A molecular investigation of the dynamics of piscine orthoreovirus in a wild sockeye salmon community on the Central Coast of British Columbia

by

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Ethics Statement

The author, whose name appears on the title page of this work, has obtained, for the research described in this work, either:

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Abstract

Many Pacific salmon (*Oncorhynchus* sp.) populations are declining due to the action of multiple stressors, possibly including microparasites such as piscine orthoreovirus (PRV), whose host range and infection dynamics in natural systems are poorly understood. First, in comparing three methods for RNA isolation, I find different fish tissues require specific approaches to yield optimal RNA for molecular PRV surveillance. Next, I describe PRV infections among six fish species and three life-stages of sockeye salmon (*O. nerka*) over three years in Rivers Inlet, BC. Screening reveals a 3% overall prevalence of PRV in this system, along with the first evidence of PRV in Dolly Varden (*Salvelinus malma*) and eulachon (*Thaleichthys pacificus*). Among sockeye, the prevalence declined by 4% from the fry to smolt stages for the 2014 and 2015 cohorts.

Keywords: piscine orthoreovirus (PRV), Pacific salmon, Rivers Inlet, wildlife epidemiology, RNA isolation

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List of Acronyms

ANOVA	Analysis of Variance
GPC	Guanidinium Thiocyanate Phenol Chloroform RNA isolation procedure
PRV	Piscine Orthoreovirus
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-qPCR	Real Time quantitative Polymerase Chain Reaction

Chapter 1.

Introduction

Pacific salmon (*Oncorhynchus* sp.) are a valuable group of species. Their anadromous life history forges a link between the resource poor freshwater environments where they are born and die, and the nutrient-rich ocean where they feed and grow. This characteristic, as well as their massive historical abundance, has caused Pacific salmon to become the foundation of numerous cultures, economies (Lichatowich, Mobrand, & Lestelle, 1999) and ecosystems on the West Coast of North America (Cederholm et al., 1999). Unfortunately, many populations of Pacific salmon have been experiencing declines in productivity throughout the southern extent of their ranges (Freshwater et al., 2017; Peterman & Dorner, 2012), and extirpation of some local populations has occurred in some cases (Gustafson et al., 2007). There is a need to understand the mechanisms of these population declines in order to preserve this suite of species. Examples of the urgency for this research come from the recent Committee on the Status of Endangered Wildlife in Canada (COSEWIC) listings for British Columbia (BC) salmon, including extensive COSEWIC listing recommendations for many Fraser River sockeye salmon (*Oncorhynchus nerka* Walbaum, 1792) populations (COSEWIC, 2003), the emergency listing of Chilko and Thompson steelhead (*O. mykiss* Walbaum, 1792) (COSEWIC, 2018), the recent DFO evaluation of southern BC Chinook (*O. tshawytscha* Walbaum, 1792) and its upcoming COSEWIC evaluation (Fisheries and Oceans Canada, 2016), and the COSEWIC listing of Southern Resident Killer Whales (*Orcinus orca* Linnaeus, 1758) which identifies a shortage of Chinook salmon as the primary cause (Fisheries and Oceans Canada, 2011).

Declining productivity of important sockeye salmon populations have been receiving a great deal of attention (Freshwater et al., 2017). In 2009, the failed Fraser River sockeye run caused significant enough concern to launch a Canadian judicial inquiry (Cohen, 2012). Trends in declining sockeye salmon productivity are evident at a broad spatial scale (Freshwater et al., 2017; Peterman & Dorner, 2012), and simultaneous declines have been occurring since the 1990s in populations from Yakutat, Alaska, to Puget Sound, Washington (Peterman & Dorner, 2012). Peterman and Dorner

(2012) suggest that while stressors such as local habitat degradation, contaminants, and predators likely exacerbate issues faced by individual sockeye populations throughout their range, inquiries are needed into stressors that act at a larger spatial scale. An example of such a stressor would be climate-change-driven changes to temperature regimes, resource availability and competitive interactions that could increase in the susceptibility of salmon to microparasite pathogens, such as fungi, bacteria and viruses. Such microparasites could be transmitted between many sockeye salmon populations at-sea and carried back into individual freshwater spawning systems resulting in similar patterns of decline across a broad area (Peterman & Dorner, 2012). Interestingly, of the Fraser River sockeye salmon populations examined by Freshwater et al. (2017), only the Harrison River population, which migrates early to the marine environment without rearing in a freshwater lake, has experienced long term productivity increase, suggesting its lack of reliance on freshwater, and/or unique early marine habitat use may segregate this population from stressors possibly including disease which are experienced by numerous other lake-type sockeye populations.

Disease-driven population declines are not without precedent. Globally, emergent infectious diseases of wildlife are garnering attention as important drivers of population decline with some taxa. The fungal disease Chytridiomycosis is a high profile example demonstrated to act synergistically with climate change to drive declines and even extinctions in amphibian populations worldwide (Pounds et al., 2006). In Africa, Canine Distemper Virus was introduced with domestic dogs, which have become a source of infection with consequences for many African predators, including critically endangered Ethiopian wolves (Haydon et al., 2006), while in North America, white-nose syndrome caused by the fungus *Geomyces destructans* has led to serious population declines in several bat species (Thogmartin et al., 2012). For sockeye salmon populations experiencing variable return strength in British Columbia, Connors et al. (2012) have presented models investigating factors that regulate population dynamics. They show that pathogens associated with Atlantic salmon (*Salmo salar*) aquaculture may interact with interspecific competition and ocean productivity, driving large-scale trends. These scenarios provide impetus to understand the distribution and consequences of pathogens in wildlife communities of concern, including Pacific salmon.

Until recently, research on the distribution and impacts of parasites on wild Pacific salmon has been quite limited and has focussed mostly on macroparasites such

as trematodes and sea lice (e.g., Jacobson et al., 2008; Krkošek et al., 2011), and the microparasites Infectious Haematopoietic Necrosis Virus (IHNV) (Amos & Thomas, 2002; Meyers, 1998) and *Renibacterium salmoninarum*, the causative agent of Bacterial Kidney Disease (BKD) (Fenichel, Tsao, & Jones, 2009; Roon, 2014). Both these latter two microparasites have been focal topics due to their demonstrated capacity to impact farmed and hatchery-reared salmonid populations (Amos & Thomas, 2002; Fenichel et al., 2009). Concerns about disease impacts to fish in culture, such as BC farmed Atlantic salmon, from endemic microparasites carried by wild fish species also prompted sporadic survey work of parasites and pathogens in marine fishes associated with salmon farms, including Pacific salmon (Kent et al., 1998; Kent, 2000). However, until recently, outside of captive environments, disease was considered to be of little consequence to wild salmon (Amos & Thomas, 2002).

A limitation faced by previous research into disease in wild salmon has been the significant challenges associated with inferring disease-induced mortality in wild fish populations (Lester, 1984; Miller et al., 2014). Unlike disease in captive salmonids, it is difficult to follow wild fish to their ultimate fates and it is rare to encounter disease-killed wild fish, either due to the action of predators selectively removing compromised individuals or the action of scavengers leaving little trace (Miller et al., 2014). For this reason, molecular paths of inquiry into disease development are being explored to probe and further understand if and how microparasites are having impact at the individual and population levels. Recently, a panel of molecular biomarkers has been developed by Miller et al. (2017) and demonstrated to discriminate between individuals who are host to latent or bacterial infections and those that are actively developing viral disease (Miller et al., 2017). Molecular tools such as this have the capacity to be used with nonlethal tissue samples, such as small pieces of gill filament (Jeffries et al., 2014), or possibly blood, and may be paired with tracking studies to correlate pathogen load and gene expression with host fate (e.g., Bass et al., 2017; Miller et al., 2014). Employing similar methods, Jeffries et al. (2014) found that gene expression markers of immune function were highly correlated with infection by IHNV infection in juvenile Chilko Lake sockeye salmon, and predicted imminent mortality in fish that were not obviously manifesting disease. These findings suggest a new avenue to investigating threats posed by microparasites to wild Pacific salmon populations.

A microparasite that has emerged as a focal point for controversy in BC and elsewhere, and a candidate for pathogen-related mortality in Pacific salmon species, is piscine orthoreovirus (PRV). PRV is a double-stranded RNA (dsRNA) virus in the family Reoviridae, subfamily Spinareovirinae (Kibenge et al., 2013). It was first described in 2010, when it was isolated from Norwegian farmed Atlantic salmon manifesting a disease known as heart and skeletal muscle inflammation (HSMI) (Palacios et al., 2010). PRV is now recognized to be ubiquitous among farmed Atlantic salmon in Norway, Scotland, Chile, the USA, and Canada (Haatveit et al., 2017), and has also been found in all species of wild Pacific salmon and trout (Kibenge et al., 2013; Marty et al., 2015; Miller et al., 2014; Morton et al., 2017; Purcell et al., 2018). The causal relationship between PRV and HSMI was recently confirmed by Wessel et al. (2017), and HSMI is currently only known to afflict farmed Atlantic salmon, and causes inflammation, lethargy, anorexia and mortality in up to 20% of cases (Kongtorp et al., 2004; Palacios et al., 2010). Outside of Norway, HSMI has been found in Atlantic salmon farmed in Scotland (Ferguson et al., 2005), Chile (Godoy et al., 2016), and very recently in BC (Di Cicco et al., 2017). However, recently several PRV-caused conditions characterized by jaundice and anemia have been described in farmed Pacific salmon species (Di Cicco et al., 2018). Variant genotypes of PRV (terminology used here *sec* Di Cicco et al., 2018) have been shown to cause disease in farmed Chinook salmon in BC (PRV-1) (Di Cicco et al., 2018), farmed coho salmon (*O. kisutch*, Walbaum, 1792) in Japan (PRV-2) (Takano et al., 2016), farmed coho salmon in Chile (Godoy et al., 2016), and rainbow trout/steelhead in Norway and Europe (PRV-3) (Olsen et al., 2015) as well as Chile (Cartagena et al., 2018).

The initial site of infection and replication of all known genotypes of PRV are the red blood cells – which in fish are nucleated (Di Cicco et al., 2018; Finstad et al., 2014). In Atlantic salmon, physiologically stressful events such as low oxygen environments or handling are proposed to trigger the virus to leak from blood cells and infiltrate other tissues resulting in the inflammation and behavioural changes that characterize HSMI (Di Cicco et al., 2018; Kongtorp et al., 2004). In farmed Pacific salmon, however, PRV ruptures from blood cells, rather than leaking, resulting in jaundice, anemia and necrosis of the liver (Di Cicco et al., 2018). This more extreme disease state suggests PRV is less well-tolerated by Pacific salmon than Atlantic salmon hosts, and raises concern about how wild Pacific salmon, with heightened physical demands, fare when infected by PRV,

and may also indicate a shorter evolutionary relationship between hosts and virus (Di Cicco et al., 2018).

Despite the evidence that PRV can develop disease states among farmed Atlantic and Pacific salmon species, clinical disease due to PRV has not been diagnosed in wild salmonids (Di Cicco et al., 2018; Garseth et al., 2013). However, PRV-1 of high sequence similarity is shared by BC farmed Atlantic and Chinook salmon manifesting disease, and Garver et al. (2015; 2016) experimentally demonstrated that PRV shedding from infected Atlantic salmon hosts is able to infect Chinook and sockeye salmon. Further, genome sequencing of PRV has shown that the same genotype that results in HSMI or JS in farmed Atlantic and Chinook salmon (PRV-1) is also carried by all species of wild salmon and trout in BC (Kibenge et al., 2013; Siah et al., 2015), Washington State, and Alaska (Purcell et al., 2018; Siah et al., 2015). Miller et al. (2014, 2017) and Di Cicco et al. Di Cicco et al. (2018) and Madhun et al. (2017), speculate disease may not be seen in wild salmonids because viral loads insufficient to cause observable disease states in farmed salmon may nonetheless put wild fish at a disadvantage in the wild, thus increasing the probability that diseased individuals fall victim to predation before they are encountered in sampling. Studies exploring the costs of infection with a wide range of taxa support the notion that direct and immune-associated costs may influence host condition decreasing survival in the absence of high quality resources (Povey et al. 2009), and/or affecting host vulnerability to additional infections or predation (Beldomenico & Begon, 2010). Survivorship analyses of radio-tagged PRV-negative and PRV-positive Chilko Lake sockeye salmon spawners supports this notion, by showing a preliminary association between PRV infection and earlier mortality of

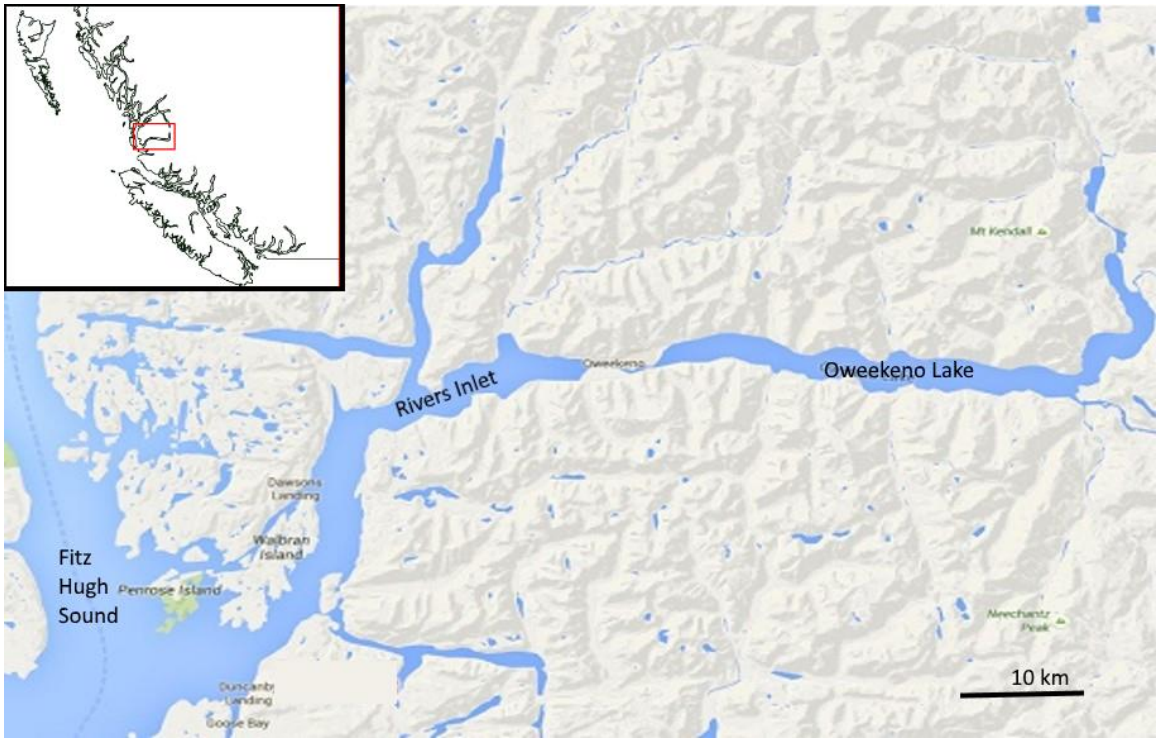

Figure 1-1. Rivers Inlet and Oweekeno Lake study region on the Central Coast of British Columbia, Canada. Inset shows location of Rivers Inlet in coastal British Columbia. Map data ©2018 Google

spawners (Miller et al., 2014). More recently, a province-wide correlational study showed that PRV infection is less frequent in spawning Pacific salmon above migration obstacles on the Fraser, Nass, and Skeena River systems which could be due to a compromised ability of these spawners to overcome migration challenges (Morton et al., 2017). These findings suggest a possibly non-trivial role of subclinical PRV infections in wild salmon that warrants the characterization of the dynamics of this virus in ecosystems.

The majority of recent wild salmon disease surveillance has been focused on Pacific salmon populations of concern from the southern region of British Columbia (Bass et al., 2017; Jeffries et al., 2014; Miller et al., 2014, 2017; Tucker et al., 2018). Many other salmon systems, however, especially those on the Central and North Coasts of British Columbia, receive less attention and our awareness of the distribution and impacts of microparasites like PRV suffers from considerable blind spots. In this thesis I seek to investigate the distribution of PRV in the Rivers Inlet salmonid community, on the

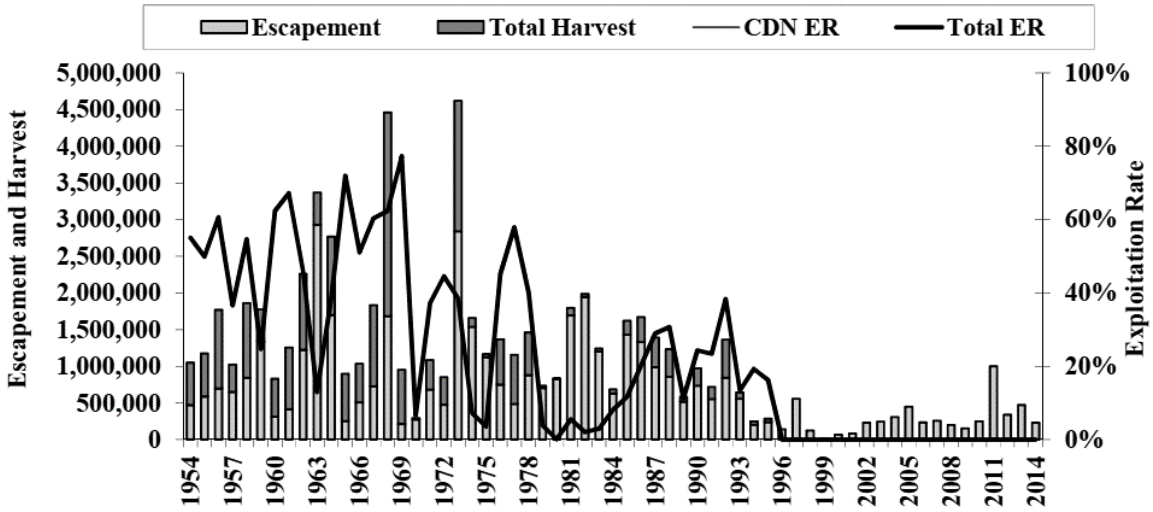

Figure 1-2. Total escapement, catch, and exploitation rate estimates for annual returns of sockeye salmon to the Rivers Inlet sockeye conservation unit. Canadian exploitation rate (CDN ER) is assumed to be equal to total exploitation rate. Reproduced with modification from English (2017).

Central Coast of British Columbia (Figure 1-1). Rivers Inlet is a 45 km long coastal fjord system joined by the 5 km long Wanukv River to Oweekeno Lake, a deep glacial lake that reaches inland to the east and north another 75 km. Once Oweekeno Lake was the rearing lake to the third largest fishery of sockeye salmon in British Columbia, with catches routinely above 1 million spawners. However, a considerable decline was noted for the sockeye salmon population by the early 1990s (McKinnell et al., 2001). After a period of adaptive management failed to abate falling numbers, the commercial fishery was completely closed in 1996. Unfortunately, declines continued despite management action and in 1999 the spawning population of sockeye was estimated to be only 3600 adults (McKinnell et al., 2001). Since this time, the spawning population has rebuilt to 200,000-500,000 individuals in recent years (English, 2017) (Figure 1-2). Currently, poor early marine survival remains the lead contender for the failure of the stock to rebuild (McKinnell et al., 2001). Disease surveillance for several salmon viruses of concern was attempted for this system in 2013, but insufficient funding prevented the screening of collected tissues (Connors, personal communication). This thesis is the first description of viral surveillance for PRV among the members of this salmonid community.

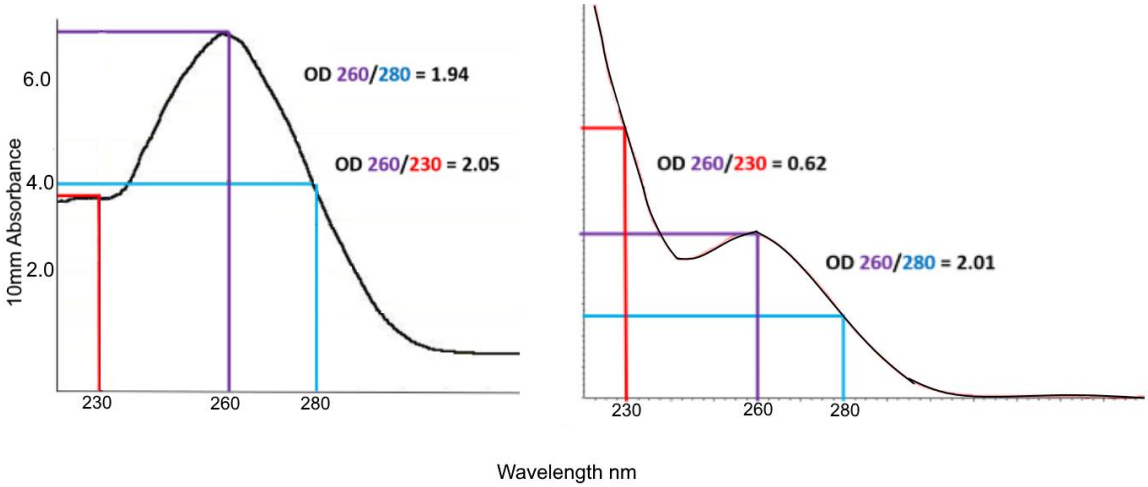

Figure 1-3. Spectrophotometrically measured RNA suspended in nuclease-free water. The left panel depicts pure RNA free from contaminants absorbing at 230 and 280 nm. The right panel shows a RNA sample contaminated with a substance absorbing at 230 nm contributing to a low 260/230 absorbance ratio.

Molecular surveillance using reverse transcriptase real time quantitative polymerase chain reactions (RT RT-qPCR) is becoming a standard method of detecting and quantifying the presence of viruses in tissues and the environment (see Bass et al., 2017; Miller et al., 2014, 2017; Vredenburg et al., 2010). This method is predicated on isolating high-quality RNA from tissues on which to perform this procedure. It is often considered ideal to find a method of RNA isolation which can be used across several tissue types, in order to provide a constant chemical background. However, as a result of differing tissue compositions, tissue-specific optimization may also be required to obtain RNA of comparable quality and quantity for downstream molecular procedures (i.e., Cirera et al., 2013; Peeters et al., 2016; Schrader et al., 2012; Schwochow et al., 2012).

In Chapter 2 of this thesis, I compare methods for the isolation of Ribonucleic Acids (RNA), the precursor molecules for protein synthesis and the building blocks of viruses such as PRV, from different field-collected salmonid tissues from Rivers Inlet in terms of RNA quality and quantity. Different tissues contain varying levels of RNA, as well as endogenous contaminants such as polysaccharides, proteins, and DNA and one RNA isolation method may not perform optimally across all tissues. The tissues being compared are field-collected blood drawn from juvenile and adult sockeye salmon, as well as pooled organs collected from adult sockeye and three trout species: cutthroat (*O.*

clarkii Richardson, 1836) and rainbow trout (*O. mykiss*), and Dolly Varden char (*Salvelinus malma* Walbaum, 1792). RNA quality is the primary metric of comparison, defined here as freedom from impurities which may inhibit accurate quantification and downstream uses of RNA, i.e., gene expression studies or pathogen screening (Fleige & Pfaffl, 2006). RNA purity is determined by measuring the absorbance (A) of light through a sample using a spectrophotometer at wavelengths of 230, 260 and 280 nm (Figure 1-3). RNA has an absorbance maximum at 260 but also absorbs at 280 nm, resulting in a A260/A280 ratio close to 2 for pure RNA (1.8-2.1 is a generally accepted range) (Fleige & Pfaffl, 2006). Absorbance at or near 280 nm, which reduces this ratio below 2, tends to be from contaminating proteins, or phenol (absorption peak at 270 nm) (ThermoScientific, 2011). Protein contamination, generally RNases, is of concern as it may degrade RNA skewing or preventing detection of target transcripts (Maniatis, Fritsch, & Sambrook, 1982). Conversely, contaminating phenol may inhibit enzymatic activity interfering with downstream applications for RNA (Maniatis et al., 1982). The ratio of absorbance at A260/A230 is also close to 2 for pure RNA. Absorbance at wavelengths near 230 nm decreases this ratio and indicates residual phenol or guanidine – both RNA extraction reagents and inhibitors of enzymatic activity – or polysaccharides which can prevent the accurate quantification of RNA concentration (ThermoScientific, 2011). These contaminants, especially guanidinium and phenol, may also inhibit enzymatic activity required for downstream uses of RNA and thus are important to quantify when comparing the performance of different isolation methods (Fleige & Pfaffl, 2006; Imbeaud et al., 2005). In Chapter 2, I additionally compare the outcome of RT-qPCR for housekeeping gene expression from smolt blood RNA isolated using two methods, to evaluate how quality differences in RNA affect gene expression. Absorbance at 260 nm also reveals RNA quantity, as one optical density unit on the spectrophotometer translates to 400 ng/μl for a 1 mm light path (Thermo Fisher Scientific, 2018). Each isolation method was additionally compared in terms of the total yield of RNA per unit input whole tissue.

Additionally, during disease surveillance, one must screen large numbers of samples to obtain accuracy and precision in estimating viral prevalence (the number of positive tests divided by the total number of tests). This places a premium on methods that allow high throughput sample processing and ease of use. Thus, in Chapter 2, I also consider the cost and time required to handle samples using each of these RNA

isolation methods, as well as their ability to be scaled-up for large numbers of samples, and the required skill level and risk posed to users. I find that one type of RNA isolation method is not ideal for all types of fish tissues one might want to interrogate, and the choice of method will be dictated by experimental requirements, laboratory resources, and user time.

In Chapter 3 I aimed to generate provisional insights into the infection dynamics and potential harm of PRV across all life stages of sockeye salmon from the Rivers Inlet system, as well as several of the other salmonid species in that community, including resident cutthroat and rainbow trout, Dolly Varden char, spawning Chinook salmon, and eulachon (*Thaleichthys pacificus* Richardson, 1836) across three years. I employed molecular methods of virus surveillance to identify the range of hosts for PRV this system, and to determine how prevalence varies within these groups by species and age over time. This is a first step to knowing whether PRV is persistent in this wild system, and whether certain hosts enable viral persistence, i.e., as reservoirs of PRV infection, which I define according to Haydon et al. (2002) as one or more epidemiologically-linked populations which maintain persistence of a pathogen and transmit that pathogen to a target population of interest – in this case, sockeye salmon.

In this Chapter I find that PRV exists at a low prevalence in this system without strong species or life-stage associations. Adult sockeye salmon and trout tested positive for PRV only in 2014, and the prevalence of PRV declined in the offspring of both 2013 and 2014 adult sockeye from the fry to smolt stages – suggesting either mortality or recovery from virus at these early life-stages. Interannual variation in PRV presence appears only in the smolt life-stage of sockeye salmon and may be driven by external inputs to the system, or potentially density changes of hosts. This work identifies two previously undescribed hosts for PRV: eulachon and Dolly Varden char, expanding our knowledge of host range for this virus.

My concluding chapter summarizes my findings and discusses the limitations of the current study and how I sought to overcome these in my analyses. I also suggest avenues for future research to address yet unanswered questions regarding the dynamics of PRV in wild salmon communities and its possible influence on wild Pacific salmon population dynamics.

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Chapter 2.

