Impacts of cumulative thermal and fishery stressors and infection development on the health and survival of adult Pacific salmon during freshwater residence

by

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B.Sc., University of Massachusetts, Amherst, 2007
M.Sc., University of Massachusetts, Amherst, 2012

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Abstract

Cumulative stressors influence the infection development, health and survival of wild Pacific salmon (*Oncorhynchus* spp.). Infectious disease is generally assumed to be the ultimate cause of death of wild adult salmon, but empirical evidence demonstrating links between infections and early mortality (i.e., prior to spawning) is lacking, especially as a function of cumulative migratory stressors. The influences of high river temperature and fishery capture and release on infection development and early mortality was explored in three Pacific salmon species. Adults were captured at river entry and held in freshwater tanks for the duration of river migration (days–weeks). Tank temperatures reflected either optimal (cool), warm (climate change scenario), or dynamic (changes in river temperature, behavioral thermoregulation) thermal conditions during migration. A subset of fish in all temperature groups was treated with a fishery bycatch release simulation (gillnet entanglement, air exposure) at the start of the holding period. We tracked shifts in physiology, immune activity and multiple infections using repeated biopsy (gill, blood) and molecular tools. Laboratory experiments were complimented by a telemetry study to assess impacts on behavior in the river. Novel application of high-throughput qPCR on nonlethally-sampled gill measured infections (bacteria, viruses, protozoa) concurrently with host immune gene expression, and was complemented by blood plasma chemistry to assess physiology. Ecologically relevant high temperatures increased mortality, infection development and stress metabolites and impaired host osmoregulatory function. Fishery stress reduced survival, especially after long entanglements and at high temperature, which reduced the capacity of individuals to resolve stress and infections. Females were more drastically affected, and mortality was delayed by more than a week. Fish with heavy infections in the river migrated more rapidly but traveled less distance. Sublethal effects of stressors included reduced migration rates and suppressed maturation indices that could delay maturity and extend river residence. Finally, river-exposed fish carried heavier infections and died sooner than those that bypassed the lower river, suggesting a causal influence of infections on early mortality. These findings support river-derived infections as causal factors contributing to the early mortality of adult Pacific salmon in fresh water and clarify its mechanisms, which comprise influences of multiple infections, sex, species, water temperature and fishery stress.

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Dedication

To my husband and daughter.

Chapter 1 - Introduction

Continued declines in Pacific salmon (*Oncorhynchus* spp.) productivity (i.e., recruits per spawner) have raised concerns among scientists and managers regarding the factors that contribute to the mortality of wild fish, especially in the context of cumulative stressors (Miller *et al.*, 2014). Climate-driven shifts in hydrology have altered the thermal experience of wild salmon (Patterson *et al.*, 2007; Petersen and Kitchell, 2001), resulting in a number of detrimental impacts that include impaired swimming capacity, decreased aerobic scope, altered phenology (e.g. egg incubation, fry emergence, migration timing) and reduced migration success of adult spawners (Eliason *et al.*, 2013; Martins *et al.*, 2011, 2012a; Reed *et al.*, 2011). Thermal stress can also compound other stressors experienced by wild salmon, such as capture-and-release from fisheries (Gale *et al.*, 2013; Raby *et al.*, 2015). Fishery "bycatch" or "discards" refers to catch that is not the target species of the fishery and must be released; in places with heavy fishing pressure and co-migration of multiple salmon species, bycatch can be a common occurrence with measurable impacts on population productivity (Baker and Schindler, 2009).

A key factor intrinsic to the survival of all animals, especially in a stress context, is infectious disease, which is often assumed to be the ultimate cause of early mortality of Pacific salmon (i.e., before spawning), in addition to its natural role in the senescence processes of semelparous fishes that die soon after spawning (Gilhousen, 1990; Groot and Margolis, 1991; Vander Wal *et al.*, 2014). Linking thermal and fisheries stressors and infectious disease processes in wild salmon populations will provide insight into how salmon productivity may change under projected river conditions and fishing regulations,

augmented by an improved understanding of the mechanisms contributing to early mortality.

Based what is known about the multiple infections carried by wild adult salmon given recent advancements in molecular tools (Miller *et al.*, 2016), this thesis explores how multiple stressors and infectious agents dictate the survival of adult salmon during freshwater migration. Although productivity declines among several salmon species and populations are likely attributable to impacts affecting all life stages, adult salmon were used as the focus of this research to comprise population level impacts that can occur due to losses of adult spawners, including those that die prior to arrival at spawning grounds (*en route*) and after arrival but without spawning (prespawn).

Adult Pacific salmon support a fishing industry and culture that are iconic components of economic and indigenous wellbeing on the Canadian west coast, undeniably threatened by the impacts of climate change (Jacob *et al.*, 2010; McDaniels *et al.*, 2010). If regulations for fishing practices are to be adapted to account for changing river hydrology or high mortality of released catch, a firm knowledge base must support those decisions, with transparent science that clearly demonstrates why and how released bycatch is impacted, beyond simply stating mortality estimates. Furthermore, inclusion of these stakeholder groups within the scientific process can improve the research itself as well as potential management outcomes (Young *et al.*, 2013). This thesis and the research conducted herein were designed to be inclusive and the output disseminated widely among user groups via presentations and workshops with First Nations and government forums in addition to publication in scientific journals, magazines, and social media. However, the content of this thesis and its chapters are structured in a classical scientific

method framework, where each chapter asks a question and proposes a hypotheses which builds on the findings of the previous chapter. Its four data chapters (2-5) describe experiments and their associated findings, collectively producing a story with a beginning, middle and end. The sixth chapter synthesizes these results to summarize the findings and propose further work that could build upon the knowledge gained. This first chapter provides background information used to structure the study design for the first and subsequent data chapters.

1.1 Pacific salmon life history

Five species of Pacific salmon inhabit the west coast of Canada: sockeye (*O. nerka*), Chinook (*O. tshawytscha*), coho (*O. kisutch*), pink (*O. gorbuscha*) and chum (*O. keta*). Pacific salmon begin their lives as eggs in fresh water, hatching into alevin with a yolk sac that that supports them until they emerge from the gravel as fry in search of food (Groot and Margolis, 1991). Fry may rear in fresh water for up to several years or immediately leave for the estuary, depending on the species and population; this variability very likely contributes to variation in infectious agents accumulated and carried to adulthood and potentially confers pathogen resistance later in life (Altizer *et al.*, 2011; Sutherland *et al.*, 2014; Zwollo, 2012). When fish are ready to leave fresh water, their physiology shifts to prepare for life in seawater; it has been recently shown that immune gene regulation also changes in preparation for smoltification in rainbow trout (steelhead; *O. mykiss*), potentially directed toward a broader array of pathogens (Sutherland *et al.*, 2014). Infectious agents have also been shown along with host immunity to influence the success of out-migration (Jeffries *et al.*, 2014a), with less

healthy individuals generally lost or consumed before they even reach the ocean (Furey, 2016).

Salmon do the majority of their growing at sea and depending on species and population will return after 1–5 years to freshwater spawning grounds. During the spawning migration, adult salmon are the target of fisheries in the ocean and rivers, with different species and populations traversing through a gauntlet of fishing gears as they navigate toward natal waters. In the Fraser River, Canada's largest salmon producing watershed, gillnets are one of the most commonly used gear types, though a wide variety are employed by recreational, commercial, ceremonial and subsistence fisheries, including tangle nets, beach seines, rod and reel, dip-nets and more. Adult salmon cease feeding prior to leaving the marine environment, relying on endogenous energy stores to fuel migration, spawning and nest defense (Kiessling *et al.*, 2004; Rand and Hinch, 1998). River migration conditions depend on timing and distance and may include challenging flows in constricted channels (e.g. Hells Gate, Fraser River, British Columbia [BC], Canada) or high temperatures during summer months or low runoff years that will likely prove more challenging in future decades, with lethal and sublethal impacts on migrants (Fenkes *et al.*, 2016; Ferrari *et al.*, 2007; Patterson *et al.*, 2007).

Upon arriving at spawning grounds, females dig a nest (redd) in the gravel and wait for courting males; courtship practices and aggressive behavior have been described in sockeye salmon, which can cause injury and modulation of sex and stress hormones (Hruska *et al.*, 2010). These interactions likely influence the development of infections, as both cortisol and testosterone can act as immune suppressants (Slater and Schreck, 1993; Tort, 2011). Cortisol levels of female salmon are generally higher than those of

males, but changes in cortisol levels can be more rapid in males (Kubokawa *et al.*, 2001); sex-specific differences in cortisol regulation likely influence pathogen defenses during spawning through the immunosuppressive effects of cortisol (Tort, 2011). Males fertilize eggs as they drop out of the water column and into the nest, which is then covered with substrate. Adults die soon after spawning, leaving their nutrients and infectious agents behind (Cederholm *et al.*, 1999; Kent *et al.*, 2014).

1.2 Stress responses and stressors of wild Pacific salmon

When fish encounter a stressor, primary, secondary, and tertiary responses are elicited that are intended to overcome and manage the threat to eventually regain homeostasis (Barton and Iwama, 1991). However, these responses can prove maladaptive if the animal cannot resolve the stress, which is more typically the case with chronic stressors (Pickering and Pottinger, 1989). The first step in a stress-induced neuroendocrine cascade is a set of primary responses that includes the release and synthesis of catecholamines like adrenaline and corticosteroid hormones like cortisol that make energy available for the “fight or flight” response (Wendelaar Bonga, 1997). Secondary responses are focused on oxygen delivery and fuel mobilization, such as respiratory and cardiovascular shifts and glucose release (Wendelaar Bonga, 1997). These responses potentially trigger osmotic imbalance due to increased membrane permeability (Wendelaar Bonga, 1997); some acute stressors have also been associated with immune cell redistribution in mammals to affected areas of the body (Dhabhar, 2002), but this is largely unexplored in fishes. If anaerobic metabolism is recruited to escape the stressor (i.e., fishery capture, air exposure, exhaustive swimming), concentrations of metabolites such as lactate increase in the body, which can be measured in blood plasma (Davis, 2002). Tertiary responses

may include recovery (i.e., regained homeostasis) or suppressed biological functions, including immune activity and maturation, due to enhanced cortisol circulation or energy exhaustion (Tort, 2011; Baker *et al.,* 2013; Mateus *et al.,* 2017). The capacity to which adult Pacific salmon can resolve stress accrued during spawning migration will likely dictate their rate of infectious disease development given that freshwater migration and reproduction (maturation and spawning) are fueled by endogenous energy stores (Groot and Margolis, 1991; Rand and Hinch, 1998) and high metabolic costs are associated with stress resolution (e.g., metabolite clearance, restoration of osmotic balance; Wendelaar Bonga, 1997). Environmental stress is highly correlated with infectious disease outbreaks in fish, suggesting that immune defenses are weakened by stress (Snieszko, 1974). Characterizing relationships between stress responses, stress resolution, immune activity, and infection development will clarify the mechanisms of mortality of adult Pacific salmon during freshwater migration. Conducting these assessments at different levels of biological organization (e.g., molecular, organismal, behavioral) will provide a comprehensive portrait of how stressors, infections and hosts interact to influence host survival outcomes.

Two major stressors affecting adult Pacific salmon are high river temperature (Caudill *et al.*, 2013; Martins *et al.*, 2011, 2012a) and fishery capture and release (Baker and Schindler, 2009; Donaldson *et al.*, 2011, 2012; Raby *et al.*, 2015). Cumulative effects of these stressors are of further concern, but rarely quantified (but see Gale *et al.,* 2011, 2013; Havn *et al.,* 2015). Bycatch is a frequent phenomenon in fisheries, with discards estimated to comprise between 10–40% of total marine catch worldwide (Davies *et al.*, 2009; Zeller *et al*., 2018). Regarding fishery capture and release, here I refer to bycatch

as fish that are not the target of the fishery and must therefore be released. The key assumption in releasing bycatch is that they will survive to spawn; however, this is not always the case, as injuries and stress accrued during the capture and release process can cause mortality, either immediately (i.e., within minutes–hours) or after a period of delay (days–weeks; Chopin and Arimoto, 1995; Davis, 2002). The impacts of fishery capture, as with most stressors, are often context-dependent, including aspects like species (Cooke and Suski, 2005), sex (Donaldson *et al.*, 2014), gear type (Donaldson *et al.*, 2011), recovery conditions (Robinson *et al.*, 2013) and water temperature (Gale *et al.*, 2013). Recent reviews have highlighted these specificities and attempted to provide information to managers on how best to utilize available science (Patterson *et al.*, 2017a,b; Raby *et al.*, 2015).

While immediate mortality following capture and release from fisheries is likely associated with cardiac collapse or anaerobiosis, delayed mortality is more likely associated with infection development. When fish encounter fishing gear, an initial "fight or flight" response is initiated, with the release of catecholamines that increase ventilation rates, oxygen transport capacity and blood glucose levels, followed by corticosteroids like cortisol to control hydromineral balance and energy metabolism (see above; Wendelaar Bonga, 1997; Davis, 2002). Different gears will also produce unique injuries: gillnets generally cause epithelial damage to gills and skin, scale and mucus loss, and potentially suffocate fish if ventilation is prevented; beach seines may cause minimal damage, but captured fish may experience mucus and scale loss on the net or incur gill damage due to air exposure if beached; hook and line fishing causes hook injury, which can occur in the mouth or gut if swallowed and, depending on how the fish is played and

landed, may also cause scale and mucus loss on substrate or gill damage due to handling or air exposure (Chopin and Arimoto, 1995; Raby *et al.*, 2015). Fishing-associated injury of released catch is quite common and has been correlated with survival and linked to sub-lethal effects (Baker *et al.*, 2013; Baker and Schindler, 2009; Casselman *et al.*, 2016). Furthermore, these injuries provide opportunities for infection by fungi and microparasites, as protective mucus, scales, and skin layers are removed (Mateus *et al.*, 2017). Collectively, an incidence of capture and release can be the most strenuous experience of a fish's life and, unfortunately, can often be followed by predation (Raby *et al.*, 2014, 2015). Indeed, recovery following capture is important for fish to regain homeostasis; conditions not conducive to recovery, such as high water temperature or challenging flows, can inhibit the clearance of metabolites and cause physiological impairment that may lead to predation or death (Farrell *et al.*, 2001; Raby *et al.*, 2015; Robinson *et al.*, 2013). Adult salmon leaving the marine environment for fresh water are also faced with osmoregulatory challenges in excess of stress responses (Shrimpton *et al.*, 2005), which may reduce the resilience of fish to capture and release after river entry (Martins *et al.*, 2011). The conditions during and following capture are therefore important to surviving a capture event, thereby emphasizing the relevance of climate-driven changes in river temperature affecting many salmon bearing watersheds (Ferrari *et al.*, 2007; Isaak *et al.*, 2012).

Within the Fraser River, BC, temperatures experienced by migrating adult salmon have been increasing for many populations, especially during the summer months (Patterson *et al.*, 2007). This thermal pattern has been observed in other river systems as well, including regulated systems that may also experience impoundment-related

warming (Caudill *et al.*, 2013; Isaak *et al.*, 2012; Macdonald *et al.*, 2012). Unregulated systems, like the Fraser River, rely solely on winter snow pack and the spring freshet to ensure adequate flow to keep temperatures low during migrations; however, projections show an earlier freshet but greater precipitation influences, driving peak flows later in the year (Morrison *et al.*, 2002). These changes will have consequences for salmon populations depending on run timing (spring, summer, fall), but overall, summer and early fall runs have already experienced warmer temperatures (Martins *et al.*, 2011, 2012b; Reed *et al.*, 2011).

For ectothermic fish like salmon, high water temperature has substantial negative effects (Fry, 1971), especially regarding aerobic scope, which can become severely limited, restricting aerobic performance for swimming (Eliason *et al.*, 2011, 2013). Chronic high temperature can also alter immune gene expression in Pacific salmon, with profiles that suggest reduced immune capacity in fish that die prematurely (Jeffries *et al.*, 2012a). Thermal tolerance has been shown to be a function of historic environmental conditions with population-level resolution among sockeye salmon (Eliason *et al.*, 2011), but can also depend on proximity to maturity and sex (Jeffries *et al.*, 2012b). Mortality associated with thermal stress has been demonstrated in laboratory (Gale *et al.*, 2014; Jeffries *et al.*, 2012a, 2014b) and field studies (Crossin *et al.*, 2008; Martins *et al.*, 2012b).

In addition to the physiological impacts of thermal stress on hosts, infectious agents are also at the mercy of environmental temperatures, the effects of which can vary depending on the agent. Infectious agents have thermal optima just like their hosts. Some organisms are more virulent at lower temperatures, such as *Flavobacterium*

psychrophilum, the agent of bacterial coldwater disease (greater virulence below 16 °C; Starliper, 2011). However, many infections amplify at high temperatures, such as *Ichthyophthirius multifiliis*, the agent of white spot disease (Noe and Dickerson, 1995), and myxozoan parasites like *Parvicapsula minibicornis* (Crossin *et al.*, 2008) and *Tetracapsuloides bryosalmonae* (Bettge *et al.*, 2009). Hence, when assessing impacts of environmental conditions like temperature, responses of both the infectious agent and host must be considered, as interactions between these three factors drive co-evolution of pathogens and hosts (Mitchell *et al.*, 2005; Wolinska and King, 2009). Co-infections, which are common in wild animals, add to the complexity of these relationships, as different agents within hosts may favor, inhibit, or have no effect on the development of others (Alizon *et al.*, 2013). Understanding the community of microorganisms affecting the host is a necessary step toward quantifying their collective responses to multiple stressors and associated impacts on salmon health and survival.

1.3 Infectious agents carried by wild adult Pacific salmon

Several recent survey studies tangential to this thesis have dramatically increased our understanding of the microparasite loads carried by adult Pacific salmon from the Fraser River watershed during their spawning migration (Bass *et al.*, 2017, unpublished data). These qPCR-derived microparasite surveys build on information derived using traditional fish health diagnostic techniques, such as histopathology (Kent, 2011; Kent *et al.*, 2013), which are generally more appropriate in culture settings than evaluations of wild fish disease (Miller *et al.*, 2014). Sick wild fish that are physiologically compromised will likely fall out of the water column or be eaten before disease can manifest to a level detectable by histology; hence, sensitive technologies that can detect earlier stages of

infection development are necessary to quantify disease dynamics in wild fish populations (Miller *et al.*, 2014).

I contributed to two infectious agent surveys, one describing Chinook salmon, which included marine and freshwater sampling locations and multiple populations (Bass *et al.*, 2017), and another that tracked one population of sockeye salmon from the Strait of Georgia to spawning grounds on the Adams and Shuswap rivers (A. Bass, unpublished data). Both studies used the same molecular technology and protocols to examine infections in lethally sampled fish, where a set of organ tissues (gill, liver, spleen, kidney, heart, muscle, brain) was homogenized and pooled (aliquots of aqueous phase pooled following homogenate centrifugation) and RNA isolated for qPCR detection of genetic sequences matching 45 infectious agents known or suspected to cause disease in BC salmon (Miller *et al.*, 2016). This approach provided a snapshot of multiple infections carried by populations at each sampling location/time, enabling us to understand temporal and spatial variation in prevalence and infectious loads via RNA expression of each agent. Importantly, lethal sampling does not capture infection development within individuals, but rather population-level changes in surviving fish with time and/or distance.

For both microparasite surveys, we used the Fluidigm Biomark™ platform, analytically validated for its use in infectious agent screening of wild salmon (Miller *et al.*, 2016). The Biomark™ platform uses nanofluidic technology to allow for thousands of reactions in a single run, with large quantities of samples to be processed for many different assays simultaneously. High-throughput quantitative polymerase chain reaction (HT-qPCR) is powerful in its sensitivity to the presence of multiple agents, even in small

amounts of tissue, and in its capacity for rapid sample processing. However, like any tool, this technology has its limitations; for example, regarding its application within these surveys, molecular detection of an organism alone does not indicate disease. Disease generally occurs when host health is observably compromised but can also occur without observable clinical signs; therefore, matching this infection screening approach with an examination of biomarkers of host physiology, immunity and other health indices can reveal processes that may lead to disease development. The surveys conducted by Bass and colleagues were instead focused on characterizing the community of microparasites carried and accumulated by adult Pacific salmon during spawning migration rather than documenting disease.

The data presented by Bass and colleagues is abundant and improves our baseline understanding of the infections currently carried by wild salmon in BC, including 20 infectious agents detected in Chinook salmon at one or more sampling occasions and 19 in sockeye salmon. One key finding was that multiple infections were common among both sockeye and Chinook salmon, including a variety of agent types such as bacteria, viruses, protozoa and others. The most prevalent agents included bacteria (*F. psychrophilum*, '*Candidatus* Branchiomonas cysticola'), myxozoa (*P. minibicornis*, *Ceratonova shasta*), microsporidia (*Loma salmonae*) and one ciliate (*I. multifiliis*), while viruses were generally less (<10%) prevalent. Many of these agents were shared by Chinook and sockeye salmon, suggesting that the shared environment dictates much of the infection dynamics of wild salmon, but falls short of demonstrating equal probability of disease development. Some agent loads correlated with physiological impairment indices (e.g. plasma cortisol, ions), suggesting the potential for disease development

given altered physiological status. However, given increasing infection intensities with time, detangling load correlations with physiological indices like osmotic imbalance from senescence processes is difficult (Jeffries *et al.*, 2011), but likely naturally intertwined.

Agents showing temporal increases in loads over time are of particular interest in terms of disease development, with several potential outcomes that may arise. Firstly, one may hypothesize that with continual increases in loads, agents may ultimately reach a threshold upon which infection-driven mortality ensues, especially after extended freshwater residence (i.e., advanced senescence). Co-evolutionary adaptations of hosts and infectious agents that delay the onset of disease (and heavy infection severity) until after spawning were likely shaped by historic migratory conditions, suggesting that alterations to these conditions may offset host-parasite balances, resulting in early mortality and population-levels effects on salmon (Altizer *et al.*, 2013; Engering *et al.*, 2013; Mitchell *et al.*, 2005). Increasing infections may be due to declining immune competence throughout freshwater residence (Dolan *et al.*, 2016). Dolan and colleagues noted that there are sequential shut-offs in the immune repertoire of adult Chinook salmon during freshwater migration that are not pathogen mediated but likely associated with the host senescence process. These immune shifts may target different agents at different stages of freshwater residence, potentially resulting in temporal changes (and a general increase) in agent virulence and infection intensities during freshwater migration and spawning (Alizon *et al.*, 2013). Studies comparing infectious loads and richness between fish measured at different time points during freshwater residence must account for temporal confounding given increases in agent loads with time (i.e., infections measured earlier may be naturally lower than those measured later). Given these baseline

infection data, we can begin to characterize how infection trajectories under optimal conditions diverge from those under suboptimal conditions using an experimental approach.

1.4 Immune responses of fish to infections

Infection trajectories are inherently affected by the immune responses elicited by the host, which are in turn influenced by environmental factors (Makrinos and Bowden, 2016). Immune responses of teleost fishes are complex and dynamic processes that comprise both innate and acquired components of the immune system (Alvarez-Pellitero, 2008; Bayne and Gerwick, 2001; Zwollo, 2017). The first line of defense against pathogens is generally the innate arm of the immune system, which includes a variety of relatively quick responses such as inflammation (e.g., immune cell migration to affected areas) and humoral components like the Complement system, which can contribute to microbial recognition and/or killing (Bayne and Gerwick, 2001; Holland and Lambris, 2002; Zou and Secombes, 2016). Innate aspects can also trigger activation of the acquired arm of the immune system, which uses cellular receptors (e.g., major histocompatibility complex [MHC]), cytokines (e.g., interleukins [IL]) and antibodies to recognize and/or destroy pathogens and protect/heal host tissues (Olsen et al., 2011; Raida et al., 2011; Raida and Buchmann, 2008; Secombes et al., 2011; Zwollo, 2017). Insight into which aspects of immunity are recruited can be gained by measuring the direction and magnitude of transcriptional changes in host immune gene regulation and can be paired with measurement of infection severities in the same tissues (e.g., Jeffries *et al.,* 2014a). However, caution in interpreting such data must be taken in that transcription is just one stage in a process that also includes translation and protein modification, and therefore

only describes changes in the transcriptional manufacturing of circulating proteins and receptors.

Acknowledging its limitations, a great deal of knowledge regarding the resource needs of the immune system can be gained through gene expression analysis, especially when measured in a tissue such as gill, which is at the forefront of host-pathogen interactions for many fish species (Magnadottir, 2010). Because gill functions as a respiratory (Hughes and Morgan, 1973), osmoregulatory (Evans et al., 2005) and immune defense organ (Secombes and Wang, 2011) and is a primary entryway for many infectious agents (ibid), I used gill as the target tissue for measuring host gene expression and pathogen community dynamics of adult salmon. These pathogen dynamics and host responses likely influence survival and are potentially affected by cumulative stressors encountered by wild adult salmon during freshwater migration. The complexity of these interactions is inherent to natural systems and requires a focused experimental approach to characterize how shifts in infectious agent communities and host responses confer disease development in wild organisms.

1.5 Thesis objectives, structure and hypotheses

This thesis describes four experiments (chapters 2-5) conducted to improve our understanding of the individual and combined impacts of thermal and fisheries stressors on infection development and health of three species of wild adult Pacific salmon: coho, Chinook and sockeye. Hypotheses pertaining to each chapter are outlined below, which utilize the current knowledge described in this introduction to ask specific questions about how interacting forces (infections, thermal and fishery stressors, host responses) influence the survival and behavior of adult Pacific salmon in fresh water. A conceptual

diagram describing the general experimental design, which pairs holding and telemetry studies with non-lethal biopsy and controlled thermal and fishery treatments, can be found in the Appendix (Fig. S1.1).

All experiments utilized the Fluidigm Biomark™ platform to examine infectious agents and host stress and immune gene expression and were complemented by blood plasma indices of host health. Laboratory-held fish were biopsied weekly (gill, blood) to track infections and host physiology and immune activity over time, while tagged fish were biopsied (gill) prior to release. For all chapters, gill was the focal tissue for molecular analysis (HT-qPCR) of host stress and immune gene expression and infectious loads, describing trajectories in genomic profiles over time (laboratory studies) or as a predictor of fish behavior in the river (telemetry study). Additionally, a pool of terminally sampled tissues from seven different organs (gill, liver, spleen, heart ventricle, head kidney, white muscle, brain) was screened using HT-qPCR to characterize infectious agent communities carried by laboratory held fish and fish sacrificed in the river (concurrent with holding studies). Infection data from multi-tissue pools was used to describe prevalence and loads of infectious agents within host populations and to isolate agents that would be evaluated in gill samples (i.e., only agents positively detected in multi-tissue pools were measured in gill). Infectious agents screened in multi-tissue pools generally included 12 bacterial, 10 viral, and 22 protozoan species, but varied slightly across experiments (see chapter tables for infectious agent species targeted in each experiment). Host gene expression was evaluated in non-lethally and lethally sampled gill tissue and included biomarkers of innate (C7, TF, IFNa) and acquired (b2m, MHCI, MHCIIb) immunity and immune regulation (IL11, IL15, IL1R, IL8, CXCR4), as well as

indicators of stress (GR2, JUN, HSC70, HSP90), ion regulation (NKA_a1b), cellular energy generation (ATP5G3C), and wound repair (MMP13; see tables within chapters for assay information and targeted gene functions). Weekly blood samples from held fish provided information pertaining to host stress responses (cortisol, glucose, lactate, hematocrit), osmoregulatory function (chloride, sodium, potassium ions) and maturation (testosterone, estradiol) to characterize host physiology and health. For each experiment, I paired the results of molecular and physiological analyses with host survival and/or migration behavior data to characterize the processes and profiles associated with early mortality and migration failure of adult Pacific salmon in fresh water.

1.5.1 Chapter objectives and hypotheses:

The first experiment (described in chapter 2) addressed an applied question relating to the survival of released sockeye salmon bycatch under realistic thermal conditions and different entanglement durations. A laboratory holding study evaluated the individual effects of capture stress and severity on wild sockeye salmon following the real-time, dynamic thermal experience of a successful migrant in the river. Fish were biopsied at the start of the experiment (gill, blood) and at death (various tissues, blood) to characterize potential mechanisms of mortality and predictive factors of longevity following release from gillnets in the river. Chapter 2 hypotheses**:** H_1) Longer gillnet entanglements will decrease longevity of adult sockeye salmon and increase infection development relative to controls. H_2) Initial biopsy samples that indicate heavy infections and poor host health (e.g., osmoregulatory impairment, heightened immune response) will be predictive of early mortality. H_3) Heavy infections will be associated with indices of poor health at death.

The results of chapter 2 and previous studies (see introduction) suggested cumulative effects of thermal and fisheries stressors on survival in fresh water, possibly due to enhanced infections and reduced host stress resolution at high temperature. The second experiment (chapter 3) tested the interaction of thermal and fisheries stressors by examining fishery treatment effects on coho salmon held in cool or warm water. Chapter 3 hypotheses: H_1) Thermal and fisheries stressors will reduce survival independently with additive effects of multiple stressors (lowest survival among thermally and capture-stressed fish). H_2) Rates of infection development will be greatest among stressed fish with additive effects of cumulative stressors. H_3) Early mortality will be associated with heavy multiple infections and poor host health, with the greatest infection intensities among cumulatively stressed fish.

The third experiment (chapter 4) incorporated behaviour by pairing telemetry with laboratory holding to identify fishery capture effects on tagged Chinook salmon in the river and held Chinook in cool or warm water. As the study design for the laboratory component of this study was almost identical to that described in chapter 3 (but with a different host species), the same set of hypotheses (H_{1-3}) also apply to chapter 4, in addition to several behavioral hypotheses. Chapter 4 hypotheses: H_{1-3}) See Chapter 3 hypotheses. H_4) Fishery stress will reduce longevity, distance traveled, and migration rates in the river. H_5) Heavy infection burdens will reduce longevity, distance traveled, and migration rates in the river.

To manipulate infection burdens for a "challenge" study, capture location was incorporated into the final experiment (chapter 5), which used marine-collected sockeye salmon as low infection "controls" for river-exposed fish from the same stock collected

one week later from the lower Fraser River. This approach manipulated host infection burdens via capture location to test whether infections accumulated in the river influenced the effects of cumulative thermal and fishery stressors during freshwater residence. Again, the initial hypotheses of chapters 3 and 4 apply to this study (different host species), in addition to several infection-based hypotheses. Chapter 5 hypotheses: $H_{1\text{-}3}$) See Chapter 3 hypotheses. H_4) River-exposed fish will carry heavier infections and show decreased longevity and resilience to stressors, especially cumulative stressors, relative to marine-collected fish. H_5) Host stress and immune gene expression will be more strongly associated with infections among river-exposed fish.

All findings are synthesized in Chapter 6, which includes a discussion of knowledge gaps, management implications, and fruitful areas of continued research.

