISA	1.1% (1)	0.10 ± 0.01	Low
		qPCR	0	–	–
MPFN	Tank 8	ELISA	0	–	–
		qPCR	0	–	–
	Tank 21	ELISA	0	–	–
		qPCR	0	–	–
	Tank 22	ELISA	0	–	–
		qPCR	0	–	–
	Tank 24	ELISA	1.1% (1)	0.11 ± 0.01	Low
		qPCR	0	–	–
MPFP	Tank 1	ELISA	1.2% (1)	0.11 ± 0.01	Low
		qPCR	0	0	–
	Tank 2	ELISA	15.1%	0.30 ± 0.02	Intermediate
		qPCR	1.2% (1)	44.80	–
	Tank 3	ELISA	0	–	–
		qPCR	0	–	–
	Tank 4	ELISA	0	–	–
		qPCR	1.1% (1)		–
	Tank 5	ELISA	0	–	–
		qPCR	0	–	–
	Tank 6	ELISA	1.1% (1)	3.07 ± 4.24	High
		qPCR	0	–	–
	Tank 9	ELISA	0	–	–
		qPCR	2.2% (2)	1.61 ± 0.69	–
	Tank 10	ELISA	2.2% (2)	0.29 ± 31	Intermediate
		qPCR	0	–	–
	Tank 11	ELISA	0	–	–
		qPCR	0	–	–
	Tank 12	ELISA	0	–	–
		qPCR	0	–	–
	Tank 13	ELISA	0	–	–
		qPCR	0	–	–
	Tank 14	ELISA	0	–	–
		qPCR	0	–	–
	Tank 15	ELISA	2.2% (2)	0.12 ± 0.06	Low
		qPCR	0	–	–
	Tank 16	ELISA	0	–	–
		qPCR	1.0% (1)	2.073	–
	Tank 17	ELISA	21.1% (20)	0.31 ± 0.03	Intermediate
		qPCR	3.2% (3)	3.36 ± 2.14	–
	Tank 18	ELISA	0	–	–
		qPCR	1.1% (1)	3.12	–

Treatment	Family	Assay	Positive Progeny	Assay Measurement	ELISA Level
MPFP	Tank 19	ELISA	4.3% (4)	0.13 ± 0.01	Low
		qPCR	0	–	–
	Tank 20	ELISA	0	–	–
		qPCR	0	–	–
	Tank 23	ELISA	3.1% (3)	0.22 ± 0.18	Intermediate
		qPCR	0	–	–
	Tank 25	ELISA	1.1% (1)	0.11 ± 0.05	Low
		qPCR	0	–	–
	Tank 26	ELISA	1.1% (1)	0.39 ± 0.45	Intermediate
		qPCR	0	–	–
	Tank 29	ELISA	0	–	–
		qPCR	0	–	–
	Tank 30	ELISA	0	–	–
		qPCR	0	–	–
	Tank 31	ELISA	1.0% (1)	0.18 ± 0.16	Low
		qPCR	0	–	–
	Tank 32	ELISA	2.3% (2)	0.11 ± 0.01	Low
		qPCR	0	–	–

Table 4.5. Bacterial counts (bacterial cells/mL^{-1}) from positive adult brood fish tissues and serums used in family assignment treatments. Bacteria numbers were estimated by a qPCR standard curve. Proportion of total progeny infected are also included for each of the positive families. (M: male, F: female, N: negative, P: positive)