A comparison of common methods of RNA isolation applied to fish tissues for the screening of piscine orthroreovirus.

Abstract

Field surveillance for RNA viruses often requires RNA isolation from multiple tissue types, each presenting different challenges. Here I compare three RNA isolation approaches for salmonid blood and pooled organ tissues across seven metrics of performance. I find that isolation methods have different strengths and weaknesses depending on the tissue. Trizol reagent performs best for adult salmonid blood, while an updated acidic phenol guanidinium chloroform method produces higher yields and purer RNA from mixed organ pools. Silica filter spin columns produce high quality RNA from juvenile sockeye blood, but Trizol produces higher yields. A trade-off is also found on the time-cost continuum. RTqPCR analysis with silica filter spin columns and Trizol-isolated RNA indicates impurities resulting from suboptimal extraction methods influence measured expression levels, thus, RNA purity and yield are important to standardize across tissue types and studies. Collectively these results indicate that there are trade-offs in the different methodologies used for RNA extraction from field-collected tissues.

2.1. Introduction

Different tissue types present differing challenges for the extraction of nucleic acids, such as RNA. Fatty tissues may inhibit the isolation of RNA (Cirera, 2013), while fibrous tissues may be RNA-poor and require much higher starting amounts to achieve the same yield (Peeters et al., 2016). Some tissues, for example, blood, brain, or spleen, may contain a greater amount of proteins including nucleases, enzymes which degrade RNA, and must be handled carefully to preserve RNA content (Schwochow et al., 2012), while polysaccharide-rich tissues like liver or plant tissues may co-precipitate starches or glycogen with RNA, making quantification inaccurate (Schrader et al., 2012). Despite the obviously unique requirements of differing tissues, it is commonly held ideal to use a single method of RNA isolation within a study to eliminate potential biases in

downstream analyses, such as gene expression, introduced by differing chemical backgrounds (Meyer et al., 2016). On the other hand, the use of a single method for different tissues can result in different levels of impurities, such as nucleases and polysaccharides, which in turn also can affect the outcome of downstream applications. Thus, RNA extraction becomes a challenge in studies that by nature require the evaluation or comparison of RNA isolated from several tissue types.

The screening of RNA from field-collected tissues by reverse transcriptase real time quantitative polymerase chain reaction (RT-qPCR) for pathogens, such as RNA viruses, may require examination of multiple tissue types (for example Miller et al., 2017). While viruses and many other pathogens may exhibit 'tissue tropism', or affinity for a specific tissue (Nomaguchi et al., 2012), some viruses, including the salmonid virus piscine orthoreovirus (PRV), affect more than one tissue type at different times over the course of an infection (Di Cicco et al., 2017, 2018; Finstad et al., 2014). In the case of PRV, peripheral red blood cells are the initial target of infection, but infection may later spread to the spleen and kidney, and subsequently, the heart, liver and skeletal muscle (Di Cicco et al., 2017, 2018; Finstad et al., 2014). When collecting and screening field-collected tissue samples, it is impossible to know *a priori* the stage of infection an organism may be in if they are not presenting outward clinical signs, let alone whether an infection is present at all. In addition, in broader assays for multiple pathogens, single tissues are often inappropriate (see methods described in Miller et al. (2014) for a multiple pathogen panel developed for screening wild Pacific salmonids). Therefore, in pathogen-surveillance and other molecular studies, it is often necessary to isolate RNA from several tissue types either separately or simultaneously as a tissue pool. In these cases, one of two approaches may be taken: i) a single type of RNA isolation method may be used and will hopefully produce RNA of sufficient quality and quantity across all tissue types being examined, thus controlling chemical background, or, ii) individual isolation methods may be tailored to different tissue types to produce RNA of optimal quality and yield thereby allowing more sensitive inquiry of each tissue.

Two of the most common approaches to RNA isolation are guanidinium-induced binding of RNA to silica fibers in a solid-state column ('spin column') and the use of acidic phenol-based solvent to extract of RNA from DNA and proteins in solution (Thermo Fisher Scientific, 2018). RNA isolation methods that involve lithium chloride

precipitation and magnetic particle-based entrapment with direct lysis are also common methods (Johnson, 2012).

Filter-based, spin column formats, such as the RNeasy kits by Qiagen, are popular due to their speed and ease of use. These procedures are based on dissolving tissue samples in a solution that contains guanidinium, a chaotropic salt that breaks hydrogen bonds in proteins, thereby inhibiting the activity of RNA nucleases (RNases) which cleave RNA. The solution is thereafter passed through a silica-based filter, in which guanidinium induces cation-bridge formation between silica fibers and RNA. The filter is then washed to remove other cellular components, followed by elution of pure RNA in a low ionic strength solution.

Extraction with an acidic-phenol solution is one of the first methods developed for isolating RNA from tissues (Kirby, 1956), and incorporates a large body of technical knowledge on adapting, fine-tuning, and troubleshooting this procedure (Kirby, 1956). In an improved version of this technique (Chomczynski & Sacchi, 1987) a tissue sample is homogenized in an acidic phenol-guanidinium solution, to which chloroform is added. The sample is centrifuged to separate it into three phases: a lower organic phase, a middle phase that contains denatured proteins and genomic DNA, and, an upper aqueous phase containing RNA. The upper aqueous phase is collected, and the RNA is separated from other cellular components by an alcohol and salt-induced precipitation. An RNA pellet is collected by centrifugation and then resuspended in RNase-free water or buffer (Chomczynski & Sacchi, 1987). This method has the additional benefit of allowing a user to retain the interphase for DNA and/or protein isolation.

Currently a variety of approaches are taken to RNA isolation for the assessment of PRV infection in salmonids in both laboratory and field-based studies. Kibenge et al. (2013) employ Trizol acid-phenol guanidinium chloroform-based extraction approaches and spin columns for RNA isolation from organ pools and gill tissues, while Finstad et al. (2014) and Dahle et al. (2015) report the use of an initial solvent extraction followed by a clean-up procedure in spin columns for salmonid blood. Garver et al. (2015; 2016), and Polinski et al. (2016) use Trizol alone for salmonid blood and kidney tissues for both viral surveillance and gene expression studies, while Miller et al. (2014; 2017) for mixed tissues, and Garseth et al. (2013a; 2013b; 2013c) for kidney, report lysing tissues with Trizol followed by a magnetic RNA-entrapment kit. While each of these studies is

internally consistent, the use of one method for different tissue types within a study may influence the sensitivity to detect virus in certain tissues if a broad range are being compared. Additionally, this diversity of approaches between studies has the potential to produce conflicting results for a single tissue, especially blood with its high levels of protein and polysaccharides.

To examine this issue, I compare the performance of three different RNA isolation protocols, a spin column method and two acidic phenol-guanidinium chloroform extraction methods, for extracting RNA from field-collected salmonid tissues for the surveillance of PRV and gene expression. My overarching question is whether one method of RNA isolation can produce RNA of uniform quality and quantity for viral surveillance across several key tissue types including: i) small and ii) large volumes of juvenile and adult sockeye salmon (*Oncorhyncus nerka* Walbaum, 1792) blood, as well as iii) a tissue pool consisting of heart, skeletal muscle, liver, kidney and spleen, dissected from adult sockeye salmon and three trout species (see Chapter 1). My specific goals were to:

i) Compare the performance of the three RNA isolation methods for each tissue type in terms of the quality (integrity and purity) and yield (amount of RNA per unit input tissue) of RNA.

ii) Compare RNA isolation protocols in terms of their cost, time investment, potential toxicity, automation potential, and scalability, i.e., whether an isolation method can be altered by increasing/decreasing reagent amounts with simple ratios to handle larger or smaller amounts of input tissues, and/or automated to accommodate mid to high-throughput sample processing.

iii) Provide further technical recommendations for improving RNA of poor quality if no single method consistently produces RNA appropriate for downstream uses.

With these inquiries I strive to resolve the conflict between conventional wisdom to use a single RNA isolation method for all tissues, and the obviously unique requirements of specific tissues in the context of experiments requiring the examination of several tissue types simultaneously.

2.2. Methods

2.2.1. Overview

Here I evaluate three different methods for the isolation of RNA from field collected fish tissues: Binding of RNA to a column silica matrix via cation bridges followed by repeated washes to remove other molecules (RNeasy commercial kit; Qiagen), separation of RNA from DNA and protein by extraction with acidic phenol, guanidinium and chloroform using a commercial reagent (Trizol reagent, Thermo Fisher Scientific), and a recently published variant of this method (Chomczynski & Sacchi, 2006). I evaluate these methods across nine metrics: RNA integrity, RNA purity, RNA yield, systematic gene expression bias, isolation method cost, sample preparation time, isolation method scalability, method variability, and user risk.

2.2.2. Sample collection

As part of a research program to monitor the prevalence of the emergent RNA virus Piscine Orthoreovirus (PRV) in a wild salmonid community in Rivers Inlet, British Columbia (BC), blood and organ tissue samples were collected from 318 salmonids comprising adult and juvenile (early saltwater smolt stage) sockeye salmon, as well as adults of: cutthroat (*O. clarkii* Richardson, 1836), rainbow/steelhead (*O. mykiss* Walbaum, 1792) and Dolly Varden char (*Salvelinus malma* Walbaum, 1792). Blood samples were collected differently for juvenile and adult salmonids. Blood was drawn in the field from smolt sockeye salmon captured by seining immediately following capture using transection of the caudal vein collecting <70 microliters (μl) of whole blood via heparinized capillary tube. Blood was expressed with a squeezable bulb into a microcentrifuge tube with 1.3 ml of RNAlater (ThermoFisher Scientific) storage solution and held on ice until it could be stored in a -20 °C freezer. Samples were transported on ice back to Vancouver from Rivers Inlet and held at -80 °C until laboratory analysis.

Adult sockeye salmon were captured in collaboration with the Wuikinuxv First Nation Fisheries Program gill net fishery. One ml of blood was drawn immediately after fish were removed from the net by caudal vein puncture with a hypodermic syringe preloaded with 10 μl of 270 mM K_2EDTA as an anticoagulant. Half a milliliter (ml) of this blood was ejected from the syringe into a 2 ml microcentrifuge tube filled with 1.3 ml

RNAlater, as suggested in the Ribopure Blood Protocol with RNAlater (Lifetech, 2011). Shortly after drawing blood, samples of heart, liver, head kidney, spleen, and skeletal muscle were also dissected out of adult sockeye and trout using aseptic technique in a field laboratory. Small pieces of organ tissue no more than 5 mm thick in one dimension, typically each 2 x 2 x 5 mm for a total of one tenth of one cubic centimeter, were immersed in no less than 10-20 volumes of RNAlater (and more often closer to 18 volumes or 1.8 ml) in a 2 ml microcentrifuge tube. All tissue types in RNAlater were held on wet ice until they could be transferred to a 4 °C refrigerator for a 24-hour incubation, and then were kept at -20 or -80 °C until RNA extraction.

2.2.3. RNA isolation procedures

RNA was isolated using one of three methods: 1) an RNA-binding spin column method, the RNeasy Micro Kit (Qiagen), 2) the guanidinium-phenol-chloroform method using the commercial Trizol reagent (ThermoFisher) (Chomczynski & Sacchi, 1987), and 3) a modified guanidinium-phenol-chloroform method (GPC) organic extraction method (Chomczynski & Sacchi, 2006).

RNeasy silica filter basket spin columns

RNA was isolated from sockeye smolt blood samples (no greater than 70 μl whole blood) using the RNeasy micro kit following the manufacturer supplied protocol (Qiagen, 2014). In brief, smolt blood samples in RNAlater were transferred from their storage tubes to 2 ml microcentrifuge tubes. Blood pellet was collected by brief centrifugation at the bottom of tubes so that RNAlater solution could be poured off. A 3.175 mm diameter tungsten bead (weighing 1 g) was then added to the blood pellet as well as 350 μl Buffer RLT (lysis buffer) from the RNeasy micro kit. Twenty-four samples at once were then homogenized on a Retsch Mixer Mill 300 at 32 Hz for 4 minutes. After homogenization another 350 μl Buffer RLT was added and the contents of the tube were transferred to a spin column in a clean 1.5 ml microcentrifuge tube. Spin columns were centrifuged for 15 s at 8000 x gravitational force (g) and the flow through was discarded. This step was repeated until all the buffer RLT and sample was passed through the spin column silica membrane. Tungsten beads were collected for sterilization and re-use. Next, 350 μl of Buffer RW1 (wash buffer) was added to spin columns. Columns were centrifuged for 15 s and the flow through was discarded. An on-column DNase digest

was then done to remove contaminating genomic DNA with DNase I supplied in the kit. For this 10 μl DNase I was added to 70 μl kit supplied Buffer RDD, and this was added to each spin column and incubated for 15 minutes at RT (RT). Then 350 μl Buffer RW1 was added to wash and again the columns were centrifuged at 8000 xg for 15. The flow through and collection tube were discarded. The spin column was then placed in a clean 1.5 ml microcentrifuge tube and 500 μl of a second wash buffer, Buffer RPE, was added and the columns were centrifuged at 8000 xg for 15 s and flow through was discarded. This was followed by the addition of 500 μl 80% ethanol to remove additional impurities in the RNA and centrifuged at 8000 xg for 2 minutes. Following this both flow through and collection tube were discarded. The spin column membrane was dried by spinning the column with its lid open in a new clean tube at maximum centrifuge speed for 5 minutes. Again, the flow through and collection tube were discarded. The spin column was placed in a new labelled 1.5 ml microcentrifuge tube and 14 μl RNase-free water was added directly to the spin column membrane. The tube was then centrifuged at maximum speed for 1 minute to elute the RNA from the spin column membrane. This step was repeated once by pipetting the eluant from the collection tube and passing it again through the membrane. Isolated RNA was then measured for purity and quantified using a spectrophotometer (in this case the Nanodrop 2000c, Thermo Fisher Scientific) and frozen at -80 °C until needed.

Trizol organic solvent extraction (Thermo Fisher)

The Trizol organic RNA extraction method was used for all tissue types: sockeye salmon smolt blood, adult sockeye blood, and organ tissue pools from sockeye and trout. For adult sockeye blood, 500 μl of mixed whole blood and RNAlater was centrifuged to recover a pellet. Adult salmonid organ tissues were pulled from RNAlater solution using sterilized forceps and a clean Kimwipe was used to express excess solution from the tissues. Using a sterile scalpel blade, equal amounts from each organ tissue were cut and weighed on a balance with a precision of 0.001 g. The total weight of pooled organ tissues did not exceed 100 mg. One ml of Trizol solution was added to both blood tissues and pooled organs in a fume hood, along with a tungsten bead, and tissues were homogenized on the Tissuelyser as above, except organ tissues were homogenized for eight minutes.

After homogenization, samples were pipetted into a new tube and allowed to incubate at RT for five minutes before 200 μl chloroform was added to each sample under a fume hood. Samples were vortexed for 15 s each. The samples were then incubated for three minutes at RT before being centrifuged at 4 °C for 15 minutes at 12000 xg to separate the aqueous and organic phases. Following this, the clear upper aqueous phase containing RNA was pipetted into a clean microcentrifuge tube.

RNA was precipitated from solution by adding 500 μl cold 100% isopropanol. Tubes were incubated for 10 minutes at RT before they were centrifuged at 12000 xg and 4 °C for 10 minutes. At this point an RNA pellet became obvious as a small white pellet at the bottom of the microcentrifuge tube. Isopropanol was poured or pipetted off the RNA pellet, taking care not to dislodge it. The pellet was washed to remove impurities by adding 1 ml of cold 75% ethanol. Each sample was briefly vortexed and then centrifuged at 4 °C for 5 minutes at 7500 xg. The ethanol pipetted off, and this step was repeated to remove any additional impurities. At the end of the second wash, tubes were briefly centrifuged at RT at 20000 xg and any remaining ethanol was carefully pipetted off the RNA pellet. Tubes were allowed to air dry at 37 °C for 5 minutes to evaporate remaining ethanol. Care was taken to avoid over-drying, as this would make the RNA pellet insoluble in water.

The RNA pellet was then resuspended in 32 μl of diethyl pyrocarbonate (DEPC)-treated nuclease-free water and heated for 10-15 minutes at 60 °C to resolubilize the pellet. A small sample was taken for purity assessment and quantification and the rest of the sample was stored at -80 °C until needed.

Acid guanidinium thiocyanate phenol chloroform extraction (GPC)

Guanidinium thiocyanate-phenol-chloroform (GPC) organic RNA extractions followed methods described in Chomczynski & Sacchi (2006), although, to work with fish tissues on the order of 50-100 mg per sample, the reaction volumes from the published protocol were scaled down by half.

Adult sockeye salmon blood and tissues were initially prepared as per the description in the Trizol isolation method. In a fume hood, 500 μl of a denaturation solution (solution D) composed of 4M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% w/v N-laurosylsarcosine, and 0.1M β-mercaptoethanol, was added to 50 mg fish

tissue (or pellet recovered from 500 μl whole adult sockeye blood) and homogenized as described above. This homogenate was transferred to a new tube and sequentially 50 μl 2M sodium acetate and glacial acetic acid solution, 500 μl water-saturated phenol, and 100 μl of 50:1 chloroform:isoamyl alcohol was added with vigorous shaking of the tube after each addition. Once all three solutions were added, each tube was shaken for 10 seconds and then allowed to incubate on ice for 15 minutes. Following incubation, the tube was centrifuged for 20 minutes at 4 °C and 10000 *g* to separate the organic and aqueous phases.

After centrifugation the aqueous phase of each sample was transferred to a clean microcentrifuge tube. To this, 600 μl isopropanol was added, and the tube was inverted to mix. RNA was precipitated by placing samples at -20 °C and incubating for a minimum of one hour. Samples were then centrifuged for 20 minutes at 4 °C and 10000 *g*, and the isopropanol was carefully discarded. The RNA precipitate was visible at this point as a gel-like pellet in the bottom of the centrifuge tube.

A second precipitation was then performed to remove more DNA and protein from the RNA. The pellet was resuspended in 300 μl of solution D and transferred to a new 1.5 ml tube. To this, 300 μl isopropanol was added, and samples were again incubated at -20 °C for a minimum of 30 minutes. Following this, samples were centrifuged for 10 minutes at 4 °C and 10000 *g* and the supernatant was discarded.

The RNA pellet was washed to remove impurities by dissolving it in 700 μl of 75% ethanol and vortexing for a few seconds. Samples were then allowed to incubate for 10-15 minutes at RT. Following incubation, samples were centrifuged for 5 minutes at 4 °C and 10000 xg and the supernatant was discarded. Samples were air dried at 35 °C and resuspended in 52 μl DEPC nuclease-free water and incubated at 60 °C to solubilize. As with the other RNA extraction methods, a small amount was taken for purity measurement and quantification and the rest stored at -80 °C until needed.

2.2.4. RNA isolation method comparisons

The RNA produced using each isolation method was compared within each tissue type across several different measures of RNA integrity, purity and yield. Further comparisons were made regarding the amount of time each protocol required, the cost

per sample, and other considerations including method scalability, equipment requirements, toxicity, and user skill requirements.

RNA integrity

RNA integrity is a measure of how intact the RNA in a sample is and can be visualized through electrophoresis on a denaturing agarose gel. I visualized RNA integrity on denaturing agarose gels made using either 3-(N-morpholino)propanesulfonic Acid (MOPS) buffer and formaldehyde, and pre-stained with ethidium bromide (Sambrook & Russel, 2001) or agarose gels with bleach (1%) in a Tris-acetate-EDTA (TAE) buffer (Aranda, LaJoie, & Jorcyk, 2012). Formaldehyde and bleach both disrupt the secondary structure of RNA, allowing separation based on differences in linear lengths by agarose gel electrophoresis.

In brief, for MOPS/formaldehyde gels a 30 ml gel was prepared by preheating 1.75 ml formaldehyde and 3 ml 10X MOPS buffer at 55 °C (Sambrook & Russel, 2001). Agarose was prepared by boiling 0.4 g in 25 ml nuclease-free water and cooling to 55 °C before mixing with MOPS/formaldehyde. The gel was cast in a form cleaned of nucleases with RNase Zap (Ambion) and allowed to cool. A denaturing solution was prepared for loading RNA composed of 550 μl formaldehyde, 150 μl formamide, 150 μl 10X MOPS buffer, 10 μl ethidium bromide (10 mg/ml; Sambrook & Russel, 2001). One to 5 μl of RNA was incubated for 15 minutes at 55 °C to denature and then cooled on ice and mixed with 1 μl 6X loading dye before loading on in gel lanes with 10 μl of denaturing solution. Gels were run for 30 minutes at 100 V in 1X MOPS buffer in a fume hood and then visualized by UV transillumination.

Bleach gels were prepared according to a protocol published by Aranda et al. (2012). Briefly, the gel was prepared by adding 0.5 g agarose in 50 ml TAE buffer solution to make a 1% gel. Once the agarose was in solution, 500 μl or 1% bleach was added, and the mixture was swirled at RT for 5 minutes. The suspension was heated to melt the agarose and allowed to cool before ethidium bromide was added to a final concentration of 0.5 ug/ml. The solution was then poured into a clean mould as above and allowed to set. One microgram of RNA was then mixed with 10X DNA loading buffer (ThermoFisher) to a final concentration of 1X. The gel was then run at 100 V for

approximately 30 minutes and then visualized with UV transillumination (Aranda et al., 2012).

Both denaturing gel types were examined for the same characteristics indicating good RNA integrity. The presence of 2 or 3 distinct bands indicates high quality RNA. The top band in eukaryotes represents 28S ribosomal RNA (rRNA) which is 4.8 kilobases (kb) in size, the middle band represents 18S rRNA at 2.0 kb, and the lowest band is a combination of 5.8 and 5S RNA (154-117 nucleotides). A band intensity for 28S:18S less than 2:1 indicates partial RNA degradation whereas smeared or absent bands indicate extensive RNA degradation. A random sample of approximately 10% of all RNA samples isolated in-house at SFU were examined for RNA integrity on one of these gel types.

RNA purity

RNA purity was measured using UV spectrophotometry on the NanoDrop 2000c (ThermoFisher Scientific), a spectrophotometer designed to handle low sample volumes (1-2 μl) with high precision and accurate quantification over a concentration range from 5-500 ng/μl (Aranda IV et al., 2009). To measure RNA purity, 2 μl of each RNA sample suspended in DEPC water was assessed on the Nanodrop 2000c (ThermoFisher). Measurements of several samples were repeated to confirm high precision of measurements. Comparisons were made between RNA isolation methods for each tissue type in terms of the average 260/280 and 260/230 ratios using Welch's two sample t-test assuming unequal variances using R version 3.4.4 (R Core Team, 2018).

Additionally, because the preferred RNA isolation method would not only have average ratio values close to two (Fleige & Pfaffl, 2006) but would also produce a majority of RNA samples measuring at or above this threshold, proportions of samples above threshold values and 95% confidence intervals of the true proportion were calculated using the Jeffrey's Interval (Brown, Cai, & Dasgupta, 2001) and reported for each isolation method for each tissue type. The proportion of samples above threshold ratio values for 260/280 and 260/230 ratios were compared in 2 x 2 contingency tables using Fisher's Exact Test for proportions. Initially, a stringent ratio requirement of 2 was imposed, but it is generally accepted that ratios of 1.8 or higher may still be adequate for downstream uses of RNA (Fleige & Pfaffl, 2006). Therefore, comparisons were also

made between the proportion of RNA samples above 1.8 for both 260/280 and 260/230 ratios. All statistics were computed in R (R Core Team, 2018).

Lastly, I was interested in comparing the RNA produced using Trizol reagent to examine how one method performs across the multiple tissues of interest. This was done in terms of both average 260/280 nm absorbance of samples and the proportion of samples above a 260/280 ratio of 2. A comparison of averages was made using a one-way ANOVA procedure to evaluate the effect of tissue type on 260/280 ratio. The ANOVA was followed by Tukey multiple pairwise comparisons to evaluate significant contrasts. Proportions were compared using Fisher's Exact Test, with adjusted *p*-values for multiple comparisons (R Core Team, 2018).

RNA yield

Concentration of RNA samples was also obtained by readings on the Nanodrop 2000c (Thermo Fisher Scientific). For the Nanodrop, 1 optical density (OD) unit translates to 400 ng/μl for a 1 mm light path (Thermo Fisher Scientific, 2018). The concentration in μg/μl was used to determine the total amount of RNA present in the final volume eluted, and then yield was calculated as the amount of RNA in μg for a given unit of input tissue – either μl for blood, or mg for organ tissues. Average yield for each isolation method by tissue type was calculated, and comparisons between RNA isolation methods were made using Welch's two sample T Test with unequal variances in R (R Core Team, 2018).

Examination of systematic gene expression bias

RNA was isolated from sockeye salmon smolt blood using the Trizol solvent extraction method (sample size =17) and the RNeasy spin column method (n = 12). These samples were treated with DNase to remove contaminating genomic DNA using the abm AccuRT Genomic DNA Removal Kit and then subject to complementary DNA (cDNA) synthesis using the abm OneScript Reverse Transcriptase Kit and oligoDT primers to create template for RT-qPCR examination of gene expression. RT-qPCR was performed using a Roche LightCycler96 platform and abm BrightGreen Master Mix chemistry with primers designed against GenBank accession AB481206 for sockeye salmon β-actin. A 1x BrightGreen assay contained 5 μl BrightGreen master mix, 0.15 μl of each 10 μM primer, 0.7 μl nuclease-free water, and 4 μl 1:3 diluted cDNA.

BrightGreen assays were performed according to abm BrightGreen protocol with the following reaction conditions: an initial denaturation of 95 °C for 10 minutes, followed by 45 cycles of denaturation at 95 °C for 15 seconds, and annealing/extension at 60 °C for 60 seconds. Data analysis was performed with Roche software which reported the average cycle threshold value for each sample across two technical replicates. The cycle threshold is the amplification cycle at which the template emerges as a signal above background fluorescence. Raw Ct values for β-actin were compared for the two isolation method groups using a two-factor ANOVA which also considered the effect of capture location to control for other systematic biases that could introduce a difference in gene expression. All statistical analyses were performed in R (R Core Team, 2018).

Isolation method cost

RNA isolation methods range widely in their cost per sample preparation. For experiments requiring a great number of RNA isolations, the method of choice may be selected as an optimization of sample quality and processing costs. To compare costs between these methods, I assembled costs of reagents required to outfit a laboratory for each of these RNA isolation methods. The quantity of reagents was selected based on readily available brands and size formats at Simon Fraser University Science Stores, and in the case of the GPC isolation method, the quantity of limiting reagent (guanidinium thiocyanate) required to make one ‘batch’ of denaturation solution according to the published protocol (Chomczynski & Sacchi, 2006). This cost per unit is an approximation, and it is expected that costs for an established laboratory with many common reagents are slightly overestimated.

Sample preparation time

The time to isolate RNA using each of the protocols was estimated from the published time steps for each protocol and experience with each method. Handling time was standardized for the simultaneous processing of eight samples for each protocol, which is a reasonable amount of simultaneous RNA isolations for an intermediate to experienced user. The amount of time to process samples was divided by eight to arrive at a ‘time-per-sample’ metric that was plotted against cost for each isolation method.