Chapter 2 - Capture severity, infectious disease processes, and sex influence post-release mortality of sockeye salmon (*Oncorhynchus nerka*) bycatch

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2.1 Abstract

Bycatch is a common occurrence in heavily fished areas such as the Fraser River, British Columbia, where fisheries target returning adult Pacific salmon (*Oncorhynchus*

spp.) *en route* to spawning grounds. The extent to which these fishery encounters reduce fish survival through injury and physiological impairment depends on multiple factors including capture severity, river temperature, and infectious agents. In an effort to characterize the mechanisms of post-release mortality and address fishery and managerial concerns regarding specific regulations, wild-caught Early Stuart sockeye salmon (*O. nerka*) were exposed to either mild (20 s) or severe (20 min) gillnet entanglement and then held at ecologically relevant temperatures throughout their period of river migration (mid – late July) and spawning (early August). Individuals were biopsy sampled immediately after entanglement and at death to measure indicators of stress and immunity, and the infection intensity of 44 potential pathogens. Biopsy alone increased mortality (males: 33%, females: 60%) when compared to non-biopsied controls (males: 7%, females: 15%), indicating high sensitivity to any handling during river migration, especially among females. Mortality did not occur until 5 – 10 days after entanglement, with severe entanglement resulting in the greatest mortality (males: 62%, females: 90%), followed by mild entanglement (males: 44%, females: 70%). Infection intensities of *Flavobacterium psychrophilum* and *Ceratonova shasta* measured at death were greater in fish that died sooner. Physiological indicators of host stress and immunity also differed depending on longevity, and indicated anaerobic metabolism, osmoregulatory failure, and altered immune gene regulation in premature mortalities. Together, these results implicate latent effects of entanglement, especially among females, resulting in mortality days or weeks after release. Although any entanglement is potentially detrimental, reducing entanglement durations can improve post-release survival.

2.2 Introduction

For wild semelparous Pacific salmon (*Oncorhynchus* spp.), lifetime fitness hinges on the survival and successful migration of adults to spawning grounds where they will deposit gametes prior to natural death (Groot and Margolis, 1991). Pacific salmon productivity is in a state of decline in many natal watersheds, especially at southern range extremes (Hinch *et al.*, 2012; Peterman and Dorner, 2012). Pre-spawning and *en route* mortality of adult Pacific salmon have likely contributed to these declines and have been attributed to several factors, including thermal and fisheries stressors encountered during freshwater migration (Donaldson *et al.*, 2011; Gale *et al.*, 2013; Gilhousen, 1990; Martins *et al.*, 2012b; Raby *et al.*, 2015). Disease processes are also known to influence the survival of wild salmon but have been notoriously difficult to study due to the logistical constraints inherent in monitoring wild animal populations under natural conditions, especially for highly migratory species (Altizer *et al.*, 2011; Miller *et al.*, 2014). With regard to adult Pacific salmon, the manner by which fisheries practices, temperature, and disease processes interact to influence the mechanisms of premature mortality remains poorly understood (Miller *et al.*, 2014).

The intense salmon fisheries of the West Coast of North America yield a strong likelihood of gear encounter by migrating adult salmon *en route* to natal streams, rivers, and lakes. Although much of this catch is retained, non-target species are often captured and viable bycatch released back to the water, depending on regulations specific to each fishery. In addition to those released, many fish get trapped in gear but escape during the fishing and landing process, displaying physical signs of entanglement at locations further upriver (Baker and Schindler, 2009; Casselman *et al.*, 2016). Depending on the

fishery, a proportion of captured and released individuals is assumed to arrive at spawning grounds and this subtotal can be counted toward spawner escapement goals set by management. There are physiological consequences of capture and release or escape from fisheries gear that contribute to post-release impairment and mortality (reviewed in Davis, 2002). Variability in these physiological responses is common within and among species and stocks (Cooke and Suski, 2005), and is associated with the severity of the capture event (Gale *et al.*, 2011), the condition of the individual at capture (Davis, 2002; Donaldson *et al.*, 2012), and the animal's ability to recover (Farrell *et al.*, 2001; Robinson *et al.*, 2013). Condition at capture and subsequent recovery are also suspected to be associated with infectious disease processes (Gilhousen, 1990; Raby *et al.*, 2015). Stress and injury caused by a gear encounter can provide opportunities for infection (Baker *et al.*, 2013; Baker and Schindler, 2009; Chopin and Arimoto, 1995), elicit enhanced immune surveillance and responses by the host (Dhabhar, 2002; Neeman *et al.*, 2012), and promote physiological disturbances such as osmoregulatory imbalance that can impair overall host health and resilience (Cooke *et al.*, 2013; Donaldson *et al.*, 2012; Gale *et al.*, 2011). Establishing linkages between physiological and infection-associated variables would aid in developing a clearer understanding of how host-parasite relationships impact the survival of released salmon bycatch and improve mortality estimates.

Environmental factors such as high water temperatures compound the effects of fisheries capture (Gale *et al.*, 2013) and have disease-associated consequences, potentially diminishing host (salmon) resilience (Dittmar *et al.*, 2014; Jeffries *et al.*, 2012a), and altering the productivity of infectious agents prior to (Chiaramonte, 2013;

Paull and Johnson, 2014) or following (Bettge *et al.*, 2009; Kocan *et al.*, 2009; Thomas and Blanford, 2003) infection. One suspected mitigation measure used by Pacific salmon faced with high river temperatures is behavioural thermoregulation, particularly in the lentic components of the migration route (Donaldson *et al.*, 2009). By residing in the cool waters near the thermocline of corridor lakes prior to arrival at spawning grounds, accumulated thermal units remain lower than if the animal remained in warmer river waters (Newell and Quinn, 2005; Roscoe *et al.*, 2010). This tactic combined with changes in river temperature during migration produces a dynamic thermal experience that likely impacts physiological and disease-associated responses to fisheries capture. The Early Stuart population of sockeye salmon (*O. nerka*), for example, migrates approximately 1200 km from the mouth of the Fraser River to spawning grounds near the Stuart Lake system (Fig. 2.1); they begin this migration earlier than any other Fraser salmon population (median historical river entry date of 7 July) while the spring freshet is still diminishing and river temperatures are concurrently rising, and are thus faced with a narrow window of optimal migratory conditions (Macdonald *et al.*, 2010; Reed *et al.*, 2011). They also migrate at the same time as some spring Chinook salmon (*O. tshawytscha*) populations, which are the target of in-river First Nations gillnet fisheries. Declining abundance of returning adult Early Stuart sockeye salmon in recent decades has raised interest in how fishery-related bycatch mortality and river conditions may affect this population's continued viability.

To characterize the mechanisms contributing to post-release mortality and address fishery and managerial concerns regarding specific regulations, we conducted a long-term holding study using wild-caught Early Stuart sockeye salmon. This project was

conducted in collaboration with First Nations user groups of the Lower Fraser Fisheries Alliance (LFFA) as well as managers and scientists of the Department of Fisheries and Oceans Canada (DFO). Concerns were raised among users and managers regarding the accuracy of the post-release mortality rate (60%) assigned by regulators to Early Stuart sockeye bycatch within the Chinook (*O. tshawytscha*) drift and set gillnet fishery that takes place during the Early Stuart sockeye migration. The primary purpose of our study was to test the variability of this post-release mortality estimate under different capture severities (i.e. entanglement durations) and a realistic thermal experience to inform fishery management and best practices of fishers. Furthermore, we sought to identify short-term effects of capture and predictive factors that distinguish fish that survive to the spawning period of Early Stuart sockeye from those that do not by using an array of physiological, environmental, and disease-associated variables. Finally, we endeavored to characterize relationships between infection intensities of potential pathogens at death with host physiology toward a mechanistic understanding of post-release mortality.

2.3 Methods

The total migration duration from ocean departure to spawning grounds for Early Stuart sockeye salmon is about 3 – 4 weeks (Crossin *et al.*, 2004; Macdonald *et al.*, 2010; Reed *et al.*, 2011). We captured individuals approximately 5 days into their upstream migration, approximately 150 river kilometers (rkm) from the mouth of the Fraser River in Yale, BC, using a 5.25-inch (13.3 cm) mesh gillnet (mesh size targeting Early Stuart sockeye). Fishing took place between 08:00 and 12:00 on July 9 and 10, 2013 and river temperature ranged between 16 – 17 °C during collection. Fish were quickly and carefully removed from the net to minimize injury and stress and immediately placed into

coolers filled with fresh river water. This type of capture was chosen as the most low-impact yet effective way of collecting Early Stuart sockeye; any observable impacts of collection (e.g. injury, lethargy) were factored into an overall condition score and incorporated into survival assessments (see below). A subset of fish was sacrificed river-side within 5 min of capture (n = 19) and sampled for gill tissue (2-3 filament tips, representing approx. 0.5 mg of tissue) and blood (approx. 2 mL from the caudal vasculature; 21-gauge needle with lithium heparinized Vacutainer®, Becton-Dickson, NJ) to provide baseline data pertaining to condition at the time of capture (details on tissue storage and handling below). Fish were then placed in aerated truck-mounted tanks filled with cool (11 – 12 °C), UV-treated, and sand-filtered water for transport to the DFO Cultus Lake Salmon Research Lab at Cultus Lake, BC (40 min transit; Fig. 2.1). Fish were dip-netted from transport tanks and sequentially distributed among eight holding tanks (approx. 8,000 L; 16 – 17 °C). Tank water at the facility was sourced from the neighboring Cultus Lake, which was sand filtered, UV-treated, flow-through (e.g. not recirculated), and temperature controlled by manipulating the proportion of water from above or below the lake's thermocline. To achieve warmer temperatures, tanks were supplemented with boiler-heated shallow lake water. Tanks had a constant inflow above 30 L/min and were outfitted with a submersible pump creating a circular flow pattern around the tank periphery (approx. 30 cm s^{-1}) which encouraged fish to slowly swim in place during holding.

Tanks were assigned to one of four treatments with two tank replicates per treatment group. The methods for this experiment were carried out under protocols approved by the Animal Care Committees of Fisheries and Oceans Canada (Pacific Region), the

University of British Columbia (certificate A11-0215), and the University of Victoria (certificate 2012-030). Treatments included (i) a severe gillnet entanglement (20 min entanglement plus 1 min air exposure), (ii) a mild gillnet entanglement (20 s entanglement plus 1 min air exposure), (iii) a biopsied control without entanglement group, and (iv) a control without biopsy or entanglement. Twenty-four to 48 h after collection, the standardized entanglement treatments were applied in the lab using an 8-inch (20.3 cm) mesh gillnet, which matches the mesh size used in the Fraser River Chinook fishery experiencing Early Stuart sockeye bycatch.

The gillnet treatment proceeded as follows: each fish was individually removed from its holding tank with a dip-net and immediately submerged in a treatment tank within the bag of the dip-net where the fish was then quickly entangled in the 8-inch mesh gillnet and then flipped out of the dip-net under water. This entanglement method was employed due to the large mesh size of the gillnet, which in our experience was too wide for Early Stuart sockeye to be caught via the gilled method. Our directive was not to quantify the causes of bycatch or encounter rates, but to understand effects of capture and release. Hence, this method was appropriate to achieve effective entanglement. After 20 min (severe) or 20 s (mild) of sustained entanglement, both the gillnet and fish were lifted out of the water and placed into a dip-net held in the air. After a 1-min air exposure, which included net removal and simulated a realistic time for bycatch landing and net removal, the fish was submerged in a flow-through, padded sampling trough for biopsy. Each fish was measured for fork length (FL; ±1 cm) and muscle lipid content (Fish Fatmeter Model–FM 692, Distell, Scotland, UK), biopsied for gill tissue (2-3 filament tips) and blood (approx. 2 mL from the caudal vasculature; 21-gauge needle with lithium

heparinized Vacutainer®, Becton-Dickson, NJ), externally tagged for identification (spaghetti-style tag, Floy®, WA), and then placed into a recovery tank. Its condition was recorded as an integer score from 0 to 6, which was a composite score of condition prior to experimental treatment (0 = no injury from the collection net and vigorous in the treatment net, 1 = mild abrasions [e.g. scale loss] but vigorous, 2 = moderately injured [e.g. skin loss] or lethargic, 3 = severely injured [e.g. bleeding or flesh loss] and lethargic) and condition following the experimental gillnet treatment (0 – 4, same criteria as above). Anesthetic was not used so as to mimic as much as possible the conditions of the fishery (see Cooke *et al.*, 2005 for evaluation and validation of biopsy without anesthetic). Water temperature was constant throughout the treatment and sampling procedures (16 – 17 °C; ≤ 2 min total time in trough). Biopsied control fish were similarly dip-netted from holding tanks, but bypassed gillnet and air exposure treatments to proceed directly to the sampling trough, henceforth following the biopsy protocol outlined above. Non-treatment air exposure associated with movement of biopsied control fish between tanks and the sampling trough was ≤ 10 s total. Non-biopsied control fish were not handled at all after collection. Experiment start for each individual corresponds to the time it entered the recovery tank; for non-biopsied control fish, start time corresponds to the earliest start time for treated fish. Due to a plumbing malfunction in their recovery tank, individuals from one holding tank (biopsied controls, n = 14) were excluded from long-term analyses. These individuals were, however, included in short-term analyses that did not relate to subsequent survival, but included condition and infection intensity at the time of the biopsy, prior to entering the recovery tank, relating to short-term effects of capture.

Tanks were checked at ≤ 4 h intervals between 08:00 and 24:00. Any individual displaying signs of morbidity (e.g. loss of equilibrium, surface gulping) was removed from the tank and euthanized; all fish surviving to the end of the spawning period, as determined by the duration of Early Stuart residence on spawning grounds and a sharp increase in (senescence-related) mortality of held fish, were euthanized. All euthanized fish were immediately biopsied as described above to preserve the integrity of RNA and blood properties. An adipose fin tissue sample was taken using a hole punch for DNA analysis. There was a gross examination of external and internal pathologies (e.g. *Saprolegnia* spp. fungus cover, organ abnormalities and lesions) while subsampling tissue from six additional major organs (liver, spleen, heart ventricle, head kidney and white muscle; brain alternated between RNA screening and histopathology) for microbe RNA screening and histopathology (data not shown).

Fish were held for the duration of their natural freshwater migration (approximately 3 weeks) and spawning period (an additional 3 weeks that included staging, spawning, and nest defense) to assess their survival during these periods associated with the experimental treatments. For the duration of the experiment, water temperature within all tanks was monitored and adjusted daily to mimic the thermal experience of a successful Early Stuart sockeye salmon that would be migrating towards spawning grounds in the same year as our study (Fig. 2.1). We constructed a thermal experience model in real time using thermal data loggers in place along the migration route (DFO Environmental Watch Program; http://www.pac.dfo-mpo.gc.ca/science/habitat/frw-rfo/index-eng.html) and migration rate estimates calculated for Early Stuart sockeye (Rand and Hinch, 1998). We estimated the geographic location of a successful migrant from the final date of collection

until the end of the spawning period, including behavioural thermoregulation utilizing cool hypolimnetic water while passing through corridor lakes (Mathes *et al.*, 2010; Newell and Quinn, 2005; Roscoe *et al.*, 2010), and adjusted tank temperatures daily to match this estimated thermal experience. Briefly, for Early Stuart sockeye captured approximately 150 rkm from the mouth of the Fraser River, traveling at a ground speed of approximately 0.8 m/s within the Fraser River mainstem (corresponding to about 4000 m^3/s discharge) and then approximately 1 m/s in the Stuart and Nechako rivers, they would reach lake systems after about 10 – 11 days from the start of the study (between July 21 – 23). To incorporate behavioural thermoregulation prior to arrival at spawning grounds, temperature was decreased to 11 – 12 °C following simulated lake arrival on July 24th. Finally, on July 28th temperature was increased to 16 °C and then lowered to 12 °C to simulate movement out of the lakes, through the river, and on to cooler spawning grounds (Macdonald *et al.*, 2012).

A subset of Early Stuart sockeye was sacrificed at spawning grounds near Takla Lake (n = 13; Fig. 2.1) and biopsied according to terminal sampling procedures described above to measure microbe prevalence on spawning grounds (7 – 8 August, 2013).

2.3.1 Laboratory analyses

Hematocrit and leucocrit values were measured immediately following blood sampling by calculating the volumes of red and white blood cell layers relative to total blood volume, respectively, after centrifugation (2 min at 10000 *g*; LW Scientific® ZIPocrit; GA, U.S.A.) in heparinized micro-capillary tubes (Drummond Scientific®, PA, U.S.A.). The remaining whole blood (approx. 2 ml) was centrifuged within the Vacutainer® for 7 min at 7000 *g* to remove plasma (Clay Adams Compact II centrifuge; NY, U.S.A.),

which was then flash frozen in liquid nitrogen for subsequent analyses of hormones and metabolites. Gill tissue and other organ tissues (liver, spleen, heart ventricle, head kidney, white muscle, brain) were preserved in 1.5 mL of RNA*later*® solution (Qiagen, MD, U.S.A.) for genomic analyses (whole brain in 3 mL). Percent lipid content of dorsal muscle was estimated using Fatmeter values and equations developed for sockeye (Crossin and Hinch, 2005). Stock identification as Early Stuart complex was confirmed via microsatellite DNA analysis of the adipose fin at the DFO Pacific Biological Station in Nanaimo, BC (Beacham *et al.*, 2004). Plasma sodium, chloride, potassium, osmolality, lactate and glucose were measured using protocols described by Farrell and colleagues (Farrell *et al.*, 2001) and cortisol, testosterone, and estradiol were examined using enzyme-linked immunosorbent assay (ELISA) kits (Neogen Corporation, KY, U.S.A.) following manufacturer's protocols.

Genomic analyses were conducted at the DFO Pacific Biological Station using high-throughput nanofluidic qPCR (Fluidigm® BioMark™ Dynamic Array, CA, U.S.A.) for quantification of relative RNA expression of disease-associated microbes and host stress- and immune-related biomarkers (Miller *et al.*, 2014, 2016; this thesis, Tables 2.1 & 2.2). Preserved tissue samples (approx. 0.5 mg each; liver, spleen, heart ventricle, head kidney, white muscle) were homogenized independently for 6 – 9 min in 600 µL TRI reagent (Ambion Inc., TX, U.S.A.) and 75 µL 1-bromo-3-chloropropane in microtubes using stainless steel beads and a MM301 mixer mill (Restch Inc., PA, U.S.A.). Whole brains were quartered and each section homogenized in 600 µL TRI reagent; 150 µL aliquots from each brain quarter were pooled into 600 µL of diluted brain homogenate prior to addition of 1-bromo-3-chloropropane. Microtubes were then manually shaken for 1 min

followed by 5 min at rest (repeated once), then centrifuged at 1500 *g* for 6.5 min. Aliquots of the aqueous phase (15 µL) from each tissue type were combined to produce a tissue pool from each individual fish. This approach potentially restricted the probability of detecting infectious agent RNA that was not suspended during centrifugation or those agents that potentially produce less of the target RNA sequence relative to others (e.g., primers/probes designed to rRNA vs. surface protein, RNA viruses, etc.). RNA was purified following manufacturer's instructions using the "spin method" for Magmax™-96 for Microarrays Kits (Albion Inc., TX, U.S.A.), with an additional DNase treatment to prevent DNA contamination. Extractions were performed using a Biomek FXP (Beckman-Coulter, ON, Canada) automated liquid handler. Quantity (A_{260}) and quality (A_{260}/A_{280} ratio) of purified RNA were examined via spectrophotometry. Total RNA in each sample was normalized to 1.0 µg per sample for pooled tissues and 0.5 µg per sample for gill due to lower RNA concentrations from non-lethal gill samples (less tissue). This process utilized spectrophotometry and the Biomek FXP automated liquid handler, using RNA/DNA free water as the diluent. cDNA was synthesized using an Invitrogen™ SuperScript™ VILO™ (CA, U.S.A.) cDNA Synthesis Kit under PCR cycling conditions of 25 ˚C for 10 min, 42 ˚C for 60 min, and 85 ˚C for 5 min.

Given the nL volumes of substrate incorporated into each qPCR reaction chamber of the Biomark™, samples must first undergo a pre-amplification step consisting of a multiplex PCR including all target assay primers to achieve high sensitivity detections (for more information see Miller *et al.*, 2016). Following manufacturer's protocols, a mix of forward and reverse primers corresponding to all targeted microbe and host biomarkers (200 nM primer mix; 1.3 µL total volume) was combined with 2.5 µL

TaqMan® PreAmp Master Mix (Applied Biosystems, CA, U.S.A.) and added to 1.3 µL cDNA; PCR cycling commenced at 95 ˚C for 10 min followed by 15 cycles of 95 ˚C for 10 s and 60 ˚C for 4 min. Any remaining nucleotides and primers were removed using ExoSAP-IT® PCR Product Cleanup (MJS BioLynx Inc, ON, Canada) cycled at 37 ˚C for 15 min then 80 ˚C for 15 min. Each sample was then diluted 5-fold with suspension buffer (TEKnova, CA, U.S.A.) so as not to overwhelm the subsequent qPCR analysis. Controls were incorporated among samples in duplicate during the extraction, pre-amplification, and qPCR steps, including both positive (pooled cDNA samples from multiple individuals) and negative controls (suspension buffer); serial dilutions of pre-amplified pooled host samples and synthetic microbe sequence clones were also included among samples on each dynamic array during the final qPCR (Miller *et al.*, 2016).

Assays for host biomarkers, microbes and three host reference genes were run in duplicate. Reference genes ensured viability of samples for host gene expression analysis (i.e. routine host gene expression). Sample (TaqMan® Gene Expression Master Mix, GE Sample Loading Reagent and pre-amplified cDNA) and assay (primer pair [9 µM], probe [2 µM], Assay Loading Reagent) mixes were individually plumbed into single reaction chambers using integrated fluidic circuits of the IFC controller prior to the qPCR cycling. The qPCR thermal cycling profile followed the GE 96 X 96 Standard v1.pcl. (TaqMan®) protocol. Passive reference dye was used to confirm that all 9216 wells contained substrate. Two probes were measured in each reaction chamber: one pertained to the target amplicon (FAM dye) and the other to microbe clone controls (NED™ dye, Applied Biosystems, Foster City, CA, USA; measured on VIC setting as closest wavelength). Any sample reaction chamber found to be VIC positive was removed as suspected clone

contamination. Cycle threshold (Ct) replicates were averaged for all samples; in the case of failed replicates, host biomarkers were assigned the single positive value, but any microbe not positive for both replicates was designated a negative detection. Relative expression of host genes was calculated according to the $2^{-\Delta\Delta Ct}$ method using the average of the 3 reference genes (Livak and Schmittgen, 2001). Predetermined total copy numbers of synthetic microbe clone dilutions were used to create a standard curve to back calculate RNA copy numbers of microbes from Ct values measured in samples. Throughout the analyses herein, host biomarker results (measured in gill only) are represented as relative expression and microbe infection intensity (RNA copy number; gill and pooled tissues) referred to as "productivity".

We measured microbe productivities via the RNA expression of each microbe. Because primers and probes were designed to different gene types with varying functions (e.g. ribosomal 16S, surface array, etc.) depending on the target species, our conclusions are limited to describing variation among hosts within each microbe species. Comparisons among species would be misleading because different target genes are expressed at different rates. We chose RNA rather than DNA quantification to include RNA viruses in our screening approach and to represent changes in active expression of living microbes as opposed to direct quantification of potentially inactive DNA. Microbe productivity as we define it here is thus a measure of the relative activity (RNA manufacturing and maintenance) of each microbe.

2.3.2 Statistical analyses

Survival analysis was used to identify differences in survival among treatment groups and sexes to the peak of the spawning period for this population (20 days post-treatment;

dpt) following treatment using the *survdiff* and *coxph* functions in the *survival* library in Program R (R Core Team, 2015; Therneau, 2014). Assumptions of the model, including proportional hazards, influential observations, and linearity, were evaluated. Survival (> 20 dpt) was also examined by treatment and sex using generalized linear models (GLMs) with a binomial response. GLMs were constructed including and excluding non-biopsied controls; this approach allowed us to examine the effect of the biopsy alone on survival and to identify survival differences between gillnetted fish and controls with and without the additional handling associated with the biopsy.

Short-term effects of capture on host physiology and microbe productivity were assessed by comparing samples taken from fish sacrificed river-side at the time of collection (immediately following capture, T0; $n = 19$) with non-lethal biopsy samples taken 1 – 2 days after fish collection (biopsied control group, T1; $n = 28$). Blood plasma indices of maturity, stress and osmoregulatory impairment at T0 and T1 were log-transformed if necessary to meet assumptions of normality. GLMs were constructed, with time and sex as predictor variables including an interaction term, and each physiological variable as the response. Principal components (PC) analysis was used to identify and characterize shifts in gene expression patterns (28 biomarkers of stress and immunity; see Table 2.1) measured in gill at T0 ($n = 20$) and at T1 ($n = 22$). Analysis of variance (ANOVA) was used to determine if the position of individuals along PC axes was correlated with sex or sampling date (T0, T1), and included an interaction term. Short-term changes in microbe productivity in gill were identified using hurdle models with a negative binomial response distribution; this approach conducts step-wise tests for differences in the presence of zeros (i.e. changes in prevalence between time points) and

continuous positive values (i.e. microbe productivity as estimated by RNA copy number in positive detections). Microbe copy numbers were log-transformed prior to all analyses. We examined the effect of microbe richness on survival to 20 dpt of gillnet treated and biopsied control fish (n = 61) using a GLM with sex and treatment as cofactors.

We used a nonparametric multivariate classification tree model to identify physiological and environmental factors associated with survival to the spawning period (20 dpt) using the *rpart* library and *cartware* functions in Program R (Compton, 2006; De'ath, 2002; De'ath and Fabricius, 2000; Therneau *et al.*, 2015). This analysis was restricted to fish that were exposed to gillnet treatments, including both severe and mild entanglements (n = 51), therefore including a mix of exposure times relevant to the fishery. The technique uses recursive partitioning to identify distinguishing variables among pre-defined groups (i.e. success or failure to survive to the spawning period). Simply, the analysis identifies the variable with the greatest power to distinguish between predefined groups, repeating this partitioning at each "branch" until terminal nodes (partitioned collections of individuals at branch tips) reach sufficient correct classification. Fifty-two variables were included in the initial partitioning (Table 2.3), which when applied for descriptive purposes can handle large variable to sample ratios. The classification tree model was constructed using the "gini" index as the splitting criteria, prior probability of group assignment was proportional to group sample sizes at each partition, and further partitioning was stopped within one standard error of the minimum relative error. Primary and surrogate splits were examined as well as variable importance regardless of incorporation in the final tree. The effectiveness of the model was examined using the Kappa chance-corrected error reduction rate. Model significance

was assessed using Monte Carlo resampling with 100 random permutations of the grouping variable (success) with the derived tree size (3 leaves) and variables (see results) of the final model; if $P < 0.05$, the correct classification rate of the original model was deemed sufficiently high relative to the distribution from random trees.

Because the purpose of our study was primarily focused on the impact of capture severity on survival, we allowed individuals to progress to the stage of morbidity prior to sacrifice and re-sampling, rather than sacrificing all individuals simultaneously. Therefore, mortality took place over an extended temporal period (weeks – months) and was furthermore confounded with treatment and sex (see survival results). Samples that were taken at death (i.e. terminal variables) are hence subject to an unknown relative influence of senescence or maturation trajectories, gillnet treatments, and sex. Comparison of terminal variables among treatment groups is therefore fraught with speculation given this temporal confounding. We therefore limit our analysis of terminal variables to characterizing trends in microbe productivities with time and qualitatively describing relationships among terminal microbe productivities and physiological variables.

To test the assumption that greater microbe productivities would be apparent in fish that die prematurely (as a proxy for advanced infection states), we used logistic and linear regression of days surviving with microbe prevalence and productivity, respectively. Linear regressions were limited to positive microbe detections with adequate sample sizes. A negative slope ($P < 0.05$) was assumed to represent higher microbe productivity in premature mortalities, suggesting potential pathogenicity, whereas a positive or zero slope would demonstrate lower or no difference in productivity in premature mortalities

relative to fish that survived to the spawning period. The latter scenarios suggest no impact of microbe productivity on the host, or possibly a decreased threshold for microbe productivity tolerated by the host. We used microbe productivity values derived from pooled tissues rather than from the gill alone for a more comprehensive representation of microbes across tissues. Relationships between microbe productivities were evaluated using Spearman's Rank Correlation. This analysis was conducted using pooled tissue data for microbes with adequate sample sizes to obtain reliable results (see Table 2.2 for prevalence information). Spearman's correlation coefficients were calculated for all complete pairs, where both observations were positive detections, between '*Candidatus* Branchiomonas cysticola' (n = 50), *Ceratonova shasta* (n = 54), *Flavobacterium psychrophilum* (n = 42), *Ichthyophthirius multifiliis* (n = 63), *Loma salmonae* (n = 57), *Parvicapsula minibicornis* (n = 82) and *Rickettsia*-like organism (RLO; n = 56). Agreement between gill and pooled tissue microbe detections was assessed by presence–absence (percent agreement) and by productivity using linear regression on positive detections with a Breusch-Pagan test for heteroscedasticity (data from individuals sampled at death; n = 83).

Relationships between microbe productivities and host biomarkers of stress and immunity were characterized using Kruskal's nonmetric multidimensional scaling (NMDS) in concert with the *envfit* function for fitting extrinsic variables in the *vegan* library in Program R (Oksanen *et al.*, 2016). NMDS is a robust unconstrained ordination method (e.g., Hülber *et al.*, 2009) that reduces the dimensionality of community data sets and establishes relationships among samples based on their composition (Minchin, 1987). We used the *metaMDS* function to create a Bray-Curtis distance matrix of individual fish

based on their microbe communities (e.g., productivities of all microbe species measured in pooled tissues at death, $n = 42$), and then characterized their relationships with host biomarkers of stress and immunity as well as days surviving, treatment, and sex. Prior to the analysis, microbe RNA copy numbers were transformed to a proportion of the total copies of each microbe species across all samples (i.e. column standardized), then expressed as a proportion of the total normalized values for each individual fish across microbe species (i.e. row standardized). The analysis was restricted to microbe species with greater than 10 positive detections to avoid a bias toward rare species. Two dimensions were included in the ordination, which was determined as the fewest possible axes with sufficient agreement between calculated and plotted distances (i.e. low stress). Species (microbe) scores were calculated as weighted averages in the 2-dimensional space. A Monte-Carlo permutation test was used to determine the significance of the ordination (McCune *et al.*, 2002; McGarigal, 2015). Genomic, clinical (blood properties, muscle lipid), and environmental (treatment, sex, days surviving) variables were fit onto the ordination using the *envfit* function, which maximizes the correlation between projected points and fitted variables. Resulting vectors represent the direction and relative strength (vector length) of the correlation; however, vector lengths for clinical variables were shortened to improve readability of the final plot. Variable goodness of fit (r^2) and "significance" (P) were assessed using permutation of environmental variables; a cut-off of $P < 0.10$ was applied for inclusion of external variables in the final descriptive diagram as demonstrating sufficient change along the ordination gradient to reliably enhance our understanding of host responses associated with microbe community structure.