Treatment	Family	Adult Sex	Mucus	Blood	Ovarian Fluid	Spleen	Liver	Kidney	*Progeny*
MNFP	Tank 27	Male	0	0	-	0	0	-	*0.13*
		Female	0	877.87	0	43.96	562.88	892.94	
	Tank 28	Male	0	0	-	0	0	0	*0.01*
		Female	0	0	1.38	20.89	169.28	193.03	
MPFN	Tank 24	Male	0	0	-	38.08	65.31	1444.24	*0.01*
		Female	0	0	0	0	0	0	
MPFP	Tank 1	Male	0	0	-	2.28	4.81	0	*0.01*
		Female	1.66	0	1.92	1.79	10.99	0	
	Tank 2	Male	20.17	0	-	0	6.86	1.20	*0.15*
		Female	11.30	0	50610.80	80618.45	55947.73	26638.76	
	Tank 4	Male	0	0	-	14.12	1.36	1.49	*0.01*
		Female	1.56	0	0	0	2.02	1.80	
	Tank 6	Male	3.28	0	-	0	2.76	3.03	*0.01*
		Female	1.73	0	0	5.60	2.14	0	
	Tank 9	Male	0	0	-	0	4.30	7.83	*0.02*
		Female	0	0	0	0	3.05	37.59	
	Tank 10	Male	1.67	0	-	3.01	6.46	4.47	*0.01*
		Female	1.91	0	1.75	0	5.28	4.05	
	Tank 15	Male	1.16	0	-	0	1.31	40.77	*0.01*
		Female	0	0	0	0	1.34	19.01	
	Tank 16	Male	3.03	0	-	0	1.14	1.40	*0.01*
		Female	11.60	0	0	0	2.79	0	
	Tank 17	Male	0	0	-	0	0	2.28	*0.21*
		Female	0	0	0	0	2.73	8.84	
	Tank 18	Male	0	0	-	0	1.55	0	*0.01*
		Female	0	0	1.38	20.89	169.28	193.03	
	Tank 19	Male	2.35	0	-	0	1.91	2.96	*0.04*
		Female	0	0	3.58	1.62	2.06	6.64	
	Tank 23	Male	0	0	-	116.53	13.55	11.75	*0.03*
		Female	0	0	2.63	26.03	11.36	11.34	
	Tank 26	Male	0	0	-	0	1.81	16.97	*0.01*
		Female	2.64	0	0	0	1.97	24.85	
	Tank 31	Male	2.35	0	-	118.92	127.60	82.82	*0.01*
		Female	4.21	0	1.82	107.83	20.57	27.73	
	Tank 32	Male	2.55	0	-	0	0	9.77	*0.02*
		Female	6.44	0	0	0	0	1.86	

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APPENDIX I

DETERMINATION OF AN AGGLUTINATION PROPERTY EXPRESSED BY *RENIBACTERIUM SALMONINARUM* ISOLATED FROM RAINBOW TROUT IN COLORADO

I.I Introduction

Renibacterium salmoninarum is a non-motile and slow-replicating, Gram-positive diplobacillus that exhibits intracellular replication and survival capabilities within host macrophages (Young & Chapman 1978; Bruno 1987; Gutenberger et al. 1997). Mechanisms allowing *R. salmoninarum* to attach and colonize host macrophages vary between bacterial strains (Lindahl et al. 1981; Bruno 1988), but can play a critical role in subclinical or chronic infections. Strains of *R. salmoninarum* with auto-agglutination properties and increased hydrophobicity of the cell surface are considered most virulent and may invade, multiply, and increase infection intensity within the host compared to strains that are non-agglutinating and do not possess a hydrophobic cell surface (Bruno 1987; Bruno & Munro 1986; Evelyn et al. 1984). These properties allow virulent strains to resist host defense mechanisms, whereas other strains may be more readily phagocytized. Visual evidence for host immune and inflammatory responses in fish with chronic infections include external lesions or blisters, exophthalmos, swollen abdomen, hemorrhaging on the abdominal wall and viscera, and nodular lesions in the kidney or other internal organs (AFS-FHS 2016).

Previous studies have suggested that a commonly used assay, direct fluorescent antibody test (DFAT), can perform differently depending on the amount of surface associated p57, which can be strain dependent (Bruno 1988, O'Farrell et al. 2000). The strain found in Colorado hatcheries is currently unknown (hereby referred to as the CO strain). The ATCC 33209 strain (referred to as ATCC strain) has similar properties as the GL-64 strain optimized for the AFS Fish Health Blue Book (2016) DFAT standard operating procedures. Therefore, I examined the potential differences in detection probability of *R. salmoninarum* with DFAT found in another study (Chapter 2) due to the agglutination properties of the CO strain versus the virulent ATCC strain.

I.II Methods

The CO strain of *R. salmoninarum* was obtained from a natural infection in Rainbow Trout at the Colorado Parks and Wildlife (CPW) Bellvue Fish Research Hatchery and the ATCC strain came from the American Tissue Culture Collection (ATCC 33209; Sanders & Fryer 1980). Bacterial cultures were stored at −80°C in 10% glycerol prior to the auto-agglutination test. Both strains were cultured in KDM broth in two replicate flasks at continual agitation and was maintained at 15°C. Subculturing of the two bacteria strains occurred every 7 days, over two passes into new media. We verified purity of the culture with a Gram stain by identifying only one bacterial morphology that is described for *R. salmoninarum* under 50 times magnification (AFS-FHS 2016). Following Bruno (1988), the bacteria from each strain were resuspended in PBS and adjusted to an absorbance level of 1.0 at 420 nm. Cell suspensions of 1.5 mL of *R. salmoninarum* in PBS obtained from the two cultures was added to four glass cuvettes for both strains and examined for auto-agglutination. Two cuvettes contained 1.5 mL of PBS for blank controls. Measurements of absorbance were collected at 30-minute intervals up to six hours with

an UV-Vis Spectrophotometer set at a wavelength of 420 nm. The temperature of the samples was maintained at 20°C between each absorbance measurement. The absorbance levels were plotted as a function of time to determine if the CO strain had similar agglutination properties as the ATCC strain.