Isolation method scalability

RNA isolation methods were also compared in terms of their ‘scalability’. This characteristic was determined by: i) whether published protocols offer the ability to alter reagent amounts based on simple ratios to handle larger or smaller amounts of input tissues, and ii) whether an isolation method can be automated to accommodate mid to high-throughput sample processing. In this latter case, there would be additional considerations concerning laboratory infrastructure.

Method variability

Ease of use was also a consideration for these RNA isolation methods. In other published works this is often reported as the difference between the coefficient of variation (COV) of some measure of RNA concentration or purity produced by experienced users versus novice users (Aranda et al., 2009). However, because multiple users of similar skill level assisted in the RNA isolation process, the coefficient of variation of the 260/230 ratio was computed for each tissue type and isolation method. Small coefficients of variation close to an ideal 260/230 ratio were taken as an indication of ease of use and consistency of acceptable quality, while small COVs further from an ideal ratio represent consistent error encountered in a method. Larger coefficients of variation demonstrated that a method required a longer learning period or was not universally accessible to laboratory assistants.

User risk

Each of the RNA isolation methods here was qualitatively compared in terms of the safety risks associated with each of the potentially hazardous reagents involved, the personal protective equipment and location procedure was required to take place (i.e., fume hood), and the amount of time a user was exposed to hazardous substances. Total procedure length was taken as a proxy for exposure time.

2.3. Results

2.3.1. Overview

The three RNA isolation methods evaluated showed performance tradeoffs with sockeye salmon blood and salmonid organ pools. Results from the comparison of gene

expression for β-actin from RNA isolated using Trizol and spin columns revealed a systematic bias in Ct value that is likely due to the purity differences in the RNA produced by these two methods. The following sections report each method's performance according to: the integrity of RNA produced, RNA quality as measured by UV spectrophotometry, downstream gene expression, user skill requirements using the coefficient of variation of 260/230 absorbance measurements, RNA yield, extraction method cost and time requirements, method scalability, and the risk of user exposure to harmful substances.

2.3.2. RNA isolation methods comparison

RNA quality: Integrity based on denaturing gel electrophoresis

The integrity of RNA isolated from sockeye salmon blood and organ tissues was visualized on denaturing agarose gels (Figure 2-1 & 2-2). Both the Trizol and GPC RNA isolation methods show varying results in terms of the clarity and presence of bands. Both methods produce RNA with bands present for 28S and 18S ribosomal RNA, and the 28S rRNA band is twice as bright as the band for 18S rRNA in most cases (Figure 2-1). The Trizol method appears to produce at least one blood sample isolated from sockeye smolt with a very clear banding pattern of the appropriate brightness ratio (BS028), while the GPC method produces faint bands for 28S and 18S rRNA for blood, and a stronger banding pattern for pooled organ sample RNA.

Trizol and RNeasy methods both show the ability to produce RNA of high quality, indicated by the 28S rRNA band at twice the intensity of the 18S band (Figure 2-2). Two RNA samples (WR315, WR402) isolated with Trizol appear slightly degraded (smeared, indistinct bands) compared to the same RNA sample isolated by RNeasy (Figure 2-2). RNeasy spin columns appear unsuitable for large (~500 μl) volumes of blood and supernatant, as evidenced by the absence of bands for RNA from the blood of adult sockeye WR354 (Figure 2-2). Lanes 1 and 2 (Figure 2-2) are an example of degraded RNA from a frozen and thawed fish specimen.

RNA purity: Absorbance ratios measured by ultra violet (UV)spectrophotometry

RNA isolated from sockeye salmon smolt blood

The RNeasy isolation method had an average 260/280 absorbance ratio that was estimated at 2.12, which was 0.29-0.41 units higher than the Trizol method (Table 2-1). This difference was highly significant using Welch's t-test (Figure 2-3). One-hundred percent of RNA samples isolated using the RNeasy method were above a 260/280 ratio of 2 while no Trizol samples achieved this benchmark, and approximately 40% more RNeasy RNA samples were above 1.8 than those isolated using the Trizol method (Table 2-2, Figure 2-3). These differences in proportions were statistically significant (Table 2-2; Fisher's Exact Test).

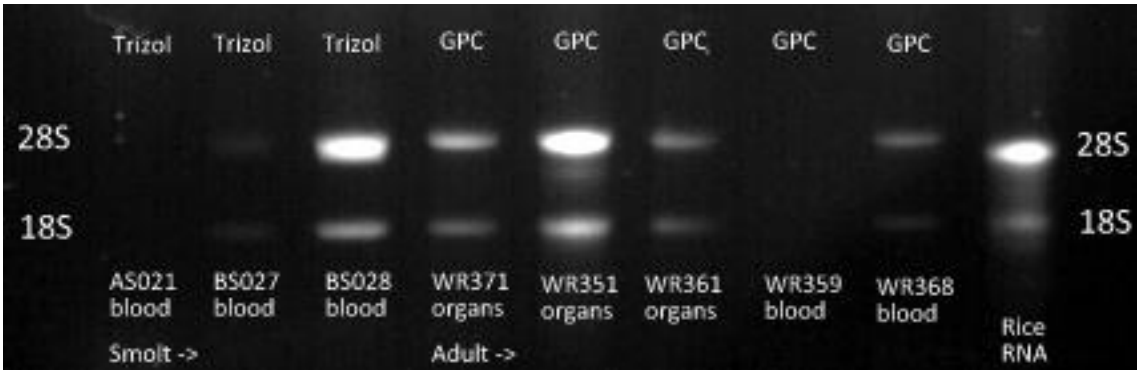

Figure 2-1. Visualization of ribosomal RNA (rRNA) integrity on a bleach 2% agarose denaturing gel. Lanes loaded with 1μg total RNA from sockeye salmon smolt blood and adult sockeye pooled organs and blood isolated using either the Trizol or GPC method. Upper markers denote 28S ribosomal RNA, while lower bands denote 18S RNA. The final column is reference RNA from rice with 26S and 18S rRNA bands.

Figure 2-2. Visualization of rRNA integrity on a formaldehyde denaturing 2% agarose gel. Lanes are loaded with 1 μg total RNA from adult salmonid organs or adult sockeye salmon blood isolated using either the RNeasy spin column or Trizol method.

Table 2-1. Summary of results of Welch's two sample t-tests comparing average ratios of absorbance at 260 and 280 nm for RNA isolated by three methods from sockeye smolt blood, sockeye adult blood, and salmonid organ pools.

Tissue	Contrast	Estimates of mean 260/280 ratio	95% CI of difference in means	t	df	*p*
Smolt blood	RNeasy, Trizol	2.12, 1.81	0.25-0.37	10.48	27.61	3.951e-11
Adult blood	GPC, Trizol	1.97, 1.98	-0.23-0.21	-0.08	34.95	0.9364
Adult organs	GPC, Trizol	2.06, 2.06	-0.04-0.03	-0.38	58.10	0.7012

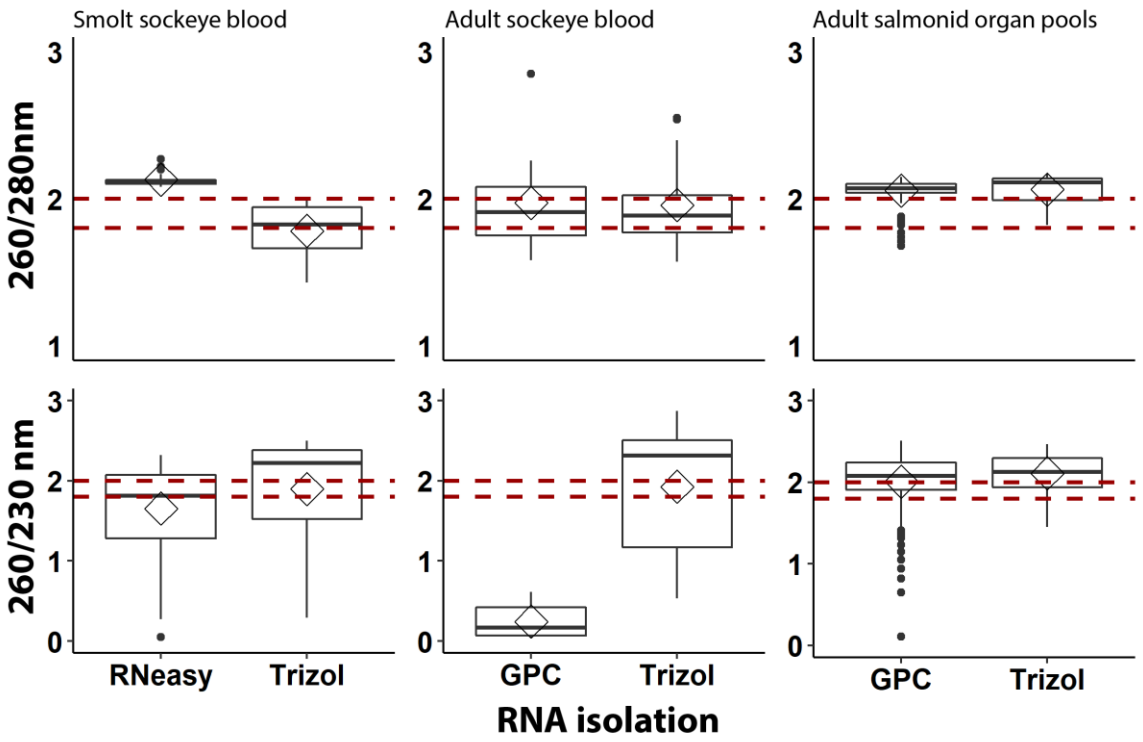

Figure 2-3. Distribution of values for 260/280 nm and 260/230 nm absorbance ratios measuring RNA purity. Boxes represent the median and interquartile range of values and whiskers extend to the most extreme data point within one interquartile range of the median. Diamonds indicate the mean value. Horizontal dashed lines are purity thresholds of 1.8 and 2.0. For the far-left column sample sizes are 29 and 32, respectively. The center column has sample sizes of 17 and 21, and the far-right column has samples of 174 and 45.

The 260/230 is also expected to be close to 2.0 if the sample contains only RNA (Wilfinger, Mackey, & Chomcynski, 1997). The Trizol method resulted in an average 260/230 close to 2.0 whereas the average of RNeasy RNA samples was below 2.0 (Figure 2-3). However, this difference in averages was not statistically significant (Table 2-3). In addition, the proportion of samples with an 260/230 ratio ≥ 2 did not differ, with nearly 40% of RNeasy RNA and 60% of Trizol samples at or above this reading (Fig. 2-3, Table 2-2). Reducing the requirement to ratios above 1.8 increases the proportion of RNeasy-isolated samples at or above the threshold value to 52% and Trizol-isolated samples to 70% (Table 2-1). This difference is again not statistically significant.

RNA isolated from adult sockeye salmon blood

RNA samples isolated using GPC and Trizol had very similar average 260/280 absorbance ratios close to the ideal ratio of 2.0 (Table 2-1; Figure 2-3). Likewise, there

was no difference in the proportion of samples with an 260/280 ratio at or above 2 with both yielding approximately 40% of samples above this threshold (Table 2-1). If the threshold for 260/280 was lowered to 1.8, 71% of GPC-isolated RNA and 62% of Trizol isolated RNA samples were above this value. This difference was not statistically significant (Table 2-2).

For purity measured by 260/230 absorbance, however, Trizol-extracted RNA had an average ratio ten times higher than that of GPC-extracted RNA (Table 2-3; Figure 2-3). This difference in average ratios was highly significant (Table 2-3). Further, the Trizol isolation method is more likely to produce RNA samples with 260/230 values at or above 2.0 than the GPC method, yielding 60% of samples above this threshold. None of the GPC-produced samples achieved this threshold (Table 2-2). Relaxing the requirement of this measurement to greater than or equal to 1.8 produced similar results, with 70% of Trizol-isolated RNA samples at or above this threshold while no GPC-produced samples meet or exceed it (Table 2-2).

RNA isolated from salmonid organ pools

Both the GPC and Trizol methods produced RNA from salmonid organ pools with high average 260/280 ratios that were not statistically different at $p = 0.05$ (Table 2-1; Figure 2-3). There was a significant difference in the proportion of samples with 260/280 ratios ≥2, however, with 90% of the GPC-produced RNA samples and 73% of the Trizol-produced samples at or above this level (Table 2-2). If the threshold was lowered to 1.8, no significant difference existed between the proportion of samples produced by either method at or above this level (Table 2-2).

Both methods also produced high average 260/230 ratios, with a marginal difference in the means when compared with Welch's t-test (Table 2-3; Figure 2-3). No differences were found between the proportions of RNA samples with 260/230 ratios measuring above 2 or above 1.8 (Table 2-2).

Table 2-2. Proportions ± 95% confidence intervals of 260/280 and 260/230 ratios above 1.8 and above 2.0 for salmon blood and organ RNA extracted by the RNeasy, Trizol and GPC methods. Samples sizes in brackets. Estimates calculated using the Jeffrey's Interval. Asterisks denote significant differences detected with Fisher's Exact Test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	Proportion of RNA samples with ratio above 2.0		Proportion of RNA samples with ratio above 1.8	
RNA from sockeye salmon smolt blood				
	RNeasy (29)	**Trizol (32)**	**RNeasy (29)**	**Trizol (32)**
260/280	1.00 (0.92-1.00) ***	0.00 (0.00-0.07) ***	1.00 (0.92-1.00) ***	0.56 (0.39-0.72) ***
260/230	0.34 (0.19-0.53)	0.52 (0.34-0.69)	0.59 (0.42-0.75)	0.69 (0.52-0.83)
RNA from sockeye salmon adult blood				
	GPC (17)	**Trizol (21)**	**GPC (17)**	**Trizol (21)**
260/280	0.41 (0.21-0.64)	0.38 (0.20-0.59)	0.71 (0.47-0.88)	0.62 (0.41-0.80)
260/230	0.00 (0.00-0.14) ***	0.62 (0.41-0.80) ***	0.00 (0.00-0.14) ***	0.67 (0.45-0.84) ***
RNA from adult salmonid organs				
	GPC (174)	**Trizol (45)**	**GPC (174)**	**Trizol (45)**
260/280	0.90 (0.84-0.94) **	0.73 (0.59-0.85) **	0.97 (0.94-0.99)	1.00 (0.95-1.00)
260/230	0.63 (0.55-0.70)	0.69 (0.55-0.81)	0.84 (0.79-0.89)	0.82 (0.69-0.91)

Table 2-3. Summary of results of Welch's two sample t-tests comparing average ratios of absorbance at 260 and 230 nm for RNA isolated by three methods from sockeye smolt blood, sockeye adult blood, and salmonid organ pools.

Tissue	Contrast	Estimates of mean 260/230 ratio	95% CI of difference in means	t	df	*p*
Smolt blood	RNeasy, Trizol	1.65, 1.90	-0.56-0.07	-1.56	58.99	0.124
Adult blood	GPC, Trizol	0.24, 2.00	-2.23- -1.3	-7.77	22.99	1.31e-07
Adult organs	GPC, Trizol	2.01, 2.11	-0.19- -0.01	-2.192	87.7	0.031

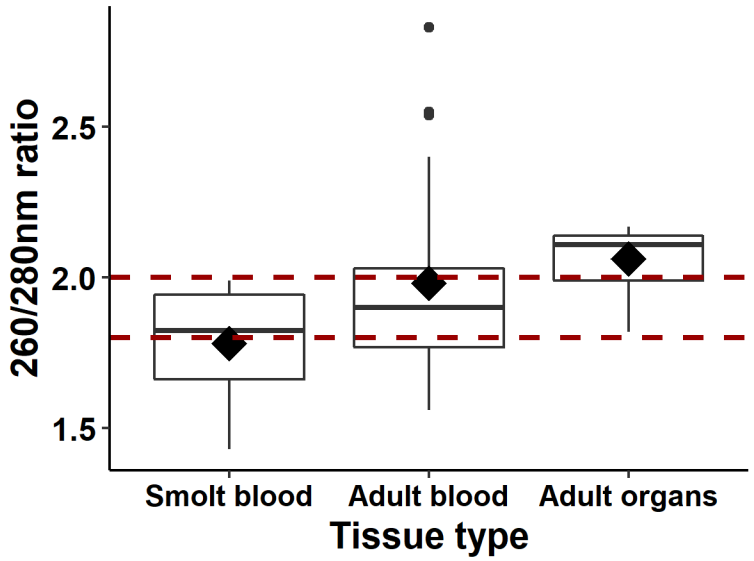

Figure 2-4. Purity measured by the ratio of absorbance at 260 and 280 nm for RNA from three salmonid tissue types isolated by Trizol reagent. Samples sizes are 32, 22, and 45, respectively. Boxplots represent mean and interquartile range while whiskers extend to the most extreme value within one interquartile range. Dots are outliers. Diamons represent mean values. Horizontal dashed lines show purity thresholds at 1.8 and 2.0.

Table 2-4. Results of one-way ANOVA testing the effect of tissue type on the mean 260/280 nm purity ratio of RNA isolated by Trizol.

Factor	DF	Sum of Square	Residual Sum of Squares	AIC	F - value	*p*
			3.86	-310.95		
Tissue type	2	1.5	5.36	-282.72	18.5	1.64×10^{-7}

Table 2-5. Tukey HSD multiple comparisons of mean 260/280 purity of RNA from three salmonid tissues isolated by Trizol reagent.

Contrast between tissue types	Difference in means	95% CI of the difference	Adj *p*-value
Juvenile blood - adult blood	-0.20	-0.34 - 0.07	0.0018
Adult organs - adult blood	0.08	-0.05 - 0.21	0.284
Adult organs - juvenile blood	0.28	0.17 - 0.39	1.0x10-7

Purity of RNA isolated by Trizol across three tissues

The Trizol method was used for each of the three tissue types, and RNA quality as measured by the 260/280 nm ratio differed across tissues in both the proportion of RNA samples above purity thresholds of 1.8 and 2.0 (Figure 2-4) and the mean values of these ratios for each tissue as determined by one-way ANOVA (Table 2-4; Table 2-5). True differences exist between the mean ratios of RNA samples extracted from smolt and adult blood, and between RNA from smolt blood and adult organ pools (Table 2-5). The proportions of smolt blood, adult blood and adult organ RNA samples above the acceptable purity threshold of 2.0 for this metric are 0, 36 and 73%, respectively. After adjusting for multiple comparisons with Fisher's Exact Test, the proportions of RNA samples above 2.0 from sockeye smolt blood and adult salmonid organ pools are found to be significantly different ($p = 4.7 \times 10^{-12}$).

Total RNA yield

RNA isolated from sockeye salmon smolt blood

On average, the Trizol isolation method produced approximately three times higher yields of total RNA from sockeye smolt blood per μl input tissue than the RNeasy method (Table 2-6; Figure 2-5). This difference was found to be statistically significant (Table 2-6).

RNA isolated from adult sockeye salmon blood

Neither the GPC nor the Trizol method produced a higher yield of total RNA than the other (Table 2-6; Figure 2-5). Interestingly, yields of total RNA isolated with Trizol from adult sockeye blood were only about a fifth of that produced from smaller starting volumes of sockeye smolt blood (Table 2-6).

RNA isolated from salmonid organ pools

Adult salmonid organ pools yielded nearly 30 times more RNA than blood samples (Table 2-6). On average the GPC method produced approximately 0.7 μg RNA per mg tissue more than the Trizol method (Table 2-6; Figure 2-5).

Table 2-6. Comparisons of total RNA yields from various fish tissues using different RNA isolation methods. Yield is reported as total RNA in μg per μl (blood) or mg (pooled tissue) of input tissue. Samples with an estimated concentration of ≤ 0.05 ng/μl were excluded from calculations.

Tissue	Contrast	Mean yield	95% CI of difference in means	t	df	*p*
Smolt blood	RNeasy, Trizol	0.12, 0.31	-0.30-0.08	-3.37	40.11	0.001
Adult blood	GPC, Trizol	0.065, 0.058	-0.028-0.042	0.39	33.56	0.6978
Adult organs	GPC, Trizol	1.93, 1.23	0.36-1.04	4.10	103.22	8.182e-05

Gene expression of RNA isolated from smolt blood by spin columns and Trizol

β-Actin expression was significantly different between the two RNA isolation methods used with sockeye smolt blood. Samples isolated with Trizol reagent had an average β-actin cycle threshold (Ct) value of 18.7 ± 0.86 (95% CI) while RNeasy isolated samples on average had a lower Ct value of 17.02 ± 0.3 (Figure 2-6). A two-way ANOVA revealed no effect of the capture location of smolts, and the model was re-run with isolation method as the only factor. RNA isolation method or a correlate was found to have a significant effect on the threshold cycle (Ct) of β-Actin (F = 11.39, df=1,27, P=0.0023; Figure 2-6).

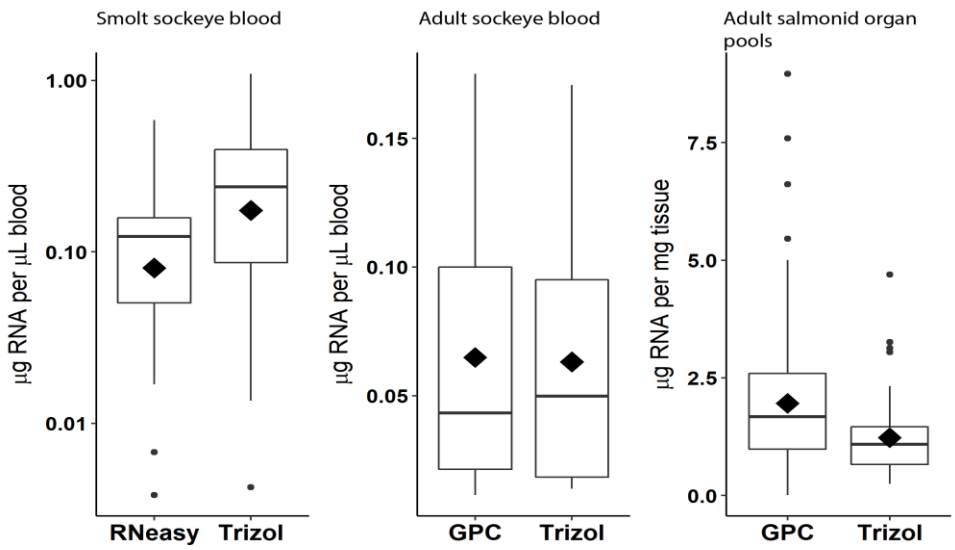

Figure 2-5. Total RNA yield in µg/µl from sockeye smolt blood, adult sockeye blood, and in µg/mg from adult salmonid organ pools produced by three RNA isolation protocols. Boxplots show the distribution of total RNA yield by isolation method. Boxes show the median and interquartile range and whiskers extend to the most extreme data point within one interquartile range of the median. Outliers beyond this are depicted by points. Diamonds show the mean value. The y-axis of the far-left panel has been $\log_{10}$-transformed to better display values. Left panel: n = 29, 32 ; center: n = 17, 21; right: n = 144, 45.

Method variability

The means and coefficients of variation were computed for the 260/230 purity measurements for each RNA isolation method within a tissue type (Table 2-7). For RNA isolated from sockeye smolt blood both RNeasy spin columns and Trizol produced similar amounts of variation about their mean ratio values indicating similar learning curves for both methods (Table 2-7).

RNA isolated from adult sockeye blood, however, shows that the GPC isolation method had a coefficient of variation twice as large as that for the Trizol isolation method, suggesting users were more inconsistent.

Similarly, RNA isolated from adult sockeye and trout tissues shows that the GPC method produced RNA with 3 times the variation in 260/230 purity results than the Trizol method. The higher 260/230 ratio and lower coefficient of variation for Trizol suggests

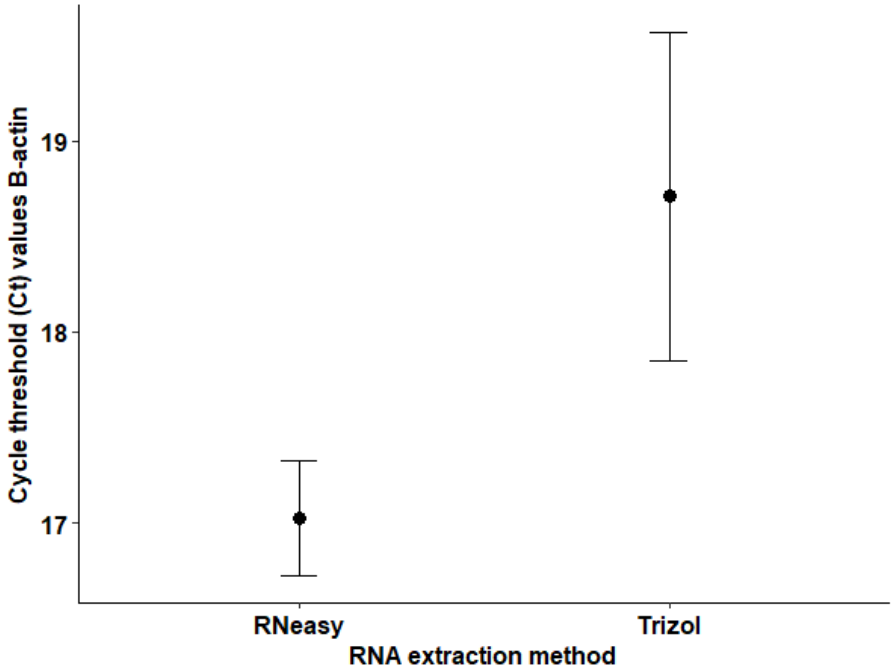

Figure 2-6. Average cycle threshold values (Ct) for β-actin expression in RNA isolated from sockeye salmon smolt blood using RNeasy spin columns (n = 12) and Trizol organic solvent extraction (n = 17). Ct values are inversely proportional to the amount of starting template. Whiskers represent 95% confidence intervals of the mean.

that this method consistently produced high quality RNA with little reagent carryover despite multiple users (Table 2-7).

Method scalability

Tissue input amounts

The upper threshold on the input amount of tissue for the three isolation methods examined varies. RNeasy Micro spin columns can individually process up to 5 mg of homogenized tissues (Qiagen, 2014). Both Trizol and the GPC organic-solvent extraction methods can handle larger quantities of tissue, between 50-100 mg per 1 ml Trizol or GPC lysis buffer (Chomczynski & Sacchi, 2012; Invitrogen 2016). These latter methods can also be scaled to accommodate larger input amounts of tissue by increasing the size of the plasticware and the amount of lysis buffer.

Table 2-7. User skill comparison. Means and coefficients of variation for 260/230 nm absorbance ratio measurements of RNA samples by RNA isolation method and tissue type.

Tissue type	RNA extraction method	Mean 260/230 absorbance ratio	Coefficient of Variation
Sockeye smolt blood	RNeasy	1.65	34.77
	Trizol	1.90	33.89
Adult sockeye blood	GPC	0.21	100.07
	Trizol	1.93	53.21
Adult salmon organ tissues	GPC	1.98	21.13
	Trizol	2.25	7.71

Automation potential

Of the three methods only the Qiagen RNeasy Micro RNA isolation kit can be automated through Qiagen's proprietary QIAcube® technology, allowing up to 12 spin columns to be processed simultaneously in low-throughput automation (Qiagen 2012). Both Trizol and the GPC method require manual processing of samples (Chomczynski & Sacchi, 2006; Invitrogen 2016).