2.4 Results

2.4.1 Survival

Based on the comparison of the control fish that were not handled at all following collection and the control fish that were biopsied, the biopsy itself had significant effects on percent mortality before the spawning period (Fig. 2.2a, Table 2.4). The low sample size of biopsied controls relative to other control and treatment groups, however, warrants caution in interpreting the survival estimates for biopsied controls. Among biopsied controls, 33% of males and 60% of females died before the spawning period, while only 7% of male and 15% of female non-biopsied controls died before the spawning period. Percent mortality further increased with both entanglement intensities: severe entanglement resulted in the greatest mortality (males: 62%, females: 90%), followed by mild entanglement (males: 44%, females: 70%). Mortality of gillnet-treated fish did not occur until 5 – 10 days after entanglement, depending on entanglement severity and sex, and mortality among biopsied controls was delayed by 10 – 15 days after the biopsy. After accounting for mortality due to capture, holding, and biopsy sampling, we can expect approximately 10 – 55% of females exposed to mild entanglement to die before the spawning period, increasing to 30 – 75% following severe entanglement, while males would be expected to show 11 – 37% mortality before the spawning period following mild entanglement and 29 – 55% mortality following severe entanglement. The minimum of each mortality range was calculated using the percent mortality of biopsied controls and the maximum using non-biopsied controls. Estimates are presented as a range of values to account for uncertainty regarding the effect of non-lethal biopsy sampling, which could be additive or masking in its impact on survival.

Evaluation of the assumptions of the survival analysis revealed that the proportional hazards assumption was violated for gillnet-treated fish (*P*-values < 0.020), wherein the risk of death due to gillnet entanglement was high in the first 10 days and then decreased. Although this information is relevant to the study, it prohibited application of the survival analysis in identifying differences in survival among treatment groups. By stratifying treatment groups, the effect of sex on survival could be evaluated (within treatments) and was found to have a significant effect ($\chi^2 = 6.9$, $P = 0.009$), with males experiencing less than half (43%, $P = 0.010$) of the daily mortality risk that females experienced (model concordance = 0.611, $r^2 = 0.069$, Likelihood ratio test $P = 0.011$). GLMs used to identify differences in survival to the spawning period showed higher mortality among biopsied controls ($P = 0.010$), 20 s gillnet ($P = 0.001$) and 20 min gillnet groups ($P < 0.001$) relative to non-biopsied controls (odds ratios = 11.6, 18.1, & 38.2, respectively), and lower mortality of males relative to females (odds ratio = 0.3, $P = 0.029$). Compared with biopsied controls, however, gillnet treatments did not significantly increase mortality (*P*-values > 0.10), though sex-specific differences were again significant (male odds ratio = 0.3, $P = 0.023$). Mortality and subsequent increases in total hazard ratios after 30 dpt (Fig. 2.2b) are attributable to senescence.

2.4.2 Short-term effects of capture

Plasma lactate, osmolality, and hematocrit were significantly lower in fish sampled 1 – 2 days following capture (T1) relative to individuals sampled immediately after gillnet capture in the river (T0, *P*-values < 0.001), while cortisol and chloride were higher at T1 relative to T0 (*P*-values < 0.001; Fig. 2.3) with no sex-specific differences. Sex hormones (estradiol and testosterone) were lower in males relative to females (*P*-

values < 0.001), and both were reduced at T1 in males and females (*P*-values < 0.001), with a more dramatic decrease in estradiol in females compared to males (interaction $P < 0.001$). Glucose levels differed between males and females ($P = 0.013$), and were elevated in females and depressed in males at T1 relative to T0 (sampling date: $P = 0.012$; interaction: $P = 0.003$).

Gene expression of targeted stress and immune biomarkers in the gill differed between fish sampled at T0 and at T1 (Fig. 2.4). PC1 explained much of the total variance (38%), and PC2 an additional 15%; a Monte Carlo randomization test identified the first two components as significant ($P < 0.001$), though only the first component showed significant associations with sampling date (ANOVA: $P < 0.001$) and interaction between date and sex ($P = 0.003$), but no main effect of sex ($P = 0.671$). Individuals sampled at T0 loaded positively on PC1, while those sampled at T1 loaded negatively. Most of the biomarkers loaded positively on PC1, suggesting enhanced positive regulation of these genes at the time of capture relative to the days following. Sex-specific differences were noted among T0 fish, but not among T1 fish, where females clustered closely and positively on PC1 unlike males that had a greater range in their positions along PC1. Positively loaded biomarkers included many aspects of the stress response such as GR2, HSP90 and SHOP21 (loadings = 0.88, 0.82 & 0.51, respectively; Iwama *et al.*, 1998; Pan *et al.*, 2002; Yada *et al.*, 2007) and several aspects of immunity such as HSC70, C3, RIG1 and CD4 (loadings = 0.97, 0.88, 0.81 & 0.77, respectively). Negatively loaded biomarkers included those associated with iron metabolism (TF, -0.39; hep, -0.21; Raida and Buchmann, 2009), immune regulation (IL11, -0.65; IL1R, -0.58;

IL15, -0.53; Secombes *et al.*, 2011) and inflammation (MMP13, -0.72; Krasnov *et al.*, 2012).

The prevalence of *F. psychrophilum* was lower at T0 than T1 ($P = 0.003$), with no significant difference in productivity between time points ($P = 0.515$; Fig. 2.5). Conversely, prevalence of *L. salmonae* at T0 was higher than at T1 ($P = 0.002$), again with no significant difference in productivity ($P = 0.093$). The productivity of *C. shasta* was lower at T1 than at T0 ($P < 0.001$) with no significant difference in prevalence ($P = 0.996$). No significant differences in prevalence or productivity of *P. minibicornis* or *Ca.* B. cysticola were identified (P -values > 0.121), though the bimodal distribution of *P. minibicornis* at T1 suggests that a subset of individuals exhibited lower productivity.

2.4.3 Factors influencing long-term survival to the spawning period

Microbe richness (total microbe species present) in gill tissue at capture was poor predictor of survival to the spawning period (GLM: $P = 0.172$). Co-infection was common both at capture and at death, with most fish carrying ≥ 3 microbe species. The multivariate classification tree model conducted on the gillnet-treated fish (20 s and 20 min) identified several variables reliably distinguishing ‘success’ and ‘failure’ to survive to the spawning period (Correct classification rate = 88%, Kappa = 75%, Monte Carlo kernel-based $P < 0.001$). Plasma lactate was identified as the primary splitting criteria at 4.6 mmol/L, with low lactate individuals more likely to survive (85% correctly classified as success). Relative expression of Mx, an interferon-induced anti-viral protein, was identified as the secondary splitter among individuals with high lactate, with higher relative expression of Mx (≥ - 0.292) associated with failure (91% correct classification) and lower expression associated with success (83% correct classification). Eighty-nine

percent of individuals with elevated plasma lactate (i.e. > 4.6 mmol/L) and relatively high Mx expression (i.e. ≥ - 0.292) at the time of capture failed to survive beyond 20 dpt. Lactate and Mx were identified as the variables of greatest importance to decreasing node impurity (normalized quantiles: 100 & 88, respectively), as well as transferrin expression, plasma glucose, CD4 and Interferon α expression, and estimated percent lipid in muscle (normalized quantiles: > 48).

2.4.4 Factors associated with mortality

Gross pathologies of many individuals at death included necrosis of skin, gill, and muscle tissues and associated *Saprolegnia* spp. fungal infections in areas where the gillnet caused constriction or injury, such as behind the operculum (Fig. 2.6). However, not all individuals that died prematurely showed external signs of poor health. Many individuals failed to develop secondary sexual characteristics or ripen. Microbes measured in pooled tissues at death showed no significant change in prevalence with days surviving (logistic regression: all *P*-values > 0.05), likely due to the high prevalence of most microbes. Relative productivity of positively detected microbes, however, did show variation with time (Fig. 2.7, Table 2.5). *F. psychrophilum* and *C. shasta* exhibited negative relationships with days surviving (*F. psychrophilum*: slope = -0.07, r^2 = 0.250, *P* < 0.001; *C. shasta*: slope = -0.07, r^2 = 0.195, *P* < 0.001). Positive relationships were identified for *I. multifiliis* (slope = 0.09, r^2 = 0.141, *P* < 0.001), RLO (slope = 0.08, r^2 = 0.168, *P* < 0.001), and *P. minibicornis* (slope = 0.16, r^2 = 0.712, *P* < 0.001). The relationship of *Ca.* B. cysticola with days surviving was positive with a shallow slope (slope = 0.04, r^2 = 0.074, *P* = 0.016), and *L. salmonae* showed no significant relationship (*P* = 0.967).

The agreement between gill and pooled tissue positive microbe detections was relatively high overall, ranging from 69 – 99% total agreement (Table 2.5). *C. shasta* exhibited the lowest total agreement, primarily attributable to negative detection in gill and positive in pooled tissue, which was the most common source of disagreement (e.g., for *F. psychrophilum, L. salmonae, Tetracapsuloides bryosalmonae, I. multifiliis*), suggesting a potential for false negatives if only the gill tissue is used for screening. Coefficients of variation between estimated copy numbers of positively detected microbes were relatively consistent and all less than one. The lowest was found in *C. shasta* (slope = 0.06, $r^2 = 0.01$, $P = 0.51$), primarily due to an outlier, though the relationship still showed high variability excluding the outlier (slope = 0.11, $r^2 = 0.22$, $P < 0.01$). *I. multifiliis* and RLO had the strongest and tightest relationships between methods (slopes > 0.43, $r^2 > 0.74$, $P < 0.01$), likely due to their isolation within gill tissue and subsequently consistent dilution in the aqueous tissue pool with other tissue types following tissue homogenization. Increased variability in the relationship between the methods with increasing productivity was evident in five out of the nine microbes evaluated (P-values < 0.01); *C. shasta, K. thyrsites* and *T. bryosalmonae* exhibited too much variability overall or too few observations to ascertain heteroscedasticity and *F. psychrophilum* showed marginal non-significance ($P = 0.08$). Spearman's rank correlations revealed a strong correlation between the productivities of *I. multifiliis* and RLO ($r_s = 0.92$) and moderate correlation between the bacteria *F. psychrophilum* and *Ca.* B. cysticola ($r_s = 0.41$). All other correlations were low ($r_s \leq |0.35|$).

Microbe prevalence in fish sacrificed at spawning grounds was similar to that of fish sacrificed riverside at Yale, BC and held in the laboratory (Table 2.2). Some microbes

showed higher prevalence at spawning grounds, including *F. psychrophilum* (100% at spawning grounds, 55% among held fish), *T. bryosalmonae* (85% spawning grounds, 4% held), and *Sphaerothecum destruens* (23% spawning grounds, 1% held).

2.4.5 Microbe productivity and host responses

NMDS analysis of pooled tissue microbe data from individuals at death (including premature mortalities and survivors euthanized at the close of the spawning period) was successful in reducing the data into 2-dimensional ordination (stress = 0.22, $P = 0.001$, Fig. 2.8). Fitted gill gene expression biomarkers significantly correlated with the ordination gradient at $P < 0.01$ for MHCIIB, JUN, IL11, B2M, TF, C7, IgMs and MMP13, at $P < 0.05$ for RIGI, Mx, SHOP21 and HSC70, and at $P < 0.10$ for HSP90, IRF1 and ATP5G3C. Clinical variables significant at $P < 0.01$ included plasma chloride and sodium, while cortisol, lactate, osmolality, hematocrit and muscle lipid were significant at $P < 0.05$. Longevity (days surviving) significantly correlated with the ordination gradient ($P = 0.004$), positively with NMDS1 and negatively with NMDS2, but treatment and sex did not (P-values = 0.13 & 0.82, respectively).

F. psychrophilum and *C. shasta* were close in ordination space, negative on NMDS1 and positive on NMDS2. Expression of TF, C7, HSP90, JUN, SHOP21, IL11 and MMP13 as well as plasma cortisol, lactate, hematocrit and muscle lipid shared ordination space with *C. shasta* and *F. psychrophilum*. *P. minibicornis* and *Ca.* B. cysticola were opposite in ordination space to *F. psychrophilum* and *C. shasta*, positive on NMDS1 and neutral on NMDS2, and associated with gene expression biomarkers RIGI (primarily *P. minibicornis*), IRF1, B2M, MHCIIB, Mx and IgMs, and the plasma variables sodium, chloride and osmolality, and host longevity. RLO and *I. multifiliis* were close in

ordination space and to *Ca.* B. cysticola, falling neutral on NMDS1 and negative on NMDS2, directly opposite to HSC70 gene expression. *L. salmonae* was isolated in ordination space from other microbes, loading negatively on both NMDS axes and positively associated with ATP5G3C expression.

2.5 Discussion

Our results provide further evidence for delayed mortality following a capture stressor and illustrate influences of entanglement duration, sex, and infectious disease processes on the survival of sockeye salmon in fresh water when exposed to a realistic temperature profile. Although severe entanglement (20 min) showed the greatest reduction in overall survival and days surviving, even a brief (20 s) encounter had a profound consequence, impacting both fish condition and survival. The 60% post-release mortality rate currently applied to Early Stuart sockeye released from Fraser River gillnet fisheries is similar to the raw mortality rates of 20 s and 20 min entangled fish (54 & 73% mortality, respectively, sexes combined), but is high after incorporating the effects of holding and biopsy (11 – 43% & 30 – 62% mortality, respectively). It should be noted, however, that entanglement treatments applied in the present study (20 s and 20 min) were on the low end of entanglement durations experienced by bycatch in actual gillnet fishery practices (e.g., set times may be > 60 min; DFO, 2002; Farrell *et al.*, 2001). Capture severity has previously been associated with enhanced mortality of released sockeye salmon in both field (Baker *et al.*, 2013; Baker and Schindler, 2009; Donaldson *et al.*, 2012) and laboratory settings (Gale *et al.*, 2011), with more severe capture conditions and injuries correlating with greater physiological disturbances. Similar responses have also been identified in coho salmon (*O. kisutch*) caught in marine gillnet fisheries (Buchanan *et al.*,

2002; Farrell *et al.*, 2001). In the present study, the bulk of mortality following entanglement in fresh water did not occur until 5 days after the event, continuing at high rates for another 5 – 7 days, then subsiding in both treatment groups. The growing body of evidence for delayed mortality following capture in fresh water (Arlinghaus *et al.*, 2007; Davis, 2002; Donaldson *et al.*, 2010, 2012; Raby *et al.*, 2012) and associated sex-specific effects (Donaldson *et al.*, 2014; Gale *et al.*, 2014; Martins *et al.*, 2012b; Robinson *et al.*, 2013) points to causal factors beyond short-term impacts, such as anaerobiosis or cardiovascular collapse, and instead toward interactions between the capture-related stress and infectious disease processes (Gilhousen, 1990; Raby *et al.*, 2015) that can differ between sexes.

In the river, a 5 – 12 day delay would place mortality far upstream of the capture location, and certainly beyond the observation of the fishery, if released bycatch continued to swim upstream at estimated ground speeds (Rand and Hinch, 1998). For this reason and because this delay period is greater than the duration of most holding studies upon which management regulations are based (e.g. 24 – 48 h), current regulations may be underestimating mortality of released catch (Patterson *et al.*, 2017a,b). Additional factors including repeated capture in heavily fished regions, areas of challenging flows (e.g. Hells Gate), and rising river temperatures may exacerbate capture stress and mortality. Mortality in the present study spiked when temperatures were > 15 ˚C, which would correspond to when individuals would be moving through the Nechako and Stuart rivers. These high temperatures could affect the virulence of microbes carried and encountered by the host as well as host resistance (Altizer *et al.*, 2013), contributing to pre-spawn mortality at the spawning grounds (Macdonald *et al.*, 2012). Although

temperature is generally inversely correlated with discharge in the Fraser River (Macdonald *et al.*, 2010; Patterson *et al.,* 2007), inclusion of multiple temperature or flow treatments would help to elucidate the roles of temperature and flow as compounding factors of fisheries capture stress.

Two control groups were included in the present study to assess the effects of holding and additional handling on survival. Mortality was high among biopsied controls, with females experiencing almost twice the mortality of males, whereas non-biopsy controls survived extremely well. This same biopsy technique has been applied in field telemetry studies on sockeye in the Fraser River and has been associated with similar or lower survival to spawning grounds (Donaldson *et al.*, 2010, 2011, 2012), but generally higher survival in marine or coastal waters (Cooke *et al.*, 2005; Martins *et al.*, 2012b). Consistently lower survival and fewer days surviving (6 – 7 days) for females in the present study could be attributed to the biopsy-sampling procedure or entanglement. All gillnet treated fish were biopsied, and biopsied controls as well as gillnet treated fish exhibited sex-specific mortality. Controlling for the effects of handling in experiments is difficult as all subjects must be tagged or handled for study. Because the sample size for our biopsied controls was low relative to other treatment groups, further investigation of the biopsy effect is needed before firm conclusions can be drawn as to its independent impact on adult salmon survival and physiology in fresh water. Collectively, these findings suggest high sensitivity to any level of handling during river migration, especially for females.

Physiological and infectious disease-associated effects of capture and handling were apparent immediately and in the days and weeks following entanglement. Enhanced

anaerobic activity following entanglement, especially when compounded by an intracellular immune response in the gill, was predictive of mortality. Although multiple infections were common at entanglement and at death, microbe species richness alone was a poor predictor of host survival. Fish that died prior to the spawning period showed elevated productivity of *F. psychrophilum* and *C. shasta* at death relative to survivors. These microbes were associated with enhanced indicators of stress, injury and inflammation and diminished immune responses in dying fish. Conversely, productivity of the myxozoan parasite *P. minibicornis* and several other microbes was positively associated with longevity and a strong immune response. Regulation of stress and immune biomarkers and blood properties at death therefore differed depending on microbe community composition, which was strongly associated with host longevity. This pattern suggests alternate responses to infections or opportunistic strategies of certain microbes that may influence post-release survival. The mechanisms of post-release mortality likely depend not only on the severity of the capture event (and likely sex), but on the condition and response of individuals prior to, during, and following capture, which are influenced by or associated with infectious disease processes.

2.5.1 Short-term responses to capture and predictive factors of mortality

Immediate and short-term responses to capture involved aspects of primary, secondary and tertiary features of the stress response (Barton and Iwama, 1991). High levels of plasma chloride following capture indicated osmoregulatory imbalance associated with a stress response and has been previously documented in adult sockeye in fresh water stressed by both warm water (Jeffries *et al.*, 2012b) and a simulated gillnet entanglement (Donaldson *et al.*, 2012). Elevated plasma cortisol was associated with shifts in microbe

community structure following capture and altered gene regulation in the gill suggestive of immunosuppressive effects. Reproductive hormones were also suppressed in the days following capture and many fish failed to develop secondary sexual characteristics, a phenomenon also observed in Alaskan sockeye salmon with injuries indicating escape from gillnet fisheries (Baker *et al.*, 2013). Although delayed maturation observed in the present study could be due to either capture or holding (Patterson *et al.*, 2004), this finding warrants further investigation into how salmon recover from an acute stressor under chronically stressful conditions. Chronic stressors such as high river temperature or discharge have been shown to reduce reproductive output of Early Stuart sockeye salmon (Braun *et al.*, 2013). Our results together with previous findings suggest that these animals are poorly equipped to manage the metabolic demands of long (> 20 min) and sometimes even brief (20 s – 3 min) capture durations, with potential carryover effects on reproduction (O'Connor *et al.*, 2014).

Both immediate responses to and condition during capture – specifically elevated plasma lactate levels (i.e. anaerobic exercise) and Mx gene expression in gill – were predictive of survival to the spawning period of Early Stuart sockeye salmon. The importance of maintaining aerobic metabolism in bycatch is evident from the high plasma lactate levels of fish destined to die prior to the spawning period (even after mild entanglement), utilizing anaerobic respiration during entanglement and unable to clear metabolites (Jain and Farrell, 2003). Strenuous exercise and air exposure are common occurrences in bycatch scenarios, and both contribute to elevated lactate levels measured in blood plasma (Cooke *et al.*, 2013; Gale *et al.*, 2011). Although microbe productivity did not emerge as an important predictor of survival, expression of Mx – an interferon-

induced antiviral protein – in gill was included in the predictive model. Elevated Mx expression has been associated with mortality of juvenile (Jeffries *et al.*, 2014a) and adult sockeye (Miller *et al.*, 2011), though the specific mechanisms driving this correlation are unknown. Viral infection was not identified as prevalent or correlated with Mx expression in this experiment, and *F. psychrophilum* and *C. shasta*, though identified as potential pathogens in this study, were not correlated with Mx expression. Despite the comprehensiveness of our screening approach, an unknown viral agent may have been present within these fish. Alternatively, expression patterns may be a relic of cleared, latent, or anticipated viral infection: freshwater resident *O. mykiss* have shown overexpression of antiviral transcripts relative to out-migrating smolts, suggesting a modulation of immune defenses toward viral pathogens in fresh water (Altizer *et al.*, 2011; Sutherland *et al.*, 2014). Enhanced expression of immune transcripts, including both cellular and humoral responses, have been documented in sockeye salmon during the spawning migration, corresponding to shifts in environmental and biotic factors (Evans *et al.*, 2011). In the present study, relative expression of Mx at death was positively associated with days surviving, which may indicate modulation of this gene's expression as part of the senescence trajectory of Pacific salmon. This temporal confounding would explain the lower Mx expression levels found in premature mortalities at death relative to survivors, with values more similar to those measured at entanglement. Regardless of its mechanism, elevated Mx expression in the presence of high plasma lactate levels was associated with an 85% chance of premature mortality, demonstrating an association between these factors that may suggest synergistic or additive effects on the likelihood of host survival.

2.5.2 The role of microbes in post-release mortality

Large-scale animal migrations have been identified as potential mitigation measures for disease epidemics that may otherwise greatly impact population dynamics (Altizer *et al.*, 2011). Infectious agents therefore likely play a measurable role in determining survival of salmon during spawning migrations. This would especially be true for semelparous Pacific salmon with a fixed energy budget to fuel their migration to natal waters where they will die soon after spawning (Rand *et al.*, 2006). Alterations to historic migratory conditions, such as increased anthropogenic activities like fishing or hydrologic changes, may offset the balance of established host-parasite relationships by lessening the effectiveness of current life history strategies in attenuating disease proliferation within and among individuals. Premature mortality of adult spawners could therefore result from a number of related causes including an upset to the community dynamics of microbes carried by the host, diminished host resilience, enhanced susceptibility to infection, or a combination of these phenomena. Distinct host responses to microbe infections may influence survival following capture, as microbe community structure at death and host gene regulation were strongly associated with longevity in the present study. Two microbes in particular, *F. psychrophilum* and *C. shasta*, exhibited pathogenic characteristics, with short- and long-term changes in their prevalence or productivity and concurrent shifts in host physiology.

Short-term increases in *F. psychrophilum* prevalence following gillnet capture may signify a means of enhanced transmission among sockeye salmon *en route* to spawning grounds, potentially increasing the influence of this bacteria in intensely fished regions. *F. psychrophilum* was present in all gillnet-treated fish at entanglement. Because the

collection and treatment nets were not disinfected between fish, *F. psychrophilum* could have been transferred via the net – a possibility that certainly exists for a real fishery. Captivity may have artificially increased the prevalence of *F. psychrophilum* following capture, but may also reflect transmission dynamics that would occur naturally among migrating individuals packed in resting pools or in pre-spawning aggregations in the river (Gilhousen, 1990). This bacteria, which has been shown to increase in prevalence toward spawning grounds (Miller *et al.*, 2014), excretes a psychrophilic protease causing lesions and necrosis in affected tissues and has been found to suppress humoral immune defenses (Barnes and Brown, 2011). Indeed, we noted lesions on many moribund fish and aspects of the complement system's membrane attack complex (C3, C4B) showed lower expression following capture. Prevalence in this study could also be considered a proxy for productivity as low RNA copy numbers, either below qPCR detection limits or in isolated regions of the gills not sampled, would produce negative detections; the likelihood of false negatives would be expected to decrease as infections intensify. Productivity of *F. psychrophilum* in the present study was also associated with decreased plasma ion levels (sodium, chloride) in both moribund and surviving fish. Osmoregulatory failure is predictive of mortality in Pacific salmon, with plasma ion and osmolality levels dropping by 20 – 40 % prior to death (Jeffries *et al.*, 2011). Our results demonstrate a link between the productivity of *F. psychrophilum* and osmoregulatory failure, which may result from opportunistic enhancement of *F. psychrophilum* in a compromised host or loss of ion homeostasis due to tissue damage caused by *F. psychrophilum* (Barnes and Brown, 2011).

Lower productivity of *C. shasta* in the days following capture could be attributed to the life cycle of this myxozoan parasite, as spores enter the host through the gills but migrate to the gut to mature (Bartholomew, 1998). Whether such pathogenesis could be initiated by acute stress is unknown; chronic thermal stress has been positively associated with ceratomyxosis and mortality of Klamath River salmon, but likely via enhanced spore densities in the river (Ray *et al.*, 2012). An intermediate polychaete host releases infectious *C. shasta* spores into the river as adult salmon begin their upstream migration (Bartholomew *et al.*, 1997); peaks or lulls in spore densities and the rate at which salmon pass through the lower river influence "dosage" and subsequent infection intensities (Stocking *et al.*, 2006). Throughout the period of collection (2 days), fish were randomized among treatment groups to reduce any time-associated bias in microbe productivities. As we did not include intestine in our tissue screen, *C. shasta* productivity measured here does not account for changes in the gut, which may have provided further information relative to its pathogenicity in Fraser River sockeye salmon. Furthermore, disagreement between detections in gill and pooled tissues suggests that pathogen screening via gill biopsy may underestimate the infection intensity of *C. shasta*. However, because *C. shasta* infects the host via the gill, this tissue provides some insight into infectious load, a key factor for host survival (Stocking *et al.*, 2006). The presence of *C. shasta* within the pooled tissues (including gill) of all prematurely morbid gillnet-treated fish points to reduced survival of infected hosts.

F. psychrophilum and *C. shasta* were most productive in the earliest mortalities at death and were associated with similar responses in both gill gene expression and plasma indices of host stress and immunity. This finding suggests either a mutualistic

relationship between these microbes, similarly opportunistic productivity in dying hosts, or equally pathogenic effects. Although our terminal analysis did not identify a significant relationship of gillnet treatment with microbe community dynamics, most premature mortalities were experimentally entangled. Among dying fish, productivities of *F. psychrophilum* and *C. shasta* (to a lesser extent) negatively or neutrally correlated with relative expression of most intra- and extracellular immune genes (e.g. IgMs, Mx, MHCIIB, B2M, IRF1), which may illustrate a relationship between immunosuppression and microbe productivity. This response may indicate an inability of the host to maintain an adequate defense against these pathogens in the days or weeks following capture. Downregulation of several immune response factors in the gill transcriptome of moribund fish has previously been described in adult sockeye salmon exposed to thermal stress, suggesting potential immunosuppression-linked mortality in stressed fish (Jeffries *et al.*, 2012a). The few immune genes that were positively correlated with *F. psychrophilum* and *C. shasta* indicated an innate pathogen defense, including compromised epithelial integrity and inflammation (MMP13, IL11; Trepicchio *et al.*, 1996; Wang *et al.*, 2005), pathogen lysis (C7; Gonzalez *et al.*, 2007), and iron metabolism (TF; Raida and Buchmann, 2009). Enhanced expression of MMP13 and IL11 have also been associated with imminent mortality of Chilko sockeye smolts at the start of seaward migration (Jeffries *et al.*, 2014a); because *F. psychrophilum* was not prevalent and *C. shasta* (as well as other agents included here) not measured in Chilko smolts, such gene regulation may be part of a more universal response to handling stress.

Other microbes such as *I. multifiliis*, RLO, *Ca.* B. cysticola and *P. minibicornis* showed increasing productivity with days surviving and more positive associations with

biomarkers of immunity at death. This association indicates an immune response from the host late in the migration rather than sooner (i.e. stronger immune responses among surviving fish during the spawning period). Mortality associated with these microbes would therefore be more likely to occur at spawning grounds instead of *en route* and thus not necessarily be attributable to capture but rather senescence. However, it is possible that some individuals that died prematurely had a decreased threshold for productivity of these microbes (Bradford *et al.*, 2010b). Their negative association with stress and wound healing biomarkers does not support an opportunistic pathogenicity in immune-compromised fish, but rather an elicited immune response by apparently non-stressed individuals in association with high microbe productivities. *I. multifiliis* and RLO were clustered closely on the NMDS and their productivities were highly correlated, which supports an endosymbiosis previously identified between this ciliate and *Rickettsia* bacteria (Sun *et al.*, 2009). Expression of the heat shock cognate 70 (HSC70), a molecular chaperone that has shown variable responses to stressors (Boone and Vijayan, 2002), was inversely correlated with *I. multifiliis* and RLO on the NMDS gradient. Although downregulation of this gene has been noted in salmon at spawning grounds (Miller *et al.*, 2009), the relationship of HSC70 with longevity was weak relative to its correlation with *I. multifiliis* and RLO productivities. The mechanism for this inverse relationship warrants further investigation. Productivity of *I. multifiliis*, RLO, *Ca.* B. cysticola and *P. minibicornis*, as well as the expression of antibody (IgMs), intracellular (Mx, B2M), extracellular (MHCIIB), and antiviral (IRF1, RIG1) host genes, plasma osmolality and ions were all positively associated with host longevity. Because survival

was impacted by gillnet entanglement and handing, capture stress cannot be excluded as a potential modifier of infectious agent communities and host responses.

Loma salmonae, a microsporidian parasite known to infect sockeye salmon in BC (Shaw *et al.*, 2000), showed lower prevalence in the days following capture. A bimodal distribution of productivities was present at collection but absent in the days following with more negative detections; low productivities could therefore have been reduced below detection limits while higher productivities remained detectable. The rupture of xenomas (spore aggregations in host cells) in gill lamellae and subsequent spore release and dispersal (Rodriguez-Tovar *et al.*, 2011) could reduce prevalence by decreasing the probability of detection in gill. Elevated expression of the inflammation-associated collagenase MMP13 gene in gill could be attributed to xenoma rupture or, alternatively, *F. psychrophilum* infection (Kent and Speare, 2005; Langevin *et al.*, 2012) or gillnet injury. *L. salmonae* displayed unique characteristics at death among the microbes evaluated, showing no significant changes in productivity with time in morbid fish. Expression of ATP5G3C was associated with *L. salmonae* productivity (and *I. multifiliis* and RLO to a lesser degree), suggesting enhanced cellular energy needs in the gills of infected fish, potentially due to ruptured xenomas and damage to gill lamellae (Kent and Speare, 2005). This parasite was not strongly associated with survival of Early Stuart sockeye in this study, though productivity measured in severely gillnetted fish at death was generally higher than in other groups (data not shown). A negative impact of *L. salmonae* infection on the survival of adult Chilko sockeye tagged in the marine environment (Miller *et al.*, 2014) may suggest that associated losses are stock-specific or occur closer to the river mouth than where our fish were collected.