I.III Results

The ATCC strain of *R. salmoninarum* resulted in clumping of the bacteria in PBS during the auto-agglutination, test with a decline of the absorbance measurements over time (Figure I.I). The clumping of the ATCC strain suggests the presence of the auto-agglutination factor (Bruno 1988; O'Farrell et al. 2000) which contrasts to the CO strain isolated from naturally infected Rainbow Trout at the CPW Bellvue Fish Research Hatchery. Absorbance levels of the isolated strain in Rainbow Trout did not vary significantly over time showing little evidence for cell clumping. The absorbance measurements observed are similar to attenuated virulent strains with non-agglutinating factors from other studies (MT-239, originally isolated from Atlantic Salmon in Scotland; Bruno 1988).

I.IV Discussion

Typical fish health diagnostic methods are optimized from strains of pathogens that are highly transmissible between hosts and thus are typically highly virulent. Various virulent factors contribute to the capabilities of the deleterious effects of the pathogens to infect and invade the hosts cells. One of the abilities of *R. salmoninarum* to colonize and infect the host tissues and cells depends on adherence of the bacteria (Kroniger et al. 2022). Adherence involves the protein p57, which is located on the cell surface providing the ability for auto-agglutination of the cells (Bruno 1988; Daly & Stevenson 1990). In contrast, attenuated virulent strains have been described in previous studies (Bruno 1988; O'Farrell et al. 2000; Elliott et al. 2013) and often

show minimal detectable p57 on the cell surface (Senson & Stevenson 1999). The presence of p57 is presumed to lead to the auto-agglutination property of the bacteria and is the detectable protein for which the DFAT assay has been optimized. Thus, without the presence of the p57 protein, the *Fluorescein*-labeled antibody to *R. salmoninarum* used in the assay cannot bind to the bacteria, resulting in a lack of or poor fluorescence. O'Farrell et al. (2000) noted tissues extracted from fish injected with an attenuated virulent strain had poor fluorescence compared to fish infected with a virulent strain and detecting the bacteria with DFAT did not occur with every injected fish. This could indicate a low specificity of DFAT to an attenuated strain of *R. salmoninarum*, and I sought to investigate the potential causes for the poor diagnostic performance I found in Chapter 2.

I tested the ability for auto-agglutination of the CO strain of *R. salmoninarum* compared to the highly virulent ATCC 33209 strain. The ATCC strain showed auto-agglutination properties, with decreased absorbance measurements over time as seen in other virulent strains of *R. salmoninarum* (Bruno 1988). By contrast, the CO strain was seen as non-agglutinating as there was little evidence for cell clumping. The low detection abilities with DFAT in this study and others described in Colorado (Kowalski et al. 2022), may be a result of the non-agglutinating properties of this strain. In addition, my study indicates the strain of *R. salmoninarum* I have isolated is not only non-agglutinating but may also be an attenuated virulent strain. The lack of noticeable clinical disease during fish health inspections in Colorado hatcheries and little evidence for disease through immune-histopathological analysis in a previous study of adult Cutthroat Trout at the CPW Poudre Rearing Unit may also suggest low virulence in the strain. High detection probabilities with qPCR found in my study and others, however, do seem reasonable. The major soluble antigen gene (*msa*) sequence detected with PCR assays are nearly

equivalent as indicated through genetic analysis of an attenuated virulent and virulent strains of *R. salmoninarum* (O'Farrell et al. 2000). Thus, I have reason to believe that Colorado may be dealing with an attenuated strain with non-agglutination properties, which would explain why my detection probabilities with DFAT are much lower than with qPCR and could describe why I observed lower vertical and horizontal transmission rates than other studies

It is possible that low within-family vertical transmission (Chapter 4) could be due to the differences in bacterial strains. Attenuated virulent strains of *R. salmoninarum* may have the limited ability to colonize the eggs from the lack of a surface protein allowing for it to occur. Several studies have suggested that the p57 has an attachment ability or inability to cells between strains (Bruno 1988; Daly & Stevenson 1989; Piganelli et al. 1999). It is therefore possible that although the adult brood fish have high bacterial loads, the bacteria may have a lower probability of successful vertical transmission during oogenesis or post-ovulation development if the strain and cannot successfully attach to cells.