Isolation method cost and time per unit

A trade off exists between cost and time for RNA extraction methods. RNeasy Micro spin column kits have the highest cost per single sample preparation at $7.17 (USD), based on the purchase price of a kit ($318.04 per 50 preparations, plus $40.51 for additional reagents (if outfitting a laboratory without prior resources). However, at 35 minutes to complete the protocol, RNeasy is also by far the fastest method. If 8 spin columns are processed simultaneously, this method yields a time per single sample preparation of 4.5 minutes (Figure 2-7). Trizol is the second most expensive and time-efficient method at $2.09 – $2.97 per sample preparation, based on 100-142 preps per 100 ml bottle of Trizol reagent ($297.16 for Trizol and additional required reagents), and 12.5 minutes per sample preparation, if 8 are processed simultaneously (Figure 2-7). The GPC method is by far the least expensive isolation method but also the most time consuming. One 250 g quantity of the limiting reagent, guanidinium thiocyanate ($468.44 for 250 g and all additional required reagents), can produce enough buffer for approximately 680 sample preparations, or $0.69 per sample. However, this method is also the most labour intensive, requiring approximately 230 minutes minimum per 1-8 samples, or 28.75-230 minutes per sample (Figure 2-7).

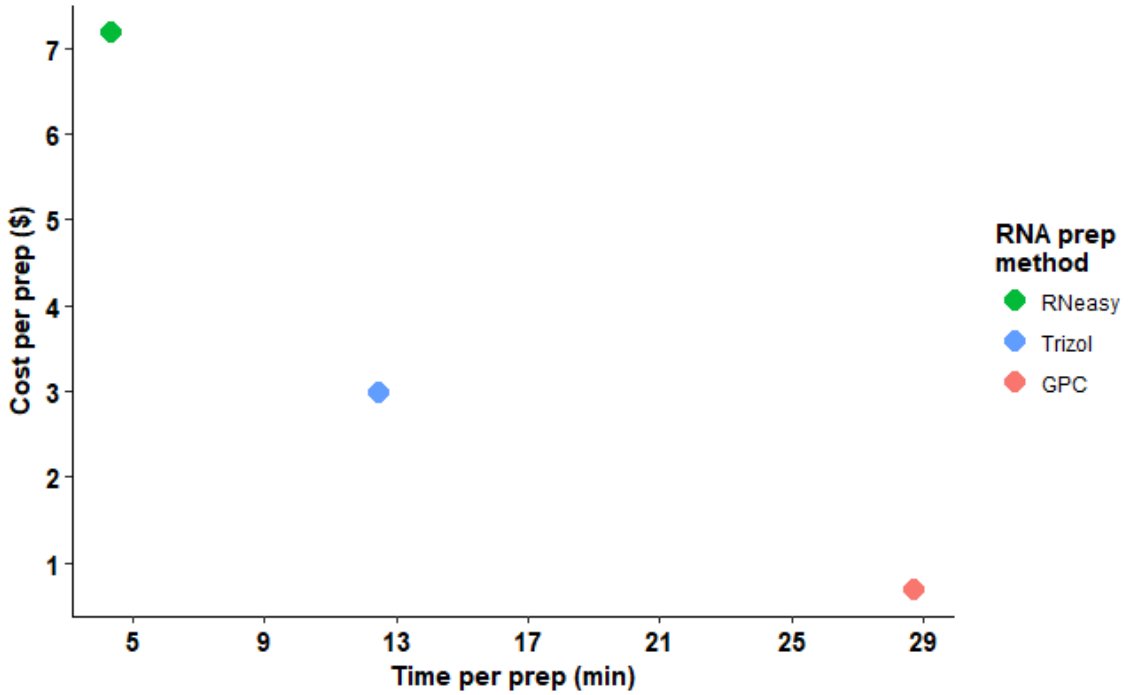

Figure 2-7. Average time to isolate RNA from salmon tissue versus the estimated cost per preparation (USD) for three RNA isolation procedures.

User risk: toxicity of reagents and exposure time

RNA binding spin columns limit user exposure to toxic elements because of the reduced time for processing and the absence of highly volatile phenol or chloroform reagents. However, Qiagen kit supplied Buffer RLT contains guanidinium thiocyanate, a category 4 toxin, category 1C irritant, and category 3 aquatic toxin (Qiagen, 2018a). Buffer RW1 also contains guanidinium thiocyanate and ethanol, a fire hazard (Qiagen, 2018b).

The GPC method contains, as expected, guanidinium thiocyanate, phenol and chloroform, as well as β-mercaptoethanol, glacial acetic acid, and sodium dodecylsulphate (SDS) (Chomczynski & Sacchi, 2012). As noted above, guanidinium thiocyanate is a skin and eye irritant with acute toxic effects if swallowed. Phenol is a category 3 acute toxin, a category 1B irritant, a category 2 mutagen and category 2 repeated exposure toxin, as well as a category 3 aquatic environmental hazard (Global Safety Management Inc, 2012). Due to its volatility it must be handled in a fume hood while wearing personal protective equipment. Chloroform is a category 4 acute oral

toxin, a category 3 acute inhalant toxin, a category 2 skin irritant, a category 2A eye irritant, a category 2 carcinogen, a category 2 reproductive toxin, and a category 1 repeated exposure toxin (Millipore Sigma, 2017). Again, this reagent requires personal protective equipment and must be handled in a fume hood. Similarly, β-mercaptoethanol, an acute toxin and irritant, and glacial acetic acid, an acute irritant and mutagen, must be handled in a fume hood due to their high volatility (Chomczynski & Sacchi, 2006). SDS is a potent eye and respiratory irritant and the powder must be handled with personal protective equipment, especially eyewear and mask to prevent inhalation (BioRad, 2015). Because the GPC method requires the assembly of all solutions prior to isolation, the user is in contact with these reagents for a prolonged period compared to the other methods. The isolation procedure itself is also lengthy thus putting the user in contact with reagents for a prolonged period (Chomczynski & Sacchi, 2006).

Trizol is a proprietary blend of acid-phenol, guanidinium isothiocyanate, and ammonium isothiocyanate, an acute toxin and irritant with a similar hazard level to guanidinium thiocyanate (Life Technologies, 2017). The Trizol procedure also requires the use of chloroform, isopropanol, and ethanol, like the GPC procedure (Chomczynski, 1993) and must be performed in a fume hood while wearing personal protective equipment. However, because Trizol comes as a premixed solution, and due to reduced processing time in comparison to GPC isolation, the Trizol method exposes users to acutely toxic substances for a shorter period than the GPC method (Chomczynski, 1993; Chomczynski & Sacchi, 2006; Life Technologies, 2017).

2.4. Discussion

Extraction of RNA is the starting place for many other molecular biology based-inquiries. In this case, RNA was isolated for later use in the screening of wild-caught fish tissues for the presence of piscine orthoreovirus, as well as gene expression, quantified by RT-qPCR. Broadly, no one method proved to be ideal for isolating RNA from sockeye salmon blood from juveniles and adults, as well as from adult salmonid organ pools. Each method produced varying results for both purity measurements and yield depending on tissue type (Figure 2-3; Figure 2-4). Across the other metrics evaluated, the GPC and spin column RNA isolation methods are opposing extremes of cost and time investment, as well as risk due to toxic exposure. Trizol, meanwhile, was a midpoint

for these three metrics, and additionally, had the lowest coefficients of variation in terms of the 260/230 nm purity measurement across all sample types indicating users quickly learned the protocol and were most consistent in its use (Table 2-7). All three methods could be scaled up to handle higher throughput sample processing either by increasing tissue input amounts (GPC and Trizol) or by automating the procedure (spin columns).

2.4.1. RNA isolation from sockeye smolt blood samples

In general, the spin column method consistently produced RNA with fewer impurities, such a proteins and phenol, than the Trizol method as measured by absorbance at 260 and 280 nm. This method had both a higher average ratio (Table 2-1) and a significantly greater proportion of samples above 2.0 (Table 2-2). No difference existed between these methods for the average absorbance ratio at 260/230 nm (Table 2-3) or the proportion of samples above 1.8 or 2.0 (Table 2-2), suggesting both methods had similarly low levels of contaminants absorbing at 230 nm. An advantage of the Trizol method with smolt blood, was its production of approximately three times more RNA per sample than the spin column methods (Table 2-6). This is a valuable characteristic when working with irreplaceable samples with limited opportunity for re-extraction of RNA, such as with limited field collected materials. One might opt in this case, to use a method like Trizol that will isolate a greater amount of RNA, and then treat it with clean up procedures, such as those described below, to improve purity. In terms of user skill, both Trizol and the spin column method had a similarly large coefficients of variation (Table 2-7), indicating that possibly working with very small volumes (≤ 70 μl) of smolt blood may be challenging at first, regardless of method. Ultimately, the downstream goals for the RNA dictate the choice method – if purity is prioritized then the spin column method provides a fast solution, but if yield is more important because of the rarity of the sample material, Trizol may be the method of choice, despite the requirement for post-processing to remove impurities.

2.4.2. RNA isolation from adult sockeye blood samples

Isolating RNA from adult sockeye blood was challenging, and neither the Trizol nor the GPC methods performed universally well. Spin columns were not evaluated with adult whole blood when a pilot investigation demonstrated they quickly became clogged (see Figure 2-2). Both GPC and Trizol produced RNA with acceptable average 260/280

absorbance ratios (Table 2-1), and both methods produced more than half of all samples with ratios above 1.8. With the 260/230 absorbance ratio, however, the Trizol method outperformed the GPC method (Table 2-2 & 2-3). This indicates substantial contamination with either extraction reagents (either phenol or guanidinium) or polysaccharides in the GPC-isolated samples – perhaps because the longer isolation procedure created more opportunity for user error. This is reinforced by the finding that the coefficient for variation of the 260/230 ratio was twice as high for the GPC as for Trizol method (Table 2-7). Relative to Trizol, the GPC protocol clearly requires more of a time investment in user practice.

Neither method produced substantial yields of RNA (Table 2-6) despite the fact that red blood cells are nucleated in fishes (Finstad et al., 2014). It is interesting to note that RNA yields were five times lower with adult blood than juvenile sockeye blood – even when restricting comparison to samples isolated using the Trizol method (Table 2-6). This may indicate that adult fish blood has less RNA than juvenile fish blood, and relatively higher protein and polysaccharide content. This has not been specifically documented, but is plausible as other authors have found juvenile fish produce more RNA in tissues such as white muscle than adults (Peragón et al., 2001). Encountering similar challenges with nucleated avian blood, Mewis et al. (2014) suggest a modified protocol with a 3:1 ratio of Trizol to fresh whole blood followed by a spin column clean up protocol. This resulted in RNA of high integrity with an average RNA integrity number (RIN) of 7.75 – and 26 ng/μl (Mewis et al., 2014). Unfortunately, the procedure described does not allow an evaluation of contaminant presence. In future, this protocol should be examined with salmonid blood, although its drawbacks involve the expense of using two isolation methods rather than one, and RNA yield may be reduced slightly, as was seen with the application of spin columns to sockeye smolt blood. From this analysis, the Trizol isolation method appears the most appropriate choice for adult salmonid blood due to the lower amount of potential PCR-inhibiting impurities absorbing at 230 nm and the more consistent user results.

2.4.3. RNA isolation from adult salmonid organ pools

Consistently higher-quality RNA was obtained from organ pools than blood samples using the same methods. With adult organ pools, there was no difference between RNA isolated with the Trizol and GPC in the average amount of contaminants

absorbing at 280 nm (Table 2-2). The GPC method outperformed Trizol, however, with 20% more samples at or above a strict threshold of 2.0 for the 260/280 absorbance ratio. This difference was lost when the requirement was relaxed to 1.8 (Table 2-2). Similarly, only a marginal difference existed in the average 260/230 absorbance ratio, with Trizol-isolated RNA 0.11 units higher than GPC (Table 2-3) and no difference existed in the proportion of samples at or above an acceptable 260/230 absorbance ratio (Table 2-2). However, in terms of RNA yield, the GPC method produced 0.7 μg more RNA per mg tissue than the Trizol method (Figure 2-5; Table 2-6). This amount, though small, is not trivial – for every two milligrams of tissue the GPC method could extract enough RNA for another potential cDNA synthesis. This increased yield may result from the lengthy isopropanol precipitations of the GPC protocol (Chomczynski & Sacchi, 2006). This supposition could be tested by adding precipitations of similar length to the Trizol method. This may be a beneficial compromise between procedures as Trizol clearly outperformed the GPC protocol in the repeatability of the 260/230 absorbance ratio (Table 2-7). As with RNA from adult sockeye blood samples, the Trizol method was only a third as variable in this metric as the GPC method.

Given the similarity of the results for GPC- and Trizol-produced-RNA from pooled organs, other metrics, such as cost, time, or toxicity, will guide user choice. Prioritizing cost, GPC is one third as expensive as Trizol per sample (Figure 2-7) but requires twice as long to process a sample and entails more setup, thus exposing users to toxic reagents for longer. Ultimately, Trizol produces purer, less variable RNA more quickly from pooled salmonid organs, while the GPC method produces greater RNA yields at a reduced cost.

2.4.4. Purity of Trizol-produced RNA across all tissues

The Trizol procedure did not produce RNA of similar quality across all three tissue types evaluated (Figure 2-4; Table 2-5). This was evaluated for the 260/280 nm purity ratio, which indicates contamination with protein and phenol. Results show that average 260/280 ratio results for smolt blood were 0.3 units lower than those for adult organ pools (Table 2-5) and no smolt blood samples isolated by Trizol were above a purity ratio of 2.0. This result underscores the need in experiments using more than one tissue type, to fine-tune isolation methods to produce RNA of comparable quality for unbiased contrasts.

2.4.5. Evaluation of downstream applications of RNA: RTqPCR housekeeping gene expression

The expression of the housekeeping gene, β-actin, demonstrated a significant difference in cycle threshold (Ct) values for samples initially isolated with Trizol versus spin columns. Spin column isolated samples produced lower Ct values indicating a greater detectable initial number of β-actin mRNA transcripts. This result suggests that impurities like phenol carryover in Trizol-isolated RNA, indicated by low 260/280 ratios, may have inhibited enzymatic activities during cDNA synthesis or RT-qPCR. Alternatively, this could indicate the presence of residual proteins, such as nucleases, which may have degraded RNA produced with the Trizol method, reducing detectable copies of β-actin. This could imply that the varying chemical background of different RNA isolation methods has led to results which many not be directly comparable with RT-qPCR (i.e., Meyer et al., 2016). If one needs to compare a target – either a pathogen or the expression of a gene of interest – between different tissue types my investigation demonstrates that impurities inhibiting downstream comparisons may result from choosing an inappropriate RNA isolation technique for a given tissue. A fine-tuned approach to RNA isolation for each tissue to obtain optimal RNA yield and quality, should allow direct comparison. Analytical approaches also exist which compensate for differing chemical backgrounds. For example, relative gene expression – the expression of a gene of interest normalized to the expression of a constitutively expressed housekeeping gene (Schmittgen & Livak, 2008) – may be used. One would expect the target and housekeeping genes within a sample to be systematically affected by their chemical background in the same way, thereby preserving their relative ratios and allowing comparison of the up or downregulation of a gene between tissues subject to different isolation methods. RT-qPCR detection of pathogens, and the estimation of viral load, does not usually rely upon relative quantification, however. In this case, precise and accurate quantification of RNA of comparable purity is needed for direct comparison of metrics such as viral copy number per microgram total RNA. To more fully explore this issue, one could artificially introduce a known quantity of target pathogen into a variety of commonly screened tissues and more specifically evaluation the effect of isolation method on the detectable amount of pathogen by tissue type.

My housekeeping gene expression comparison of Trizol and spin column produced RNA from smolt blood demonstrates the effects of a low 260/280 purity ratio

on downstream uses of RNA but does not preclude the comparison of samples produced using different RNA isolation methods. Rather, it cautions against comparisons between RNA of differing quality.

2.4.6. Recovery of RNA with low purity measurements

It is more difficult to optimize RNA extraction from some tissue types than others, i.e., blood from adult salmonids. Fortunately, additional options exist for the handling or improvement of RNA with poor 260/280 or 260/230 absorbance ratios.

Improving low 260/280 nm absorbance ratios

If protein contamination is likely, samples are most simply handled by constraining potentially problematic enzymatic activity with cold temperatures (Maniatis et al., 1982). Efficient work with samples on ice can stave off degradation without directly addressing contamination. However, this only slows degradation and samples stored for long periods are likely to degrade, even at colder temperatures. Converting RNA into more stable cDNA is also an option for longer term integrity of samples with a low 260/280 ratio. Additionally, RNA samples may be treated with a proteinase digestion procedure to denature harmful RNases (Maniatis et al., 1982).

Removal of carryover reagents such as phenol will also improve the 260/280 absorbance ratio. Blended protocols, e.g., the addition of a spin column-based clean-up procedure following an initial organic solvent-based RNA extraction are sometimes used for this purpose (as in Finstad et al. 2014; Mewis et al., 2014). This procedure pairs the thorough lysis of difficult tissue types by Trizol with the RNA-specific adhesion of spin columns. A disadvantage of this approach is that it increases both the material costs and the time required for RNA extraction. Alternatively, extraction using 1-butanol has been shown to improve 260/280 and 260/230 absorbance ratios, as well as RNA integrity (Krebs, Fischaleck, & Blum, 2009). Chloroform, already part of the published Trizol and GPC protocols (Chomczynski, 1993; Chomczynski & Sacchi, 2006b), may also be used for a second extraction to remove trace phenol (Kirby, 1956). Both chloroform and butanol are less soluble in water than phenol and more easily separated.

Improving low 260/230 nm absorbance ratios

If RNA samples are suspected of containing contaminating chaotropic salts (e.g., guanidinium thiocyanate) or possibly polysaccharides, monovalent cations like sodium acetate can be used to selectively precipitate RNA leaving contaminants in solution (Walker & Lorsch, 2013). These precipitations involve cold 70% ethanol and are lengthy, taking longer than an hour, however they have been found to improve 260/230 ratios from 1.4 to closer to 2.4 in this work. They have the disadvantage of slight reductions in RNA yield, however, and may also reduce the 260/280 ratio slightly, but only if impurities skewed light absorbance leading to a falsely high ratio.

2.4.7. Conclusions

I have found that no single RNA isolation protocol I evaluated is able to produce RNA of ideal purity and quantity for juvenile and adult sockeye blood, and salmonid organ pools. A study must ultimately make choices about RNA isolation technique based on the nature of inquiry, i.e., what tissues are being used and what comparisons are being made, as well as the time and resources of the laboratory. In this study, I found that conceding certain compromises in quality, the Trizol extraction method consistently produced high yields of RNA and had a low coefficient of variation in terms of purity measured by absorbance at 260 and 230 nm. It also sits at a mid-point for cost and time investment, as well as toxic exposure concerns for the user. If RNA isolation from tissues is individually optimized, however, spin columns produced the most consistently high-quality RNA from small volumes of sockeye salmon smolt blood, while Trizol-extracted RNA from adult sockeye blood had consistently lower contaminant carryover than RNA isolated by the GPC method. However, the GPC method produced higher yields of RNA and a greater proportion of high purity samples from pooled organs. I also show that suboptimal isolation approaches affect RNA purity, and downstream molecular results from RNA. For this reason, care should be taken in comparing results of analyses based on RNA isolated using different methods, and supplementary materials with transparency regarding RNA quality and quantity would be helpful in making safer contrasts (Bustin et al., 2009).

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Chapter 3.

Interannual dynamics and host range of piscine orthoreovirus (PRV) in the wild salmonid community of Rivers Inlet

Abstract

Many Pacific salmon (*Oncorhynchus* sp.) populations are declining due to the action of multiple stressors including disease. Piscine orthoreovirus (PRV) is ubiquitous among farmed salmon and widespread in wild Pacific salmon populations but its host range and infection dynamics in natural systems are poorly understood. I carried out three years of viral surveillance in Rivers Inlet, BC using RT-qPCR to document PRV infections among six fish species including three life-stages of sockeye salmon (*O. nerka*). I found a 3% overall prevalence of PRV in fish samples, and the first evidence of PRV in Dolly Varden (*Salvelinus malma*) and eulachon (*Thaleichthys pacificus*). Among sockeye, the proportion of PRV-positive fish declined by ~4% from fry to smolts in the 2014 and 2015 cohorts. The highest overall PRV prevalence was observed in 2014 (~10%). This is the first description of PRV dynamics in a wild Pacific salmonid community and suggests directions for future research.

3.1. Introduction

Many once-abundant populations of Pacific salmon (*Oncorhynchus* sp.) are experiencing declines in number or productivity (number of adults per spawner) throughout their ranges, including parts of British Columbia (BC), Canada (Freshwater et al., 2017; Peterman & Dorner, 2012; Riddell et al., 2013; Schindler et al., 2013). As a result, conservation concerns for these populations are escalating with numerous, recent conservation policy recommendations (COSEWIC, 2018; Fisheries and Oceans Canada, 2016). As Pacific salmon are so integral to many ecosystems and current economic activities, there is a need to understand the diverse drivers that could be responsible for these observed declines.

Conservation concerns regarding sockeye salmon (*Oncorhyncus nerka* Walbaum, 1792) populations due to declines in numbers and productivity as well as increased mortality have garnered attention in recent years – exemplified by the response to the failure of the 2009 Fraser River runs (Cohen, 2012; Connors et al., 2012; Freshwater et al., 2017; Peterman & Dorner, 2012; Ruggerone & Connors, 2015). Trends in declining sockeye salmon productivity are proposed to act at a broad spatial scale, and simultaneous declines have been observed since the 1980s across Fraser River populations (Freshwater et al., 2017) and since the 1990s in populations from Yakutat, Alaska, to Puget Sound, Washington (Peterman & Dorner, 2012). Peterman and Dorner (Peterman & Dorner, 2012) suggest that while stressors such as local habitat degradation, contaminants, and predators likely exacerbate problems faced by individual sockeye populations, the shared pattern of declines implicates larger scale stressors. The broad scale effect of infectious disease transmitted between sockeye populations at sea has been proposed as a potential stressor (Peterman & Dorner, 2012) and investigated by several authors (Connors et al., 2012; Kent, 2011; Miller et al., 2011; 2014). Connors et al. (2012) found that patterns of shifts in sockeye salmon production were correlated with ocean productivity, interspecific competition with pink salmon (*O. gorbuscha* Walbaum,1792), and potential exposure to pathogens from Atlantic salmon (*Salmo salar* Linnaeus, 1758) aquaculture, as well as interactions among these factors. Investigating the unusual mortality of Fraser River sockeye salmon, Miller et al. (2011) found a significant correlation between increased mortality and a transcriptomic signature that functional analyses suggested was related to viral infection. Other research has demonstrated a higher odds of predator-induced mortality for juvenile sockeye salmon with increasing microparasite (i.e., virus, bacteria or fungi) loads and diversity, and a higher odds of in-river migration failure for adult sockeye carrying microparasites (Miller et al., 2014). These analyses provide impetus for inquiries into the distribution and consequences of microparasites in Pacific salmon populations, and their capacity to influence survival and productivity trends in populations of concern.

The population of sockeye salmon in Rivers Inlet on the Central Coast of BC (Figure 1-1) has experienced major declines in numbers and productivity since the 1990s (McKinnell et al., 2001; Peterman & Dorner, 2012). Once this system was one of the most productive runs of sockeye salmon in BC, supporting the third-largest fishery for sockeye in the province with catches plus escapement routinely above one million

spawners (McKinnell et al., 2001). By the mid-1990s the run had diminished to only a few thousand spawners, and currently, Fisheries and Oceans Canada estimates returns remain below 200,000 spawners. As suggested in analyses of sockeye salmon population dynamics (Connors et al. 2012), it is possible that this precipitous population decline may be linked to pathogens contracted from aquaculture facilities. Rivers Inlet salmon may encounter aquaculture-based pathogens directly, from aquaculture facilities located near Klemtu, 175 km north on the Central Coast, or, indirectly, by association with other salmon populations, such as those from the Fraser River, which have a much higher exposure to Atlantic salmon aquaculture (Morton et al., 2017). Disease surveillance for salmon viruses of potential concern was attempted for this system in 2013, but insufficient funding prevented the screening of collected tissues (Connors, pers. comm.). Here I present the first description of viral surveillance for the aquaculture-associated piscine orthoreovirus (PRV) in this wild Pacific salmonid community.

While pathogens with high virulence (*i.e.,* those that inflict a high degree of harm upon their hosts) are devastating in high-density aquaculture environments, their virulence makes them less likely to be successfully transmitted in lower density populations of wild Pacific salmon (Miller et al., 2014). Those pathogens of moderate harm which are a chronic nuisance on salmon farms may be of greater concern to wild salmon populations due to their higher transmission probability (Miller et al., 2014). Viral pathogens, in particular, are of concern, as few effective treatments exist for delivery in aquatic systems (Crane & Hyatt, 2011). PRV is one such pathogen (Miller et al., 2014). It is a double-stranded RNA virus in the family Reoviridae (Di Cicco et al., 2018; Kibenge et al., 2013; Palacios et al., 2010), which causes long term, chronic infections in farmed Atlantic and Pacific salmon (Di Cicco et al., 2017, 2018; Garver et al., 2016) and may manifest a disease known as Heart and Skeletal Muscle Inflammation (HSMI) in Atlantic salmon, with approximately 20% mortality during outbreaks (Di Cicco et al., 2017).

PRV is a non-enveloped virus with two capsids bearing icosahedral symmetry (Kibenge et al. 2013). Its double-stranded, linear, RNA genome contains 10 segments of different sizes: large segments L1-L3; medium segments M1-M3; and small segments S1-S4 (Kibenge et al. 2013). Segments L1 and S1 are fundamental to our understanding of PRV. L1 is a conservative sequence coding for a structural core shell protein and is used frequently in assays for viral detection, while S1 codes for a major outer clamp

capsid protein and many published sequences exist for phylogenetic analyses (Siah et al. 2015; Kibenge et al. 2013).

While its origin in BC is currently undetermined, PRV was originally described from Atlantic salmon with HSMI in Norway (Palacios et al., 2010). Recently, this virus has also been shown to cause disease states in farmed Pacific salmon species in Norway, Japan, BC, and Chile (Cartagena et al., 2018; Di Cicco et al., 2018; Godoy et al., 2016; Olsen et al., 2015; Takano et al., 2016) and is found to infect a broad host range including all Pacific salmon and some additional marine fishes (Di Cicco et al., 2018; Kibenge et al., 2013; Morton et al., 2017; Purcell et al., 2018; Wiik-Nielsen et al., 2012, this thesis). Its capacity for long term, chronic infection in farmed salmon makes PRV a candidate pathogen of concern. Additionally, its broad host range may allow this virus to harbour in infection reservoirs, defined here as one or more epidemiologically linked populations that can sustain a viral infection permanently and pass it to a defined target population of interest (Haydon et al., 2002), in this case sockeye salmon.