Collectively, these changes in microbe productivity and prevalence suggest a dynamic community and warrant further investigation into their natural trajectories throughout the entire migration period. Microbe prevalence in the laboratory was similar to that measured at spawning grounds, though sample sizes were limited. However, some pathogenic microbes including *F. psychrophilum* and *T. bryosalmonae* (etiological agent of proliferative kidney disease; Longshaw *et al.*, 2002) were more prevalent at spawning grounds; fish held in the relatively sterile laboratory environment may have therefore been protected from additional infectious agents affecting fish in the river, which could have enhanced their survival (Benda *et al.*, 2015). Causal relationships between microbe infections and changes in host responses have yet to be established and will require an improved understanding of the baseline trajectories of microbe productivities and host responses over time in wild fish.

2.5.3 Sex-specific differences

Females showed more susceptibility to impairment imposed by handling stress, with unique physiological changes relative to males and consistently greater (and earlier) mortality among biopsied controls and gillnet treated fish. Given that biopsied controls experienced high sex-specific mortality, extrapolating this sex effect beyond experimental handling is speculative. However, sex-specific effects have been repeatedly documented in response to capture stress with respect to survival and physiological effects (Donaldson *et al.*, 2014; Gale *et al.*, 2014; Martins *et al.*, 2012b; Robinson *et al.*, 2013). Martins and colleagues (2012a) noted, for example, that females exhibited lower survival when compounding stressors (e.g. temperature and hydraulic challenges) were acting simultaneously. Individuals in the present study were exposed to a similar thermal

experience to those traveling in the river, which corresponds to an increase in temperature with time in the first 1 – 2 weeks of the migration until thermal refugia can be accessed (Rand and Hinch, 1998). Female sockeye salmon have been shown to perform poorly when exposed to chronic high temperatures, with lower survival among females held at 19 ˚C relative to those held at 13 ˚C and overall lower survival relative to males (Jeffries *et al.*, 2014b), but few transcriptomic differences between sexes. Sex-specific differences in gene expression were identified at the time of collection in the present study, with females exhibiting more consistently elevated expression of immune-related gene transcripts in gill than males, suggesting that females may differ in their response to migratory stress in the river. As a secondary stress response, short-term differences in glucose levels following capture were also sex-specific, pointing to a stress-related release of glucose needed to support aerobic respiration in females that is absent in males (Barton, 2002), though possibly due to holding rather than capture (Donaldson *et al.*, 2011). Causal factors for higher female mortality during river migration have yet to be fully characterized.

2.6 Conclusions

Post-release mortality of adult salmon has repeatedly been shown to be context-specific and dependent on numerous factors (Raby *et al.*, 2015). The mechanisms of mortality following capture are therefore complex and include internal and external influences (Fenkes *et al.*, 2016). In addition to previously identified factors, such as temperature and sex, our results support a role for capture severity and infectious disease processes in impacting the survival of released bycatch in fresh water. Anaerobic activity in concert with an intracellular immune response was associated with decreased long-term survival

to the spawning period. The benefits of removing bycatch from the net quickly and under-water, though already embraced by some fishers as best practices, should be widely disseminated among user groups. The degree to which temperature impacts post-release mortality rates deserves further investigation, as the majority of premature mortality occurred 5 – 12 days after entanglement, when temperatures were at their highest. Early Stuart sockeye navigate a balance of challenging migratory conditions, when the timing of river entry coincides with high flows often accompanied by lower temperatures or high temperatures with generally more manageable flows (Morrison *et al.*, 2002). Projected climate-associated changes to Fraser River hydrology include a shift in the timing of the spring freshet to earlier in the year, which would result in elevated river temperatures during the historic migration period of Early Stuart sockeye (Morrison *et al.*, 2002; Patterson *et al.*, 2007). These changes have been associated with elevated levels of both *en route* and pre-spawn mortality, likely due to stress and disease processes as well as enhanced energy consumption by migrating sockeye salmon utilizing a fixed energy budget (Crossin *et al.*, 2008; Fenkes *et al.*, 2016; Gilhousen, 1990; Macdonald, 2000; Martins *et al.*, 2012b).

The observed delay in post-release mortality not only has implications for how managers assign mortality rates to release fisheries but alludes to its causes. Interactions between anaerobic activity, changes in microbe community dynamics, and shifts in local (gill gene expression) and systemic (plasma hormones and metabolites) stress and immune responses affect the chances of survival following capture. Although our experimental design prohibited direct comparison of treatment groups at death, most fish exposed to severe gillnet entanglement died prematurely, showing higher productivities

of *F. psychrophilum* and *C. shasta* and demonstrating response profiles distinct from survivors. A more complete understanding of the natural trajectory of infectious agent productivities and host responses will improve our understanding of how stressors modulate these factors throughout migration and should be a top priority for future research. Our findings offer insight into the linkages between physiology, infectious agents, and post-release survival following gillnet capture, thereby improving our understanding of the mechanisms contributing to mortality of released catch.

Table 2.1 Primer and probe sequences corresponding to stress and immunity biomarkers and three reference genes evaluated via qPCR on adult sockeye salmon (*Oncorhynchus nerka*). Assay type classifies genes by their association with immunity, stress, or a mortality-related signature (MRS) predictive of migration failure of wild salmon (Miller *et al.*, 2011). References and qPCR efficiencies are provided; in house designs were conducted by the Molecular Genetics Laboratory at the Pacific Biological Station, Nanaimo, BC.

Assay name	Gene information	Assay type	EST/Accession#	Primer and probe sequences	Efficiency	source
B2M	Beta 2-microglobulin	Immune	AF180490	F - TTTACAGCGCGGTGGAGTC	0.92	(Haugland *et al.*, 2005)
				R - TGCCAGGGTTACGGCTGTAC		
				P - AAAGAATCTCCCCCCAAGGTGCAGG		
C3	Complement factor	Immune	U61753, AF271080	F - ATTGGCCTGTCCAAAACACA	0.93	(Raida and Buchmann, 2009)
				R - AGCTTCAGATCAAGGAAGAAGTTC		
				P - TGGAATCTGTGTGTCTGAACCCC		
CD4	Cell receptor	Immune	AY973028	F - CATTAGCCTGGGTGGTCAAT	0.83	(Raida and Buchmann, 2008)
				R - CCCTTTCTTTGACAGGGAGA		
				P - CAGAAGAGAGAGCTGGATGTCTCCG		
CD83	Cell receptor	Immune	AY263794	F - GATGCACCCCTTGAGAAGAA	0.76	(Raida *et al.*, 2011)
				R - GAACCCTGTCTCGACCAGTT		
				P - AATGTTGATTTACACTCTGGGGCCA		
Hep	Hepcidin	Immune	AF281354.1	F - GAGGAGGTTGGAAGCATTGA	0.82	(Raida and Buchmann, 2009)
				R - TGACGCTTGAACCTGAAATG		
				P - AGTCCAGTTGGGGAACATCAACAG		
IFNa	Interferon-α	Immune	AY216595	F - CGTCATCTGCAAAGATTGGA	0.78	(Ingerslev *et al.*, 2009)
				R - GGGCGTAGCTTCTGAAATGA		
				P – TGCAGCACAGATGTACTGATCATCCA		
IgMs	Immunoglobulin	Immune	S63348, AB044939	F - CTTGGCTTGTTGACGATGAG	0.79	(Raida *et al.*, 2011)
				R - GGCTAGTGGTGTTGAATTGG		
				P - TGGAGAGAACGAGCAGTTCAGCA		

IL-11	Cytokine	Immune	AJ535687	F - GCAATCTCTTGCCTCCACTC	0.79	(Raida and Buchmann, 2008)
				R - TTGTCACGTGCTCCAGTTTC		
				P - TCGCGGAGTGTGAAAGGCAGA		
IL-15	Cytokine	Immune	AJ555868.1	F - TTGGATTTTGCCCTAACTGC	0.82	(Raida *et al.*, 2011)
				R - CTGCGCTCCAATAAACGAAT		
				P - CGAACAACGCTGATGACAGGTTTTT		
IL-1R	Cytokine	Immune	AJ295296	F - ATCATCCTGTCAGCCCAGAG	0.80	(Raida *et al.*, 2011)
				R - TCTGGTGCAGTGGTAACTGG		
				P - TGCATCCCCTCTACACCCCAAA		
IRF1	Interferon regulatory factor 1	Immune	CB511515	F - CAAACCGCAAGAGTTCCTCATT	0.74	In house
				R - AGTTTGGTTGTGTTTTTGCATGTAG		
				P - CTGGCGCAGCAGATA		
MHCI	Major histocompatibility complex I	Immune		F - GCGACAGGTTTCTACCCCAGT	0.81	(Ingerslev *et al.*, 2009)
				R - TGTCAGGTGGGAGCTTTTCTG		
				P - TGGTGTCCTGGCAGAAAGACGG		
MHCII-B	Major histocompatibility complex IIβ	Immune	AF115533	F - TGCCATGCTGATGTGCAG	0.80	(Raida and Buchmann, 2008)
				R - GTCCCTCAGCCAGGTCACT		
				P - CGCCTATGACTTCTACCCCAAACAAAT		
MMP13	Matrix metalloproteinase	Immune	213514499	F - GCCAGCGGAGCAGGAA	0.81	(Tadiso *et al.*, 2011)
				R - AGTCACCTGGAGGCCAAAGA		
				P - TCAGCGAGATGCAAAG		
Mx	Antiviral protein	Immune		F - AGATGATGCTGCACCTCAAGTC	0.81	(Eder *et al.*, 2009)
				R - CTGCAGCTGGGAAGCAAAC		
				P - ATTCCCATGGTGATCCGCTACCTGG		
RIG-I	Retinoic acid inducible gene I	Immune	NM_001163699	F - ACAGCTGTTACACAGACGACATCA	0.81	(Larsen *et al.*, 2012)

				R - TTTAGGGTGAGGTTCTGTCCGA		
				P - TCGTGTTGGACCCCACTCTGTTCTCTC		
SHOP21	Salmon hyperosmotic protein 21	Immune	CA054269	F - GCGGTAGTGGAGTCAGTTGGA	0.76	In house
				R - GCTGCTGACGTCTCACATCAC		
				P - CCTGTTGATGCTCAAGG		
TF	Transferin	Immune	D89083	F - TTCACTGCTGGAAAATGTGG	0.81	(Raida and Buchmann, 2009)
				R - GCTGCACTGAACTGCATCAT		
				P - TGGTCCCTGTCATGGTGGAGCA		
ATP5G3-C	ATP synthase	MRS	CB493164	F - GGAACGCCACCATGAGACA	0.79	In house
				R - CGCCATCCTGGGCTTTG		
				P - AGCCCCATTGCCTC		
C4B	Complement factor	MRS	CB518123	F - TCCAACCACATCGCATTATCC	0.73	In house
				R - ATCTCTGACACCACTGACCACAA		
				P - ATAGACAGGCTTCCC		
C7	Complement factor	MRS	CA052045	F - ACCTCTGTCCAGCTCTGTGTC	0.84	In house
				R - GATGCTGACCACATCAAACTGC		
				P - AACTACCAGACAGTGCTG		
EIF4E	Initiation factor	MRS	CA051191, CB496372	F - TCTGGAAACCCACACACAAAGA	1.00	In house
				R - GCGTTTTGAGGTTTGCATGTT		
				P - CCTGCCATAGCCACAC		
KCTD1	Potassium channel tetramerizationdomain	MRS	CA062065	F - TGTTTGTTAAAAGGGGACACAGTG	0.88	In house
				R - GTGAAGTGTTATCTGGGCTGAAAG		
				P - CTCCAAGGCTGAAAT		
MCSF	Macrophage colony stimulating factor	MRS	CA061415	F - GCTCTCTCAATCCTTGGCTTTAC	0.85	In house
				R - ACCAGCATAATTGAAAACCAGAGG		

				P - CTCAATGTCCTCAATGCT		
GR-2	Glucocorticoid receptor	Stress		F - TCCAGCAGCTATGCCAGTTCT	0.84	(Yada *et al.*, 2007)
				R - TTGCCCTGGGTTGTACATGA		
				P - AAGCTTGGTGGTGGCGCTG		
HSC70	Heat shock cognate 70	Stress	CA052185	F - GGGTCACACAGAAGCCAAAAG	0.75	In house
				R - GCGCTCTATAGCGTTGATTGGT		
				P - AGACCAAGCCTAAACTA		
Hsp90	Heat shock protein 90	Stress	CB493960, CB503707	F - TGGGCTACATGGCTGCCAAG	0.80	In house
				R - TCCAAGGTGAACCCAGAGGAC		
				P - AGCACCTGGAGATCAA		
JUN	Transcription factor	Stress	CA056351	F - TTGTTGCTGGTGAGAAAACTCAGT	0.79	In house
				R - CCTGTTGCCCTATGAATTGTCTAGT		
				P - AGACTTGGGCTATTTAC		
78d16.1		Reference	CA056739	F - GTCAAGACTGGAGGCTCAGAG	0.84	In house
				R - GATCAAGCCCCAGAAGTGTTTG		
				P - AAGGTGATTCCCTCGCCGTCCGA		
COIL-P84-2		Reference	CA053789	F - GCTCATTTGAGGAGAAGGAGGATG	0.83	In house
				R - CTGGCGATGCTGTTCCTGAG		
				P - TTATCAAGCAGCAAGCC		
MRPL40		Reference	CK991258	F - CCCAGTATGAGGCACCTGAAGG	0.76	In house
				R - GTTAATGCTGCCACCCTCTCAC		
				P - ACAACAACATCACCA		

Table 2.2 Abbreviations, names, and types of microbes suspected or known to cause disease in Pacific salmon in British Columbia, Canada, evaluated via qPCR on adult sockeye salmon (*Oncorhynchus nerka*). Prevalence values describe percent positive detections among Early Stuart sockeye collected in the Fraser River at Yale, BC (n = 107; includes individuals sacrificed river-side at collection and those held for up to 40 days) and among those sacrificed at spawning grounds (n = 13; near Takla Lake, 7 – 8 Aug, 2013). Primer and probe sequences with references and qPCR efficiencies are provided; in house designs were conducted by the Molecular Genetics Laboratory at the Pacific Biological Station, Nanaimo, BC.

Assay abbreviation	Microbe full name	Type	Prevalence *en route* and held	Prevalence at spawning grounds	Primer and probe sequences	Efficiency	Reference
ae_hyd	*Aeromonas hydrophila*	Bacterium	1	15	F - ACCGCTGCTCATTACTCTGATG	0.91	(Lee *et al.*, 2006)
					R - CCAACCCAGACGGGAAGAA		
					P - TGATGGTGAGCTGGTTG		
ae_sal	*Aeromonas salmonicida*	Bacterium	0	0	F - TAAAGCACTGTCTGTTACC	0.96	(Keeling *et al.*, 2013)
					R - GCTACTTCACCCTGATTGG		
					P - ACATCAGCAGGCTTCAGAGTCACTG		
re_sal	*Renibacterium salmoninarum*	Bacterium	0	0	F - CAACAGGGTGGTTATTCTGCTTTC	0.93	(Powell *et al.*, 2005)
					R - CTATAAGAGCCACCAGCTGCAA		
					P - CTCCAGCGCCGCAGGAGGAC		
c_b_cys	*Candidatus Branchiomonas cysticola*	Bacterium	96	100	F - AATACATCGGAACGTGTCTAGTG	0.90	(Mitchell *et al.*, 2013)
					R - GCCATCAGCCGCTCATGTG		
					P - CTCGGTCCCAGGCTTTCCTCTCCCA		
ye_ruc	*Yersinia ruckeri*	Bacterium	0	0	F - TGCCGCGTGTGTGAAGAA	0.93	(Glenn *et al.*, 2011)
					R - ACGGAGTTAGCCGGTGCTT		
					P - AATAGCACTGAACATTGAC		
fl_psy	*Flavobacterium psychrophilum*	Bacterium	55	100	F - GATCCTTATTCTCACAGTACCGTCAA	0.80	(Duesund *et al.*, 2010)
					R - TGTAAACTGCTTTTGCACAGGAA		
					P - AAACACTCGGTCGTGACC		
pch_sal	*Piscichlamydia salmonis*	Bacterium	0	0	F - TCACCCCCAGGCTGCTT	0.87	(Nylund *et al.*, 2008)
					R - GAATTCCATTTCCCCCTCTTG		
					P - CAAAACTGCTAGACTAGAGT		

pisck_sal	*Piscirickettsia salmonis*	Bacterium	0	0	F - TCTGGGAAGTGTGGCGATAGA	0.95	(Corbeil *et al.*, 2003)
					R - TCCCGACCTACTCTTGTTTCATC		
					P - TGATAGCCCCGTACACGAAACGGCATA		
rlo	*Rickettsia-like organism*	Bacterium	78	69	F - GGCTCAACCCAAGAACTGCTT	0.89	(Lloyd *et al.*, 2011)
					R - GTGCAACAGCGTCAGTGACT		
					P - CCCAGATAACCGCCTTCGCCTCCG		
sch	*gill chlamydia*	Bacterium	0	0	F - GGGTAGCCCGATATCTTCAAAGT	0.95	(Duesund *et al.*, 2010)
					R - CCCATGAGCCGCTCTCTCT		
					P - TCCTTCGGGACCTTAC		
vi_ang	*Vibrio anguillarum*	Bacterium	0	0	F - CCGTCATGCTATCTAGAGATGTATTTGA	0.96	In house
					R - CCATACGCAGCCAAAAATCA		
					P - TCATTTCGACGAGCGTCTTGTTCAGC		
vi_sal	*Vibrio salmonicida*	Bacterium	0	0	F - GTGTGATGACCGTTCCATATTT	0.91	In house
					R - GCTATTGTCATCACTCTGTTTCTT		
					P - TCGCTTCATGTTGTGTAATTAGGAGCGA		
aspv	Atlantic salmon paramyxovirus	Virus	0	0	F - CCCATATTAGCAAATGAGCTCTATCTT	0.92	(Nylund *et al.*, 2008)
					R - CGTTAAGGAACTCATCATTGAGCTT		
					P - AGCCCTTTTGTTCTGC		
pmcv	Piscine totivirus (CMS)	Virus	4	15	F - TTCCAAACAATTCGAGAAGCG	0.92	(Løvoll *et al.*, 2010)
					R - ACCTGCCATTTTCCCCTCTT		
					P - CCGGGTAAAGTATTTGCGTC		
ver	Viral encephalopathy and retinopathy virus	Virus	0	0	F - TTCCAGCGATACGCTGTTGA	1.02	(Korsnes *et al.*, 2005)
					R - CACCGCCCGTGTTTGC		
					P - AAATTCAGCCAATGTGCCCC		
vhsv	Viral hemorrhagic septicemia virus	Virus	0	0	F - ATGAGGCAGGTGTCGGAGG	0.86	(Garver *et al.*, 2011)
					R - TGTAGTAGGACTCTCCCAGCATCC		
					P - TACGCCATCATGATGAGT		
omv	Salmonid herpesvirus	Virus	0	0	F - GCCTGGACCACAATCTCAATG	0.95	In house

					R - CGAGACAGTGTGGCAAGACAAC		
					P - CCAACAGGATGGTCATTA		
sav	Salmon alphavirus	Virus	0	0	F - CCGGCCCTGAACCAGTT	0.99	(Andersen *et al.*, 2007)
					R - GTAGCCAAGTGGGAGAAAGCT		
					P - TCGAAGTGGTGGCCAG		
ven	Viral erythrocytic necrosis virus	Virus	0	0	F - CGTAGGGCCCCAATAGTTTCT	0.96	James Winton, pers. comm.
					R - GGAGGAAATGCAGACAAGATTTG		
					P - TCTTGCCGTTATTTCCAGCACCCG		
pspv	Pacific salmon parvovirus	Virus	0	0	F - CCCTCAGGCTCCGATTTTTAT	NA	In house
					R - CGAAGACAACATGGAGGTGACA		
					P - CAATTGGAGGCAACTGTA		
prv	Piscine reovirus (HSMI, CMS)	Virus	0	0	F - TGCTAACACTCCAGGAGTCATTG	0.85	(Wiik-Nielsen *et al.*, 2012)
					R - TGAATCCGCTGCAGATGAGTA		
					P - CGCCGGTAGCTCT		
ihnv	Infectious haematopoietic necrosis virus	Virus	0	0	F - AGAGCCAAGGCACTGTGCG	0.87	(Purcell *et al.*, 2013)
					R - TTCTTTGCGGCTTGGTTGA		
					P - TGAGACTGAGCGGGACA		
cr_sal	*Cryptobia salmositica*	Parasite	0	15	F - TCAGTGCCTTTCAGGACATC	0.89	In house
					R - GAGGCATCCACTCCAATAGAC		
					P - AGGAGGACATGGCAGCCTTTGTAT		
ce_sha	*Ceratonova shasta*	Parasite	96	85	F - CCAGCTTGAGATTAGCTCGGTAA	0.93	(Hallett and Bartholomew, 2006)
	(formerly *Ceratomyxa shasta*)				R - CCCCGGAACCCGAAAG		
					P - CGAGCCAAGTTGGTCTCTCCGTGAAAAC		
de_sal	*Dermocystidium salmonis*	Parasite	1	0	F - CAGCCAATCCTTTCGCTTCT	0.90	In house
					R - GACGGACGCACACCACAGT		
					P - AAGCGGCGTGTGCC		
fa_mar	*Facilispora margolisi*	Parasite	1	0	F - AGGAAGGAGCACGCAAGAAC	0.92	In house
					R - CGCGTGCAGCCCAGTAC		

					P - TCAGTGATGCCCTCAGA		
gy_sal	*Gyrodactylus salaris*	Parasite	0	0	F - CGATCGTCACTCGGAATCG	0.89	(Collins *et al.*, 2010)
					R - GGTGGCGCACCTATTCTACA		
					P - TCTTATTAACCAGTTCTGC		
ic_mul	*Ichthyophthirius multifiliis*	Parasite	83	100	F - AAATGGGCATACGTTTGCAAA	0.9	In house
					R - AACCTGCCTGAAACACTCTAATTTTT		
					P - ACTCGGCCTTCACTGGTTCGACTTGG		
ku_thy	*Kudoa thyrsites*	Parasite	10	23	F - TGGCGGCCAAATCTAGGTT	0.91	(Funk *et al.*, 2007)
					R - GACCGCACACAAGAAGTTAATCC		
					P - TATCGCGAGAGCCGC		
lo_sal	*Loma salmonae*	Parasite	79	77	F - GGAGTCGCAGCGAAGATAGC	0.93	In house
					R - CTTTTCCTCCCTTTACTCATATGCTT		
					P - TGCCTGAAATCACGAGAGTGAGACTACCC		
my_arc	*Myxobolus arcticus*	Parasite	18	23	F - TGGTAGATACTGAATATCCGGGTTT	0.89	In house
					R - AACTGCGCGGTCAAAGTTG		
					P - CGTTGATTGTGAGGTTGG		
my_ins	*Myxobolus insidiosus*	Parasite	0	0	F - CCAATTTGGGAGCGTCAAA	0.83	In house
					R - CGATCGGCAAAGTTATCTAGATTCA		
					P - CTCTCAAGGCATTTAT		
my_cer	*Myxobolus cerebralis*	Parasite	0	0	F - GCCATTGAATTTGACTTTGGATTA	0.99	(Kelley *et al.*, 2004)
					R - ACCATTCATGTAAGCCCGAACT		
					P - TCGAAGCCTTGACCATCTTTTGGCC		
ne_per	*Neoparamoeba perurans*	Parasite	0	0	F - GTTCTTTCGGGAGCTGGGAG	1.05	(Fringuelli *et al.*, 2012)
					R - GAACTATCGCCGGCACAAAAG		
					P - CAATGCCATTCTTTTCGGA		
nu_sal	*Nucleospora salmonis*	Parasite	0	0	F - GCCGCAGATCATTACTAAAAACCT	0.94	(Foltz *et al.*, 2009)
					R - CGATCGCCGCATCTAAACA		
					P - CCCCGCGCATCCAGAAATACGC		

pa_ther	*Paranucleospora theridion*	Parasite	13	23	F - CGGACAGGGAGCATGGTATAG	0.92	(Nylund *et al.*, 2010)
					R - GGTCCAGGTTGGGTCTTGAG		
					P - TTGGCGAAGAATGAAA		
pa_pse	*Parvicapsula pseudobranchicola*	Parasite	0	0	F - CAGCTCCAGTAGTGTATTTCA	0.95	(Jørgensen *et al.*, 2011)
					R - TTGAGCACTCTGCTTTATTCAA		
					P - CGTATTGCTGTCTTTGACATGCAGT		
pa_kab	*Parvicapsula kabatai*	Parasite	2	8	F - GTCGGATGATAAGTGCATCTGATT	0.97	In house
					R - ACACCACAACTCTGCCTTCCA		
					P - TGCGACCATCTGCACGGTACTGC		
te_bry	*Tetracapsuloides bryosalmonae*	Parasite	4	85	F - GCGAGATTTGTTGCATTTAAAAAG	0.89	(Bettge *et al.*, 2009)
					R - GCACATGCAGTGTCCAATCG		
					P - CAAAATTGTGGAACCGTCCGACTACGA		
pa_min	*Parvicapsula minibicornis*	Parasite	100	100	F - AATAGTTGTTTGTCGTGCACTCTGT	0.88	(Hallett and Bartholomew, 2009)
					R - CCGATAGGCTATCCAGTACCTAGTAAG		
					P - TGTCCACCTAGTAAGGC		
sp_des	*Sphaerothecum destruens*	Parasite	1	23	F - GCCGCGAGGTGTTTGC	0.89	In house
					R - CTCGACGCACACTCAATTAAGC		
					P - CGAGGGTATCCTTCCTCTCGAAATTGGC		
sp_sal	*Spironucleus salmonicida*	Parasite	0	0	F - AACCGGTTATTCGTGGGAAAG	0.91	In house
					R - TTAACTGCAGCAACACAATAGAATACTC		
					P - TGCCAGCAGCCGCGGTAATTC		
ic_hof	*Ichthyophonus hoferi*	Parasite	0	0	F - GTCTGTACTGGTACGGCAGTTTC	0.91	(White *et al.*, 2013)
					R - TCCCGAACTCAGTAGACACTCAA		
					P - TAAGAGCACCCACTGCCTTCGAGAAGA		
na_sal	*Nanophyetus salmincola*	Fluke	0	0	F - CGATCTGCATTTGGTTCTGTAACA	0.88	In house
					R - CCAACGCCACAATGATAGCTATAC		
					P - TGAGGCGTGTTTTATG		

Table 2.3 Variables included in the multivariate classification tree analysis using survival to the spawning period of Early Stuart sockeye (>20 days post-treatment) as the grouping factor. Full microbe names can be found in Table 2.2

Type	Variables
Environmental/morphological	Gillnet exposure time, Sex, Total condition score, Length, Stock
Microbes	c_b_cys, ce_sha, fl_psy, ic_mul, lo_sal, pa_min, rlo
Gene expression biomarkers of stress and immunity	ATP5G3C, B2M, C3, C4B, C7, CD4, CD83, EIF4E, GR2, hep, HSC70, Hsp90, IFNa, IgMs, IL11, IL15, IL1R, IRF1, JUN, KCTD1, MCSF, MHCI, MHCIIB, MMP13, Mx, RIGI, SHOP21, TF
Clinical variables (hormones, metabolites, ions and other physiological indicators)	Chloride, Osmolality, Sodium, Potassium, Muscle lipid, Cortisol, Estradiol, Testosterone, Glucose, Lactate, Hematocrit, Leucocrit

Table 2.4 Sample sizes (*n*), mean days surviving (± standard error), and percent mortality prior to the spawning period for female (F) and male (M) Early Stuart sockeye by treatment

	n		Days surviving		Mortality prior to spawning period	
Treatment	F	M	F	M	F	M
Severe gillnet	10	16	9.4 ± 1.8	16.3 ± 2.8	90%	62%
Mild gillnet	10	16	17.0 ± 2.8	24.4 ± 2.7	70%	44%
Biopsied control	5	9	23.4 ± 3.5	29.6 ± 2.9	60%	33%
Control	13	14	31.2 ± 2.4	36.0 ± 1.2	15%	7%
Total	**38**	**55**	**20.7 ± 1.5**	**25.9 ± 1.4**	**55%**	**38%**

Table 2.5 Agreement between gill and pooled tissues in quantification of presence and relative productivities. Total agreement and sources of error in presence/absence data are shown as percents; relationships between calculated productivities are shown as the slope (β), r^2, and p-values from linear regression of gill and pooled values (predictor and response, respectively). Breusch-Pagan tests describe the heterosckedasticity of the linear relationships. Only positive values were included in the linear regression models

			Agreement between gill and pooled tissues (%)				Linear regression of positives			Breusch-Pagan test	
Microbe	Abbrev.	Total agreement	Gill + Pool +	Gill - Pool -	Gill + Pool -	Gill - Pool +	β	r^2	*P*	BP	*P*
A. salmonicida	ae_sal	99	1	98	0	1	NA	NA	NA	NA	NA
Ca. B. cysticola	c_b_cys	98	95	2	1	1	0.29	0.81	< 0.01	28.5	< 0.01
C. shasta	ce_sha	69	66	2	0	31	0.06	0.01	0.51	0.3	0.59
C. salmositica	cr_sal	98	0	98	0	2	NA	NA	NA	NA	NA
D. salmonis	de_sal	99	0	99	1	0	NA	NA	NA	NA	NA
F. psychrophilum	fl_psy	84	49	35	1	14	0.29	0.97	< 0.01	3.2	0.08
I. multifiliis	ic_mul	94	75	19	1	5	0.45	0.74	< 0.01	25.1	< 0.01
K. thyrsites	ku_thy	98	11	87	0	2	0.22	0.29	0.13	0.6	0.43
L. salmonae	lo_sal	80	65	14	4	17	0.33	0.90	< 0.01	25.7	< 0.01
P. minibicornis	pa_min	99	99	0	0	1	0.23	0.55	< 0.01	14.8	< 0.01
Rickettsia-like organism	rlo	99	67	31	0	1	0.43	0.83	< 0.01	16.9	< 0.01
S. destruens	sp_des	95	0	95	2	2	NA	NA	NA	NA	NA
T. bryosalmonae	te_bry	94	8	86	0	6	0.43	0.44	0.11	0.5	0.46

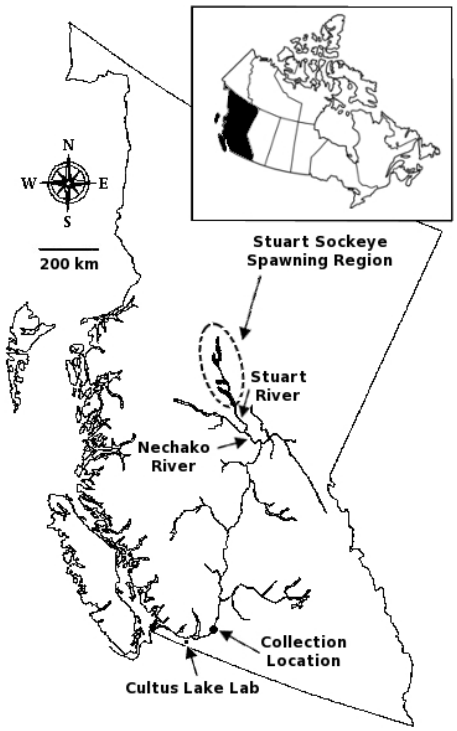

Figure 2.1 British Columbia, Canada, and the Fraser River watershed. Early Stuart sockeye enter the Fraser River in early to mid-July, migrating approximately 1200 km to spawning grounds (dashed circle) in the interior of the province. Fish pass through the Nechako and Stuart rivers before reaching corridor lakes (shown in black, from north to south: Takla, Trembleur, and Stuart). Locations of collection (Yale, BC) and experimental holding (DFO Cultus Lake Salmon Research Lab) are shown.