It is well known that *R. salmoninarum* is capable of surviving without a host for up to 21 days when water conditions are favorable (Austin & Rayment 1985; Evelyn 1988; Balfry et al. 1996). The bacteria are likely to bind to feces that are extruded from the fish and settle in the water. *Renibacterium salmoninarum* has been known to have a hydrophobic surface that allows it to have an affinity for organic matter which can be found within fecal matter (Austin & Rayment 1985; Gurijala & Alexander 1990; Balfry et al. 1996). However, this hydrophobic surface has also been known to be variable in different strains of the bacteria. Virulent strains of *R. salmoninarum* that typically express the p57 surface have a hydrophobic cell surface (Piganelli et al. 1999). Previous studies have tested the hydrophobic nature of various strains by salt aggregation assays, adherence to hydrocarbons and nitrocellulose filters, and have shown

that the strains without a hydrophobic surface tend to be less virulent (Bruno 1988; Daly & Stevenson 1989; Bandin et al. 1989; Piganelli et al. 1999). Thus, if the strain found at the PRU during our study is less virulent, it may not be binding well to the fecal matter coming from infected fish, thus limiting the success of horizontal transmission through fecal ingestion. Although I did successfully detect *R. salmoninarum* in one fish (Chapter 3), if the strain detected at the PRU is an attenuated strain, transmission may have been limited because the bacteria did not have a favorable affinity to the feces; the suspected primary route of horizontal transmission on the unit.

Our use of an auto-agglutination test to determine the potential virulence of the strain of *R. salmoninarum* found in inland trout of Colorado is interesting but needs to be followed up with an end-point mortality experiment comparing the CO strain to a virulent strain, as well as a test of the hydrophobicity of the CO strain as this also contributes to the overall virulence.

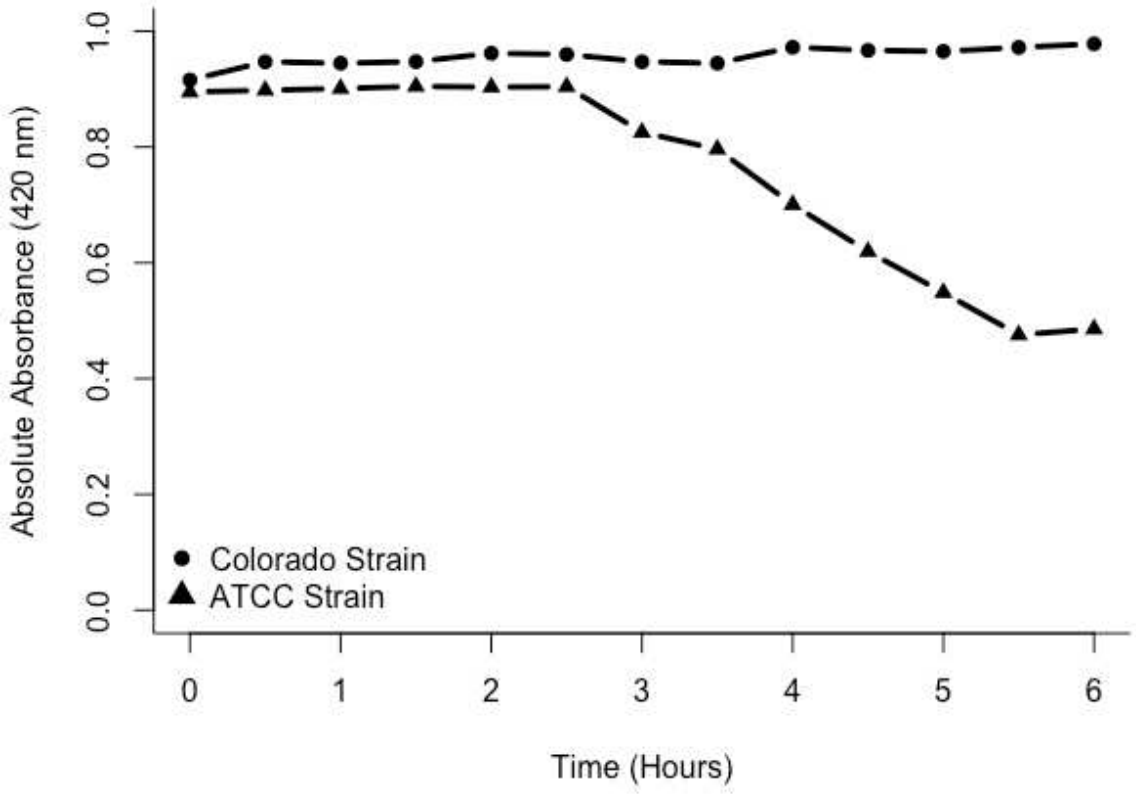

Figure I.I. Change of absorbance measurements over six hours ATCC 33209 strain and the CO strain found in Rainbow Trout from the Colorado Parks and Wildlife Bellvue Fish Research Hatchery.

I.V References

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