PRV-caused disease has not yet been demonstrated in wild salmon populations (Di Cicco et al., 2018). However, Garver et al. (2016) experimentally found that PRV of genotype one (PRV-1), found in BC, readily transmits from Atlantic salmon to sockeye salmon. This strain of virus is responsible for outbreaks of both HSMI (Di Cicco et al., 2017) and Jaundice Anemia in farmed Chinook salmon (*O. tshawytscha* Walbaum, 1792) in BC (Di Cicco et al., 2018), demonstrating its pathogenicity. Recent research by Morton et al. (2017) has found that wild salmonid populations sampled above significant spawning migration challenges, *i.e*., Hells Gate Canyon on the Fraser River, had reduced frequencies of PRV infection compared to those sampled below the obstacles. This may imply that wild salmon carrying PRV are less able to navigate these obstacles. Miller et al. (2017) provide additional rationale for investigation of PRV in wild salmon by also speculating that viral levels which would be considered subclinical in farmed salmon may be enough to compromise the swimming performance of wild salmon thereby making them more vulnerable to predators and less able to endure migratory stresses.

Initially gathered wildlife epidemiological data is often sparse and rarely published but is necessary to ultimately construct an accurate understanding of the ecology of wildlife disease (Haydon 2002). Here I examined PRV infection dynamics in sockeye salmon and co-occurring species in Rivers Inlet on the Central Coast of BC to

help address potential knowledge gaps in the ecology of PRV in wild salmon communities. Using data from molecular screening for PRV from 2014-2016 I document the proportion of PRV positive sockeye salmon across life stages, as well as in spawning Chinook salmon and adults of three trout species (rainbow trout (*O. mykiss* Walbaum, 1792), cutthroat trout (*O. clarkii*, Richardson, 1836), and Dolly Varden char (*Salvelinus malma*, Walbaum, 1792)), which were investigated as potential reservoirs of infection, and in spawning eulachon (*Thaleichthys pacificus* Richardson, 1836). Identifying species and life-stage affinities with PRV infection is a valuable first step towards the characterization of competent hosts (i.e., hosts able to support and transmit infections (Johnson, Ostfeld, & Keesing, 2015)) and infection reservoirs (competent hosts that incur little damage due to infection (Dobson, 2004) and are epidemiologically connected to a vulnerable host of interest (Haydon et al., 2002)). This knowledge will ultimately allow for a clearer understanding of both PRV transmission dynamics and host-specific vulnerabilities.

In addition, I examine patterns over time in PRV prevalence from the fry to adult life-stages of sockeye salmon as well as in adult trout. If, as with Infectious Haematopoietic Necrosis Virus (IHNV), PRV targets early life-stages of sockeye and results in mortality or infection clearance, this should be evident from the distribution of virus-positive results across age classes. Furthermore, identification of yearly variation lays a foundation for understanding the biological and environmental variables that influence PRV prevalence. Collectively, this research provides baseline epidemiological data and contributes to a clearer understanding of the geographic and species distribution of PRV and its dynamics in a salmonid community from the Central Coast of BC.

3.2. Methods

3.2.1. Overview

Surveillance for PRV was conducted for six species of fish in Rivers Inlet in 2014-2016: sockeye salmon (2014-2016), Chinook salmon (2014-2015), cutthroat trout, rainbow trout/steelhead (2014-2016), Dolly Varden char (2014-2016), and eulachon (2015). In 2015, an additional sample of trout were also collected in 9 other Central Coast lakes. Molecular viral screening was done at the Atlantic Veterinary College and

in-house by Taqman probe-based reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR). Regular end-point PCR and Sanger sequencing were used to confirm positive tests.

Salmonid sampling did not constitute an extensive, rigorous surveillance of wild salmonids due to the logistical and financial constraints of working in such a remote area. Rather, this was an exploratory study which found evidence of PRV presence in a salmonid community for which no information currently exists. Methods are geared for generally identifying patterns of viral infection among species and life-stages and generating hypotheses that can be addressed through future inquiry.

3.2.2. Ethics statement

All salmon and trout were sampled under federal permits XR_154_2014 and XR_162_2015, provincial permit Hrushowy-NA15-167159 and under collaborative research agreements with the Wuikinuxv First Nation Stewardship and Fisheries Programs and Heiltsuk First Nation Integrated Resource Management Department. Fish were additionally provided for viral screening by Indigenous food fishers and recreational fishermen. Animal Use Field protocols were approved by Simon Fraser University Animal Use Permit 1100S-13.

3.2.3. Field Sampling methods

Several methods were used to collect salmonids of different species and life stages. Methods were adapted to suit life-stages and capture environments.

Sampling for sockeye fry and yearlings in freshwater

Young-of-year (YOY) sockeye salmon fry were sampled in the Wanukv River and Oweekeno Lake (Figure 1-1) using a small beach seine in 2014, a small trawl net in 2015, and fixed Fyke nets with live boxes in 2016. Methods were adapted over time as capturing sockeye fry proved logistically challenging due to the high volume and large amounts of debris on the Wanukv River, as well as the distance up-lake to other natal systems. As no genetic population structure exists within the sockeye population of Oweekeno Lake (Nelson et al., 2003), and all YOY and yearling sockeye mix in the main

lake basin, different capture methods were not thought to target different local populations.

Beach seining occurred in June of 2014 in the Wanukv River. The seine was 10 m long and 1.5 m deep with a stretched mesh of ½" for the outside panels and ¼" in the bunt. Up to 200 fish were collected in a single set. Fish were carefully collected in the bunt end of the net, the total catch estimated, and a sub-sample dipped from the bunt with a small aquarium net. The rest of the catch was released. Due to time constraints in 2014, only three successful sets were completed retaining 21 YOY sockeye fry.

A small trawl net (15 ft long with a 1 m x 1 m net opening) towed behind a 20 ft research vessel on Oweekeno Lake was also tested as a sampling option in June of 2014, catching 5 YOY fry between 3 sets. In 2015, the trawl net was successfully used at night and became the main method of capturing YOY and yearling sockeye in Oweekeno Lake. It was fished after nautical twilight using methodology adapted from Johnson (1956) and Ruggles (1965). Trawling was done in the first basin of Oweekeno Lake where yearling sockeye gather before swimming into tidewater. At the end of a tow, the trawl was manually hauled up vertically to collect any fish in the cod-end. The number of smolt and YOY sockeye were counted and up to 10 fry and 10 smolts were retained from a tow. In total, 48 sets were made between March and June 2015 and 155 YOY and 112 yearling sockeye were retained. Sampling by trawl was also attempted in May 2016, but after 20 unproductive attempts it was abandoned in favour of Fyke net trapping. Fyke nets were deployed in natal streams from March to May of 2016. These nets had a metal framed mouth 0.61 m tall by 1.2 m wide and wings 6 m long and 0.61 m deep. Nets were 5 m long and composed of 1/8" stretched mesh held open by metal hoops 0.61 m in diameter. The bunt end of each net was connected by PVC pipe to a live box, measuring 1.2 m x 0.80 m x 0.61 m. A baffle with ¾" diameter holes provided a refuge for fry from larger, incidentally trapped fishes. Fyke nets were deployed in the afternoon and checked 10-14 hours later, catch was enumerated, and a small sub-sample of sockeye fry retained. Fyke nets were deployed 8 times catching 139 YOY sockeye. Unfortunately, the lack of success in capturing a simultaneous trawled-sample of fry requires a cautious comparison of infection rates of YOY fry from 2014 and 2015 with fry from 2016.

Sockeye smolts Oncorhynchus nerka

Sockeye smolts were collected in Rivers Inlet using a small beach seine and a large purse seine in 2014, 2015 and 2016. Seining for smolts with the large seine was done in June in 2014, and May to June in 2015 and 2016. The large purse seine measured 61 m long by 6.1 m deep, with five panels of ¾", ½", and ¼" stretched mesh in the 'bunt' section. Dimpling schools of fish were targeted. The purse seine was deployed by attaching a float to one end that was thrown behind the boat. The boat was driven in a semi-circle around the school, and the float collected and brought on board. The purse-line was drawn to close the net, and working from either end, the webbing was pulled in until only a shallow pouch of the bunt remained in the water. Fish caught in this pouch were enumerated, and a small subsample of no more than 10 smolts retained by aquarium net. Additional catch was released with minimal handling. In 2014, 15 sets were completed with 11 sockeye retained, while in 2015 22 sets were made retaining 112 smolts, and in 2016 78 sets were made capturing 124 smolts.

Adult sockeye Oncorhynchus nerka

Adult Oweekeno Lake sockeye were gill-netted in the Wanukv River in collaboration with the Wuikinuxv First Nation Fisheries Program in all three years. Gill nets were 15 or 30 m long and 2.5 m deep with a mesh of 5 ¼". Drift fishing was done from a 25 ft aluminum jet boat operated by Wuikinuxv Fisheries. Three successive sets were made, and 1-5 adult sockeye were retained from each set. If no sockeye were obtained in the first three sets, fishing continued until sockeye were caught or until it was determined there were no fish present at the time. Effort for gill-netting began in late June of each year and continued into early August. While gill-netting occurred everyday throughout the run, we participated with sampling 2 days per week to obtain 30-50 adult sockeye throughout the run.

Adult Chinook Oncorhynchus tshawytscha

Adult Chinook salmon were gill-netted in a similar way to sockeye in collaboration with the Wuikinuxv First Nation Fisheries Program and the Percy Walkus Wanukv Chinook Hatchery Program. A gill net 15 m long and 2.5 m deep with a mesh size of 8" was used. Fish were caught on several successive days in mid-October of 2014 and 2015. In both years, viral surveillance sampling was done on a random sample of 10 males and 10 females.

Trout O. mykiss, O. clarkii, Salvelinus malma

Trout were collected throughout 2014, 2015 and 2016. In all three years trout were fished with rod and reel from small boats and from shore in the Wanukv River and various locations throughout Oweekeno Lake. Members of the Wuikinuxv First Nation assisted by donating hook and line caught trout. In 2015 and 2016, two 6-panel gang nets were used to catch trout. Each net was 91.4 m long and 2.5 m deep. Panels had mesh of 1”, 3”, 2”, 3.5”, 1.5”, and 2.5” to catch a range of trout sizes. A floating net and a sinking net were typically deployed together. The floating net fished the top 2.5 m while the sinking net was set at the depth of the thermocline, determined by YSI meter. Nets were deployed in late afternoon or evening and retrieved 12 hours later. Nets were also used to sample 40 trout from each of 9 additional lakes throughout the Central Coast in 2015 (Figure 3-1, Table 3-1). Because lakes were accessed by foot, lakes were reasonably matched in terms of proximity to tidewater and elevation.

Figure 3-1. Sampling locations in the Rivers Inlet region, including additional Central Coast lakes: 1) Kisameet, 2) Namu, 3) Elizabeth, 4) Doris, 5) Elsie, 6) Sandell, 7) Allard, 8) Caroline, 9) Walkus. Map data ©2018 Google.

Table 3-1. Names and coordinates of Central Coast BC lakes sampled for salmonids throughout the Central Coast region.

Waterbody	Coordinates (Lat/Long)	Species sampled
Elsie Lake	51.546481/ -127.737862	*O. clarkii, S. malma*
Elizabeth Lake	51.714313/ -127.779404	*O. clarkii, S. malma*
Kisameet Lake	51.966216/ -127.871757	*O. clarkii, S. malma*
Namu Lake	51.857089/ -127.854763	*O. clarkii, S. malma*
Oweekeno Lake	51.688922/ -127.153229	*O. nerka, O. clarkii, O. mykiss, S. malma*
Allard Lake	51.473664/ -127.331414	*O. clarkii*
Caroline Lake	51.381728/ -127.311158	*O. nerka, O. clarkii, S. malma*
Doris Lake	51.596204/ -127.671944	*O. clarkii, O. mykiss*
Sandell Lake	51.573664/ -127.445891	*O. clarkii, O. mykiss, S. malma*
Walkus Lake	51.647410/ -126.618333	*O. nerka, O. clarkii*

Eulachon Thaleichthys pacificus

Eulachon were donated to this project for screening by Wuikinuxv First Nation food fishers collected from the banks of the Wanukv River after fish had spawned in January 2015. An additional sample came from the Klina Klini River in Knight Inlet collected by members of the Namgis First Nation the same month. In both cases fish were held in -20 °C household freezers until they could be sent for virus screening at the Atlantic Veterinary College.

3.2.4. Tissue sampling for viral surveillance

All fish captured alive were immediately euthanized by a lethal dose of Tricaine mesylate (MS-222). Because of the remote locations of capture, any fish that could not be sampled immediately was held on wet ice in a cooler for transport to a field lab or transferred to a refrigerator. All fish had organ tissues sampled within 36 hours of capture, and most were sampled within 12 hours of capture.

Juvenile sockeye

Juvenile sockeye were placed on ice after euthanasia in 2014 and 2015 and transported back to a laboratory for measurement and sampling. Fish were bagged individually and frozen whole in a -20 °C - -30 °C freezer. Fish were rinsed before individual packaging to minimize cross contamination. Tools were sprayed with 95% ethanol and flamed between fish. In 2016, blood was drawn from a random subsample

of sockeye smolts to compare detection of PRV in this tissue with organ tissues (see Chapter 2).

Adult salmon and trout

Organ tissue collection

Organ tissues were dissected from adult salmonids in all three years. Tissues were stored in RNAlater and incubated for 24 hours at 4 °C, and then placed at -20 °C until analysis. Disposable surfaces were used to minimize cross-contamination between fish. Tools were divided into 'outside' and 'inside' sets for organ removal to minimize introductions of external virus particles. Small 2 x 2 x 5 mm pieces of liver, spleen, heart, head kidney, and skeletal muscle were collected with a clean scalpel and placed into 5-10 volumes of RNAlater in microcentrifuge tubes. Notes were made about the condition and quality of the body cavity and organs. After each use tools were cleaned by sequential immersion in 20% bleach, water, and 70% ethanol, then flamed. Gloves were changed between handling the inside and outside of each fish and between fishes.

Adult salmonid blood sampling

In 2016, blood was also drawn from adult sockeye and trout. Briefly, freshly-caught fish were cleaned with antiviral wipes on the ventral surface of the caudal peduncle. A 1.5-inch hypodermic needle on a 10 ml syringe preloaded with 100 μl Potassium-2-EDTA anticoagulant was inserted into the caudal vein. One ml of blood was collected and split between two vials filled with 1.5 ml RNAlater. Vials were inverted and placed on ice.

3.2.5. Viral screening

RNA was extracted from tissue samples and screened for viral presence/absence at one of two laboratories: the Atlantic Veterinary College at the University of Prince Edward Island, or in-house at Simon Fraser University in the Mattsson Lab by the author.

Atlantic Veterinary College

Whole juvenile fish and pooled tissues in RNAlater were sent to the Atlantic Veterinary College (AVC) in 2014, 2015, and 2016 by overnight courier on ice. For each

specimen, screening was conducted on total RNA extracted with Trizol reagent and/or RNeasy spin columns. RNA isolation, quality control, and RT-qPCR testing for PRV was conducted with Taqman probe-based chemistry as described in Kibenge et al. (2013). A more detailed description can be found in Appendix A. The Kibenge Laboratory also screened for three additional salmon viruses: Infectious salmon anemia virus (ISAV), Salmon alphavirus (SAV), and Piscine myocarditis virus (PMCV). Methods and results for these viruses are described in Appendix B.

In-house screening for PRV

The expense associated with sending samples away for screening inspired the development of in-house assays for PRV for materials collected in 2016. See Appendix C for details describing the development of methods.

RNA isolation and DNase treatment

RNA was isolated from 50-100 mg pooled tissue or whole fry and 70-300 μl blood as described in Chapter 2, using Trizol reagent (Invitrogen), RNeasy silica-filter spin columns (Qiagen), or a Trizol-like reagent (GPC) (Chomczynski & Sacchi, 2006). RNA was eluted in 14-32 μl nuclease-free water. Quantity and RNA purity was determined using a NanoDrop 2000c (Thermo Fisher). RNA was deemed suitable for downstream applications if 260/280 and 260/230 absorbance ratios were > 1.8. If ratios were lower, RNA was re-precipitated using sodium acetate and ethanol.

Pure RNA was DNase-treated with the abm AccuRT genomic DNA removal kit. RNA was diluted to 0.3 μg/μl and 2 μg was used with 2 μl AccuRT reaction mix in a final volume of 8 μl. The solution was incubated for 5 minutes at room temperature (RT) before adding 2 μl of reaction stopper.

cDNA synthesis and RTqPCR assays

Two μg (9.1 μl) DNase-treated RNA was reverse-transcribed into complementary-DNA (cDNA) using the abm OneScript Reverse Transcription kit with in-house designed gene-specific primers (Appendix C) for PRV and oligoDT primers for the housekeeping gene according to manufacturer's directions. The cDNA was diluted 1:3 in a final volume of 60 μl. Gene-specific cDNA synthesis primers were designed to flank qPCR primer regions on both PRV sense and antisense strands. This enhanced

sensitivity of the assay by doubling the number of detectable PRV transcripts as well as specifically synthesizing cDNA of the desired primer annealing sites.

RT-qPCR assays also relied on an in-house designed primer and probe set which targeted a conserved region of PRV genome segment L1 (Table 3-2). Primers were designed as described for cDNA primers (Appendix C) with the additional specification that they fall within reverse-transcribed regions. In-house designed PCR primers were validated against material from Atlantic salmon head kidneys (an organ that frequently returns high Ct values for PRV in infected fish, e.g., Garver et al. (2016)) that screened positive using three published primer sets (Table 3-2) and yielded DNA sequencing reads with high identity to published PRV genotype one sequences (Table 3-3).

SYBR-green-based RT-qPCR was performed on 4 μl cDNA from field-collected samples as described in Chapter 2 to validate methods and for housekeeping gene assays. Detailed reaction conditions can be found in Appendix C. The sockeye salmon β-actin-encoding gene was used for housekeeping and primers were designed against

Table 3-2. Primers and probes used for in-house PRV screening of salmonid material at Simon Fraser University.

Primer name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
B-Actin_nerka	CCAAAGCCAACAGGGAGAAG	AGGGACAAGACGGCCTG	NA
PRV L1_flSH	ACCCCGTTACCAGTTCAACC	AGGGATGGGGAGGTCTTCAA	FAM-ACCACACGAGCGCACTCAGA-BHQ1
PRV L1_Pal (Palacios et al. 2010)	TGCTAACACTCCAGGAGTCATTG	TGAATCCGCTGCAGATGAGTA	NA
PRV L1 (Haugland et al. 2011)	CCCCATCCCTCACATATGGATA	GGTGAAATCATCGCCAACTCA	NA
PRV S1_Fin (Finstad et al. 2014)	TGCGTCCTGCGTATGGCACC	GGCTGGCATGCCCGAATAGCA	NA

GenBank accession AB481206. A high-resolution melt curve was used to assess if single products were formed. Products were confirmed by visualization on a 2% agarose gel. Samples were run as duplicate technical replicates. Triplicate positive (template from confirmed PRV-positive Atlantic salmon) and negative controls (water)

were included. Assays were considered successful if unknown fish samples successfully amplified the housekeeping gene, technical duplicates produced Ct values within 0.5 cycles of each other, positive controls exponentially amplified products of appropriate size with Ct values less than 35, and no product was amplified in no template controls.

Taqman probe-based PRV screening targeting PRV genome segment L1 was conducted using 3 μl cDNA template (details in Appendix C). As above, samples were run as technical duplicates, with triplicate negative and positive controls. Criteria for successful assays are the same as above. In addition, results of housekeeping gene assays were used to determine the success of cDNA synthesis.

A PRV positive control was made from cDNA produced from the head kidneys of six store-bought farmed Atlantic salmon that tested positive for PRV using three published primer sets (Finstad et al., 2014; Haugland et al., 2011; Palacios et al., 2010). A purified 143 base pair PCR product from PRV genome segment S1 was sent for confirmatory Sanger sequencing, and read identity was confirmed by searching sequences against the Genbank database using the nucleotide Basic Local Alignment Search Tool (BLASTn) (Altschul et al., 1990) (Table 3-3).

3.2.6. Statistical analyses

Comparison of PRV positive proportions in sockeye salmon and resident trout

This was an exploratory analysis, the objective of which was to generate provisional insights about PRV infection dynamics in the Rivers Inlet salmonid community among different life stages of sockeye salmon, as well as three resident trout species, Chinook salmon, and eulachon sampled 2014-2016. In keeping with general principles for interpreting exploratory analyses, these results will require further investigation.

Ideally, a mixed effects logistic regression model would have been employed to look at the effects of species, life-stage, and brood year on likelihood of carrying a PRV infection. However, this method depends upon relatively large numbers of both positive and negative results. In this case, although the sample sizes would normally be considered large ($n > 20$), so few positive tests were found that a logistic model was

inappropriate. Instead, PRV positive proportions were calculated cumulatively for each species and life-stage of sockeye salmon and trout, and yearly for each species and life-stage. Ninety-five percent confidence intervals of the positive proportion were estimated using the Jeffrey's method, recommended for cases with small numbers of positive or negative results (Brown, Cai, & Dasgupta, 2001). Proportions were compared using Fisher's Exact tests. All statistical analyses were performed in R version 3.4.4 (R Core Team, 2018). The Fisher's test was chosen because it is suitable for comparisons between categories with few successes or failures (Routledge, 1992). This test is based on a relatively simple model without complex interactions between factors and focusses on identifying the main factors of importance. Because the p-values produced in this test are generated using a highly discrete, exact hypergeometric distribution, they are conservative. A mid-p-value was used for the interpretation of results to reduce conservatism and strengthen the power of the test to detect patterns (Routledge, 1992, 1994). If a Fisher's Test yielded significant results for a 2 x 3 or greater contingency table, multiple comparisons were made to identify important contrasts and *p*-values were adjusted with Bonferroni corrections.

I assumed independence between individual salmonid and eulachon samples to satisfy underlying assumptions for the calculation of confidence intervals for proportions. While fish caught spatially and temporally close together are more likely to experience the same pathogens (Bakke & Harris, 1998), effort was made to sample fish at intervals in time and space, and to retain few fish from any one sampling event. Additionally, at the time of capture, it is impossible to determine without longer term tagging and tracking whether individuals caught in proximity have had an enduring close association, or merely a transient one. For these reasons I decided it was appropriate to treat samples independently, at least to generate preliminary inferences.

3.3. Results

3.3.1. In-house methods validation

RNA isolated from six Atlantic salmon head kidneys was found to be positive for PRV using three different published PRV primer sets. Purified PCR products were sent for confirmatory Sanger sequencing and reads had 81-100% sequence identity to published sequences of genome segment S1 (Table 3-3). All three commonly used

primer sets for PRV produce bands of the expected size for PRV in the same three Atlantic salmon (Figure 3-2). Primers by Palacios et al. (2010) and Finstad et al. (2014) give the same result for five out of six samples, and primers by Haugland et al. (2011) and Palacios et al. (2010) agree over four samples. This provides evidence that all six Atlantic salmon used as PRV positive controls for in-house virus screening can be detected as positive using published methods. Moreover, pooled RNA from these six Atlantic salmon yielded positive results from the in-house designed Taqman RT-qPCR assay for a 197-base fragment of PRV genome segment L1, giving raw Ct values of 28.91 and 30.13. Water and fish previously testing negative for PRV by the Atlantic Veterinary College (AVC) did not amplify PRV products using the in-house assay. Follow up visualization of PCR products on a 2% agarose gel demonstrates a strong band of the expected size for the PRV-positive Atlantic salmon, and no bands appear in the negative controls (Figure 3-3).

Table 3-3. Nucleotide BLAST (BLASTn) somewhat similar sequence matches for four of six PRV genome segment S1 RT-qPCR products from store bought Atlantic salmon used as positive controls.

Sample name	PRV isolate & identity	GenBank Accession match	Query coverage	Percent identity	E value	Isolated host
AT1_fwd	V/P1.1 sigma 3 protein (OCPSigma3) gene	KX844958	83%	85.5%	1.34x10-17	rainbow trout
AT1_rev	A.3.5-168_G860 outer clamp gene	MH093983	12%	95%	3x10-21	Atlantic salmon
AT2_fwd	P.3-120_G417 outer clamp gene	MH093990	9%	81%	1x10-6	Chinook salmon
AT2_rev	P.3-37_G446 outer clamp gene	MH093989	19%	99%	5x10-29	Chinook salmon
AT3_fwd	P.3-120_G417 outer clamp gene	MH093990	18%	94%	1 x10-24	Chinook salmon
AT3_rev	P.3-120_G417 outer clamp gene	MH093990	35%	100%	3x10-41	Chinook salmon
AT4_fwd	P.3-120_G417 outer clamp gene	MH093990	29%	97%	7x10-31	Chinook salmon
AT4_rev	A.3.5-168_G860 outer clamp gene	MH093983	36%	100%	1x10-40	Atlantic salmon

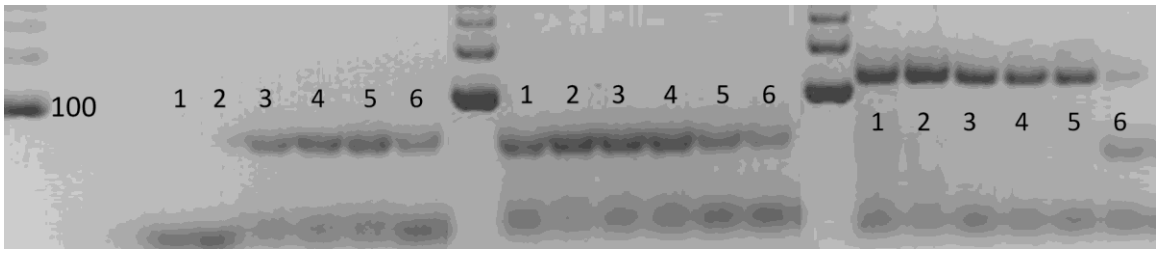

Figure 3-2. Results of RT-PCR for three different regions of PRV in samples isolated from six store-bought Atlantic salmon head kidneys visualized on a 4% high resolution agarose gel run in 1X TBE buffer. From left to right primers used are: Haugland et al. (2011) for a 64-base fragment of genome segment L1 (primers used by AVC in RT-qPCR), Palacios et al. (2010) for a 59-base fragment of genome segment L1, and Finstad et al. (2014) for a 143-base fragment of genome segment S1.

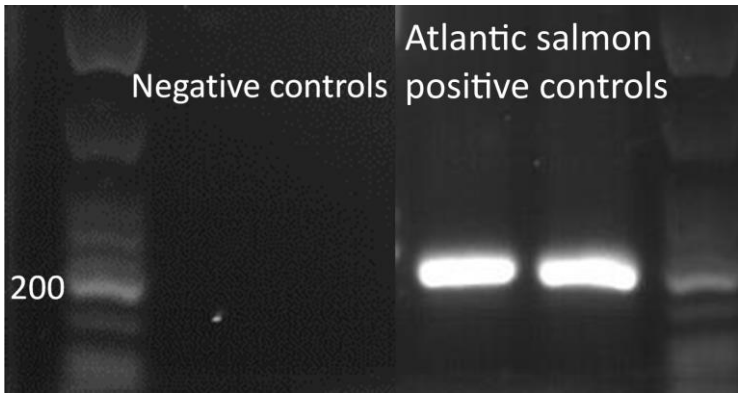

Figure 3-3. Results of Taqman probe-based RT-qPCR assay using in-house designed primers for a 197-base pair fragment of PRV genome segment L1. Results of Taqman probe-based RT-qPCR assay using in-house designed primers for a 197-base pair fragment of PRV genome segment L1.Templates are pools of Atlantic salmon PRV positive material previously shown in Figure 3-2. Results are visualized on a 2% agarose gel run in 1X TBE buffer.