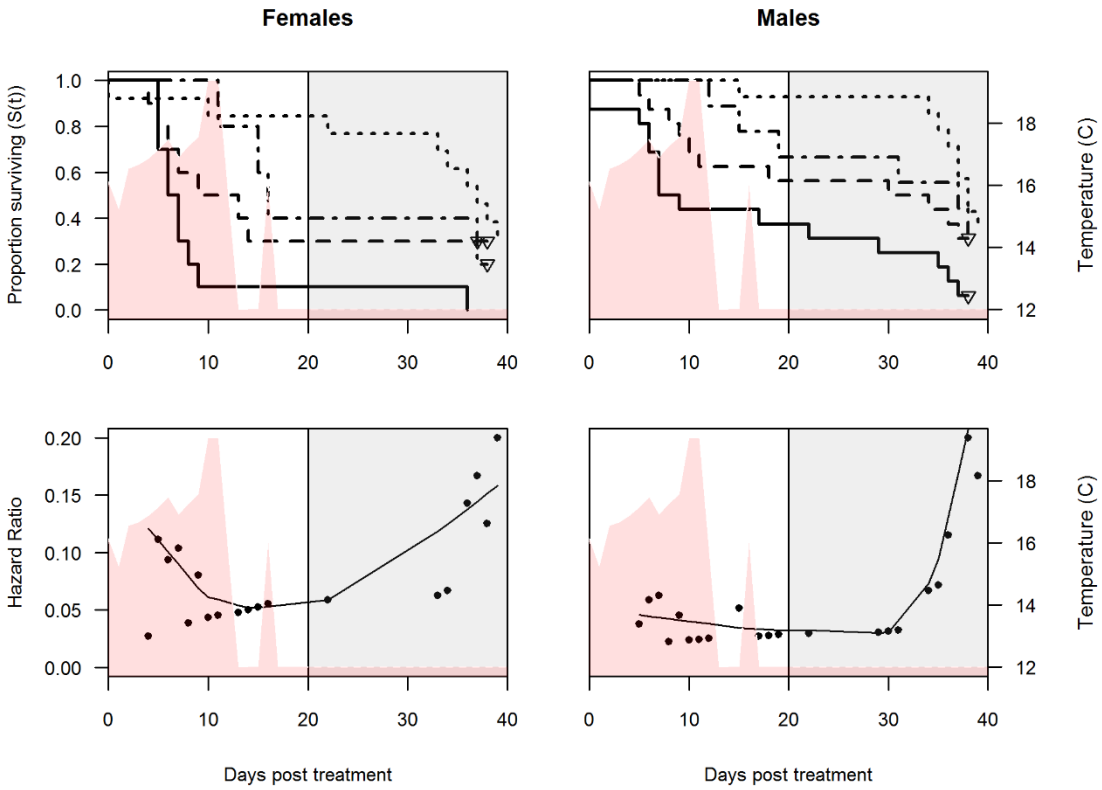

Figure 2.2 Kaplan-Meier survival curves are shown for female (top left) and male (top right) Early Stuart sockeye exposed to severe gillnet entanglement (20 min plus 1 min air exposure, *n* = 26; solid), mild gillnet entanglement (20 sec plus 1 min air, *n* = 26; dashed), biopsied controls (n = 14; dot-dashed), and non-biopsied controls (*n* = 27; dotted). Triangles are censored data points. The grey shaded area corresponds to the spawning period of this population including nest defense. The red shaded area shows the temperature (°C) of all holding tanks through course of the study, which follows the modeled thermal experience of an Early Stuart sockeye migrant in the Fraser River in 2014. Daily hazard ratios for females (bottom left) and males (bottom right) are plotted as a function of time (all treatments combined) with a solid line lowess smoothing function. Hazard ratios correspond to the number of mortalities divided by the total survivors on each day a mortality occurred.

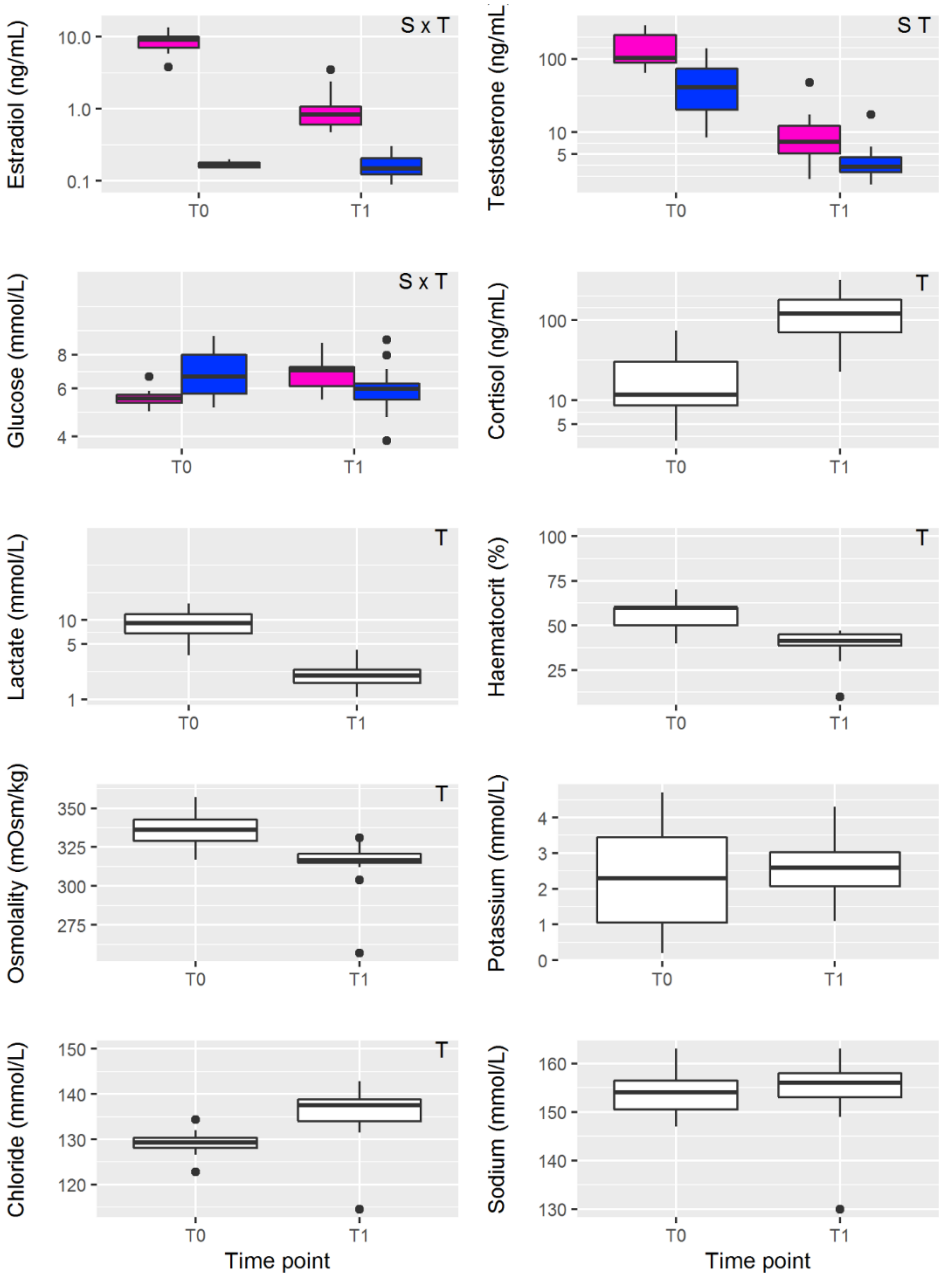

Figure 2.3 Box plots illustrating blood plasma indices of maturation (estradiol and testosterone), metabolic stress (glucose, cortisol, lactate, haematocrit), and osmoregulatory and ionic imbalance (osmolality, potassium, chloride, sodium) measured in Early Stuart sockeye at the time of gillnet capture (T0; n = 19) and 2 days following gillnet capture (T1; n = 28). Estradiol, testosterone, cortisol, and glucose models included a significant sex factor showing differential changes for females (pink) and males (blue) at each time point. Letters at the top right of each plot denote significant differences ($P < 0.05$) between treatments (T), sexes (S), or an interaction between the terms (S × T).

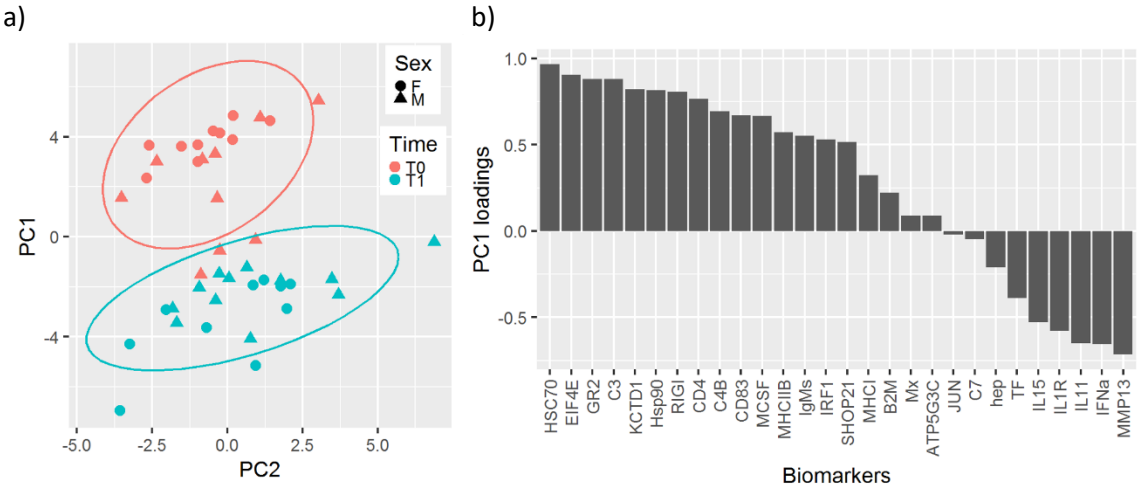

Figure 2.4 a) Principal components analysis of gene expression in gill tissue (28 biomarkers of stress and immunity) at the time of gillnet capture (T0; orange) and 2 days following capture (T1; blue). Ellipses represent 95% confidence intervals for each group cluster. b) Principal component loadings of genomic biomarker variables.

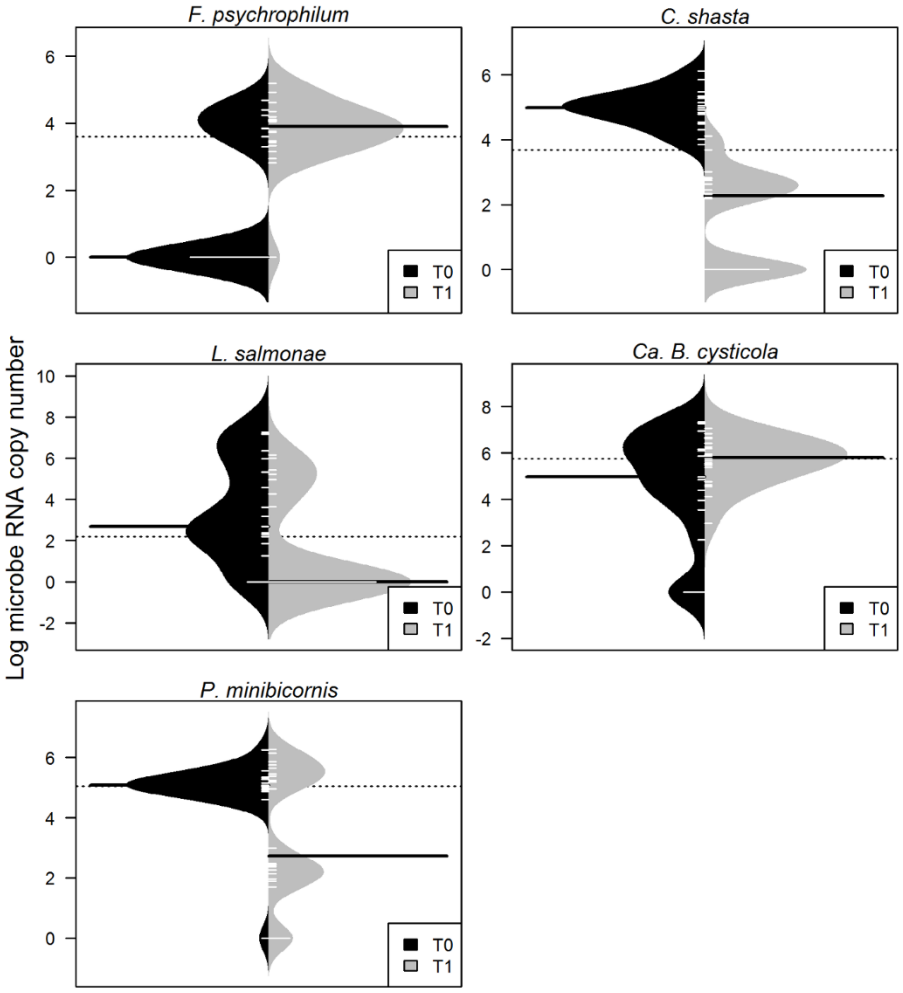

Figure 2.5 Beanplots of microbe productivity (log RNA copy number) at the time of gillnet capture (T0, *n* = 19; black) and 2 days following gillnet capture (T1, *n* = 22; grey) of Early Stuart sockeye in the Fraser River in Yale, BC. Polygons represent nonparametric density estimates, white bars represent total samples corresponding to RNA productivity, solid black bars represent the median productivity per time point (including negative detections), and dotted lines mark the overall median productivity. Significant differences in prevalence ($P < 0.05$) were identified for *F. psychrophilum* and *L. salmonae*, while *C. shasta* productivity differed between time points. Only microbes with sufficient total positive samples in one or both groups could be included in the analysis. Microbe productivities were measured from a small gill tissue biopsy (2-3 filament tips), normalized to 0.5 µg/µL of RNA per sample after RNA purification.

Figure 2.6 Three examples of Early Stuart sockeye exposed to experimental gillnet entanglement: a) a prematurely moribund male showing severe necrosis and *Saprolegnia* spp. fungal infections, b) a surviving male lacking secondary sexual characteristics and mild gillnet scarring posterior to the operculum, and c) a surviving male with well developed secondary sexual characteristics and ventral gillnet scarring anterior to the dorsal fin.

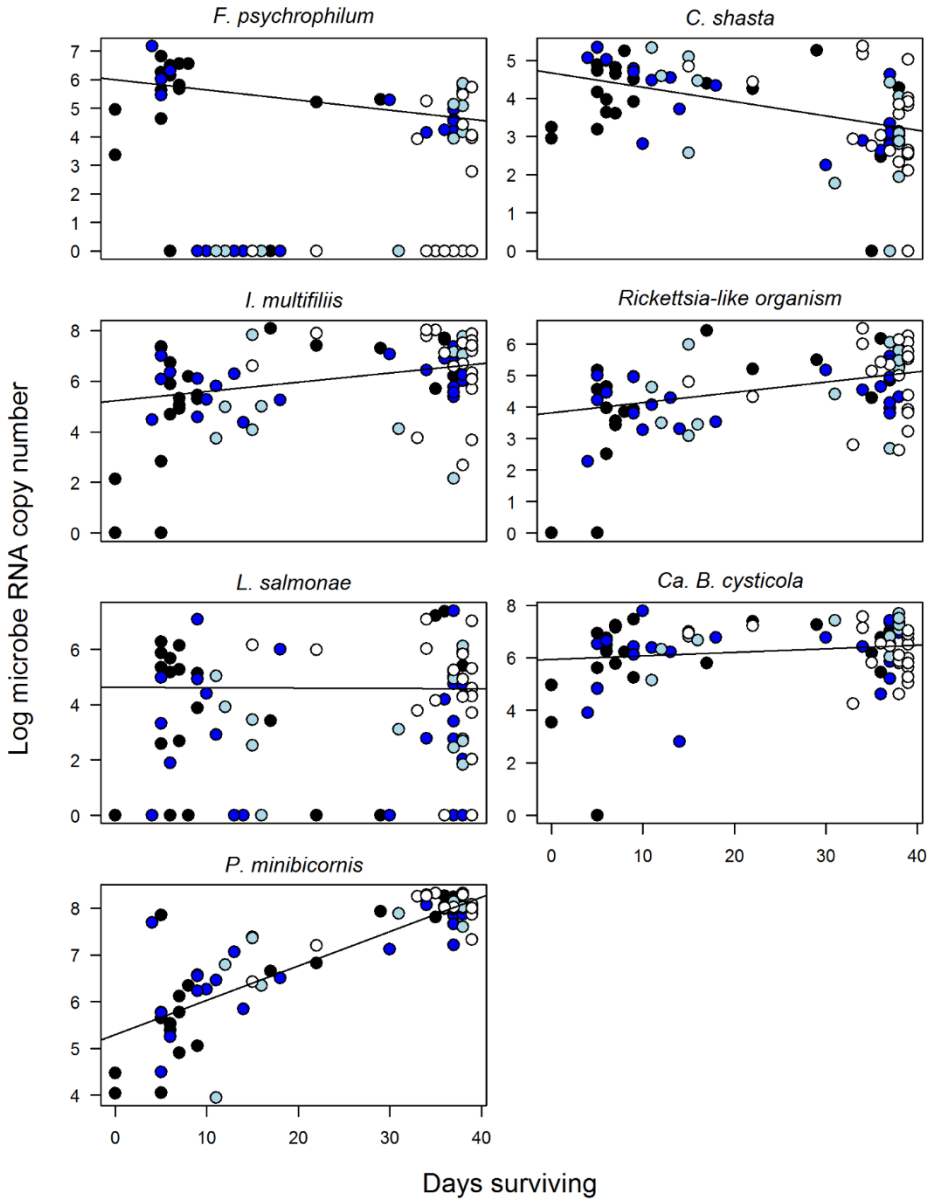

Figure 2.7 Relative productivity (log RNA copy number) of *Flavobacterium psychrophilum*, *Ceratonova shasta*, *Ichthyophthirius multifiliis*, Rickettsia-like organism, *Loma salmonae*, *Candidatus* Branchiomonas cysticola and *Parvicapsula minibicornis* as a function of days surviving for adult Early Stuart Sockeye Salmon. Each point represents the microbe burden of an individual at death; color corresponds to treatment, with severe (20 min) entanglement in black, mild entanglement (20 s) in dark blue, biopsied controls in light blue, and non-biopsied controls in white. Screening for microbes was conducted using a pool of aqueous phase from seven homogenized tissues including gill, liver, spleen, head kidney, heart, white muscle, and brain (alternated every other individual). All relationships (linear models on positive detections) were significant ($P < 0.05$), except for *L. salmonae* ($P = 0.97$).

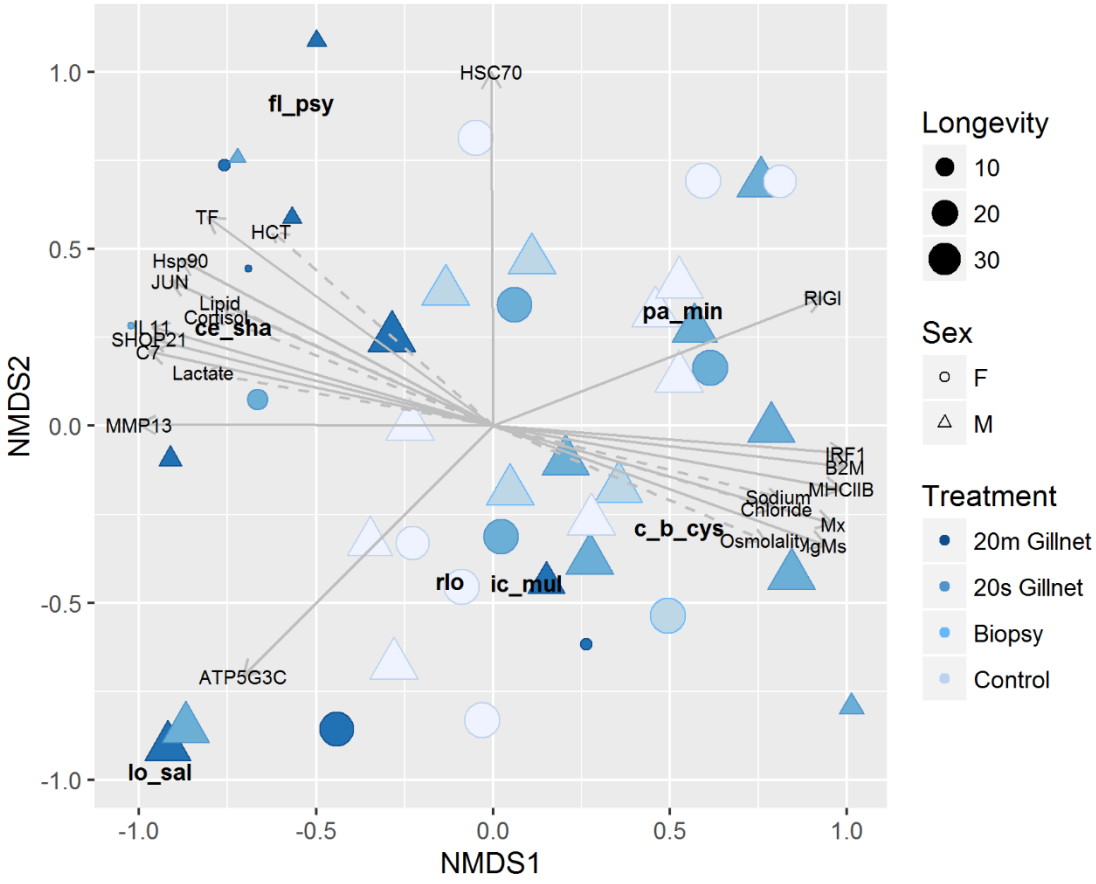

Figure 2.8 Nonmetric multidimensional scaling (NMDS) plot of microbe productivities within the pooled tissues (gill, liver, spleen, heart, kidney, muscle, brain) of 42 Early Stuart sockeye. Vectors represent correlated ($P < 0.10$) host gene expression and plasma/muscle biomarkers of stress, condition and immunity. Shapes designate sex (● = females, ▲ = males), and color represents the severity of handling and experimental gillnet treatment, with lightest to darkest as non-biopsied controls, biopsied controls, 20s gillnet treated, and 20 min gillnet treated fish, respectively. The size of points represents longevity, with the largest points living the longest.

Chapter 3 - Cumulative effects of thermal and fisheries stressors reveal sex-specific effects on pathogen development and early mortality of adult coho salmon (*Oncorhynchus kisutch*)

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3.1 Abstract

Multiple stressors are commonly encountered by wild animals, but their cumulative effects are poorly understood, especially regarding influences on pathogen development. Empirical study of the potential linkages among pathogens, stressors and host survival would improve our knowledge and management of wild animal populations. Through repeated biopsies and controlled application of chronic thermal stress and acute gillnet entanglement and air exposure, we characterized the effects of cumulative stressors on pathogen development of adult coho salmon (*Oncorhynchus kisutch*) returning to their natal watershed. High-throughput qPCR examined the potential loadings of 35 infectious agents in a pool of terminally sampled tissues and the expression of 17 host immune genes in gill, while chemical analysis of blood described host physiology. Temporal increases in pathogen richness and infection intensities were concurrent with decreases in the expression of most immune gene biomarkers in the gills of fish sampled in the river. Thermal stress in laboratory experiments increased delayed mortality (>one week) of both males and females (8 & 28% mortality, respectively) and enhanced mortality associated with handling and biopsy (~40% in both sexes). Experimental gillnetting further enhanced mortality at high temperature (73%) but only among females. Blood physiology and gene expression at high temperature were consistent with a reduced resilience to infections, including osmoregulatory impairment and suppressed female maturation and immune aspects, while gillnetting enhanced physiological impacts in females after one week. Females had more severe infections at death and reduced capacity to recover from stressors. Cumulative effects of multiple stressors on female

mortality are likely a function of impaired host resilience and enhanced infections at high temperature.

3.2 Introduction

Evidence is mounting for a functional role of infectious agents in the ecology of all wild organisms, with influences on food webs (Buck and Ripple, 2017; Miller *et al.*, 2014; Selakovic *et al.*, 2014), migration behavior (Altizer *et al.*, 2011; Johns and Shaw, 2016), and other ecological facets that have collectively evolutionary implications (Vander Wal *et al.*, 2014). As a result, interest has grown in how anthropogenic and climate-associated stressors will alter current host-pathogen relationships in wild ecosystems. Moreover, the stressors wild animals face throughout their lives affect their resilience and resistance to contribute to natural variation in infectious disease development within and among populations (Johnson *et al.*, 2015). Indeed, the energetic and physiological costs of migration, for example, contend with disease resistance to affect the likelihood of survival and reproductive success, and can be compounded by additional stressors (Altizer *et al.*, 2011). Thus, understanding how a species' ecology and relevant stressors influence infectious disease development and host survival is the cornerstone of disease ecology and crucial to effective natural resource management.

Physiological stress is known to alter immune competence, with variable immune responses depending on the type of stressor (Bowers *et al.*, 2008) and disease outcomes that are pathogen-dependent (e.g. viral vs bacterial; Hori *et al.*, 2013). Importantly, concurrent stressors are commonly experienced by wild animals but are rarely quantified for their cumulative effects (Gale *et al.*, 2013; Johnson *et al.*, 2012), especially regarding disease impacts (Miller *et al.*, 2014). Given that climate change is expected to alter host-

pathogen relationships directly and compound other stressors (Altizer *et al.*, 2013; Burge *et al.*, 2014; Lohmus and Björklund, 2015; Miller *et al.*, 2014), evaluations of multiple stressors are increasingly needed to account for the context-specific nature of disease development, which comprises internal (genotype, immune portfolio, host condition) and external (environment) forces to influence survival outcomes (Mitchell *et al.*, 2005).

Pacific salmonids (*Oncorhynchus* spp.) are an excellent model for an evaluation of cumulative stressors and infection development given the comprehensive knowledge base describing their physiology (e.g. Cooke *et al.*, 2012; Farrell *et al.*, 2008; Patterson *et al.*, 2016) and their frequent exposure to multiple stressors (Miller *et al.*, 2014). Furthermore, novel genomic tools recently applied to Pacific salmon in British Columbia (BC), Canada, have vastly improved our understanding of the infectious agents carried by wild salmon (Bass *et al.*, 2017; Miller *et al.*, 2014), the genomic and physiological responses that accompany those agents (Jeffries *et al.*, 2014a; Miller *et al.*, 2017; Teffer *et al.*, 2017; this thesis, Chapter 2), and how host genomic profiles predict migration success (Miller *et al.*, 2011). Juvenile Pacific salmon migrate to the marine environment to feed and grow until adults return to fresh water to reproduce (Groot and Margolis, 1991); the spawning migration couples starvation with the energetic demands of reproduction and swimming while immune competence nature diminishes and infection intensities increase (Bass *et al.*, 2017; Dolan *et al.*, 2016; Rand and Hinch, 1998). Although death occurs naturally after spawning, mortality prior to spawning during the river migration eliminates any lifetime fitness.

During river migration, two major stressors are currently affecting adult Pacific salmon: high river temperature and fisheries non-retention (i.e. capture and release or

escape), which are increasingly concurrent. A prime example is the Fraser River in BC, the largest salmon producing river in Canada, which has shown significant climate-driven warming in recent decades (Patterson *et al.*, 2007). While terminal fishing (commercial, recreational and ceremonial) targets certain salmon stocks and species, the likelihood of non-target species being caught and subsequently released as bycatch is high because different Pacific salmon species co-migrate through its mainstem and tributaries toward spawning grounds. Non-retention causes stress and injury (Davis, 2002; Raby *et al.*, 2015), making released catch more susceptible to infections (Svendsen and Bøgwald, 1997). Given that salmon released from fisheries are more likely to die when rivers are warm (Martins *et al.*, 2011, 2012b) and carry severe infections of multiple pathogens (Teffer *et al.*, 2017; this thesis, Chapter 2), we tested the hypothesis that thermal stress amplifies the impacts of capture on disease development and mortality because empirical evidence for such a cumulative effect is lacking.

Our objectives for this experiment were to: 1) characterize and quantify the development over time of pathogen communities and host immune responses within individual adult Chilliwack River coho salmon (BC, Canada) during their river residency prior to spawning through repeated gill biopsy, and 2) describe the individual and cumulative impacts of chronic high temperature and acute gillnet entanglement stressors on the development of pathogenic infections and fish mortality using a laboratory experiment that permitted controlled application of stressors (e.g. Jeffries *et al.*, 2014b; Teffer *et al.*, 2017; this thesis, Chapter 2). To ground our experimental results regarding pathogen burdens and immune responses of fish in the river, we conducted point sampling at the Chilliwack River Hatchery concurrent with our experiment. High-

throughput quantitative polymerase chain reaction (qPCR) applied to tissue biopsies was used to simultaneously characterize the trajectory of the pathogen community composition (prevalence) and structure (relative infection intensity) with host immune responses (gene expression). These genomic data were complemented by an array of physiological indices to provide a comprehensive characterisation of the effects of multiple infections and stressors on host health prior to spawning.

3.3 Methods

3.3.1 Hatchery sampling and laboratory analysis

Tissues from wild adult Chilliwack River coho salmon were obtained in 2012 by terminally sampling fish during their river residence at the Chilliwack River Hatchery, Chilliwack, BC during the "middle" burst of this population's migration (Jeremy Mothus, Hatchery Manager, personal communication) on 18-Oct (H0; n = 9), 8-Nov (H1; n = 10), and 26-Nov (H2; n = 11; Table 2.1a). Fish were collected by dipnet from raceways and euthanized via cerebral concussion before removing tissue samples from major organs, which included: gill (5-6 gill filament tips), white muscle proximal to the lateral line just posterior to the dorsal fin, liver, spleen, heart ventricle, head kidney, and brain (every other fish brain alternated with histology, data not shown). Tissue samples (~0.5 mg) were preserved in 1.5 mL of *RNAlater*® (Ambion, Inc., Austin, TX, USA) solution (whole brain 3 mL) at 4 ˚C for 24 h then at -80 ˚C until analysis. These tissues allowed simultaneous characterization of the trajectory of the host immune responses along with the community composition and structure of the infectious agents which comprise microorganisms such as viruses, bacteria and parasites known to affect Pacific salmonids (Miller *et al.*, 2016).

Over a three-day period (17-19 Oct), 240 coho salmon (silver with minimal scale loss) were transported from the raceways at the Chilliwack River Hatchery to the Cultus Lake Salmon Research Lab, Cultus Lake, BC (30 min transport) in truck-mounted, aerated tanks (10 ˚C; Table 2.1b). Fish were sequentially distributed among twelve 8,000 L holding tanks (10 ˚C) at low densities (n < 25 tank^{-1}). Sex, which was unknown at the time of collection as fish had not yet developed sex-specific morphologies, was determined visually during necropsy at the termination of the experiment and sex ratios ranged between 60-85% female in each tank. Tanks were supplied with sand-filtered, UV-treated water from Cultus Lake in a flow-through system. The temperature in each tank was controlled by varying the proportion of water from above or below the thermocline in the lake and by adding boiler-heated water. A submerged pump produced a low velocity current around the tank periphery to encourage fish to orientate during holding (approx. 1 body length s^{-1}). Twenty-four hours after the final transport, the temperature of half of the tanks was increased over a two-day period from 10 ˚C (low) to 15 ˚C (high), which is regarded as an extremely high temperature scenario for a coho spawning migration (Barnes and Magnusson, 2000). Both temperature groups were divided into three treatment groups (each replicated in two tanks): gillnet-treated with biopsy, biopsied controls (control for gillnet treatment), and non-biopsied controls (control for biopsy), which generated genomic, physiological and mortality data in a longitudinal design over 14 days with biopsy sampling (e.g. Cooke *et al*., 2005; Teffer *et al.*, 2017; this thesis, Chapter 2) at days 0, 7 and 14.

Gillnet treatment was a standardized representation of capture and release from a gillnet fishery in the Fraser River watershed and progressed as follows: each fish was

removed from its holding tank using a dipnet and quickly submerged in a smaller flow-through tank where the opening of the dipnet faced a wide frame with a 5.25-inch (13.3 cm) mesh gillnet mounted within it. Upon exiting the bag of the dipnet, the fish was caught in the gillnet. If the fish escaped, the timer was stopped until entanglement had been achieved. After 20 s of sustained underwater entanglement, the fish and gillnet were pulled from the water and into a dipnet held in air for 1 min while the fish was detangled from the net (simulating release by fishers). The fish was then submerged in a foam-lined, flow-through sampling trough, where it was sampled for blood (approx. 2 mL from the caudal vasculature; 21-gauge needle with lithium heparinized Vacutainer®, Becton-Dickson, NJ, USA) and a small gill biopsy (2-3 gill filament tips from consistent location across fish and sampling events, ~0.5 mg tissue, preserved in 1.5 mL RNA*later*®), implanted intraperitoneally with a small (12 mm) passive integrated transponder (PIT; Biomark Inc., Boise, ID, USA) and examined for injuries. Total time in the trough was approx. 2 min, after which the fish was placed into a recovery tank until all individuals in the holding tank had been treated and sampled, then all fish were returned to holding. Biopsied controls proceeded directly from holding tanks to the sampling trough and followed the same biopsy procedure as gillnet-treated fish. After seven days of holding, gillnet-treated fish and biopsied controls were biopsied again, following the protocols described above (no gillnet treatment). Non-biopsied controls were left untouched until morbidity or the termination of the experiment. Any fish that showed signs of morbidity during the experiment (gulping, loss of equilibrium) were euthanized by cerebral concussion and biopsy sampled according to the protocols described above for hatchery sampled fish. Additionally, blood was taken and various morphological measurements,

including length (post-orbital hypural; POH±1.0 mm), organ weights and gross pathology (e.g., fungus cover, lesions, macroparasites) were recorded. After 14 days, surviving fish in all treatment groups (excluding half of the non-biopsied controls) were euthanized and processed according to sampling protocols outlined for moribund fish. A subset of non-biopsied controls were excluded from our terminal pathogen analysis for use in a pilot study examining long-term effects of thermal stress on pathogen development (see Miller *et al.*, 2014).

3.3.2 Molecular analysis and blood properties

Prevalence and infection intensities of up to 35 disease agents were measured in a pool of seven terminally-sampled host tissues (gill, liver, spleen, head kidney, heart ventricle, white muscle, brain) while host gene expression as well as infection intensities were measured in gill biopsies using high-throughput qPCR on the Fluidigm BioMark™ platform (see Miller *et al.*, 2016, validated for use as a screening tool for infectious agents in wild and cultured salmon). Assay information including primer and probe sequences, gene functions, and references is outlined in Tables S3.1 and S3.2 of this thesis.

For hatchery-sampled fish, both gill and a multi-tissue pool were evaluated for infection status on three lethal sampling dates. For laboratory held fish, infection development and host gene expression were evaluated in nonlethally-sampled gill from three non-lethal sampling dates (i.e., changes within individuals over time) and infectious agents were also measured in a multi-tissue pool at death. As this experiment was performed prior to full validation and development of the Biomark™ platform, some aspects of the analysis differed from those described in Miller *et al.*,(2016), including

several alternate primer and probe sequences (see Table S3.1), fewer controls (pre-amplified positive, no reverse transcriptase cDNA, cDNA negative, and several artificial construct positive controls not included), and only RNA was evaluated (no DNA extraction from homogenate).

Tissue samples were thawed and trimmed to normalize weights (~0.5 mg), transferred into sterile microtubes with stainless steel beads, and homogenized in tri-reagent (600 µL; Ambion, Inc., Austin, TX, USA) and 1-bromo-3-chloropropane (75 µL) using a MM301 mixer mill (Retsch Inc., Newtown, PA, USA). After centrifugation (1500 *g*, 6.5 min), the aqueous phase (100 µL) was transferred into 96-well plates for RNA purification using Magmax™-96 for Microarrays Kits (Ambion Inc., Austin, TX, USA) following manufacturer's protocols for the "spin method" with a DNase treatment after the first wash. To create a tissue pool, organs were homogenized separately and aliquots of aqueous phase from each tissue in equal volumes were pooled for each individual prior to RNA purification (water was substituted where brain was absent). RNA quantity (A_{260}) and quality ($A_{260/280}$) were assessed using spectrophotometry and samples were normalized to 1 µg RNA per well prior to cDNA synthesis using Invitrogen™ SuperScript™ VILO™ (Carlsbad, CA, USA) cDNA Synthesis Kits under PCR cycling conditions of 25˚C for 10 min, 42˚C for 60 min, and 85˚C for 5 min. As recommended by the manufacturer (Biomark™), a preamplification step consisting of a multiplex PCR including all primers to be included in the qPCR was completed prior to qPCR. A mixture of 200 nM primer mix, TaqMan® PreAmp Master Mix (Applied Biosystems, Foster City, CA, USA) and cDNA was cycled at 95˚C for 10 min followed by 15 cycles of 95˚C for 10 s and 60˚C for 4 min, followed by treatment with ExoSAP-IT® PCR

Product Cleanup (Affymetrix, Santa Clara, CA; 37˚C for 15 min, 80˚C for 15 min) and 5-fold dilution (TEKnova suspension buffer, Hollister, CA, USA). Negative controls were included from homogenization forward and serial dilutions of host and pathogen pre-amplified cDNA were included in the qPCR (artificial constructs only available for a subset of pathogens, see Table S3.1). Sample (TaqMan® Gene Expression Master Mix, GE Sample Loading Reagent and pre-amplified cDNA) and assay (primer pair [9 µM], probe [2 µM], Assay Loading Reagent) mixes were loaded into reaction chambers using the integrated fluidics circuit controller and qPCR cycling was completed following the GE 96 X 96 Standard v1.pcl. (TaqMan®) protocol. Passive reference dye (ROX) confirmed the presence of sample in each well, one probe (with NED™ dye, Applied Biosystems, Foster City, CA, USA) was included to detect artificial constructs in samples (i.e. lab contamination), and a second probe (with FAM dye) quantified target amplicons. Pathogen assays with detections of the VIC-labeled probe or those not detected in duplicate (with FAM-labeled probe) were considered failed. Quantification cycle (Cq) is reported as the average of assay duplicates; host genes are reported as relative expression derived from the $2^{-\Delta\Delta Ct}$ method using two reference genes (Livak and Schmittgen, 2001) and pathogens are reported as "relative load", calculated by subtracting the Cq from 40 (maximum Cq).

Hematocrit and leucocrit were measured in the field following blood sampling via centrifugation (2 min at 10000 *g*; LW Scientific® ZIPocrit; Lawrenceville, GA, USA) in heparinized microcapillary tubes (Drummond Scientific®, Broomall, PA, USA). Plasma was extracted and flash frozen in liquid nitrogen after centrifugation at 7000 *g* for 7 min (Clay Adams Compact II centrifuge; Becton-Dickson, Sparks, MD, USA). Ions (chloride,

sodium, potassium), metabolites (lactate, glucose) and osmolality were measured according to (Farrell *et al.*, 2001) and hormones (cortisol, estradiol, testosterone) were measured using enzyme-linked immunosorbent assay (ELISA) kits (Neogen Corporation, Lansing, MI, USA) following manufacturer's protocols.

3.3.3 Statistical analysis

All statistical tests were completed using R Statistical Software (R Core Team, 2015). Results from qPCR analysis of gill and pooled tissues from fish sacrificed at the Chilliwack River Hatchery were used to identify temporal and sex-specific differences in infectious agent richness (number of unique pathogens), relative loads of individual pathogens (40–Cq), and relative infection burden (RIB; Bass *et al.*, in review), which is a composite score incorporating aspects of pathogen richness and relative loads via the equation,

$$RIB = \sum_{i \epsilon m}^{m} \frac{L_i}{Lmax_i},$$

where for a given fish, the relative load of the i^{th} infectious agent (L_i) is divided by the maximum load within the population for the i^{th} infectious agent ($Lmax_i$) and then summed across all agents (m) infecting the given fish. Analysis of variance (ANOVA) was used to test for significant differences in each disease metric between sampling dates and sexes (with an interaction term) and post hoc Tukey tests for individual differences between groups. Host immune responses were evaluated using permutational multivariate analysis of variance (PERMANOVA) with sampling date, sex and an interaction term as predictors; principal components analysis (PCA) and environmental fitting (*envfit*

function, *vegan* library) were used to graphically represent the data and describe the relationships of gene biomarkers to predictor variables (Oksanen *et al.*, 2016).

For laboratory data (held fish), impacts of temperature, treatment and sex on survival were evaluated using survival analysis and Cox proportional hazards (*survival* package, Therneau, 2014), including and excluding non-biopsied controls to account for possible impacts of nonlethal biopsy on survival. Following an initial model that included both sexes to test for overall sex, treatment and temperature effects, males and females were evaluated separately to determine hazard ratios associated with stressors within sexes. Exponents of coefficients (e^{β}) represent the relative daily hazard of mortality. Effects of thermal and fisheries stressors, sex and time on disease metrics measured in gill were assessed using linear mixed effects (LME) models (*nlme* package, Pinheiro *et al*., 2017; Zuur *et al*., 2009). Three discrete gill sampling events (T0: 22-23 Oct, T1: 29-30 Oct, T2: 5-6 Nov) were included in the analysis, as well as samples taken from moribund fish that died in the interim between sampling events (T0.5, T1.5); for all time-dependent analyses (LME, survival analysis), data from samples taken from fish that died prior to the termination of the study were grouped into T1 if the fish died prior to T1, or into T2 if the fish died after T1. Inclusion of data from moribund fish improved the comprehensiveness of our analysis beyond just survivors. Missing values in the response variable (e.g., negative detections) were excluded from each analysis. Differences between treatments, temperatures and sexes over time were tested for each disease metric in a repeated measures framework. The following equation describes the full mixed effects model,

$$D_i = \beta_1 H + \beta_2 G + \beta_3 S_i + \beta_4 T + \beta_5 (H \cdot G) + \beta_6 (H \cdot S_i) + \beta_7 (H \cdot T) + \beta_8 (G \cdot S_i) + \beta_9 (G \cdot T) + \beta_{10} (S_i \cdot T) + (\alpha + T | \mu_i) + \varepsilon_i$$

$$\mu_i \sim N(0, \sigma^2), \varepsilon_i \sim N(0, \sigma^2),$$

where D_i is the disease metric (i.e., richness, RIB, or relative load of an individual agent) for individual i, β_1 is the coefficient of the fixed effect of high temperature (H), β_2 is the coefficient of the fixed effect of gillnet treatment (G), β_3 is the coefficient of the fixed effect of the sex of individual i (S_i), β_4 is the coefficient of the fixed effect of time (T), β_5 is the coefficient of the interaction between high temperature and gillnet treatment, β_6 between high temperature and sex, β_7 between high temperature and time, β_8 between gillnet treatment and sex, β_9 between gillnet treatment and time, and β_{10} between sex and time. A random intercept (α) and slope ($T|\mu_i$) describe individual variability (μ_i) in the response variable and its relationship to time; μ_i and the residual error (ε_i) are normally distributed with a mean of zero and variance σ^2. Random intercepts and/or slopes were included to control for individual variation and autocorrelation across sampling events within individuals. Optimal random effects (intercept, slope or both) were identified using top-down model comparison and Akaike's Information Criterion (AIC), varying the random component of each full fixed effects model (Zuur *et al.*, 2009). Fixed effects (treatment, temperature, sex, time) and all two-way interactions were then evaluated beginning with the most complex model and then removing non-influential fixed effects (low t-value and high P-value). Stepwise reduction was repeated sequentially until the reduced model contained only significant factors and interactions (and nonsignificant main effects of components of significant interactions) with reduced AIC. AIC values, β estimates for fixed effects with standard errors and P-values are reported from the most parsimonious models. Where significant interactions prevented further model reduction to test the significance of main effects, P-values were derived from t-values. Intra-class

correlation coefficients (ICC) are also reported, which describe the proportion of total variance due to individual differences.

Effects of stressors and sex on infection intensities in pooled tissues of survivors at T2 were evaluated using ANOVA on richness, RIB, and relative loads for highly prevalent agents (>70%) and logistic regression on presence-absence data for agents with lower prevalence (<70%). Temperature, treatment and sex with all possible interactions were included as predictor variables in ANOVAs, while only main effects were examined for logistic regressions due to low power (few positive samples). The influence of pathogens on survival was quantified using survival analysis with time-dependent covariates. Because few fish died at low temperature, this analysis was conducted using high temperature fish only (biopsied controls and gillnet-treated). Sex and treatment were included as constant covariates, while time-dependent covariates included RIB and load information from highly prevalent agents. Because nonlethal biopsy was conducted weekly, survival times were grouped by week (i.e. survival to T1 or T2). Relative loads were transformed into a binary response: 0 for negative or low loads (<average load of held population) or 1 for high loads (>average load).

The gene expression and blood analyses included only samples taken from live fish at the first and second sampling events (T0, T1); this approach targeted changes occurring in the first week of holding that prefaced and may have influenced delayed mortality (occurring mostly after T1). Changes in host immune responses were evaluated using PERMANOVA to evaluate the relative influences of temperature, treatment (and their interaction), fate (survival 14 d), and RIB at T0 and T1. PCA and environmental fitting (*envfit*) were used to visually represent gene biomarker relationships to predictor

variables (temperature, treatment, fate, RIB) while generalized linear models (GLMs) related predictor variables to the first 2-3 PC axes (cumulative variance explained > 50%). Tests were run for males and females separately to account for sex-specific differences, thereby characterizing treatment effects within each sex. To assess immediate and delayed physiological responses to stressors, ANOVA was used to test for significant differences in blood properties due to thermal and fishery stressors (with an interaction) at T0 and at T1. Analyses were performed for males and females separately to account for sex-specific differences in blood chemistry and each time point was evaluated independently. Cortisol, lactate, testosterone and glucose were log-transformed to meet normality assumptions.

3.4 Results

3.4.1 Pathogen community trajectories and host responses in the river

Eleven infectious agents were positively detected out of the 35 that were evaluated in multi-tissue pools from 30 coho salmon at the Chilliwack River Hatchery. *Ceratonova shasta* and *Parvicapsula minibicornis* were found in all fish, while *Ichthyophthirius multifiliis* was found in nearly all fish (*n* =27 fish). *Kudoa thyrsites* (22 fish) and *Rickettsia*-like organism (RLO; 15 fish) had at least 50% prevalence, while the remaining 7 infectious agents had a lower prevalence: *Paranucleospora theridion* (8 fish), *Tetracapsuloides bryosalmonae* (5 fish), piscine reo-virus (PRV; 2 fish), *Aeromonas salmonicida* (1 fish), *Ichthyophonus hoferi* (1 fish), and *Sphaerothecum destruens* (1 fish). Total positive detections for each agent as a function of total positive detections of all agents on each sampling date suggested a relatively static community composition over time (Fig. 3.1). Richness per fish ranged between 3–6 agents and RIB ranged

between 1.69–5.80 across sampling events, but neither changed significantly over time or between sexes ($P > 0.05$). The relative loads of the freshwater myxozoans *P. minibicornis* and *C. shasta* increased over time ($P < 0.001$ and $P = 0.006$, respectively) due to significant increases only in females (Tukey tests for H1 vs H2: *C. shasta*: $P = 0.030$; *P. minibicornis*: $P = 0.001$; Tukey tests for H0 vs H2: *C. shasta*: $P = 0.056$; *P. minibicornis*: $P = 0.002$). Females also carried higher loads of another myxozoan, *K. thyrsites* ($P = 0.001$), in pooled tissues and at a higher prevalence than males (F=57%, M=17%).

In gill tissue from hatchery fish, the only significant difference in infections detected was an increase in *P. minibicornis* relative loads in gill with time ($P = 0.019$), primarily between H1 and H2, but with no sex-specific effect ($P = 0.473$). PERMANOVA identified distinct immune profiles in gill depending on collection date (r^2=0.161, $P = 0.003$) and sex-specific differences (r^2=0.118, $P = 0.020$) that demonstrated high variability among females relative to males, especially at later sampling dates. RIB and relative loads of highly prevalent agents (*C. shasta* and *P. minibicornis*) explained little variation in immune gene expression patterns (r^2-values<0.060, *P*-values>0.05). Immune genes were generally grouped by their functions in the PCA (PC1: 22% variance explained, PC2: 11%), with biomarkers of adaptive immunity (CD83, CD4, MHCI, MHCIIb) and cytokines (IL1R, IL15, IL11) associated with the first and second samplings (H0 and H1), while biomarkers of antiviral activity (Mx) and iron metabolism (Hep) clustered with the third sampling (H2). Most immunity biomarkers and several other anti-viral indicators (IRF1, IFNa) were negatively associated with H2, suggesting an overall downregulation of these genes with time.

3.4.2 Cumulative effects of multiple stressors on survival, infections and host health

3.4.2.1 Fish survival

At 10˚C, 85–100% of both sexes survived 14 days in all treatment groups (Table 3.1b), with no significant effect of either biopsy or fishery simulation. In contrast, survival at 15˚C was differentially affected by treatment and sex. For males, survival of non-biopsied controls was high (92%), while gillnet treatment and biopsy significantly and similarly reduced male survival to ~60%, suggesting no effect of the fishery simulation. For females, survival of non-biopsied female controls was significantly reduced at 15˚C (72%), and further reduced by biopsy (59%) and again by gillnet treatment (27%). Overall, mortality was delayed by more than one week after study start, with most fish (~98%) surviving to the second sampling occasion (T1).

The significance of treatment and sex effects on survival were assessed in a two-stage survival analysis (Likelihood ratio test *P*-values<0.050; Fig. 3.2). The first set of models evaluated survival relative to non-biopsied controls, which indicated significant effects of high temperature ($P < 0.001$), gillnet treatment ($P = 0.001$), and sex-specific differences that showed a greater overall daily hazard (of mortality) for females relative to males ($e^{\beta}=2.0$, $P = 0.040$). Among females, high temperature increased the daily mortality risk to 15.6-times that of low temperature females ($P < 0.001$). Gillnet-treated females experienced 3.7-times the daily hazard of non-biopsied controls ($P = 0.005$), but biopsied and non-biopsied controls did not significantly differ ($e^{\beta}=1.5$; $P = 0.396$). Mortality was minimal among males, which reduced our power to characterize differences using survival analysis, and a marginal violation of the proportional hazards assumption ($P =$

0.050) was evident, wherein a significant negative effect of high temperature (e^{β}=6.9, P = 0.013) likely decreased with time among males.

In the second set of models, which excluded non-biopsied controls and included a temperature-treatment interaction term, temperature significantly affected survival (P = 0.008) and enhanced the effect of gillnet treatment (interaction: P = 0.028), but no significant overall sex-effect was detected (P = 0.109). However, females consistently demonstrated more severe responses to stressors than males. High temperature significantly reduced female survival (e^{β}=3.9, P = 0.008) and marginally (but non-significantly) enhanced the impact of gillnet treatment (e^{β}=10.0, P = 0.058). Excluding the temperature-treatment interaction from the model, females at high temperature experienced 13.1-times the daily hazard of females at low temperature (P < 0.001) and gillnetting increased the daily hazard to 2.3-times that of biopsied controls (P = 0.014). Differences in hazard ratios of females depending on the inclusion of non-biopsied controls suggests an additive effect of handling (biopsy) on female survival in the presence of additional stressors. No interaction term was included in the male survival model due to minimal male mortality and only temperature significantly reduced male survival (e^{β}=6.9, P = 0.013).

3.4.2.2 Pathogen dynamics during holding and it relationship to a fish's fate

Results from LME analysis of relative loads and composite infection metrics in repeated gill biopsies showed impacts of temperature, treatment and sex, but the strength and nature of these relationships varied depending on the metric and time (Fig. 3.3; model coefficients in Table 3.2). Sex-specific differences were identified only in *I. multifiliis*, with higher loads overall in females. All metrics increased with time and most

were enhanced at high temperature (excluding *C. shasta*). Importantly, *P. minibicornis* and richness reached maximum levels sooner at high temperature (i.e. significant negative temperature-time interactions, suggesting greater temperature-driven differences at T0 and T1 than at T2). Survival analysis with time-dependent covariates identified significant effects of sex ($e^{\beta} = 2.59$, $P = 0.016$) and *P. minibicornis* loads ($e^{\beta} = 2.48$, $P = 0.021$; Model concordance=0.73, Likelihood ratio test $P = 0.013$) on survival at high temperature, with greater mortality risk among females and individuals with higher *P. minibicornis* loads in gill.

In pooled tissues of survivors at T2, 11 agents were positively detected (excludes *Myxobolus arcticus* due to the bias of intermittent brain inclusion in qPCR analysis), with a wide range in prevalence (2–100%; Fig. S3.1, *P*-values of factor effects in Table 3.3). Richness, RIB, and loads of *C. shasta*, *I. multifiliis*, *Rickettsia*-like organism (RLO) and *K. thyrsites* were increased in the pooled tissues of survivors after 2 weeks of chronic thermal stress ($P \leq 0.016$). Females carried higher loads of *P. minibicornis*, *I. multifiliis*, *Rickettsia*-like organism (RLO) and *K. thyrsites* in pooled tissues as well as higher richness and RIB ($P \leq 0.001$). Sex-specific treatment effects were apparent in *I. multifiliis* and RLO loads, with a positive effect of biopsy sampling in males only (i.e., sex-treatment interactions). Logistic regression identified a positive influence of high temperature on the prevalence of *T. bryosalmonae*, while biopsy sampling and gillnetting increased the prevalence of *A. salmonicida*, and biopsy decreased the prevalence of *P. theridion*. Low prevalence agents with too few detections to assess statistically included PRV (2%), *Nucleospora salmonis* (2%), *S. destruens* (2%), and *I. hoferi* (3%).

3.4.2.3 Host immune responses over time

High temperature was the primary factor influencing gill immune gene expression in both sexes (Fig. 3.4; model coefficients in Table 3.4). Among females at T0, PERMANOVA identified temperature, RIB and fate (survival 14 d) as significant ($P < 0.05$) explanatory variables describing immune gene expression patterns, though coefficients of variation were low ($r^2 < 0.10$). The GLM identified PC1 as negatively associated with high temperature and RIB in females at T0. Most immune biomarkers loaded negatively on PC1, suggesting an overall upregulation of these genes in females with high RIB and/or exposed to increasing temperature for 48 h (Fig. 3.4). At T1 (one week later), the influence of thermal stress on immune gene regulation in females increased ($r^2 = 0.18$) and a weak association with fate was still apparent ($r^2 = 0.03$), but with no significant association with RIB. High temperature, early mortality and the expression of MMP13, IL11 and Hep all negatively associated with PC1, while most immune biomarkers loaded positively, suggesting primarily iron sequestration and tissue repair responses in dying and thermally stressed females. High temperature was also associated with the loadings of immunoglobulin (IgMs) and cellular immune components (CD4, MHCI, b2m) on PC3, possibly signifying thermal stress responses that are independent of fate.

Among males at T0, temperature was the only significant factor associated with overall gene expression, but the GLM identified negative relationships of both high temperature and gillnet treatment with PC1 (Table 3.4). Like females, most immune biomarkers negatively loaded on PC1, indicating enhanced immune gene expression in males exposed to chronic thermal stress and immediately after gillnet entanglement. At T1, the

influence of temperature on immune gene expression in males increased (r^2 = 0.28) and a weak influence of RIB was also apparent (r^2 = 0.05). RIB correlated with PC1, PC2 and PC3, demonstrating associations between high RIB and iron regulation (Hep, TF), humoral immunity (IgMs, C3) and tissue repair (MMP13), while cellular immune components (e.g., receptors) were generally characteristic of low RIB. Temperature was positively associated with cell receptors (CD4, CD83, MHCIIb), interferon response regulators (IRF1, IFNa) and IgMs on PC3, but negatively associated with anti-viral (Mx, RIG1) and iron metabolism (TF, Hep) gene loadings. Variation along PC3 also supported a temperature-treatment interaction in males (β = -1.61±0.67, P = 0.021), suggesting antagonistic treatment effects (i.e. thermal response signal reduced and more variable in gillnetted males).

3.4.2.4 Initial and sustained stress responses over time

Thermal and fishery stressors caused an immediate stress response in both sexes, but females demonstrated more severe physiological disturbances and minimal capacity to resolve the stress after one week (Fig. 3.5; coefficients in Table 3.5). At T0, evidence of thermal stress in females at 15 ˚C (after 48 h of increasing temperature from 10 ˚C) included elevated cortisol, hematocrit, lactate, leucocrit, chloride, sodium, potassium and osmolality, as well as depressed estradiol and testosterone relative to females at 10˚C. Immediate responses to gillnet treatment were temperature-dependent in females: at 10 ˚C, gillnetting increased hematocrit, lactate and potassium, while at 15 ˚C, gillnetting increased hematocrit and sodium relative to biopsied controls, and also muted thermally-driven decreases in estradiol. After one week, females maintained a significant stress response at high temperature and also showed signs of osmoregulatory impairment, with

elevated cortisol, glucose, lactate and potassium, as well as depressed estradiol, testosterone, osmolality, chloride and sodium relative to fish at 10 ˚C. Delayed effects of gillnet treatment in females were apparent after one week, which were mostly independent of temperature and included elevated cortisol, glucose and lactate, and depressed leucocrit, estradiol and testosterone (especially at 15 ˚C) relative to biopsied controls.

Males at T0 had relatively mild responses to thermal stress that included increased lactate and potassium and decreased testosterone at 15 ˚C. Gillnet treatment immediately increased hematocrit and lactate in males regardless of temperature. Males showed an enhanced thermal stress response after one week at 15 ˚C, with elevated cortisol, glucose, lactate and potassium, and depressed testosterone, osmolality and sodium relative to fish at 10 ˚C. The only significant response to gillnet treatment detected in males at T1 was elevated lactate and only at 15 ˚C.

3.5 Discussion

This study is the first to examine relationships among pathogen loads, immune gene profiles and plasma characteristics in coho salmon. Our controlled application of relevant cumulative stressors revealed disease-associated mechanisms of premature mortality of adults in fresh water and provides empirical evidence for an interaction between thermal and fisheries stressors that primarily affects females. Sex-specific differences in pathogen loads, immune profiles and blood characteristics indicated poor recovery of females from thermal and capture stress and lower defenses against multiple infections, with suppressed maturation indices and circulating blood leucocytes that prefaced mortality. Our finding that stressor-induced mortality was typically delayed by a week or more is

consistent with previous studies of Pacific salmon in fresh water (Donaldson *et al.*, 2012; Jeffries *et al.*, 2014b; Patterson *et al.*, 2017a; Teffer *et al.*, 2017; this thesis, Chapter 2). Survival was high overall at low temperature, suggesting that both sexes were resilient to capture stress when temperatures were cool, with divergent immunity and physiology depending on thermal experience. Our survival results confirm earlier laboratory findings where ecologically relevant high temperatures reduced survival of sockeye (*O. nerka*) and pink salmon (*O. gorbuscha*; Jeffries *et al*., 2012b, 2014b), and exacerbated the impacts of gillnet entanglement, especially among females (Teffer *et al.*, 2017; this thesis, Chapter 2). Disease-associated mechanisms of mortality were apparent at high temperature, with higher prevalence and loads of most infectious agents in multiple tissues and evidence of osmoregulatory impairment and chronic stress. Although mortality patterns would predict further increases in pathogen loads among gillnetted fish at high temperature, no further increase was detected, indicating that infection intensity was not the sole contributor to mortality or thresholds for infection were lowered. The combined impacts of severe physiological impairment, immune modulation and enhanced infection intensities likely contributed to the increased mortality of gillnet-treated females at high temperature. Our results demonstrate relevant roles of pathogen dynamics and host response patterns during freshwater residence that are predictive of early mortality of coho salmon, influenced by multiple stressors and sex-specific.