3.3.2. Patterns in PRV infection of Rivers Inlet/Oweekeno Lake salmonids

Overall, a total of 508 salmonids and eulachon were sampled between 2014-2016 and screened for PRV by the AVC and in-house at SFU (Tables 3-4 and 3-5). The overall prevalence of PRV in species consistently sampled across all three years was 3% (1.7-4.9%). One positive sample from 2014, isolated from adult sockeye WR211, was confirmed positive by additional screening for PRV genome segment S1. Purification and sequencing of this product yielded a 1081 nucleotide sequence with 99% sequence identity (E = 0) to GenBank PRV sequence KC473452.1 isolated from an

Atlantic salmon host. This screening also yielded the first PRV positive results for Dolly Varden char (2014) and Eulachon from both Rivers Inlet and Knight Inlet (2015) (Table 3-5).

PRV infection presence: Species and life stage correlates

Associations between PRV and the different life-stages of sockeye salmon were investigated by comparing the cumulative PRV positive proportions of all sockeye salmon fry (3%, 95% CI: 1-9%), smolts (1.5%, 0.4-4%) and adults (2.1%, 0.4-6%) (Table 3-4). None of the sockeye salmon life-stages was significantly more infected with PRV than the others (Fisher's Exact Test, *mid-p* > 0.05). Comparing the cumulative proportions of each sockeye age-class with resident trout also failed to reveal a greater association between PRV and any one of these categories (Fisher's Exact Test, *mid-p* > 0.05).

Adult Chinook salmon, adult sockeye salmon, trout, and Rivers Inlet eulachon were compared in terms of their PRV positive proportion to probe for preliminary evidence of increased susceptibility of one of these species to PRV. Only adults and only fish sampled in 2015 were used in this comparison as eulachon samples were restricted to this year, and Chinook salmon were not sampled in 2016. A significant difference was found from the 2x4 contingency table comparison of PRV positive proportions across these four species (Fisher's Exact Test, mid-*p* = 0.016). However, after adjustment for multiple comparisons, only the contrast between adult sockeye (0%, 95% CI: 0-6%) and eulachon (15%, 5-31%) was close to significance (mid-*p* = 0.065; Figure 3-4).

Table 3-4. Summary of sockeye salmon and trout of three species screened for PRV segment L1 by year and age class. Number screening positive in brackets. Confidence intervals calculated using Jeffrey's Interval.

Year	Sockeye				Trout	Totals by year	PRV %	95% CI*
	Fry	Smolts	Adults	All				
2014	26 (1)	11 (2)	28 (2)	65 (5)	18 (3)	**83 (8)**	**9.6**	4.7-17.4
2015	39 (2)	80	41	160 (2)	9	**169 (4)**	**2.4**	0.8-5.5
2016	24	114 (1)	28	166 (1)	22	**188 (1)**	**0.5**	0.06-2.5
Total	89 (3)	205 (3)	97 (2)	391 (8)	49 (3)	**440 (13)**	**3**	1.7-4.9
PRV %	**3**	**1.5**	**2.1**	**2**	**6**			
95% CI*	1.0-9	0.4-4	0.4-6	1.0-4.0	2.0-15			

Table 3-5. Sample sizes for Chinook salmon and eulachon screened by the Atlantic Veterinary College in 2014 and 2015. Number screening positive for PRV genome segment L1 in brackets. Confidence intervals calculated using Jeffrey's Interval.

Year	Chinook	Rivers Inlet eulachon	Knight Inlet eulachon
2014	21	-	-
2015	20 (1)	27 (4)	19 (1)
Total	41 (1)	27 (4)	19 (1)
PRV %	**2**	**15**	**5.3**
95% CI*	0.3-11	5.0-31	0.6-22.1

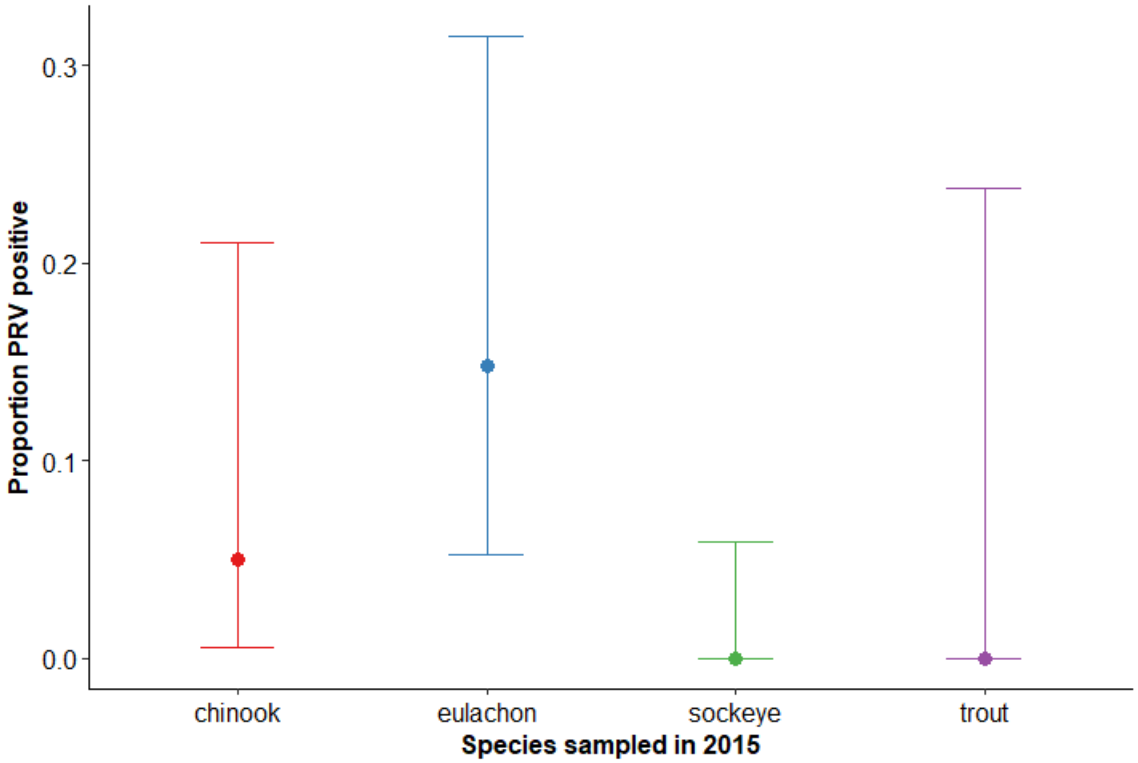

Figure 3-4. Comparison of PRV prevalence in adults from four species sampled in 2015. Error bars represent the 95% confidence interval of the true population proportion estimated using the Jeffreys' Interval method for small n.

PRV presence in sockeye salmon and trout: Interannual patterns

The yearly PRV positive proportion of all sockeye salmon life-stages and trout was 9.6% (95% CI: 4.7-17.4%) in 2014, 2.4% (0.8-5.5%) in 2015, and 0.5% (0.06-2.5%) in 2016 (Table 3-4). Using a Fisher's Exact test, these years were found to be significantly different in their overall positive proportions (mid-$p < 9.796 \times 10^{-5}$). Performing pairwise comparisons between all years with a Bonferroni adjustment revealed that this difference was driven by a significantly greater PRV positive proportion in 2014 than in each of 2015 (Difference: 7.2%, 95% CI of the difference: 4-12%; Odds Ratio (OR) 8.82, 95% CI:1.7-87.4, mid-*p*-value = 0.0014) and 2016 (Difference: 9.1%, 95% CI: 4.6-14.9%; OR: 17.2, 2.2-773, mid-*p*-value=0.0002) (Figure 3-5). There was no difference in the proportion of fish testing positive for PRV between 2015 and 2016 (mid-$p = 0.36$). This result is attributable to a greater proportion of sockeye smolts with PRV positive results in 2014 than in subsequent years (Figure 3-5). Smolts in 2014 tested positive at 18.2% (4-43.6%) higher than in 2015 (OR: ∞, 95 CI: 1.4-∞, adjusted mid-$p = 0.02$) and 17.3% (3.9-42.7%) higher than in 2016 (OR: 23.5, 95 % CI: 1.1-1475.4,

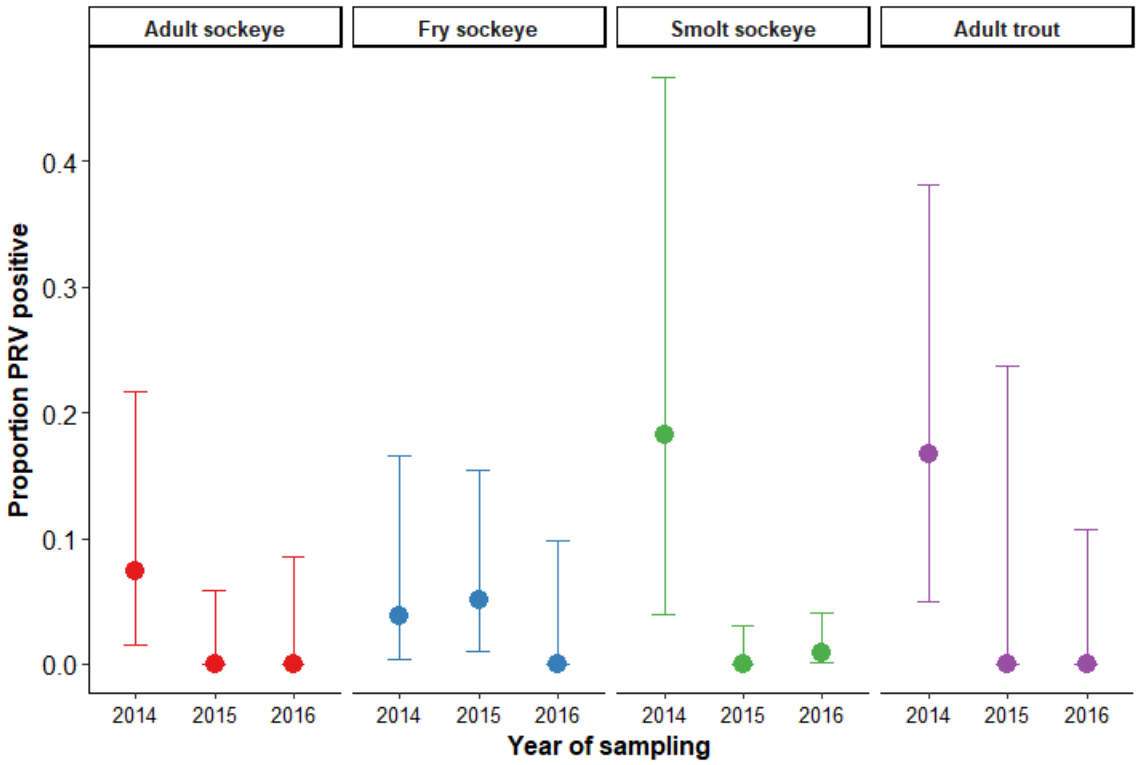

Figure 3-5. Proportion testing positive for PRV in adult, smolt, and fry sockeye salmon, as well as three species of resident trout in Rivers Inlet and Wuikinuxv Lake from 2014-2016. Error bars represent the 95% confidence interval of the population proportion estimated using the Jeffreys' Interval.

adjusted mid-*p* = 0.03). No statistically significant difference was found between the proportions of fry, adult sockeye, or trout from year to year, despite 2014 being the only year both adult sockeye and resident trout yielded positive test results (Figure 3-5).

The PRV positive proportion decreased over time between the fry and smolt stages of sockeye. The pooled PRV prevalence for fry hatched in the winters of 2014 and 2015 was 4.6% (95% CI:1.3-11.8%) and it fell by 4.1% (95% CI: 1.2-9.4%) to 0.52% (0.06-2.4%) in the smolt stages sampled in 2015 and 2016 (Fisher's Exact Test, Odds Ratio: 9.2, 0.7-491.2, mid-*p* = 0.027; Figure 3-5).

Central Coast Lakes 2015

Ten trout (adult *O. clarkii*) were tested from each of the 9 additional Central Coast lakes sampled between June and August in 2015. None of the trout in any of these lakes tested positive for PRV. A 95% confidence interval produced with the

Jeffreys' interval shows that the true proportion could lie between 0-21.7 % for any of these lakes.

3.4. Discussion

The salmonid community of Rivers Inlet is not naïve to infection by PRV, for which positives were found in every fish species and age-class sampled between 2014 and 2016. An adult Rivers Inlet sockeye salmon testing positive for PRV in 2014 yielded a sequence for genome segment S1 which was a 99% sequence match to GenBank sequence KC473452 (E = 0) isolated from an Atlantic salmon from BC's Discovery Islands, indicating an epidemiological link to PRV outside this system. For species surveyed over all three years, sockeye and trout from Rivers Inlet had a 2% and 6% prevalence for PRV, respectively. PRV showed no age-specific prevalence structure among sockeye fry, smolt and adult life-stages, and in salmonids surveyed in 2015, there was only a marginally higher prevalence of PRV in eulachon than sockeye salmon. This work did, however, identify both Dolly Varden char and eulachon as previously undescribed susceptible hosts for this virus.

3.4.1. Associations of PRV with life-stages and species

No previous explicit research has been conducted on the infection susceptibilities of different life-stages of Pacific salmon to PRV. When all three years of PRV presence data from Rivers Inlet are considered together, no consistent association between PRV infection level and life-stage of sockeye salmon was revealed. This agrees with Morton et al. (2017), who found life-stage was not an important predictor of PRV presence in a province-wide screening of Pacific salmon from nine regions of BC. Garseth et al. (2013), however, found a 10 cm increase in body length resulted in a 1.2 times greater odds of PRV infection for Atlantic salmon sampled over three years in Norway. Madhun et al. (Madhun et al., 2018; 2017) also found that while PRV infections were present in all production stages of escaped Norwegian farmed Atlantic salmon, the likelihood of infection increased with the size of fish, a proxy for duration in an aquaculture facility before escape, and thus duration of exposure to heightened infection pressure. As few positive results were found for Rivers Inlet sockeye and trout, infection associations with fish age are difficult to determine and may require larger sample sizes. Additionally, RT-

qPCR positive results reported here do not differentiate between actively progressing infections and asymptomatic carriers with latent infections. Miller et al. (2017a) employed a principle components analysis of viral disease-related gene expression biomarkers to differentiate between these states, while other studies have relied on immunoassays to detect antibodies to PRV indicating recovery from infection (Teige et al., 2017). Such convergent methods would provide clarification of susceptible life-stages and the interpretation of age-specific PRV infection dynamics.

PRV prevalence, while not found to significantly associate with a sockeye life-stage, was found to decrease over time within the two sockeye juvenile cohorts that could be examined. A 4.1% decrease in the proportion of PRV infections from the fry to smolt stages was observed when juveniles in the 2014 and 2015 cohorts were considered together (Figure 3-5). By examining the cohorts together, a detailed examination of year-specific effects is prevented. Nevertheless, these results suggest hypotheses to pursue:

i) Sockeye salmon may be acquiring PRV infection at the alevin or fry stage. If so, it is important to establish whether declines in prevalence indicate recovery from infection or infection-related mortality before reaching the smolt stage;

ii) If PRV is horizontally transmitted, then fry must be acquiring infections within the lake from conspecifics in the cohort ahead of them, a resident non-sockeye host, or an environmental reservoir. In this scenario, pulses of PRV introduced on anadromous adult salmonids would be indirectly mediated through one of these routes.

As changes in prevalence result from fluctuations in the rate of infection as well as mortality and recovery, the first hypothesis could be examined with a better characterization of these rates. An increased frequency of sampling for virus at early life-stages of sockeye would provide information on the rate of new infections (Brenkman et al., 2008), while use of immunoassays, as described above, with RTqPCR could provide estimates of recovery versus mortality. A longer time series with more frequent measurement of PRV infection rates would also allow more precise estimates of the true differences in the infected proportion at each life-stage. Clarity on this issue has important implications for understanding the long-term persistence of PRV in Rivers Inlet sockeye. A decline in PRV infection prevalence from fry to smolt stage may indicate that individuals are recovering or dying faster than new infections can establish. Thus,

without additional infection pressure from repeated virus introductions or a species acting as an infection reservoir, PRV would be predicted to 'fade out', or be lost from this system. A longer-term longitudinal study could provide valuable further insight into these scenarios.

The second hypothesis would also be aided by a better appreciation of infection rates for different age classes of sockeye and different species. Additionally, by identifying among which groups new cases are appearing and pairing this with genotyping of PRV, one can begin to unravel the likeliest transmission pathways in this system (Breyta et al., 2017). Environmental sampling of PRV in sediments and water could also be included in such an investigation, as it has been shown experimentally that non-enveloped reoviruses are incredibly persistent, surviving as long as 30 days in desiccated matrices (Howie, Alfa, & Coombs, 2008).

A goal of this research was also to better understand the host range of PRV and to search for heightened associations between PRV and certain species. Such information would be useful in assessing how diversity might affect infection risk (Dobson, 2004) and to identify potentially important virus transmitters in a system – *i.e*., abundant species with high infection rates (Dobson, 2004). From my data, PRV did not appear to have a higher affinity for infecting either trout or sockeye, with the cumulative infected proportion for sockeye ranging from 1 to 4% and from 2 to 15% for the three trout species combined. Trout were previously hypothesized as a potential infection reservoir due to their apparently high abundance in Oweekeno Lake, year-round presence, and a previous study which found a high PRV infection level (76%, 13 of 17) in cutthroat trout from Cultus Lake (Kibenge et al., 2013; Morton et al., 2017). However, extensive sampling of trout throughout nine additional Central Coast lakes in 2015 failed to reveal PRV broadly infecting cutthroat or rainbow trout, or Dolly Varden char, suggesting that in this region a trout reservoir for PRV is unlikely. Nonetheless, while trout may not be functioning as a reservoir, this work has expanded the known host range of PRV to include Dolly Varden.

In addition to sockeye salmon, rainbow and cutthroat trout, and Dolly Varden, this work also identified eulachon as hosts to PRV. This finding that PRV can infect eulachon aligns well with previous results by Wiik-Nielsen et al. (2012a) also found another species of smelt, capelin (*Mallotus villosus*), RT-qPCR positive for PRV in a survey of

marine fish from the North Sea. Interestingly, eulachon yielded a marginally significantly higher prevalence of PRV relative to the estimate for adult sockeye in 2015. As eulachon from the Klinaklini River in Knight Inlet also tested positive for PRV in 2015, one wonders if this is a common occurrence. Due to conservation concerns about this culturally valuable species, this finding would be worthy of follow up. Additionally, a single Chinook salmon from the Wanukv River population tested positive for PRV, although survey coverage of Chinook was not extensive in this study (only 8% of fish tested were Chinook). Purcell et al. (2018) recently found that the largest proportion of PRV positives (29%) from their survey of 2252 fish came from Chinook salmon sampled throughout three regions in Alaska and Washington State. Within BC, Marty et al. (2014) and Siah et al. (2015) similarly found high associations of PRV with Chinook and coho salmon (*O. kisutch* Walbaum, 1792). Further, in a recent survey of microparasites in juvenile salmonids from coastal BC, Miller et al. (2017b) found a higher association between PRV and juvenile Chinook salmon (4% of 1876), than juvenile sockeye (1% of 630). Di Cicco et al. (2018) has recently identified PRV as the causal agent of Jaundice Anemia (Di Cicco et al., 2018)/Jaundice Syndrome (Garver et al., 2015), a disease which affects Chinook salmon in BC aquaculture, but has not yet been described in wild populations. These results suggest future surveys for PRV should make Chinook salmon a high priority.

That PRV has been found to infect multiple fish species in this community and others (Morton et al., 2017; Wiik-Nielsen et al., 2012a) has implications for predicting features of viral dynamics. When a pathogen has density-dependent transmission, as is likely for PRV since it has no presently known vector, if increased diversity of susceptible hosts increases host density, this may amplify the likelihood of disease outbreak and persistence (Dobson, 2004). Future studies should pursue a clearer understanding of the host range of PRV and differing infection and recovery rates of host species in a community.

3.4.2. Interannual patterns in PRV prevalence

PRV infection prevalence was assessed within each species or life-stage by year to determine patterns in prevalence over time. The year 2014 emerged with greater prevalence for all groups considered together than that measured in both subsequent years (Figure 3-5; Table 3-4). In addition, in 2015 and 2016, only juvenile sockeye that

were offspring of adults in 2014 returned positive results (Figure 3-5). This may suggest that returning adult salmonids have an important influence on the PRV dynamics within Oweekeno Lake. This preliminary finding warrants further investigation over a much longer time series. The larger proportion of PRV positives observed in 2014 was largely driven by higher proportions of infected smolts in this year, with nearly 20% more positives than in 2015 and 2016. This could have been due to sampling error as relatively few smolts (n=11) were captured in only three sampling events; however, PRV positive smolts came from two separate sampling events occurring many kilometers and nearly two weeks apart. Sample sizes were eight to ten times greater in 2015 and 2016 and did not replicate this result. This adds weight to the notion that PRV prevalence may fluctuate over time. Again, more fine-scaled temporal sampling might help to relate incidence of new infections with features of sockeye population dynamics or environmental variables that could influence these patterns.

3.4.3. Rivers Inlet PRV infections within a coast-wide context

I found that Rivers Inlet sockeye had a cumulative PRV prevalence of 2% (95% CI:1-4%) over the three years of this study, and an overall prevalence 3% (1.7-4.9%, Table 3-4) for both sockeye and trout. This is lower than infection levels for farmed Atlantic salmon – prevalence of 20-100% was reported from a single farm by Di Cicco et al., (2017) and 95% for a large two-year sample of BC farmed Atlantic salmon Morton et al. (2017). Results for Rivers Inlet are similar to findings by Morton et al. (2017) for regions distant to high levels of salmon aquaculture in BC (5% for North coast salmon), but higher than that found for other remote regions by Marty et al. (2014): 0.6% (1/180) for BC and 0% (0/120) for Alaska sockeye. Similarly, Purcell et al. (2018) found a 0% PRV prevalence (0/788) for Alaskan and Washington State sockeye sampled in 2013 and 2014. However, results for Rivers Inlet sockeye are lower than prevalence reported by Miller et al. (2014) for adult sockeye from the Chilko and Late Shushwap Fraser River populations (29%). It is possible, as suggested by Morton et al. (2017), that these differences in PRV prevalence among wild salmon populations exist due to relative exposure of populations to salmon aquaculture facilities. Rivers Inlet, while remote to high densities of aquaculture activity in southern BC, is relatively close to a small cluster of aquaculture facilities roughly 175 km to the north. Exposure to these facilities could occur during a sockeye smolt's first winter at sea, as trawl surveys have shown juvenile

Rivers Inlet sockeye have a tendency to overwinter inshore locally on the Central Coast (Welch et al., 2004). Temporal and spatial knowledge of PRV prevalence and HSMI/Jaundice Anemia outbreaks at these and other salmon aquaculture facilities, could provide more definitive epidemiological evidence for testing this hypothesis.

Biases naturally exist in this type of study due to both sampling error and assay sensitivity (Brenkman et al., 2008) that must be overcome to strengthen any future study. It has been assumed that the fish screened in this study constitute a random sample of the population and that fish infected by PRV are just as likely to be caught as fish without PRV. However, in practice this is unlikely to be true. PRV is an infection of concern because it may compromise wild fishes, altering their behaviour, making them more vulnerable to predation (Miller et al., 2014). Among the positive samples I obtained from Rivers Inlet, most fish had low levels of infection as evidenced by qPCR Ct values relative to the high viral loads reported for many farmed Atlantic salmon screened with the same or similar methods. These discrepancies in PRV load between wild and farmed fish have also been noted by other researchers (e.g., Garver et al., 2016; Marty et al., 2015) who argue it indicates low pathogenicity of PRV in Pacific salmon. Conversely, it may also indicate a truncated distribution of infected individuals from the loss of highly infected individuals due to predation mortality (Lester, 1984; Miller et al., 2014). This conclusion is supported by recent findings by Di Cicco et al. (2018) indicating Pacific salmon may have more severe pathological responses to PRV than Atlantic salmon. Determining which of these alternatives is at work should be high priority in future research.

The observations made through this study raise numerous hypotheses about the species associations and temporal and spatial distribution of PRV within Rivers Inlet and the West Coast of North America. To address these hypotheses and the challenges faced by the present study additional inquiry will be needed. Studies with a longer time-series of sampling in order that trends might be elucidated; the pairing of immunoassays with RT-qPCR to better estimate rates of infection, recovery, and mortality; the sampling of a broader range of potential hosts and the incorporation of virus genotyping; and the relating of these patterns to viral dynamics external to this system are all directions which may assist in addressing unanswered questions about the ecology of PRV.

Whether PRV presence in this community may lead to population-level impacts for sockeye cannot be determined by this present investigation. A decline in the infected proportion from the fry to smolt stages in two consecutive years may indicate mortality or recovery from virus, and further investigation is needed. To answer population level impacts it would also be important to determine whether PRV-related mortality is greater than the expected mortality level for any life-stage of sockeye in this system. Experimental investigations of how PRV-infected salmonids fare compared to uninfected individuals when faced with resource competition and predation will be helpful in these predictions. Further, the pairing of such experimental results with field-collected data on sockeye abundance and PRV distribution and infection load could be useful in the development of models to predict higher-order impacts of this virus (i.e., Krkošek et al., 2011).

3.5. Conclusions

Wild Pacific salmon populations are in decline in many places throughout their range, yet the ability of pathogens such as PRV to cause population-level impacts is still poorly understood and significantly hampered by a lack of basic epidemiological data. Here I have obtained the first information on PRV prevalence in a large, understudied region of the west coast of North America. This was also the first study to monitor changes in the abundance of PRV over time within different species and life-stages of salmon anywhere on the west coast of North America. I have found preliminary evidence that PRV is present in all life-stages of sockeye salmon and five other fish species, including a first description of PRV in two new host species, Dolly Varden and eulachon. These data provide a benchmark for PRV infection in the Rivers Inlet salmonid community, against which both changes in the environment and policy regarding salmon aquaculture can be measured in terms of PRV prevalence. In light of concerns about the health impacts of PRV on Pacific salmon (Di Cicco et al., 2018), this epidemiological evidence warrants further attention.

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Chapter 4.

Synopsis and extensions

In my thesis I present the first longitudinal reporting of the prevalence of piscine orthoreovirus (PRV) in a Pacific salmon ecosystem. My approach is unique for looking at the age-specific prevalence of PRV at the fry, smolt, and adult stages of sockeye salmon, and for including the adults of four other salmonid species, as well as eulachon, in surveillance. With this data, I have contributed to the collective knowledge of the spatial distribution of PRV on the West Coast of North America, and highlights Rivers Inlet, an important sockeye salmon system, which has suffered poor productivity since the 1990s (McKinnell et al., 2001; Peterman & Dorner, 2012). In this final chapter, I first provide a synopsis of the key findings of my two data chapters on RNA isolation and virus surveillance. Next, I discuss the rationale behind my decisions, and lastly, I suggest future research opportunities to advance the study of PRV dynamics in salmonid communities.