3.5.1 Sex-specific differences in infection patterns and host responses

Sex-specific differences observed in the present study demonstrate alternate responses of females to stressors and infections relative to males. Female salmon have lower cardiac and metabolic capacity relative to males (Clark *et al.*, 2009; Sandblom *et al.*,

2009), which are likely mechanisms contributing to reduced survival under adverse migratory conditions (e.g., high temperature, hydraulic challenges; Martins *et al*., 2012b; Roscoe *et al*., 2011), and are consistent with reduced disease resistance during spawning migration. Furthermore, at high temperature, female sex hormones were initially depressed (after two days of rising temperature) and continued to drop, especially after gillnetting. This phenomenon may indicate suppressed or inhibited maturation and has been demonstrated in wild sockeye salmon following gillnet entanglement (Baker *et al.*, 2013; Teffer *et al.*, 2017; this thesis, Chapter 2) and during chronic high temperature exposure (Mathes *et al.*, 2010). Though captivity can inhibit maturation (Patterson *et al.*, 2004), decreases were not uniform across treatment groups. Cumulative stressors could therefore result in pre-spawn mortality of females if maturation, ovulation and spawning cannot be achieved before senescence and disease development take hold.

The physiological shifts imposed by chronic thermal stress in females likely overwhelmed or masked their immediate responses to gillnet treatment, suggesting that coping strategies for acute stress were deficient or delayed in females at high temperature. Indeed, female responses to gillnet treatment were stronger after one week, just prior to the onset of mortality. Our results indicated that high temperature generally enhanced components of adaptive immunity (cell surface receptors, antibodies) and reduced anti-viral activity (Mx) in gill regardless of survival, whereas high temperature effects in fish that would die also included biomarkers of iron sequestration and tissue repair. Increased infection intensities and altered immunity at high temperature combined with an inability to resolve physiological stress may serve as possible mechanisms for the additive effects of multiple stressors on female mortality. Indeed, sex-specific differences

in glucose metabolism previously identified in sockeye salmon (Teffer *et al.*, 2017) were also found in the present study, with higher plasma glucose levels after one week in females at high temperature suggesting enhanced energy mobilization relative to males. These findings support a limited capacity of female Pacific salmon to cope with multiple stressors during spawning migration and demonstrate links between energy needs, infection intensities, maturation indices, and mortality. Whether physiological impairment results from or contributes to infection development and immune modulation is unknown, but these trends warrant further examination.

3.5.2 Pathogen communities and immune responses at the hatchery

Multiple infections were a common finding in gill and pooled tissues of hatchery-sampled fish, a common trait of wild animals with inherent complexity for characterizing pathogen virulence (Kinnula *et al.*, 2017; Sofonea *et al.*, 2015). The pathogen community (i.e., RIB) among fish in the river was rather static over time despite increased loading by several agents (myxozoans). Hatchery data were produced from independent lethal sampling events that do not encapsulate infection development within individuals, but rather population-level shifts among surviving fish. Given that held fish demonstrated increases in RIB with time, it is unlikely that RIB was maintained at low levels by targeted immune responses. Rather, removal of ripe fish by hatchery staff for spawning may artificially reduce RIB among surviving fish because RIB is temporally confounded with maturity. Sex-specific differences in pathogen dynamics were primarily restricted to myxozoan parasites in pooled tissues, with consistently higher loads carried by females (*K. thyrsites*, a marine and freshwater myxozoan), or increasing loads with time at the population level in females only (*C. shasta* and *P. minibicornis*, freshwater myxozoans).

These findings are similar to those described for Chinook salmon (*O. tshawytscha*) in southwestern BC (Bass *et al.,* 2017). The absence of significant differences over time and between sexes in gill loads of hatchery-sampled fish supports previous comparisons in sockeye salmon (Teffer *et al.*, 2017) and demonstrates an incomplete or alternate microbe community structure represented in gill relative to other tissues. Such differences should be incorporated into data interpretations and assessed according to the ecology of each agent (e.g., tissues of primary infection).

3.5.3 Cumulative effects of thermal and capture stressors on pathogen dynamics

By taking repeated individual biopsies over time, we detected temporal shifts in pathogen community structure in gill characterized by increases in richness and loads that differed depending on temperature and, in the case of *I. multifiliis*, sex of the host. Significant increases in RIB were apparent at high temperature and with time, but impacts of acute gillnet capture stress on RIB were not observed, which may require more than two weeks or a more severe stressor to manifest (Teffer *et al.*, 2017; this thesis, chapter 2). The degree to which a stressor affects host immune responses can differ depending on the pathogen (Hori *et al.*, 2013), suggesting that infectious agent community composition as well as the types of stressors encountered are relevant to disease-induced mortality of wild animals (Sofonea *et al.*, 2015). Notably, both *I. multifiliis* and *C. shasta* levels in gill at high temperature remained slightly lower for gillnetted fish, which may signify effective host responses targeted toward these parasites following acute stress, despite the immunosuppressive effects of chronic thermal stress (Campisi *et al.*, 2002; Dhabhar, 2002; Mateus *et al.*, 2017; Wendelaar Bonga, 1997). As these fish were also more likely to die, successfully maintaining lower infection levels

may prove deleterious for semelparous fish by causing epithelial damage during the immune response (e.g. inflammation hypertrophy, hyperplasia, lamellar fusion in gill) or facilitating enhancement of other pathogenic infections (*P. minibicornis*; Bradford *et al*., 2010a; Buchmann *et al*., 2001; Sofonea *et al*., 2017). Thermal enhancement of *C. shasta* in pooled tissues but not in gill may signify accelerated migration of spores from gills to the gut (via the blood) at high temperature (Bartholomew *et al.*, 1997; Okamura *et al.*, 2015), thereby contributing to pathogenesis, but this hypothesis requires further study. Load increases of several agents in response to stressors differed between gill and pooled tissues, suggesting that gill biopsies alone may be inadequate to characterize infection development of some agents, such as *C. shasta*, that do not mature in the gill.

Our analysis identified a negative association of *P. minibicornis* loads with survival at high temperature. *P. minibicornis* is a myxozoan parasite endemic to the Fraser River that has been linked to premature mortality of adult sockeye salmon (Bradford *et al.*, 2010b; Jones *et al.*, 2003). Thus, our findings provide further evidence for a role of *P. minibicornis* in premature mortality of stressed salmon by confirming temperature as a driver of *P. minibicornis* infection intensity in salmon (Wagner *et al.*, 2005) and lending support to the idea that high pathogen loadings during the late stage of freshwater residence lead to mortality of adult coho salmon (Miller *et al.*, 2014). Despite its association with kidney disease, *P. minibicornis* has been documented in the gill of sockeye salmon (Bradford *et al.*, 2010b) and diseased fish show respiratory stress (Bradford *et al.*, 2010a), with negative impacts on exercise recovery (Wagner *et al.*, 2005). Given that high loads of *P. minibicornis* in gill were associated with high temperature and mortality in the present study, but loads in pooled tissues (including

kidney) of survivors showed no temperature effects, gill infections of *P. minibicornis* may be more relevant to survival (Bradford *et al.*, 2010b), further emphasizing the importance of infection locale within the body.

Shifts in osmoregulatory and stress indices and reduced aspects of immune gene expression demonstrated the highest level of physiological impairment in gillnetted females held at high temperature. RIB in gill was more strongly associated with the immune profiles of males after one week than that of females, which may signify more targeted responses of males to infections. Because RIB was not strongly associated with female immunity after one week and was not the primary predictor of mortality, it is likely that female mortality was not solely pathogen driven, but rather a function of both enhanced infections and impaired physiological resilience. Additionally, not all infectious agents contributing to mortality were comprised by our metric, as our evaluation did not comprise the full array of agents impacting wild Pacific salmon populations (Miller *et al.*, 2016), and most notably does not include some newly discovered viruses in BC salmon (K. Miller, unpublished data). Furthermore, our experiment was not designed to identify the nature of inter-pathogen interactions that may have influenced survival, but instead provides crucial insight for future challenge work to include a range of environmental conditions and coinfections.

3.5.4 Immune and physiological responses to pathogens and multiple stressors

Our results add to growing evidence for decreased immune activity of Pacific salmonids late in freshwater residence, suggested to be due to senescence processes in advance of mortality rather than immune suppression by pathogens (Dolan *et al.*, 2016). Demonstration of this phenomenon in Chinook salmon (*O. tshawytscha*) in the upper

Willamette River basin (Dolan *et al.*, 2016), Early Stuart sockeye salmon from the Fraser River (Teffer *et al.*, 2017; this thesis, Chapter 2), and Chilliwack River coho in the present study provides evidence for conservation of this trait across river basins, latitudes, and species. Stressor effects on immune gene expression were apparent and primarily attributable to thermal stress, characterized by increased expression of most immune genes after just 1-2 days of rising temperature followed by a decrease in most aspects of immunity after one week. Pink and sockeye salmon showed similar increases in immune gene expression that were maintained after 5-7 days of chronic thermal stress (Jeffries *et al.*, 2014b), suggesting species-specific or season-dependent differences in the timing of immune modulation. Most immune biomarkers were decreased in coho salmon after one week at high temperature, likely due to immunosuppressive effects of chronic thermal stress (Barton and Iwama, 1991; Dittmar *et al.*, 2014), and included the interferon-induced antiviral protein Mx. However, Mx was positively associated with premature mortality and gillnetting, consistent with previous work on sockeye salmon (Teffer *et al.*, 2017; this thesis, Chapter 2). The only virus positively detected in this population was PRV and then only two individuals. The observed expression of Mx (and RIG-1) could be a relic of a cleared infection or a component of a conserved response to acute stress (Zwollo, 2012) or possibly senescence, or may be a response to one of the novel viruses recently identified in BC Chinook and sockeye salmon (K. Miller, unpublished data).

Differences in immune gene expression between gillnetted and non-gillnetted females at high temperature indicate alternate responses to enhanced infectious loads. After one week, gillnetted fish showed positive association with biomarkers of iron regulation (Hep, TF), anti-viral and intracellular responses (Mx, RIG-1) and inflammation (MMP13,

IL11), but lower and more variable expression of most other aspects of immunity including extracellular pathogen recognition (e.g., IgMs, MHCIIb). Parasites associated with mortality (e.g., *P. minibicornis*) would contribute to an inflammatory response, but not iron metabolism or intracellular responses (Buchmann *et al.*, 2001; Okamura *et al.*, 2015). RLO is an alphaproteobacteria associated with strawberry disease (Metselaar *et al.*, 2010), producing characteristic red lesions observed in our study fish, but is not considered pathogenic in culture settings (Olson *et al.*, 1985). Modulation of iron metabolism and inflammation are characteristic of bacterial infection (Raida and Buchmann, 2009) and as an intracellular pathogen, RLO may have the potential to recruit the intracellular immune components observed. RLO was not evaluated in gill, but as an endosymbiont of *I. multifiliis* (Sun *et al.*, 2009) with highly correlated loads in Pacific salmon (Bass *et al.*, 2017), the responses of these agents were likely similar. Importantly, our analysis, though comprehensive, was completed prior to the inclusion of several assays on the BioMark platform (Miller *et al.*, 2016), including the bacteria *Flavobacterium psychrophilum* and '*Candidatus* Branchiomonas cysticola', both of which are highly prevalent among Chilliwack River Hatchery Chinook salmon (Bass *et al.*, 2017) and very likely driving the bacterial responses we observed in coho salmon.

The physiological effects of thermal and fishery stressors impacting wild adult salmon have been well studied (Cooke *et al.*, 2012; Farrell *et al.*, 2008; Hinch *et al.*, 2012; Martins *et al.*, 2012a; Patterson *et al.*, 2017a; Raby *et al.*, 2015), but data showing linkages between physiological variables and disease development are scarce (but see Bass *et al.*, 2017; Miller *et al.*, 2014; Teffer *et al.*, 2017; this thesis, Chapter 2). High temperature effects on plasma ions were apparent, causing decreases in chloride and

sodium, especially among gillnetted fish. These shifts indicate different osmoregulatory responses to individual and combined stressors that can influence longevity (Hruska *et al.*, 2010; Jeffries *et al.*, 2011) and are very likely pathogen driven (Bass *et al.*, 2017; Bradford *et al.*, 2010a; Buchmann *et al.*, 2001). A stress response was apparent in fish at high temperature, with cortisol, lactate, glucose and hematocrit significantly increased at the start of the study. Gillnetting enhanced muscle activity, with initial elevation and subsequent clearance of metabolites (e.g., lactate) that is consistent with previous studies (Farrell *et al.*, 2001; Raby *et al.*, 2012; Teffer *et al.*, 2017). Temporal changes in glucose were temperature-dependent, increasing only at high temperature and especially among gillnetted females. Impaired exercise recovery of infected fish relative to healthy fish (Wagner *et al.*, 2005) and the potential for thermal influences on this relationship (Kocan *et al.*, 2009) have implications for migration success, especially if rivers are warm.

Our results provide novel experimental data describing the disease ecology of an ecologically, culturally and economically valued fish species. Fisheries bycatch is a prevalent phenomenon comprising an estimated 40% of global marine catches across a wide array of species (Davies *et al.*, 2009) and the effects of climate change are expected to impact wild animals globally, especially regarding temperature (Poloczanska *et al.*, 2013; Root *et al.*, 2003). The context-specific nature of animal responses to multiple stressors warrants continued research into their cumulative effects and associated disease development, especially in the face of climate change (Altizer *et al.*, 2013). Our application of multiple stressors and examination of an array of infectious agents affecting Pacific salmon with host responses over time is a necessary step toward comprising the complexity of disease dynamics in wild ecosystems.

Table 3.1 A) Sample sizes by date and sex for coho salmon sacrificed at the Chilliwack River Hatchery, and B) sample sizes by temperature, treatment and sex for coho salmon transported, treated and held at the DFO Cultus Lake Salmon Research Lab, Cultus Lake, BC.

A)

Time point	Date	Female	Male
H0	18 Oct	5	4
H1	8 Nov	5	5
H2	26 Nov	8	3

B)

		Female		Male	
Temperature	Treatment	*n*	% mortality	*n*	% mortality
Low	Gillnet	28	3.6	12	0.0
	Biopsy	26	11.5	14	14.3
	Control	27	3.7	13	0.0
High	Gillnet	30	73.3	10	40.0
	Biopsy	27	40.7	12	41.7
	Control	29	27.6	12	8.3

Table 3.2 Parameters (β±s.e.m) from linear mixed effects models describing changes in infection metrics over time including infectious agent loads, richness (total unique agents) and relative infection burden (RIB) in gill. Only significant parameters for time (T), high temperature (H) and interactions between terms are shown with ΔAIC and intra-class correlation coefficients (ICC). All models were fit with a random intercept; no significant effect of gillnet treatment was identified.

Infection metric	ΔAIC	ICC	Sex	T	H	H × T
P. minibicornis	9.49	0.35		β=4.56±0.61	β=5.01±1.77	β=-1.59±0.74
				$P < 0.001$	$P = 0.005$	$P = 0.036$
C. shasta	5.55	0.14		β=2.37±0.31		
				$P < 0.001$		
I. multifiliis	7.69	0.14	β=2.54±0.92	β=3.18±0.50	β=6.44±0.96	
			$P = 0.007$	$P < 0.001$	$P < 0.001$	
Richness	5.72	0.20		β=0.90±0.08	β=1.23±0.23	β=-0.25±0.11
				$P < 0.001$	$P < 0.001$	$P = 0.018$
RIB	9.30	0.18		β=0.71±0.04	β=0.66±0.08	
				$P < 0.001$	$P < 0.001$	

Table 3.3 Prevalence and relative loads (mean ± s.e.m) of positively detected infectious agents in a pool of seven tissues collected from 132 Chilliwack River coho salmon at death after laboratory holding and experimentation. Tissues included gill, liver, spleen, heart, head kidney, white muscle, and brain (alternated every other fish). Significance values ($P < 0.05$) pertaining to the influence of sex (S), high temperature (H), treatment (non-biopsied controls, biopsied controls, and gillnet-treated groups included; G), and interaction terms in infectious loads (agents with ≥70% prevalence) or presence-absence (agents with ≥20% prevalence) were derived using analysis of variance and generalized linear models, respectively, on surviving fish after 14 days of holding.

Infectious agent	Positive detections	Prevalence (%)	Mean relative load (± s.e.m.)	S	H	G	S×G
C. shasta	132	100	27.86 ± 2.85		$P < 0.001$		
P. minibicornis	132	100	22.80 ± 2.95	$P < 0.001$			
I. multifiliis	128	97	22.59 ± 5.85	$P = 0.001$	$P < 0.001$	$P = 0.051$	$P = 0.005$
Rickettsia-like organism	104	79	19.24 ± 4.69	$P < 0.001$	$P < 0.001$		$P = 0.002$
K. thyrsites	93	70	18.00 ± 3.89	$P < 0.001$	$P < 0.001$		
T. bryosalmonae	43	33	18.04 ± 3.22		$P < 0.001$		
P. theridion	35	27	12.99 ± 4.45			$P = 0.027$	
A. salmonicida	30	23	14.11 ± 4.96		$P = 0.016$	$P = 0.028$	
I. hoferi	4	3	16.88 ± 8.65				
S. destruens	3	2	17.49 ± 4.22				
N. salmonis	2	2	25.03 ± 3.55				
Piscine Orthoreovirus	2	2	16.98 ± 5.91				

Table 3.4 Results from permutational multivariate analysis of variance (perMANOVA) and generalized linear models (GLM) describing the relationships of stressors, survival and relative infection burden (RIB) with immunity in male and female coho salmon. Gill biopsies were taken at the start of the study (T0) and after one week (T1). GLMs used high temperature (Temp) and gillnet treatments (with an interaction), RIB, and fate (survival 14 d) as predictors of principal component (PC) analysis axes describing host immune gene expression. Only significant parameters and coefficients (β±s.e.m.) are shown; percentages represent variance explained by each PC. Gene loadings in the PCA are shown in Fig.3.5.

	T0		T1			
	perMANOVA	PC1	perMANOVA	PC1	PC2	PC3
Female		(28%)		(27%)	(18%)	(11%)
Temp	$r^{2=}$0.08	β=-2.68±0.78	$r^{2=}$0.18	β=-1.64±0.68		β=1.51±0.47
	P = 0.001	P = 0.001	P < 0.001	P = 0.019		P = 0.002
RIB	$r^{2=}$0.04	β=-0.91±0.38				
	P = 0.024	P = 0.022				
Fate	$r^{2=}$0.03		$r^{2=}$0.03	β=-1.18±0.50		
	P = 0.042		P = 0.048	P = 0.023		
Male		(27%)		(23%)	(18%)	(14%)
Temp	$r^{2=}$0.12	β=-2.13±0.88	$r^{2=}$0.28			β=3.23±0.48
	P < 0.001	P = 0.021	P < 0.001			P < 0.001
Gillnet		β=-2.01±0.91				
		P = 0.034				
RIB			$r^{2=}$0.05	β=0.97±0.41	β=-1.26±0.47	β=-0.81±0.29
			P = 0.030	P = 0.022	*P = 0.011*	P = 0.008
Temp×Gillnet						β=-1.61±0.67
						P = 0.021

Table 3.5 Effects of high temperature (H), gillnet entanglement (G) and their interaction (H×G) on blood properties measured in female and male coho salmon sampled at the start of the study (T0) and after one week (T1). *F*-statistics of only significant parameters derived from analysis of variance are shown.

	T0			T1		
	H	G	H×G	H	G	H×G
Female						
Cortisol	F=20.3			F=24.7	F=4.1	
	$P < 0.001$			$P < 0.001$	$P = 0.046$	
Estradiol	F=38.8		F=6.7	F=176.1	F=13.6	
	$P < 0.001$		$P = 0.012$	$P < 0.001$	$P < 0.001$	
Testosterone	F=12.0			F=102.2	F=10.8	F=8.8
	$P < 0.001$			$P < 0.001$	$P = 0.001$	$P = 0.004$
Hematocrit	F=18.2	F=15.9				
	$P < 0.001$	$P < 0.001$				
Leucocrit	F=7.4				F=11.7	
	$P = 0.008$				$P = 0.001$	
Lactate	F=30.1	F=14.4	F=8.5	F=18.3	F=6.0	
	$P < 0.001$	$P < 0.001$	$P = 0.005$	$P < 0.001$	$P = 0.019$	
Glucose				F=30.0	F=6.8	
				$P < 0.001$	$P = 0.010$	
Osmolality	F=6.3			F=6.3		
	$P = 0.014$			$P = 0.010$		
Chloride	F=12.2			F=9.9		
	$P = 0.001$			$P = 0.002$		
Sodium	F=9.3	F=5.8	F=4.4	F=21.2		
	$P = 0.003$	$P = 0.018$	$P = 0.040$	$P < 0.001$		
Potassium	F=11.6	F=15.3	F=5.1	F=38.9		
	$P = 0.001$	$P < 0.001$	$P = 0.027$	$P < 0.001$		
Male						
Cortisol				F=9.7		
				$P = 0.003$		
Testosterone	F=13.3			F=35.9		
	$P = 0.001$			$P < 0.001$		
Hematocrit		F=4.6				
		$P = 0.038$				
Lactate	F=11.2	F=6.0		F=20.2		F=5.9
	$P = 0.002$	$P = 0.019$		$P < 0.001$		$P = 0.019$
Glucose				F=6.0		
				$P = 0.019$		
Osmolality				F=7.5		
				$P = 0.009$		
Sodium				F=30.8		
				$P < 0.001$		
Potassium	F=4.7			F=13.8		
	$P = 0.036$			$P = 0.001$		

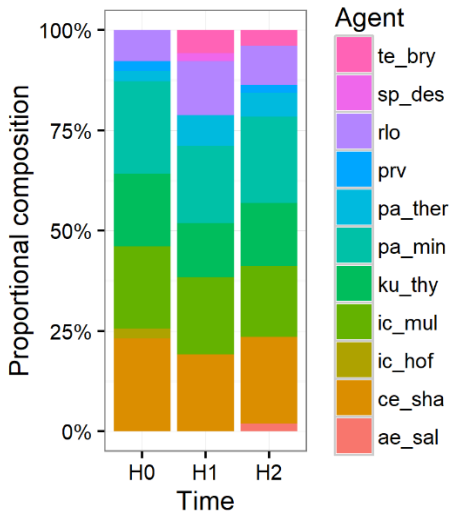

Figure 3.1 Proportional prevalence (total positive detections for each agent divided by the total positive detections of all agents detected on each sampling date) measured in a pool of seven tissues from coho salmon sampled on 18-Oct (H0, *n* = 9), 8-Nov (H1, *n* = 10), and 26-Nov 2012 (H2, *n* = 11) at the Chilliwack River Hatchery. The pool of organ tissues included gill, muscle, liver, spleen, head kidney, heart ventricle, and brain (every other individual). Infectious agent abbreviation codes can be found in Table 3.2.

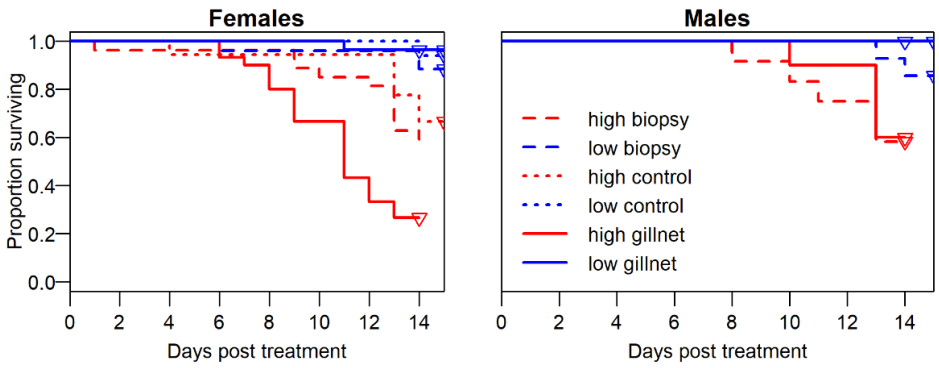

Figure 3.2 Kaplan Meier curves showing the survival of female and male Chilliwack River coho salmon (*Oncorhynchus kisutch*). Solid lines represent fish exposed to a standardized gillnet treatment (20s entanglement in water plus 1 min air exposure with biopsy), dashed lines represent biopsied controls, and dotted lines represent controls that were not biopsied. Colors depict water temperatures maintained throughout holding, with blue as the current average during migration (i.e. "low"; 10 ˚C) and red as an ecologically relevant "high" (15 ˚C).

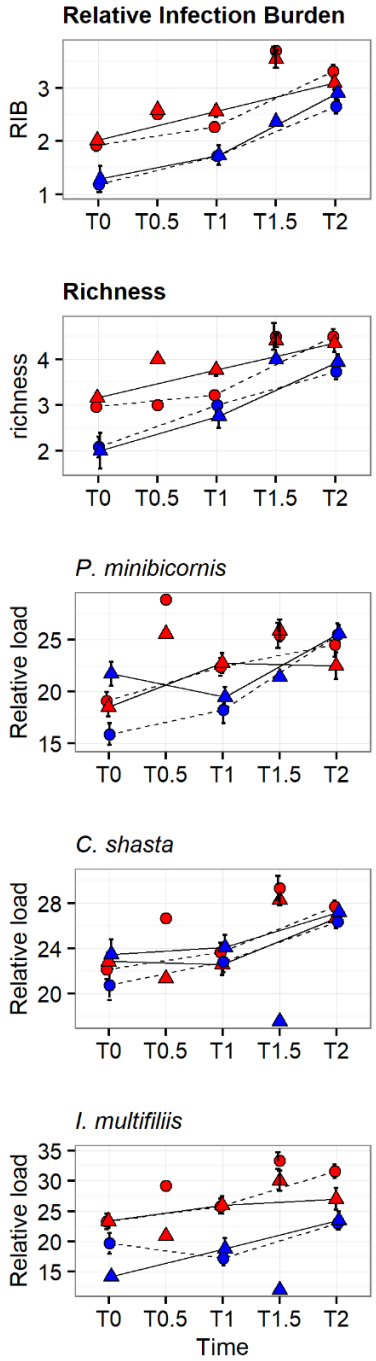

Figure 3.3 Pathogen richness, relative infection burden (RIB), and the relative loads of three prevalent infectious agents (*Parvicapsula minibicornis*, *Ceratonova shasta* and *Ichthyophthirius multifiliis*) measured using qPCR in gill biopsies from Chilliwack River coho salmon (*Oncorhynchus kisutch*). Nonlethal biopsies were taken at the start of the study (T0) and after 1 week (T1); all surviving fish were sacrificed and sampled after 2 weeks (T2) and fish that died prematurely were sampled at morbidity in the interim between live-sampling events (T0.5, T1.5). Color represents thermal experience (blue = 10 ˚C, red = 15 ˚C) and shape and line type indicate treatment (▲ and solid line=gillnet treatment; ● and dashed line=biopsied controls). Mean ± s.e.m.

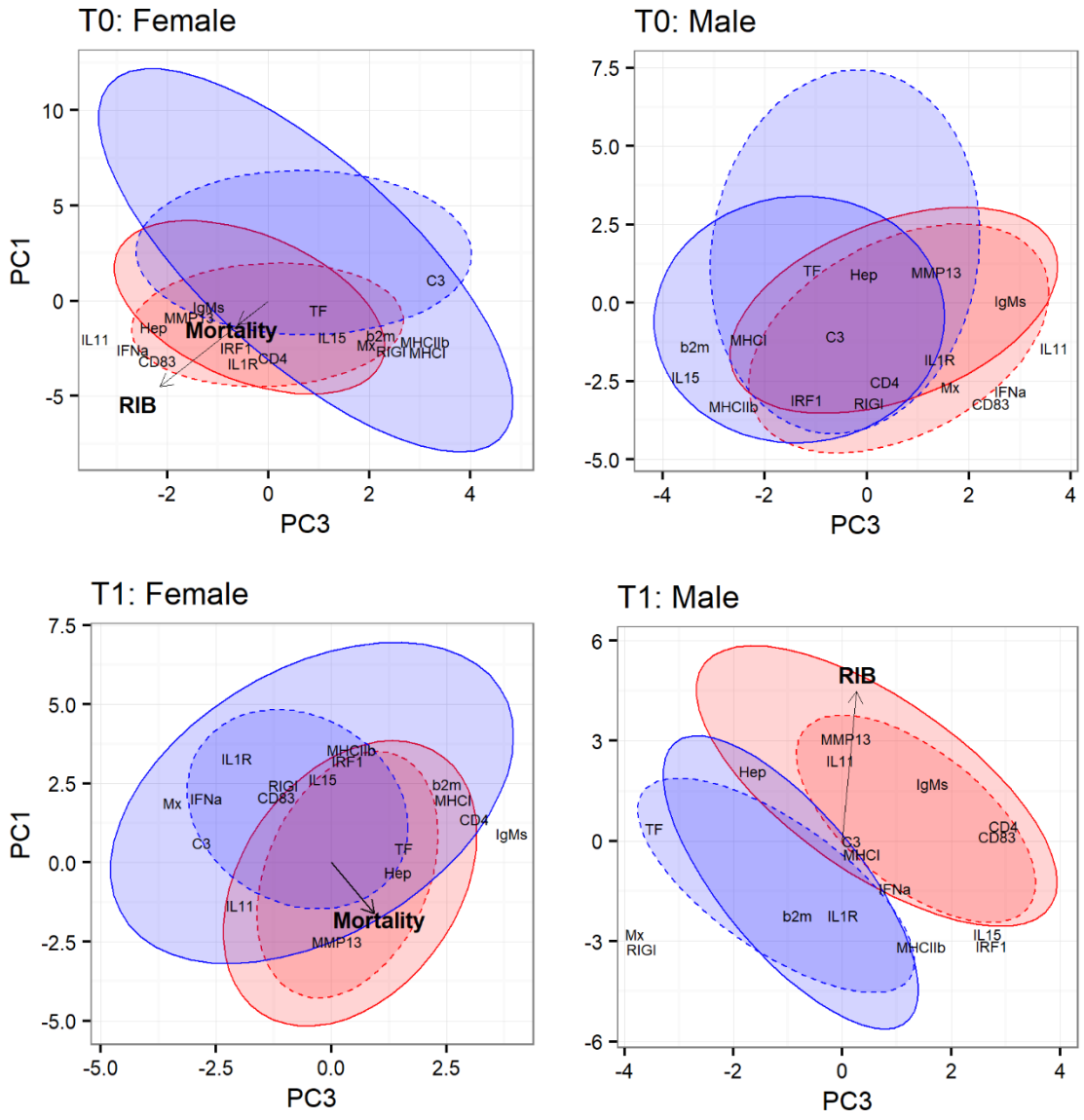

Figure 3.4 Principal component analysis (PCA) of the relative expression of 17 immune-related genes measured in the gill of Chilliwack River coho salmon (*Oncorhynchus kisutch*). Females and males are plotted separately and were sampled at the start of the study (T0) and after 1 week (T1). Ellipses depict 95% confidence intervals for each temperature-treatment group: blue = 10˚C, red = 15˚C; solid line = gillnet treatment, dashed line = biopsied control. Vectors represent directionality and strength of significant correlations ($P < 0.05$) between relative infection burden (RIB) and fate (mortality) with gene expression profiles; temperature significantly influenced gene expression in all analyses.