4.1. Synopsis

In Chapter 2, I found, contrary to common advice to standardize RNA isolation methods, no single RNA isolation approach performed optimally for all three tissue types I examined. In fact, to produce sufficient amounts of RNA free from impurities, such as contaminating polysaccharides and proteins, my data suggests one must fine-tune methods for each tissue type being examined. The requirement that a method produces RNA that is free from impurities which might inhibit downstream uses, the methods themselves should be of greatest concern. My comparison of RT-qPCR results for the gene encoding sockeye salmon β-actin showed that the isolation method with poorer 260/280 absorbance ratios, indicating contaminating proteins, also had higher Ct values indicating a lower amount of detectable target sequence. I argue that the presence of impurities, rather than the methods themselves, are responsible for this discrepancy. With regard to optimal methods identified through my work, I found that small volumes of sockeye salmon smolt blood produced less protein or phenol contaminated RNA when a spin column procedure was used compared to the Trizol reagent. Spin columns limited RNA yield though, while Trizol produced three times more RNA for the same tissue. In

contrast, RNA isolated from adult sockeye blood had less reagent or polysaccharide carryover when isolated with Trizol than with a recent variant on this method (GPC) (Chomczynski & Sacchi, 2006), although both methods had comparable yields (Figure 2-5). Lastly, in working with salmonid organ tissue pools consisting of heart, liver, kidney, muscle and spleen, the GPC protocol produced higher RNA yields and a greater proportion of samples free from protein or DNA at a more stringent purity threshold than the Trizol method. The three RNA extraction methods also differed in their apparent user-friendliness, cost per sample, protocol length, and toxic exposure risk. Ultimately, the choice of RNA isolation method comes down to the tissue types being queried, prior user experience, and the resources of a given laboratory.

In Chapter 3, I show that PRV is present in Rivers Inlet salmonids and add two new species, Dolly Varden char and eulachon, to the list of hosts for this virus. Additionally, sequencing of a positive result for an adult sockeye sampled in 2014 links PRV in Rivers Inlet epidemiologically to PRV carried by farmed Atlantic salmon in the Discovery Islands of southeastern BC in 2013 (Genbank Accession: KC473452, sequence identity: 99%, E=0). PRV in Rivers Inlet had an overall proportion of 3% (95% CI: 1.7-4.9%) for sockeye and trout over the three years of surveillance (Table 3-4). This prevalence is three times higher than that found for sockeye from Alaska to Washington State reported by Purcell et al. (2018) and Marty et al. (2015) for the period from 2012-2013. However, Miller et al. (2014) found a prevalence nearly ten times as high in Fraser River Chilko and late Shushwap sockeye populations in 2010, while Purcell et al. (2018) found high PRV prevalence among Alaskan and Washington State Chinook and coho salmon in 2012-2013. These results imply spatial and temporal structure to PRV prevalence along the west coast of North America and suggest certain species may be more predisposed to carrying infection than others. Within Rivers Inlet sockeye and trout, PRV prevalence appeared to change over time, with a greater proportion of positive results coming from individuals in 2014 than in subsequent years (Figure 3-4), especially among sockeye salmon smolts. However, a longer times series is needed to validate temporal trends. Interestingly, I also found the proportion of PRV positive results in young-of-year sockeye fry fell from 4.6% in 2014 and 2015 to 0.52% in the smolt stages of these cohorts in 2015 and 2016 (Figure 3-5). This may indicate PRV-infected fry are recovering or succumbing to infection between the fry and smolt stages. Also, since few smolts were found infected in 2015 and 2016, relative to fry, it may be that few

new infections are acquired after the fry life-stage. These patterns suggest intriguing hypotheses warranting further investigation.

4.2. Extensions of this research

In this thesis I have provided information regarding RNA isolation techniques for salmon tissues key to RT-qPCR monitoring for microparasites, and document the prevalence of the controversial virus, PRV, across several species and life-stages of salmonid from Rivers Inlet, BC, generating the first provisional insights into the dynamics of this virus in a natural Pacific salmon community. During this research, many logistical considerations dictated research decisions and in the following section I put these decisions into context, discuss if and how they have influenced my interpretation of the data, and what opportunities exist for extending this research forward.

In my assessment of RNA isolation methods applied to salmon tissues I made important findings regarding method suitability to different tissue types. However, not all isolation methods were tested against all tissues. Instead I only assessed tissues of specific value to the molecular surveillance for PRV, i.e., organs implicated in disease processes for this virus (Di Cicco et al., 2017, 2018). RNA isolation methods too were selected based on methods common to published works involving molecular surveillance for PRV and other salmon pathogens (e.g., Finstad et al., 2014; Garseth, Ekrem, & Biering, 2013; Garver et al., 2016; Kibenge et al., 2013). Isolation method selection was further restricted by time and cost constraints, but still provides a reasonable comparison of methods easily accessible to most researchers approaching this issue. Numerous opportunities exist to extend comparisons of RNA quality and yield beyond these tissues and RNA isolation methods. Currently, many studies reporting PRV loads across several tissue types appear to employ single approaches to RNA isolation, without providing more detailed information on the 260/280 and 260/230 absorbance ratios (e.g., Garver et al., 2016; Marty et al., 2015; Polinski et al., 2016). One concern is that certain tissues with greater fat, polysaccharide, or protein content may be systematically underrepresenting PRV load by RT-qPCR if isolation methods are not being optimized. Future work examining this issue will guide more accurate molecular surveillance for PRV and other microparasites, as well as provide sounder RT-qPCR data regarding tissue tropism and viral disease progression.

Due to the volume of samples assessed during this project, numerous volunteers were involved in isolating RNA. I used a coefficient of variation metric for the 260/230 absorbance ratio measuring polysaccharide or reagent contamination of RNA, which allowed the assessment of whether users were generally able to produce consistent high-quality results using a given extraction method. Potentially though, a better metric of the learning curve would be a comparison of RNA quality produced by experienced and inexperienced users (similar to Aranda et al., 2009), or a measurement of the time it takes a user to begin consistently producing high-quality RNA. Given the time constraints of most studies, how quickly a protocol can be operationalized to generate data is a valuable consideration and a worthwhile metric to quantify for quick reference in planning future studies.

The virus surveillance methods employed to screen fish tissues for PRV changed over time because of study resources. Laboratory methods in-house initially followed methods used by the Kibenge laboratory at the Atlantic Veterinary College (Kibenge et al., 2013) to increase comparability of results. Unfortunately, PCR amplicon contamination prevented the continuance of this approach and new cDNA PRV-specific primers and a new RT-qPCR assay was developed to avoid false positive results (see Appendix C). With this approach I replicated negative results for 2016 sockeye smolts and both my RT-qPCR primers and those used by Kibenge et al. (2013) detected positives in store-bought Atlantic salmon (Figures 3-1 and 3-2). Unfortunately, I was unable to replicate previously positive results for Rivers Inlet materials in two instances. This may be a result of the implied low viral loads of these samples, given that Ct values were in the mid to high 30s. With low numbers of target DNA transcripts, stochastic events early in PCR amplification cycles can lead to a failure to exponentially amplify target sequences leading to false negative or non-repeatable results (Sambrook & Russel, 2001). In this context, this may have resulted in lower estimates of PRV prevalence in 2016, aside from sockeye smolts screened by the Kibenge Laboratory.

A relatively large total number of salmonids and eulachon were captured, processed and screened for PRV in the three years of this study (n=508). Because this was a cross-sectional survey producing point prevalence of PRV across several species and life-stages, however, a trade-off was made in the decision to sample for breadth of species and life-stages, rather than depth within a single group (although some species in the community were still excluded in order to allow greater depth of sampling in the

focal categories). This sampling approach provided a reasonable way of detecting a high PRV prevalence, should it have existed in any group, but compromised the power of this study to make highly precise estimates of PRV prevalence for most groups and timepoints. This has the consequence of making my data potentially conservative in its estimation of PRV prevalence – especially when sampled groups had relatively low numbers and yielded only negative results, e.g., resident trout from Oweekeno Lake in 2015 (n=9, 0% PRV). For this reason, my data should generally be regarded as providing lower limits of the likely PRV prevalence rate in this system. However, in some cases groups with high numbers of individuals screened, e.g., 2015 and 2016 sockeye smolts, also yielded low prevalence results (n= 80 and 114, 0 and 0.9% PRV, respectively), allowing greater confidence that this sampling adequately characterized the true population prevalence of PRV. In future, power analyses initially assuming low rates of infection should be employed to gauge sampling levels of fish in each category. Additionally, by employing non-lethal blood sampling, a higher level of sampling would be less onerous and pose less of an impact to the study system.

During the three years of field collections, I endeavoured to increase the sample sizes of groups that were not well represented in 2014. This resulted primarily in an evolution of the methods used to capture young-of-year sockeye salmon fry. In 2014, fry were sampled using a beach seine in the Wanukv River. However, few fry were caught with this approach and in subsequent years, trawling on Oweekeno Lake (2015) and fyke net trapping in natal streams feeding the lake (2016) were substituted as more effective methods of capture. I do not think this change in locations or capture methods led to strong biases in the data, and particularly did not lead to an overestimation of PRV infection prevalence, for several reasons: i) Genetic variability across 10 microsatellite loci suggests Rivers Inlet consists of a single, well-mixed, sockeye salmon population (Nelson et al., 2003). Movement of spawning adults between natal systems should allow for the distribution of microparasites. ii) Many lake outlet populations of sockeye are known for positive rheotaxis (swimming upstream into the natal lake) (Smith, Margolis, & Wood, 1987) therefore it is likely that many juvenile sockeye hatched in the Wanukv River, outlet to Oweekeno Lake where seining took place in 2014, swim upstream to rear in the main lake basin with juveniles from other natal systems. Trawling in 2015 took place near the lake outlet in the main basin, possibly still capturing Wanukv-hatched juveniles; and iii) Trawling in the main lake basin in 2015 sampled aggregated juveniles

from many natal streams before they left the lake. In 2016 fyke-netting changed the location and developmental stage at which sockeye fry were caught but sampled in a diverse set of natal systems throughout the lake, hopefully providing a similar cross section to that from trawling. While the same diversity of locations was hopefully represented in 2016, the possibility remains that PRV may have been underrepresented in samples collected by fyke netting. This scenario depends on the location of PRV transmission. If PRV is only acquired once juvenile sockeye assume a pelagic lifestyle, then sampling exclusively in natal streams would lead to conservatism in PRV prevalence estimates for fry in 2016. This will remain an outstanding question without further research. However, sampling in 2014 in the Wanukv River yielded positive PRV results for young-of-year fry, and three trout in the same year tested positive from natal stream systems in the second and third basins of Oweekeno Lake. This suggests that PRV is well-distributed throughout Oweekeno Lake and PRV-infected salmonids can be detected in natal systems. Going forward, it would be informative to nonlethally sample sockeye fry both in natal streams and the lake basin in the same year to resolve where PRV transmission first takes place.

The results of the current study also indicate some important considerations for future monitoring of PRV in wild stocks. Significant conservation concerns exist around eulachon, a fish of considerable cultural and ecological importance (COSEWIC, 2011). The finding of PRV positive results in both Rivers and Knight Inlet eulachon warrants additional investigation to establish the frequency of infection and potential consequences of this virus for eulachon in BC. Likewise, given the known consequences of PRV to Chinook salmon in captivity (Di Cicco et al., 2018), a finer-scaled investigation of PRV infection in wild and hatchery-raised Chinook in Rivers Inlet would be valuable, especially given the costly efforts currently underway to enhance both Wanukv River and Chuckwalla/Kilbella Chinook populations in this system (English, English, & Roias, 2017). Lastly, infection in non-salmonids like eulachon raise the question of the host range for PRV. Future surveys including abundant local species such as three-spine stickleback (*Gasterosteus aculeatus*) will add to our understanding of the community dynamics of this virus.

It is beyond the scope of the current thesis to comment on PRV transmission routes or population-level impacts, and several outstanding questions remain about the epidemiology and disease ecology of PRV in natural systems. These would be valuable

to address with future observational and experimental studies, as well as with the synthesis of empirical data and models to predict larger-scale features of PRV dynamics. For example, transmission dynamics of PRV will be better characterized when studies employ sampling on a finer time-scale to estimate incidence – or the rate of development of new PRV cases – as well as immunoassays for acquired immunity and genotyping of PRV. Incidence is valuable for understanding environmental correlates with the development of new cases, such as location, temperature or host diversity (Viana et al., 2014) while acquired immunity will assist in estimating recovery rates. Viral genotyping can demonstrate connectivity between hosts, and paired with incidence can indicate routes of virus transmission (Breyta et al., 2017). This study found the PRV strain infecting an adult Rivers Inlet sockeye in 2014 had a 99% sequence match for a variable genome segment to PRV isolated from a farmed Atlantic salmon from 2013 demonstrating epidemiological connectivity. Additional phylogenetic studies, perhaps utilizing a greater number of PRV genomic regions, will hopefully clarify these relationships and yield helpful insights.

Whether PRV infections lead to population-level impacts for sockeye in this system, or any wild Pacific salmon community, remains an important outstanding question. A decline in the infected proportion from the fry to smolt stages from two years of this study was observed but does not necessarily indicate mortality. If mortality is confirmed, one must also understand this mortality is above the level normally expected for sockeye fry in this system and how mortality could interact with other density-dependent determinates of juvenile sockeye growth and survival. In order to contribute to a population-level decline, PRV must be shown to cause mortality above the normal background that may be due to predators, fishing, and resource limitation (Krkošek, 2017). Assessing this scenario will require more detailed information of the current population estimates of different age classes of sockeye, age-specific mortality, and resource abundance. Experimental studies which address if and how and at what viral load PRV alters a host's competitive ability or predation risk could be paired with models of density-dependent population growth and predator-prey-parasite equilibrium models to generate helpful insights as to the higher order consequences of this infection for Pacific salmon populations (Krkošek et al., 2011).

Here I have provided the first description of the presence of the RNA virus PRV in a wild Pacific salmonid community, remote to the largest putative reservoir of PRV on

the west coast of North America –salmon farms. I have shown that the choice of molecular methods of inquiry into the study of microparasites affecting wildlife species – beginning with RNA isolation – is dependent on the tissue types being handled as well as the resources and goals of a laboratory. Though this research experienced limitations due to the logistic challenges of both field collections and laboratory processing of samples, through it I have identified several future research avenues into the dynamics, species and age class associations, and harm of PRV. The extent to which wild host communities influence PRV dynamics, spread, and evolution remains an important unanswered question. The results and hypotheses generated by this thesis are preliminary, but they highlight a pressing need for additional observational and experimental inquiry into this widespread virus.

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Appendix A.

Molecular methods used by the Atlantic Veterinary College and additional virus screening protocols and results

RNA isolation and quality control practiced by the Atlantic Veterinary College

Whole juvenile fish or pools of organs from individual adults were weighed and macerated to a 10% suspension w/v in phosphate buffered saline (PBS) with 10x antibiotics. Samples preserved in RNAlater were washed three times with PBS and homogenized prior to total RNA extraction. Total RNA was isolated using a modified total RNA extraction protocol that combines Trizol RNA extraction with column-based purification using the RNeasy mini Kit (Qiagen). Briefly, total RNA was isolated from samples using 1.25 ml of Trizol Reagent (ThermoFisher Scientific) and 375 μl of sample volume. The extracted RNA was eluted in 20–50 μl of nuclease-free water. The eluted RNA was tested immediately following quantitation or was stored frozen at -80°C until use (Kibenge et al., 2013). A NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) was used to assess concentration of total RNA by the absorbance at 260 nanometers (A260), and purity based on the 260/280 absorbance ratio. Quality was additionally assessed by RT-qPCR amplification of elongation factor one alpha (ELF-1α - GenBank accession number FJ890356) after DNase treatment and cDNA synthesis (see below). RNA was considered suitable for viral testing if amplification of ELF-1α yielded cycle threshold (Ct) values <30 (Kibenge et al., 2013).

cDNA synthesis and RTqPCR

One-step RT-qPCR was run on the LightCycler 480 (Roche Applied Science), version 4.0. The threshold cycle (Ct) was determined by use of the maximum-second-derivative function in LightCycler software. The OneStep RT-PCR kit (Qiagen) and Roche LightCycler 480 RNA master Hydrolysis Probe kit (Roche Diagnostics) were employed for all RT-qPCR reactions according to the manufacturer's specifications.

Sequences for primers and probes used for all viral screening and internal control are presented in Table A1.

Table A1. RT-PCR and RT-qPCR primers and probe sequences for all virus screening done by the Kibenge Lab at the Atlantic Veterinary College, University of Prince Edward Island.

Primer name (Author)	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-3')
Chinook ELF-1α (Kibenge et al. 2013)	GGTCACCACCTACATCAAGAAGA	CCAACCAGAGATGGGCACAAA	FAM-TGGCTACAACCCTGCCACTGTC-BHQ1
PRV L1 (Haugland et al. 2011)	CCCCATCCCTCACATATGGATA	GGTGAAATCATCGCCAACTCA	FAM-ATGTCCAGGTATTTACC-BHQ1
PRV L1 3'-1 (Kibenge et al. 2013)	CACTCACCAATGACCCAAATGC	TTGACAGTCTGGCTACTTCGG	
PRV L1 3'-2 (Kibenge et al. 2013)	CTGAACTGCTAGTTGAGGATGG	GCCAATCCAAACAGATTAGG	
PRV S1 (Kibenge et al. 2013)	GATAAAGACTTCTGTACGTGAAAC	GATGAATAAGACCTCCTTCC	
ISAV (Snow et al. 2006)	Sequences unavailable		
SAV (Hodneland & Endresen 2006)	CCGGCCCTGAACCAGTT	GTAGCCAAGTGGGAGAAAGCT	FAM-CTGGCCACCACTTCGA-MGB
PMCV (Haugland et al. 2011)	AGGGAACAGGAGGAAGCAGAA	CGTAATCCGACATCATTTTGTGA	FAM-TGGTGGAGCGTTCAA-MGB

The final concentrations of primers and probe for the housekeeping gene (ELF-1α) were 900 nM for each primer and 250 nM for the probe in a final volume of 25 μl. The following thermal cycling parameters were used: 1 cycle of RT for 3 min at 63°C, followed by denaturation at 95°C for 3 s, and 45 cycles of denaturation at 95°C for 15 s, annealing and detection at 60°C for 1 min and extension at 72°C for 1 s. Ct values above 30 and no Ct values were defined as degraded or negative and these samples were considered unfit for further testing if re-extraction and repeated RT-qPCR yielded the same results.

RTqPCR reaction conditions and confirmatory testing for piscine orthoreovirus

Screening for PRV L1 was done using primers and probes by Haugland et al. (2011), with the same reaction conditions used by Palacios et al. (2010), using 8 μl of template RNA. The following concentrations were used: 400 nM primer, 300 nM probe and 1.25 mM MgCl2. The following thermal cycling parameters were used: 1 cycle of RT for 30 min at 50°C followed by denaturation at 94°C for 15 min, and 45 cycles of denaturation at 94°C for 15 s, annealing at 54°C for 30 s and amplification and detection at 72°C for 15 s. Samples with Ct values below 40 and with an exponential amplification curve were considered positive. Ct values between 40.1 and 45 were considered tentative negatives, and a sample was deemed negative if there was no amplification of product (resulting in no Ct value) (Kibenge et al. 2013). A positive control from cell culture does not exist because of the difficulty in propagating PRV in a cell line, therefore a previously determined positive sample with low Ct value was used as a positive control. Confirmation of samples determined to be positive was done by end-point RT-PCR and Sanger sequencing. Samples with a Ct value below 40 were subjected to end-point RT-PCR targeting the 3' portion of the L1 genome segment. RT-PCR was done using the OneStep RT-PCR kit (Qiagen). Briefly, the reaction mixture contained 1 μl of total RNA, 4 μl of 5X Qiagen OneStep RT-PCR buffer, 0.8 μl of dNTPs, 0.5 μM (final concentration) of each primer pair, and 0.8 μl of QIAGEN OneStep RT-PCR enzyme mix in a final volume of 20 μl. Thermal cycling conditions were as follows: an initial RT cycle of 50°C for 40 min and 95°C for 10 min; then 40 cycles of 95°C for 30 s, 54°C for 30 sec, 72°C for 70 s; and a final extension cycle of 72°C for 10 min. Amplified products were analyzed by electrophoresis on 1% agarose gel for bands of the appropriate size (Kibenge et al. 2013).

Confirmatory sequencing was done by purifying genome segment S1 RT-PCR products using High Pure PCR Product Purification Kit (Roche) (Kibenge et al., 2013). PCR products were directly sequenced by ACGT Corporation (Toronto, Ontario, Canada) and the identity of sequence results were confirmed using a nucleotide search in Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (Altschul et al., 1990).

RTqPCR for additional viruses screened by the Atlantic Veterinary College

Infectious Salmon Anemia Virus

Screening for Infectious salmon anemia virus (ISAV) was conducted using primers and probe designed against ISAV segment 8 (Snow et al., 2006) and used a European genotype ISAV standard as a positive control. Samples are determined to be positive if Ct ≤ 34.20 ± 1.05 (Snow et al., 2006).

Salmon Alphavirus

Salmon Alphavirus (SAV) was screened for using Taqman PCR primers and probe designed for all alphaviruses targeting a region in the 5' end of the nsP1 gene (Hodneland and Endresen, 2006. Samples are determined to be positive if Ct ≤ 37.5 (Hodneland & Endresen, 2006).

Piscine Myocarditis Virus

The RT-qPCR assay for piscine myocarditis virus (PMCV) used the primer-probe set sequences developed by Haugland et al. (2011) targeting the open reading frame 2 (ORF2) of PMCV. The sample is considered positive when the fluorescence signal increases above threshold cycle (Ct), and if the Ct value is ≤35.

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Appendix B.

Results of surveillance for other salmon viruses

Fish tissues collected in Rivers Inlet were also screened for three other salmon viruses of concern: (Infectious Salmon Anemia Virus (ISAV), Salmon Alphavirus (SAV), and Piscine Myocarditis Virus (PMCV)) (methods described in Appendix A). Sockeye were screened for Salmon Alphavirus (SAV), and Infectious Salmon Anemia Virus (ISAV), in 2014-2016, although only smolt-stage sockeye were screened for these other viruses in 2016. Trout were screened for SAV and ISAV in 2014, but not thereafter. Chinook salmon were screened for SAV, ISAV in 2014 and 2015, and PMCV in 2014. Eulachon were screened for ISAV in addition to PRV in 2015.

Infectious Salmon Anemia Virus

In sockeye salmon, the overall proportion testing positive for ISAV in 2014 was 1.2% (95% CI: 0.13-5.6%) (Figure B1). In 2015, this proportion was slightly higher at 3.1% (1.2-6.7%) (Figure B2), and in 2016, 0.88% (0.09-4%) tested positive for ISAV (Figure B3). The ISAV positive proportion was driven by sockeye fry in 2014 (3.8%, 0.4-16.6 %); smolts (3.8%, 1.1-9.7 %) and adults (4.9%, 1.0-14.7 %) in 2015; and smolts (0.88, 0.09-4%) in 2016 (Figures B1-B3).

No trout tested positive for ISAV in 2014 (0, 0-12.8%) (Figure B1) and none were screened for this virus in 2015 or 2016. Likewise, none of the spawning Chinook sampled in 2014 tested positive for ISAV (0, 0-11%; Figure B1), although 5% (0.54-21.1%) of spawning Chinook tested positive for ISAV in 2015 (Figure B2). ISAV was not assessed for Chinook in 2016. Wanukv (Rivers Inlet) eulachon were only assessed for ISAV in early 2015 (Figure B2) and 7.4% returned positive results (95% CI: 1.6-21.7%). Klinaklini River eulachon showed a 5.3% positive rate for ISAV (95% CI 0.6-22%) (not shown).

Salmon Alphavirus

SAV was assessed in sockeye salmon in all three years, although in 2016 only smolts were tested. In 2014, 1.6% (0.17-7.1%) of all sockeye tested positive for this virus (only adult sockeye returned positive results) (Figure B1), while in 2015, no positive tests were found (0-1.6% true population proportion; Figure B2). In 2016, 0.88% (0.095-4.0%) of sockeye smolts tested positive for SAV (Figure B3).

Resident trout in 2014 had a higher raw proportion positive for SAV than sockeye at 11.1% (2.4-31.1%) (Figure B1) and were not screened for this virus in 2015 or 2016. Chinook screened for SAV in 2014 did not yield positive results (FigureB1). The true SAV positive proportion was estimated to be 0-11.1% in 2014 (Figure B1) and 0-11.7% in 2015 (Figure B2). Eulachon were not assessed for this virus.

Piscine Myocarditis Virus

Only spawning Chinook from 2014 (n=20) were screened for PMCV and yielded a positive proportion of 4.8% (0.52-20.1%).

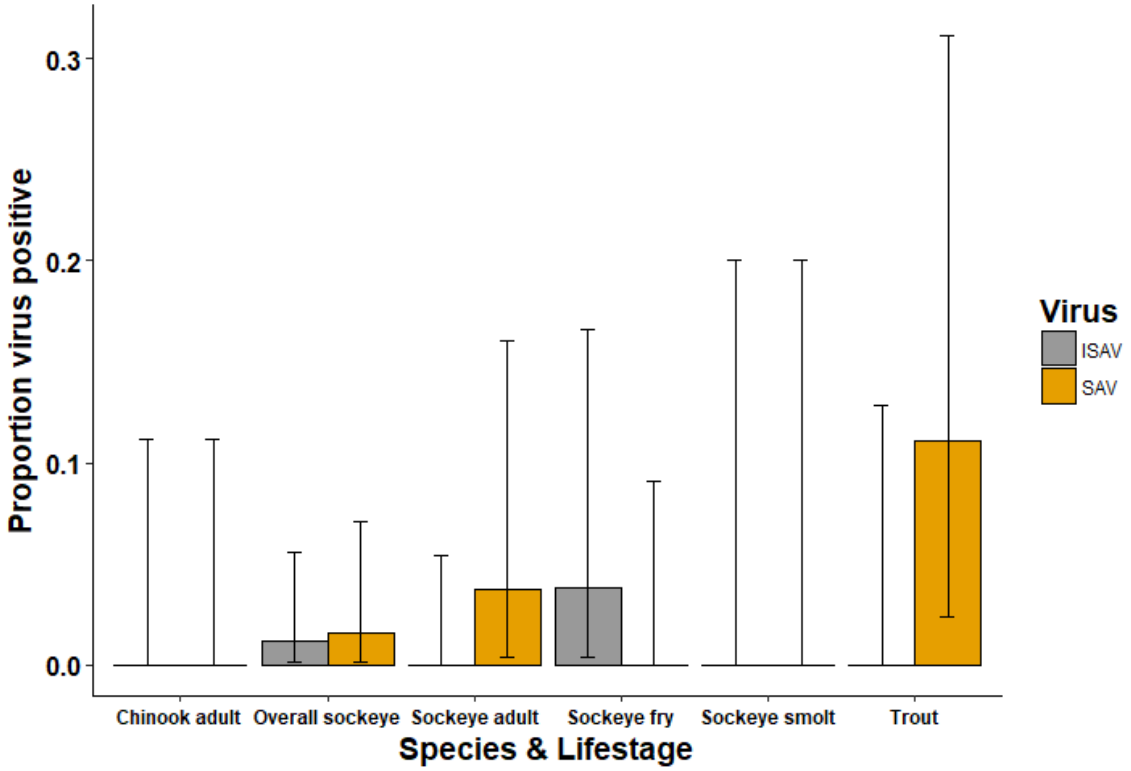

Figure B1. **Proportions of salmonids testing positive by RT-qPCR assay for ISAV and SAV in 2014. 95% confidence limits calculated with Jeffrey's interval for small sample sizes.**

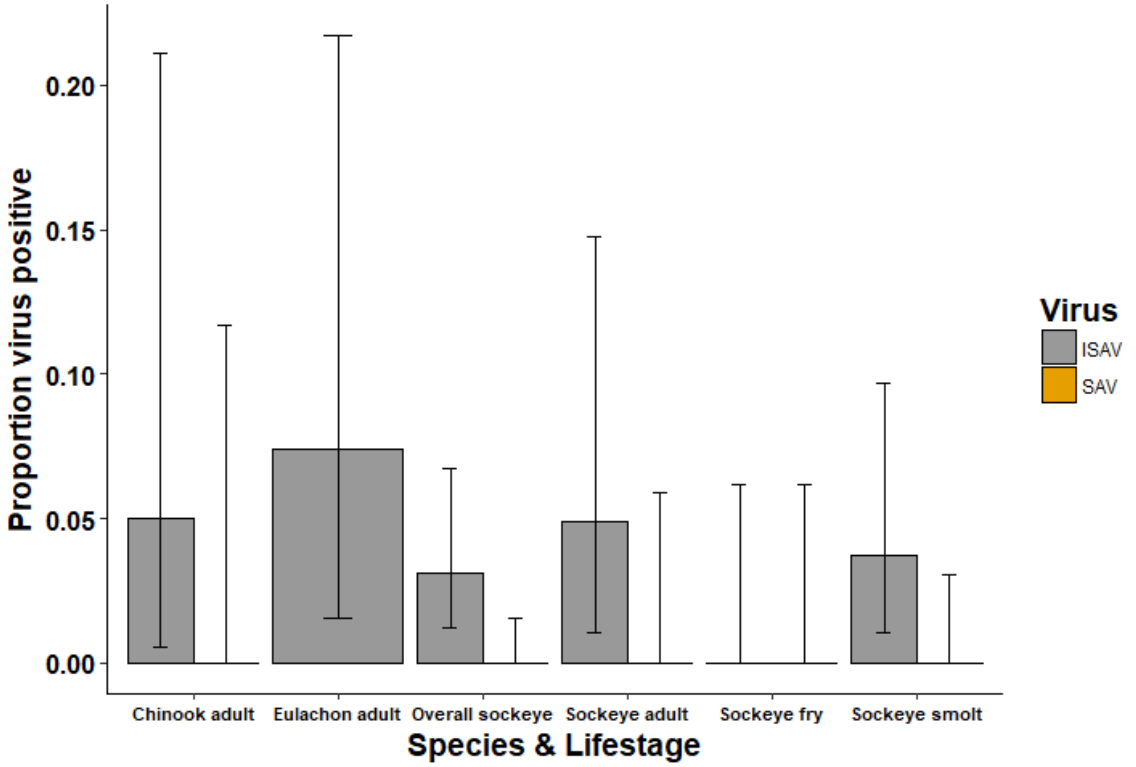

Figure B2. **Proportions of salmonids and eulachon testing positive by RT-qPCR assay for ISAV and SAV in 2015. 95% confidence limits calculated with Jeffreys interval for small sample sizes.**

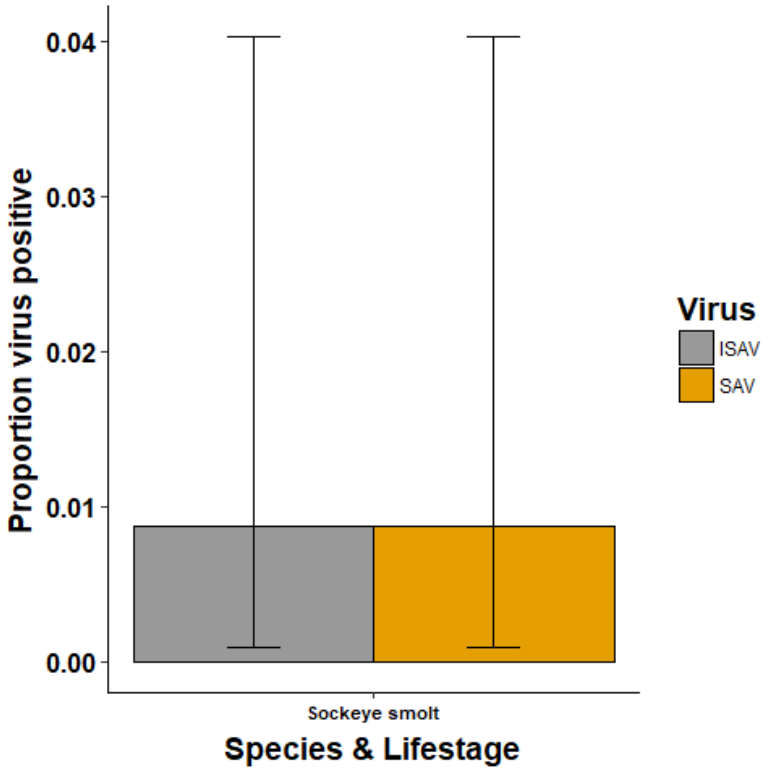

Figure B3. **Proportions of sockeye salmon smolts testing positive by RT-qPCR assay for ISAV and SAV in 2016. 95% confidence limits calculated with Jeffreys interval for small sample sizes.**

Appendix C.

In-house molecular methods development

Gene-specific primer design

Gene-specific cDNA synthesis primers were designed to flank qPCR primer regions on both PRV sense and antisense strands. This design enhanced sensitivity of qPCR assay by doubling the number of detectable PRV transcripts as well as specifically synthesizing cDNA for only the desired primer annealing sites. Three pairs of gene-specific cDNA primers were designed for PRV; two sets flanking qPCR primer annealing sites on the L1 genome segment (an in-house designed primer/probe site, and the primer/probe site designed by Palacios et al. (2010)) and one set flanking the qPCR annealing site on genome segment S1 designed by Finstad et al. (2014). These three sites were chosen to allow the validation of RTqPCR PRV positive results using multiple primers for PRV. Primers were designed by accessing PRV sequences for the genome segments of interest through GenBank. Sequence files were assembled in BioEdit sequence alignment software (Hall, 1999) and uploaded to TCoffee Multiple Sequence Alignment (MSA) server (Notredame, Higgins, & Heringa, 2000). A MSA was done to identify highly conserved regions of sequence among 18 different isolates of PRV genome segment L1 and 14 different isolates of genome segment S1 (Table C1). This alignment of several PRV isolates was done to identify conserved primer binding sites. MSA output was viewed and trimmed in Jalview sequence alignment visualizer (Clamp et al., 2004)(Clamp et al., 2004), and exported to BioEdit to generate a consensus sequence. This consensus sequence was uploaded to the online Primer3 (Untergasser et al., 2012) primer selection tool. Primer3 generated several primer pair options and the highest ranked pair of each gene-specific primer was ordered through Eurofins Scientific (Table C1).

Primers for RT-qPCR were designed as described above with the additional specification that they were located within the regions reverse-transcribed by the gene-specific cDNA primers. Suitable primers were validated through the screening of known positive material isolated from Atlantic salmon head kidneys.

Table C1. PRV gene specific primers used for cDNA synthesis in-house at Simon Fraser University.

Primer name	Forward Primer (5'-3')	Reverse Primer (5'-3')	GenBank sequences used for alignment
PRVL1_SHout 1	ACCCCGTTACCAGTTCA ACC	AGAAAGGAGCGAAACGA GCA	JX502839, JX502838, JX502837, KC795565, KX851983, KX851982, KT429750, KT429740, KT429730, KC795585, KC795584, KC795583, KC795582, KC795581, KC795579, KC795577, KC776256, KC715679
PRVL1_SHout 3	CCGGGTTCTCTGGTCT CAAC	TCGGACAACAGCTTCCA CTC	
PRVS1_SHout 3	GAGATGACACAGCTGC AGGA	TTCCTTGCGTCATTTTGA TG	KT456503, KT456504, KC473452, KU131593, KU131592, KT456500, KC473454, KU131591, KT429756, KT429736, JN991006, GU994022, KX844956, KC795571

Complementary-DNA (cDNA) synthesis

A 1x reaction mixture for cDNA synthesis contained 2 μg (9.1 μl) RNA, 0.5 μM oligoDT primers, 10 nM of each gene-specific primer and 1 μl of deoxynucleic triphosphates (dNTPs) in a final volume of 14.5 μl. This mixture was heated to 65 °C for 5 mins and incubated on ice for one minute. To this, 4 μl of 5x reverse transcriptase buffer, 0.5 μl RNase OFF RNase inhibitor, and 1 μl OneScript Reverse Transcriptase was added. Synthesis was performed by incubating tubes for 50 mins at 42 °C. Reaction was stopped by incubating at 85 °C for 5 mins and chilling on ice.

Reaction conditions for Brightgreen RT-qPCR assays

A 1x BrightGreen assay contained 5 μl BrightGreen master mix, 0.15 μl of each 10 μM primer, 0.7 μl nuclease-free water, and 4 μl diluted 1:3 cDNA. BrightGreen assays had the following reaction conditions: initial denaturation at 95 °C for 10 minutes, and 45 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 60 seconds.

Reaction conditions for IDT Prime Time probe-based RT-qPCR assays

A 1x reaction mixture contained 5 μl of Prime Time master mix, 0.25 μl of forward and reverse primers (final conc. 250 nM), 0.3 μl Taqman dual-quenched FAM labelled probe (150 nM), 1.2 μl nuclease-free water, and 3 μl of diluted 1:3 cDNA. Reaction conditions were an initial denaturation/polymerase activation step at 95 °C for 3 minutes, and 45 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 1 minute.

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Appendix D.

Antiviral gene expression of 2016 sockeye smolts

Antiviral gene expression assays of sockeye smolt relative Mx expression

I used antiviral innate immunity gene expression as a proxy for viral impacts to smolts as numerous publications have shown that mounting an innate immune response is costly to both vertebrates and invertebrates (e.g., as reviewed in Lochmiller & Deerenberg, 2000). In order to assess the antiviral gene activity of sockeye smolts, I collected a balanced sample of out-migrating smolts from the head of Rivers Inlet, and six weeks later, from regions close to the mouth of the inlet in 2016, constituting a before-and-after sample of the same population. Garver et al. (2016) showed that juvenile sockeye salmon may maintain persistent PRV infections for 41 weeks. Thus, it is unlikely that fish would have recovered from an infection between the time of the first sampling and that of the second. I drew blood for gene expression and viral screening from a random subsample of these smolts and extracted RNA from this blood using the RNeasy method described in Chapter 2. Using BrightGreen relative RT-qPCR assays, as described above, I targeted the Mx1 gene (Mx), an interferon-induced gene in the antiviral JAK/STAT innate immunity pathway which is one among a panel of genes which is shown to be strongly related to viral disease development by Miller et al. (2017).

The expression of Mx was normalized against expression of the constitutively expressed housekeeping gene β-actin using the negative delta Ct method (Schmittgen & Livak, 2008). β-actin was chosen as an appropriate housekeeping gene after Dahle et al. (2015) found that its expression remained stable in experimentally infected Atlantic salmon in comparison to uninfected controls, while other typically chosen housekeeping genes (EF1a and 18s rRNA) are systematically altered in their expression by PRV infection.

For my analysis, relative Mx expression was first assessed for correlation with PRV expression intensity in positive screening smolts. To test for differences in the average relative expression of Mx between smolts collected near the head of Rivers Inlet (recent ocean entry) with those collected near the mouth of Rivers Inlet (longer marine

duration) I used a Welch's T-Test with unequal variances using the 't.test' function in R (R Core Team, 2018).

To test whether smolts fighting a viral infection would be trading off resources that could be used for growth and development in favour of mounting an antiviral immune response, I tested whether smolts with a relatively higher overexpression of Mx would be smaller in terms of their fork length (mm) than those fish with no antiviral gene expression and/or PRV infection. To test for an effect of Mx expression on growth an ANCOVA model was fit to sockeye smolt relative Mx expression data using fork length (mm) as the continuous predictor variable. 'Capture location' was included as a covariate in the initial model to test for differences in this relationship between the inlet head and inlet mouth sockeye smolts. Model fitting was carried out using the R functions 'aov' and 'lm' and residual vs. fork length plots were examined to determine whether model assumptions were being met. All statistics were carried out using R open source statistical software (R Core Team, 2018).

Results & Discussion

Changes in gene expression of sockeye salmon smolts during down-inlet migration

The expression of Mx was assessed for a subsample of sockeye smolts for which blood samples were collected in the spring of 2016. None of these fish tested positive for PRV in RT-qPCR screening by either the AVC or in-house at SFU (30 smolts were screened by both), but expression of Mx measured by RT-qPCR was found to be differentially expressed relative to β-actin among smolts.

Figure D1 shows the distribution of relative expression values for 2016 sockeye smolts captured at Rivers Inlet head and mouth. No difference was found between the average relative expression of Mx between early versus late marine smolts, with mean ± 95 % CI relative Mx expression of $7.5x10^{-3} \pm 4.5x10^{-3}$ for inlet head smolts and $3.7x10^{-2} \pm 5.5x10^{-2}$ for inlet mouth smolts ($t = -1.1518$, $p > 0.05$; Welch's two sample t-test with unequal variances). One smolt from the group captured at the inlet mouth had a Ct value for β-actin which was 5 cycles below the group average, giving it a relative Mx expression nearly 200-1000x higher than all other smolts assessed. This was

determined to be an outlier due to RNA degradation, poor efficiency of reverse transcription, or possibly a failure of the RT-qPCR for β-actin and was excluded from figures and all subsequent analysis.

An ANCOVA linear model including both fork length (FL) and the interaction between FL and the covariate 'capture location', a proxy for marine residence time, was fitted to relative Mx expression data for both groups of sockeye smolts:

*Relative Mx Expression = FL + Capture Location + FL*Capture Location*

This model had a significant interaction term ($p = 0.03$, Table D1), suggesting that a single linear model does not describe the relationship between relative Mx expression and smolt length in both groups. Figure D2 shows the residuals for this model plotted against the fork length of both groups of smolts. It appears that variance in residuals is greater for fish with fork length above approximately 85 mm for the inlet head smolts, this is also primarily the lower end of the range of the inlet mouth smolts. Mx expression is likely limited by resources and a mechanistic explanation for this pattern is provided in the discussion section. This also further supports the fitting of separate models for each of the two groups.

A highly significant linear relationship was found between inlet head smolt relative Mx expression and fork length ($F_{1,13}= 9.358$, $p < 0.01$, adjusted $R^2 = 37.4$). This shows that larger smolts from the inlet head were expressing higher levels of innate antiviral gene activity than smaller smolts. Table D2 displays the coefficients and significance for this model. In contrast, a linear model fit to the smolts collected from the inlet mouth found no relationship between relative Mx expression and fork length ($p > 0.05$) (Table D3). Figure D3 shows a scatterplot of relative Mx expression against fork length by smolt group. Trend lines are fitted for each group separately to display the trends suggested by these linear models.

While I was not able to test a relationship between antiviral gene expression and PRV infection status in out-migrating sockeye salmon smolts from 2016, I did find that sockeye salmon smolts may experience a period of heightened vulnerability to viral infections during their early marine residence which may be related to critical size and environmental resources. Early marine sockeye salmon smolts captured near the head of Rivers Inlet appeared to have a positive relationship between antiviral gene

expression and fork length, which was not present for smolts captured lower in the inlet (Figure D3). Rivers Inlet has a strong freshwater influence for nearly two thirds of its length, and has been found to be very resource-poor in terms of zooplankton, compared to the lower inlet that has a strong oceanic influence (Buchanan, 2006). Such a paucity of forage in the earliest marine environment of the inlet could prevent sockeye salmon smolts without sufficient resources from mounting an effective antiviral immune response due to the principle of allocation (Downs & Stewart, 2014). This principle suggests that when resources are limited, a trade-off will occur in energy investment between expensive, competing processes such as immune function, growth, and survival (Lochmille, 2000). A critical fork length of 80-82 mm may exist for food-limited inlet head smolt sockeye salmon, above which the relationship between size and antiviral immune function begins to break down (Figure D3). For lower inlet smolt sockeye salmon, no such relationship appears to exist, even for smolts which fall below the fork length threshold seen in the upper inlet smolts, which may reflect the relatively higher abundance of zooplankton resources and the lack of necessity to make trade-offs. This has implications under climate change scenarios, which for this region are predicted to be large swings in precipitation, freshwater runoff, winds and coastal storms (Okey et al., 2014). As these factors are strong influences on plankton bloom phenology, they may lead to the decoupling of phytoplankton bloom and zooplankton community development from sockeye salmon smolt out-migration timing (Tommasi et al., 2013). This may have the compounded effect of increasing sockeye smolt vulnerability to infection, morbidity, and mortality, as it can be inferred that food limitation may affect the ability of smolts to effectively mount immune responses to viral pathogens (Miller et al., 2014). Research into the Rivers Inlet plankton community already shows large interannual variability in the phenology of the spring phytoplankton bloom (Tommasi et al., 2013), placing urgency on more intensive research to quantify the relationships between phytoplankton bloom dynamics, temperature and salmon health status in Rivers Inlet.

Table D1. Sum of Squares table for the full Analysis of Covariance model (*Relative Mx Expression = FL + capture location + FL*capture location*) (AS29 outlier removed)

	DF	Sum of Squares	Mean Squares	F value	p
Fork length	1	0.0001135	0.0001135	1.037	0.3186
Capture location	1	0.0000252	0.0000252	0.231	0.6355
FL*Capture location	1	0.0005764	0.0005764	5.270	0.0307
Residuals	24	0.0026252	0.0001094		

Table D2. Coefficient estimates and significance for the linear model for relative Mx expression by fork length (mm) for inlet head smolts.

Coefficients	Estimate	Std error	t value	P (>|t|)
Intercept	-0.052	0.02	-2.662	0.01955
Fork Length	0.00077	0.0003	3.059	0.00914

Table D3. ANOVA table for linear model of relative Mx expression of inlet mouth smolts vs fork length (mm).

Coefficients	Estimate	Std error	t value	P (>|t|)
Intercept	0.0606077	0.0479832	1.263	0.233
Fork Length	-0.0005292	0.0005186	-1.020	0.329

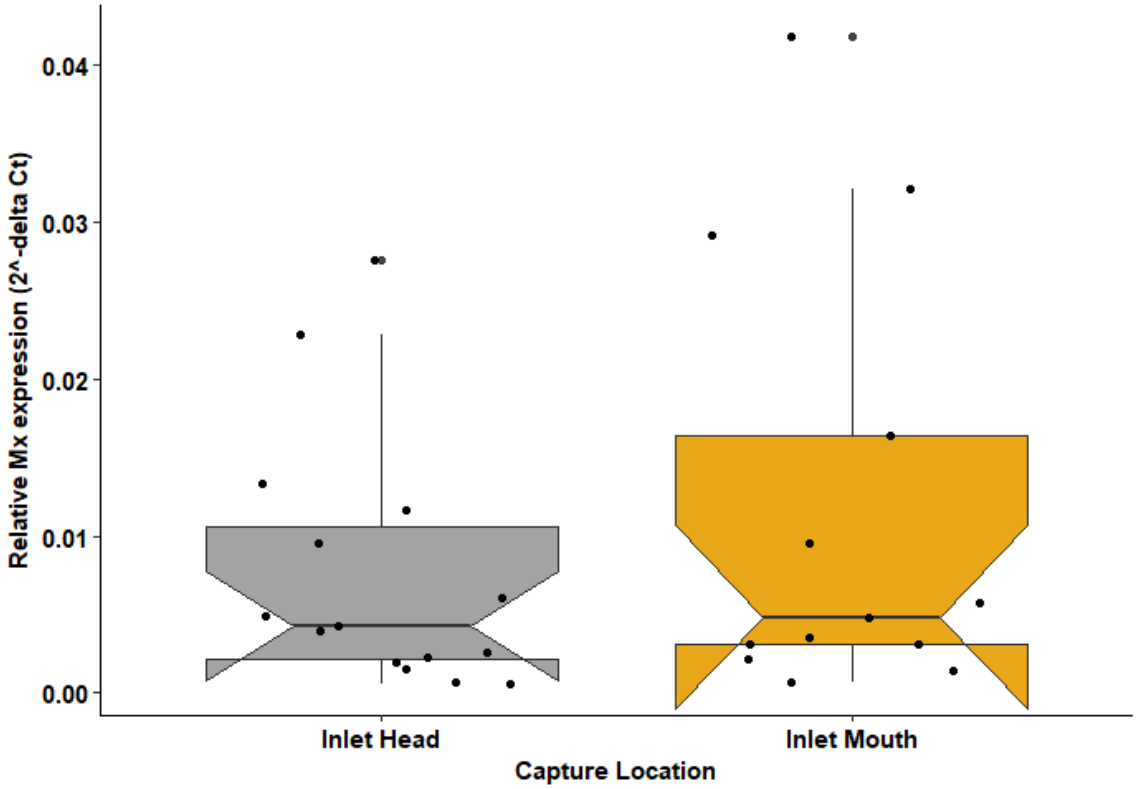

Figure D1. Distribution of relative Mx expression levels for sockeye smolts captured at Rivers Inlet head and mouth. Boxplots represent the interquartile range of antiviral expression values while whiskers extend to the most extreme data point within one interquartile range of the median. Notches represent a 95 % confidence interval of the median, and if they do not overlap there is strong evidence of a difference in medians. Points are raw data.

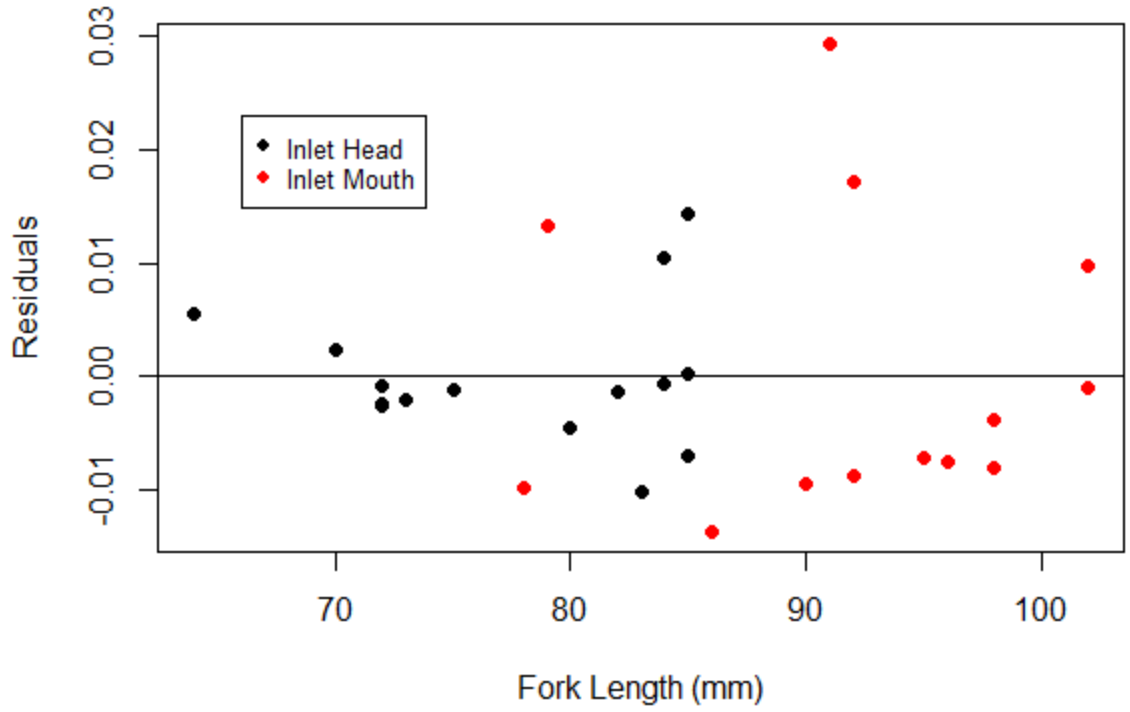

Figure D2. **Residuals from the fit of the full Analysis of Covariance model for sockeye smolts captured at the inlet head and inlet mouth.** Residuals for inlet head smolts appear to increase in variance about zero at around 85 mm fork length.

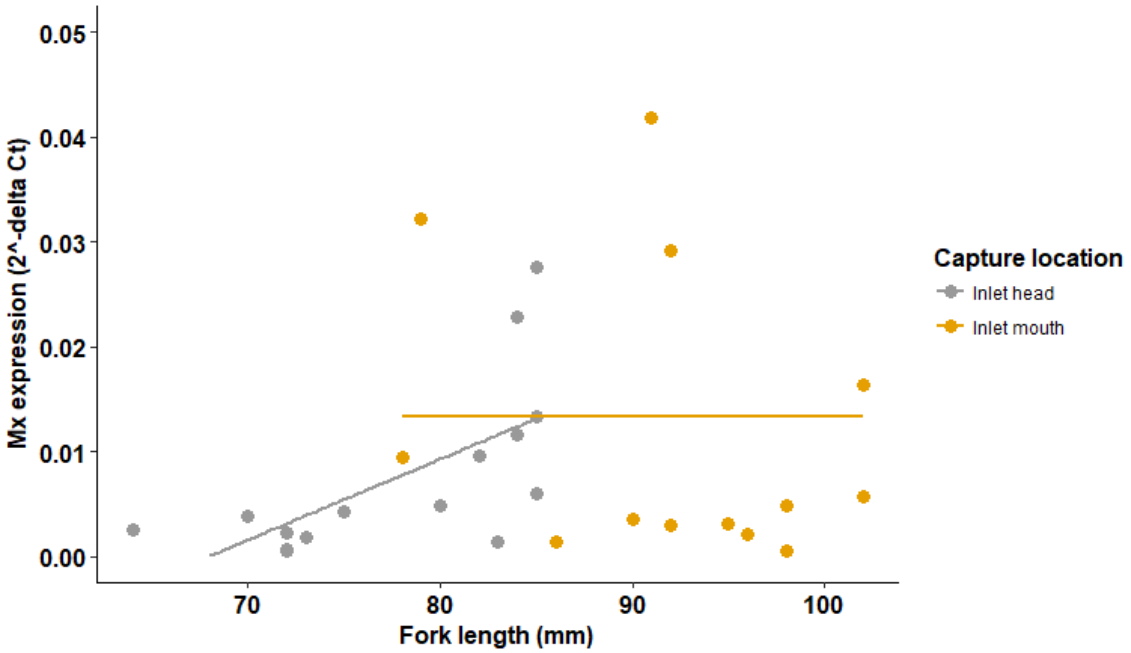

Figure D3. Relationship between relative Mx expression and size (fork length) in sockeye smolts collected at Rivers Inlet head and mouth. A positive linear relationship exists for relative Mx expression and fork length in Inlet head smolts, while no relationship exists between these variables in smolts collected at the inlet mouth.

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