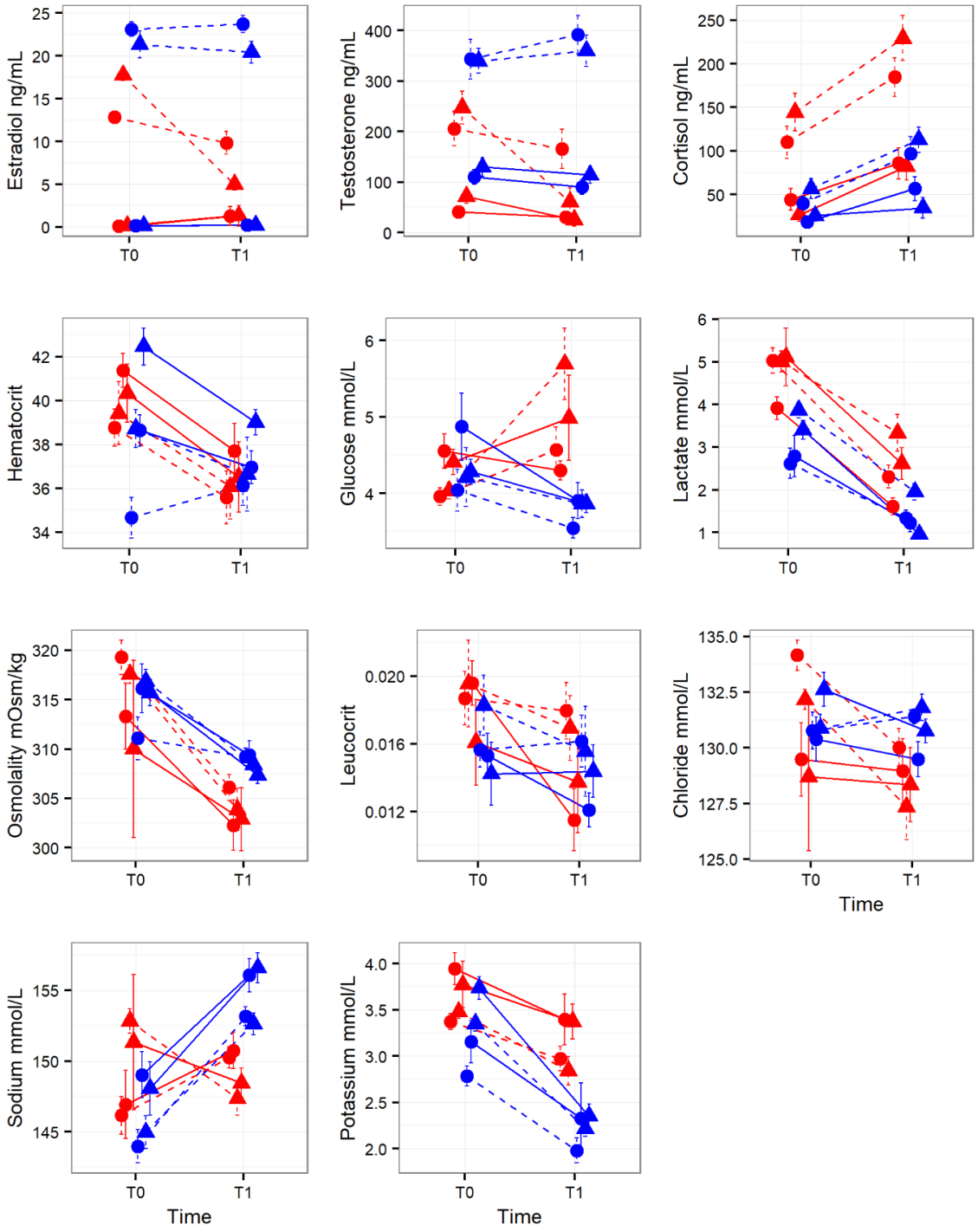

Figure 3.5 Hormones (cortisol, estradiol, testosterone), metabolites (glucose, lactate), ions (chloride, sodium, potassium), relative blood cell volumes (hematocrit, leucocrit) and osmolality measured in the blood of Chilliwack River coho salmon (*Oncorhynchus kisutch*). Fish were sampled at the start of the study (T0) and after 7 days (T1). Colors designate temperature groups (red=15 ˚C, blue=10 ˚C), shapes represent treatment (▲=gillnet treatment, ●=biopsied control), and lines differentiate sexes (solid = male, dashed = female). Mean ± s.e.m.

Chapter 4 – Influences of infections, host responses, and multiple stressors on the survival and behaviour of adult Chinook salmon (*Oncorhynchus tshawytscha*)

Adapted from: Teffer, Amy K.[1,2,*], Bass, Arthur L.[2], Miller, Kristi M.[3], Patterson, David A.[4], Juanes, Francis[1], Hinch, Scott G.[2], *Canadian journal of Aquatic and Fisheries Sciences*, in revision

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4.1 Abstract

Infectious disease dynamics of wild Pacific salmon are poorly understood and may play a prominent role in recent declines of Chinook salmon populations. Multiple stressors influence migration success of adult salmon, such as rising river temperatures and capture and release by fisheries, and likely modulate infection development. To understand how these factors impact survival and migration behaviour of adult salmon in fresh water, we conducted simultaneous holding and telemetry studies with gillnet

treatments and nonlethal gill biopsy and blood sampling. Laboratory fish were held and treated in either cool (9 °C) or warm (14 °C) water. High temperature reduced survival but did not amplify simulated gillnetting effects. Gillnetting reduced migration rate and distance traveled upriver and increased infection burdens but had no effect on longevity. Heavy infections were associated with reduced longevity and faster migrations. Blood properties and immunity were associated with stressors, survival, and infection burden. These results demonstrate multiple impacts of infectious agents, improve our predictive capability regarding how stressors can reduce migration success and longevity, and add to the growing knowledge of disease dynamics in wild Pacific salmon.

4.2 Introduction

Little is known about infectious disease dynamics of wild Pacific salmon (*Oncorhynchus* spp.) and associated impacts on population productivity (Riddell *et al.,* 2013, Miller *et al.,* 2014). The unique life history of Pacific salmon comprises multiple life stages and long-distance migrations spanning thousands of kilometers across marine and freshwater habitats (Groot and Margolis, 1991). These characteristics add complexity to host-parasite relationships and make infectious disease development and associated mortality difficult to observe in the wild (Bakke and Harris, 1998). Challenging environmental conditions and gradients likely influence infection development and host stress and immune responses to enhance the likelihood of mortality during migration (Snieszko 1974; Miller *et al.,* 2014).

Chinook salmon (*O. tshawytscha*) is one of several species of Pacific salmon to have experienced population declines within Canada (Riddell *et al.,* 2013) and the United States (Myers *et al.,* 1998; Heard *et al.,* 2007; Hoekstra *et al.,* 2007). In British Columbia,

Canada, habitat degradation and overfishing contributed to declines of Fraser River Chinook salmon starting in the 1950s, with some recovery following enhanced regulations put forth by the Pacific Salmon Treaty in the 1980s (Fraser *et al.,* 1982; DFO 1999). Since then, however, declines have been observed over recent generations (Riddell *et al.,* 2013). Continued losses despite management efforts suggest that some causes are beyond those alleviated by current regulatory influence. Infectious disease processes enhanced by mounting environmental and anthropogenic stressors may help to explain these continued declines (Miller *et al.,* 2011; Teffer *et al.,* 2017; this thesis, Chapters 2 & 3).

Although many factors can contribute to salmon population declines (Hoekstra *et al.,* 2007) with influences affecting all life stages, *en route* and prespawn mortality of adult Pacific salmon during their once-in-a-lifetime spawning migration can have drastic population level effects (Bowerman *et al.,* 2016), including increased extinction risk (Reed *et al.,* 2011; Spromberg and Scholz, 2011). Adult salmon cease feeding prior to entering fresh water and rely on endogenous energy reserves to fuel migration and spawning, after which rapid senescence results in natural death (Groot and Margolis, 1991; Kiessling *et al.,* 2004; Hruska *et al.,* 2010). This life history strategy places a great deal of weight on migration and spawning success, as failure to arrive at spawning grounds and spawn reduces an individual's fitness to zero. Efforts to improve our predictive capabilities with respect to early mortality of adult salmon can aid fishery managers in decision making and must be informed by studies describing its associated mechanisms (Macdonald *et al.,* 2010) and cumulative effects of multiple factors (Miller *et al.,* 2014).

Stressors beyond those historically encountered during migration, such as high river temperatures (Morrison *et al.,* 2002; Martins *et al.,* 2011; Altizer *et al.,* 2013) and fishery non-retention (i.e. capture and release; Baker and Schindler 2009; Raby *et al.,* 2015; Teffer *et al.,* 2017; this thesis, Chapters 2 & 3), can cause a stress response in returning adults. High temperature, for example, can cause osmoregulatory impairment, increased metabolic stress, and altered immune gene expression in adult sockeye salmon (Jeffries *et al.,* 2012a). A stress response can therefore alter host-pathogen relationships by reducing the resilience and resistance of hosts to infections, depending on the type and magnitude of the stressor (Barton 2002; Bowers *et al.,* 2008; Mateus *et al.,* 2017), but chronic high temperature can also directly increase pathogen replication (Ewing *et al.,* 1986; Bettge *et al.,* 2009) or infectious dose in the environment (Stocking *et al.,* 2006). Fishery non-retention is an example of an acute stressor and occurs when captured fish are not the target species of a fishery and are released (i.e., bycatch, discards). Air exposure and handling during the release process contribute to oxygen deprivation, equilibrium loss, physiological stress and reduced antimicrobial defenses (Svendsen and Bøgwald 1997; Gale *et al.*, 2011; Raby *et al.*, 2015). Water temperature plays a large role in determining post-release survival of Pacific salmon in addition to its strong independent effects on physiology and survival (Martins *et al.*, 2011, 2012b; Jeffries *et al.*, 2012b; Gale *et al.*, 2013). Co-occurrence of these stressors is expected to increase as water temperatures experienced by adult Pacific salmon continue to rise due to climate change (Ferrari *et al.*, 2007; Isaak *et al.,* 2012). The degree to which individual and combined stressors impact disease development in wild adult salmon will be a function of fish condition as well as the responses and interactions of an array of infectious agents.

Pacific salmon, like all organisms, accumulate infectious agents throughout their lives, with a spike in pathogen richness following freshwater re-entry (Benda *et al.,* 2015; Bass *et al.,* 2017). These infections then develop at different rates depending on resistance factors, such as pathogen recognition capability (Bayne and Gerwick 2001; Alvarez-Pellitero 2008; Dolan *et al.,* 2016), physiological resilience including injury, aerobic capacity and energy density (Hinch *et al.,* 2012; Gale *et al.,* 2014; Mateus *et al.,* 2017), as well as pathogen type and co-infection (Cox 2001; Hori *et al.,* 2013). Our knowledge of stress-dependent disease dynamics of wild Pacific salmon is in its infancy, especially regarding multiple stressors experienced by migrants (but see Miller *et al.,* 2014; Teffer *et al.,* 2017; this thesis), with far more disease information available from culture settings (Kurath and Winton, 2011). However, recent technological developments have brought new tools, such as high-throughput quantitative polymerase chain reaction (HT-qPCR), to the forefront of disease ecology. This technology facilitates rapid quantification of dozens of gene transcripts at once, targeting both the host and infectious agents in very small amounts of tissue, for a comprehensive evaluation of multiple infections and pathways to disease development in wild animals.

A recent study used HT-qPCR to describe the pathogen community carried by adult Chinook salmon during their return migration in southwestern BC, sampling multiple populations and sites in marine and freshwater habitats (Bass *et al.,* 2017). This work, in addition to identifying 20 unique bacteria, viruses, protozoa, and other microparasites in the tissues of 82 adult Chinook salmon, demonstrated sex-specific differences in infectious loads and correlated infection intensities of several agents with indices of morbidity and advanced senescence. Furthermore, prevalence and loads of different

agents were spatially variable across marine and freshwater collection sites and temporally variable within one site (Chilliwack River Hatchery, Chilliwack, BC, Fig 4.1). This variability could be due to pathogen-induced mortality (loss of diseased hosts from the population due to pathologies from heavy infections or immune/inflammatory responses), effective host immune responses (reduced loads and detection probabilities over distance/time), or life cycle characteristics of individual pathogens (fresh-/saltwater tolerance, timing of shedding/spore release). Empirical study of how pathogen loads change over time within individuals is needed to clarify these trends.

The findings of Bass and colleagues (2017) provide baseline data for an assessment of infection responses of adult Chinook salmon to high river temperature and capture and release from fisheries. Adult Chinook salmon *en route* to spawning grounds are frequently captured as bycatch in sockeye and other salmon fisheries and must be released. The extent to which physiological stress and injuries associated with this interaction alter migration behaviour, infection development and longevity of Chinook salmon is unknown. The Fraser River is the largest producer of Chinook salmon in Canada, which are distributed throughout most of the watershed (Fraser *et al.,* 1982), returning in three run-timing groups (spring, summer, fall) that comprise four major stock complexes (Parken *et al.,* 2008). The fall timed run is dominated by “ocean type” Harrison River fish that generally spawn from mid-October to mid-November. Harrison and Pitt River fish comprise the source population for the fall Chinook run returning to the Chilliwack River Hatchery, which was sampled on two occasions (10 and 21 Oct 2014) by Bass and colleagues. Given that temperatures in the Fraser River are increasing as a result of climate change and will likely affect fall run Chinook salmon in coming

decades (Patterson *et al.*, 2007; Hague *et al.*, 2011), cumulative impacts of fishery and thermal stressors are increasingly relevant.

We conducted a two-phase study to examine independent and cumulative effects of high water temperature and fisheries capture and release on infection development, physiology, migratory behaviour and survival of adult Chinook salmon. The first phase of our study used a laboratory holding approach with repeated biopsy of individuals to track changes in infections and host responses over time. The second phase worked to compensate for potential impacts of holding. We tagged and released biopsied fish into the lower Fraser River to relate infectious loads with longevity and behaviour in the river and the likelihood of returning to hatchery "spawning grounds." For both experiments, we exposed a subset of fish to a standardized gillnet entanglement treatment, a common gear-type used in the Fraser River. Furthermore, laboratory fish were treated and held in either cool water typical of historic river temperatures during migration or warm water representing a climate change scenario, allowing for evaluation of interacting effects of multiple (fishery and thermal) stressors. Our objectives were to: 1) evaluate the effects of gillnet entanglement and air exposure on survival, behaviour and migration success, 2) evaluate the impact of thermal stress on survival and potential interactions with a fishery stressor, 3) determine how stressors modulate pathogen productivity over time, 4) relate stressors, survival, infection intensities and host immune and stress responses to characterize differences in response profiles of survivors relative to early mortalities, and 5) use pathogen loads to predict migration behaviour in the river, evaluating sex-specific effects and any influence of fishery stressors.

4.3 Methods

To better understand the stress-related disease dynamics of adult Chinook salmon, we conducted laboratory holding and telemetry studies of adult Chinook salmon from the Chilliwack River Hatchery in October and November of 2013. Returning adult salmon enter the hatchery from a neighbouring creek where they can hold or move freely between the river and raceway until maturity, at which time they are collected from raceways, sacrificed and spawned by hatchery personnel. Although differences in the pathogen dynamics and immune competency between wild and hatchery-produced salmon may exist and are currently under investigation (K. Miller, unpublished data), hatchery-produced fish are released as juveniles and experience similar environmental conditions in both marine and freshwater habitats as wild fish do (Naish *et al.,* 2007). Therefore, pathogen burdens of hatchery-produced and wild adult salmon are likely similar early after freshwater re-entry, making hatchery-produced fish an effective model for understanding pathogen dynamics in salmon populations.

For both holding and telemetry studies, fish in good condition (vibrant, minimal scale loss) were dip-netted from raceways and immediately placed into truck-mounted tanks for transport to the Cultus Lake Salmon Research Laboratory, Cultus Lake, BC, or to the telemetry release location (approx. 40 min transport, Fig 4.1). Collection for laboratory holding took place on 9 & 10 Oct 2013 while collection for tagging took place throughout the freshwater residence period of this population (3, 7, 17, 24 Oct 2013). Transport tanks were filled with cold (8–9 ˚C), sand-filtered and UV-treated water, equipped with air stones and monitored continuously for dissolved oxygen and temperature.

4.3.1 Laboratory holding

Upon arrival at the Cultus Lake laboratory, fish were sequentially distributed among 12 holding tanks (8000–10000 L) of equal temperature to the hatchery (~9 ˚C); tanks were equipped with air stones and a submerged pump, which provided a slow current around the tank periphery to encourage fish to swim during holding (approximately 1 body length s^{-1}). After 48 h acclimation, the temperature of half of the tanks was increased incrementally over 48 h from 9 ˚C to 14 ˚C. Two temperature groups represented either a 'cool' thermal experience, which reflected current Chilliwack River and hatchery temperatures during migration (~9 °C), or a 'warm' thermal experience (~14 °C) that represented potential maximum temperatures encountered during migration and those expected to affect Chinook salmon populations under projected changes to the hydrology of the Fraser River watershed (Barnes and Magnusson 2000, Morrison *et al.*, 2002).

Four days after collection (timepoint henceforth referred to as "T1"), three tanks from each temperature group were exposed to standardized gillnet entanglement and air exposure treatment: each fish was dip-netted from its holding tank and immediately submerged in a treatment tank, with the opening of the dip-net facing a taught 8-inch monofilament gillnet mounted within a wide frame; upon exiting the bag of the dip-net, fish immediately swam into and were "caught" in the gillnet. After 20 s of sustained entanglement under water, the fish and gillnet were removed from the water and held in air for 1 min while the fish was disentangled from the net. Following air exposure, the fish was placed into a foam-lined trough with water flowing continually over the body and gills. Blood was extracted from the caudal vasculature (21-gauge needle, lithium heparinized Vacutainer®), a small gill biopsy (2-3 gill filament tips) was taken using

sterile end cutters and preserved in 1.5 mL RNA*later*® solution, a "spaghetti"-style tag (Northwest Marine Technology, Shaw Island, WA) was secured in the dorsal musculature, and any external wounds were recorded. The fish was placed into a recovery tank for up to 30 min and then put back into its holding tank. Time in the trough was approx. 2 min and water temperature throughout treatment, biopsy and recovery was the same as the holding temperature. Control fish were similarly dip-netted from holding tanks but immediately submerged in the sampling trough for blood and gill sampling following the procedures described above, with no gillnet or air treatment.

Holding tanks were monitored at ≤4 h increments from 08:00–24:00 for fish condition and water quality. Any fish displaying signs of morbidity (lethargy, gulping, loss of equilibrium) was removed and euthanized by cerebral concussion and cervical dislocation. Four days after nonlethal biopsy and treatment all surviving fish were sacrificed (timepoint henceforth "T2"); this time frame was in alignment with the telemetry component of the study, given that most tagged fish completed their migration from the release location to the hatchery within four days. Blood was again extracted from the caudal vasculature and tissue samples (~0.5 mg) were collected from various organs using sterile tools and included gill (2–5 gill filament tips), muscle (at lateral line even with dorsal fin, including skin, red and white muscle), liver, spleen, heart (ventricle), head kidney, and brain (every other fish). Blood and tissue sampling was conducted as above for prematurely morbid fish (only moribund or freshly dead) and all sacrificed fish. Tissue samples were preserved in 1.5 mL RNA*later*® solution (whole brain in 3 mL), allowed to fix at 4 ˚C for 24 h and then frozen at -80 ˚C until analysis. Length, weight and individual organ weights were recorded as well as aspects of gross

pathology and condition, such as the presence of macroparasites and lesions, scale loss and injuries, and organ discoloration.

4.3.2 Telemetry

For the telemetry component of our study, we aimed to test whether fisheries capture and release, and infectious agents (measured in nonlethal gill samples taken at release) were associated with longevity, migratory success, and migration time. Longevity was defined as the number of days surviving in the Chilliwack River following release, migratory success was defined as detection at the Tamihi Rapids radio receiver (rkm 28, Fig 4.1), which was considered the downstream extent of suitable spawning habitat (snorkeling observations, Fisheries and Oceans Canada [DFO] Stock Assessment, personal communication, 2013), and migration time was calculated as the difference between the time of the first detection at rkm 28 and the time of release.

Fish were transported by truck from the hatchery to a release location 8 km upstream from the mouth of the Chilliwack River (Fig. 4.1). Temperature in the river during the study ranged from 8 – 12 °C. No more than 15 fish were held in a transport tank at one time and dissolved oxygen was monitored to maintain proper concentration (8- 11 mg L^{-1}) during transport and as fish were removed from the tank for tagging. No fish were held in the transport tank for longer than 2.5 h. Tagged fish were released into the Chilliwack River with the expectation that they would be motivated to return to the hatchery from where they were captured. Treatments (gillnet and control) were alternated between fish. Simulated gillnet capture and gill and blood biopsy were conducted as described for the holding study. Gillnet treatment took place in a 1000 L tank that was continuously fed with river water. Control fish (not gillnet treated) were taken directly to the tagging

trough for biopsy. Following biopsy, a gastric radio tag (Pisces 5®, Signa Eight Inc, Newmarket, ON; 43 mm length x 16 mm diameter, 15.2 g in air) was inserted into the stomach of all fish, just past the esophageal sphincter with the wire hanging out of the mouth. A visual identification "spaghetti" tag was looped through the musculature posterior to the dorsal fin for recovery of fish at the hatchery and by anglers.

Six fixed radio telemetry receivers (Orion®, Sigma Eight Inc), each equipped with a 3-element Yagi antenna, were positioned along the Chilliwack River, similar to the layout used by Nelson *et al.*, (2005; Fig 4.1). Additionally, a receiver was placed immediately upstream of the hatchery to detect fish moving further upstream, and another was placed immediately downstream of the hatchery attraction channel to detect fish entering and leaving. Detection ranges were tested using a radio tag to ensure complete coverage of the river channel. Stationary receiver efficiency was calculated for the four middle receivers based on detections of tagged fish (> 95%) at adjacent upstream and downstream receivers – this could not be estimated for the furthest downstream or upstream receivers. Mobile tracking was performed on a weekly basis throughout October and the first week of November using a Lotek SRX 600 (Newmarket, Ontario, Canada) with a truck mounted 5-element Yagi antenna. At study completion, the entire river between the hatchery and confluence with the Fraser River was walked with a mobile receiver to determine final locations of radio tags. Mobile tracking data were used to determine maximum upstream detection location, to calculate antenna efficiency, and to visualize detection data to aid in interpretation of movement patterns.

Telemetry data were filtered to remove non-target frequency-code combinations. False positives for a given frequency-code combination were filtered automatically in R

statistical software (R Core Team, 2015), removing detections that occurred less than five seconds apart (the tag burst rate) or more than two minutes apart. Next, plots of detections by time for each individual were observed to identify remaining false positives. Longevity in the river was determined by observing individual detection plots. We assumed that rapid downstream detections (equating to a rough approximation of river velocity) indicated moribund or deceased individuals. These rapid downstream movements were easily recognizable in plots and were not followed by subsequent upstream movements. Survival times were assigned to the first detection in the series of downstream detections. An intensive recreational fishery overlaps with the fall Chinook migration on the Chilliwack River. If reported tags could not be censored at any fixed station prior to capture (i.e., fish captured prior to any detections at stationary receivers), they were removed from further analyses.

4.3.3 Laboratory analysis

Haematocrit (HCT) was measured in the field by centrifuging (2 min at 10000 *g*; LW Scientific® ZIPocrit; GA, U.S.A.) blood in heparinized micro-capillary tubes (Drummond Scientific®, PA, U.S.A.). Vacutainers® of whole blood were centrifuged at 7000 *g* for 7 min (Clay Adams Compact II centrifuge; NY, U.S.A.) and extracted plasma was flash frozen in liquid nitrogen. All plasma analyses were conducted at the DFO West Vancouver Laboratory, West Vancouver, BC. Plasma chloride, sodium, potassium, lactate and glucose concentrations and osmolality were measured following protocols described in Farrell *et al.,* (2001) and cortisol, estradiol and testosterone were measured using enzyme-linked immunosorbent assay (ELISA) kits (Neogen Corporation, KY, U.S.A.) according to manufacturer's protocols.

Gene expression was measured using the Biomark™ platform for HT-qPCR (Miller *et al.,* 2016) in the Molecular Genetics Laboratory at the DFO Pacific Biological Station, Nanaimo, BC. Primer and probe sequences and assay efficiencies can be found in Table S4.1. We first screened for 45 infectious agents in a pool of terminally sampled organ tissues (holding study only); positively detected pathogens were then measured in nonlethally and lethally sampled gill tissue along with 17 host immune genes (C3, C7, CD4, CD83, GR2, IFNa, IgMs, IL11, IL15, IL1R, MHCI, MHCIIb, MMP13, Mx, RIG.I, TF) and 2 reference genes. Processing protocols for HT-qPCR followed those described in Bass *et al.,* (2017). To summarize, each tissue sample was homogenized independently using a MM301 mixer mill (Restch Inc., PA, U.S.A.); aliquots of aqueous phase from seven lethally sampled organs (holding study only) were pooled for each individual (one organ tissue pool per fish) prior to RNA purification, including lethally sampled gill. All gill samples (nonlethally and lethally sampled) were also homogenized and extracted independently to quantify host and pathogen gene expression at the start of holding and telemetry studies and at the close of the holding study. RNA quantity and quality were assessed by spectrophotometry using A_{260} and $A_{260/280}$. Following RNA normalization across samples (0.5 µg for gill, 1 µg for pooled tissues), cDNA was synthesized (Invitrogen™ SuperScript™ VILO™, CA, U.S.A.) and all primers were incorporated into a multiplex PCR prior to qPCR as per manufacturer's protocols for the Biomark® platform, followed by ExoSAP-IT® PCR Product Cleanup (MJS BioLynx Inc, ON, Canada) and 5-fold dilution (TEKnova suspension buffer, CA, U.S.A.). Assay and samples mixes were loaded into qPCR chambers using the integrated fluidics circuit

controller and qPCR was completed using the "GE 96 X 96 Standard v1.pcl." (TaqMan®) cycling.

Positive and negative controls were included at each step in the protocol to detect any cross contamination among sample wells (negative controls) and any false negative detections (positive controls). Serial dilutions of host template and artificial clone constructs containing the primer sequence of the 45 pathogen assays under examination (Miller *et al.,* 2016) to quantify assay efficiency and calculate RNA copy number for infectious agents under examination. Two probes were quantified by the qPCR; the first detected contamination by clone constructs (NED™ dye, Applied Biosystems, Foster City, CA, USA, detected with VIC setting as closest wavelength), which could then be removed from subsequent analysis; the second quantified target primers of host and pathogen genes (FAM dye). Infectious agent assays not detected in duplicate were failed. Quantification cycle (Ct) is reported as the average of assay duplicates and relative expression of host biomarkers was derived according to Pfaffl (2001) using two reference genes. Infectious agent loads are presented as RNA copy number calculated using standard curves of clone dilutions.

4.3.4 Statistical analysis

High levels of mortality prior to initial nonlethal biopsy and treatment (see results) raised questions about the role of pathogens in mortality following collection and transport; therefore, we compared infectious loads in the gill of early mortalities (prior to T1) with those of fish that survived to T1 (low temperature controls) using nonparametric Kruskal Wallis rank sum tests to account for uneven sample sizes. This analysis and all of those described for held fish were restricted to males only due to the low number of

females collected for the laboratory holding study (approx. 20%; Table 4.1a), which likely reflected hatchery sex ratios during collection. Infectious agent metrics under evaluation included relative loads (RNA copy number) of pathogens, infectious agent richness (number of positively detected agents), and relative infection burden (RIB; Bass *et al.*, in review) in pooled tissues. RIB is a composite metric of multiple infection burden using qPCR data and was derived by the following equation:

$$RIB = \sum_{i \epsilon m}^{m} \frac{L_i}{Lmax_i},$$

where for a given fish, the relative load of the i^{th} infectious agent (L_i) is divided by the maximum load within the population for the i^{th} infectious agent ($Lmax_i$) and then summed across all agents (m) infecting the given fish. Relative loads (RNA copy number) and RIB were log-transformed prior to analysis and all tests comparing relative loads omitted negative detections, using only positive detections from prevalent pathogens.

The impact of gillnet treatment and high temperature on the survival of held fish was quantified using survival analysis and Cox proportional hazards (*survival*, R statistical package; Therneau 2014). We used linear mixed effects models to quantify the effects of gillnet treatment and high temperature on changes in infectious agent loads, richness and RIB measured in gill tissue at T1 and T2 (repeated measures). Random effects either allowed the response variable (infectious agent metrics) to vary across individuals but remain constant over time (random intercept) or allowed the response variable and its relationship with time to vary among individuals (random intercept and slope). Optimal random effects were chosen for each metric using the full set of fixed effects

(temperature, gillnet treatment, time, and all two-way interactions) by comparing second order Akaike's Information Criteria (AICc) and significance of model differences with analysis of variance (ANOVA; $P < 0.05$ corrected for "testing on the boundary"; Zuur *et al.*, 2009). Random intercept variance or intercept and slope variance and their correlation coefficients are reported for each model. Top-down model selection was then applied to identify fixed factors contributing to variation in disease metrics, whereby beginning with the full model (all factors and interactions), low t-value and high p-value parameters were removed and the reduced model compared using ANOVA; factors that did not significantly increase model likelihood ($P < 0.05$) and reduce AICc when included were excluded from the subsequent model. The final model included only parameters that significantly contributed to variation in the data or were components of a significant interaction term (Zuur *et al.*, 2009); reported ΔAICc values correspond to differences between the full model and the fully reduced model.

To characterize the relative contribution of high temperature, gillnet treatment, and fate (survival 4 days = travel time to spawning grounds) to the variation in blood properties measured in held males at T1 (immediately following gillnet treatment and after 48 h of increasing temperature), we used ANOVA with blood properties as response variables. Where sample sizes were uneven, non-parametric (Kruskal-Wallis) tests were also applied to ensure there was no impact of low samples sizes on test results; no difference between parametric and non-parametric approaches were identified and only ANOVA results are presented. Response variables were log- transformed if necessary to meet assumptions of normality and all interaction terms were included. Unbalanced sample sizes were unavoidable due to mortality at 14 ˚C (9 ˚C: Control Survivor $n = 7$, Gillnet

Survivor n = 15, Control Mortality n = 5, Gillnet Mortality n = 10; 14˚C: Control Survivor n = 4, Gillnet Survivor n = 4, Control Mortality n = 9, Gillnet Mortality n = 10). ANOVA was also used to characterize the explanatory power of thermal and fisheries stressors on blood properties measured in surviving males at T2 with an interaction term (9 ˚C: Control n = 12, Gillnet n = 15; 14 ˚C: Control n = 5, Gillnet n = 5).

A binomial generalized linear model (GLM) was used to identify contributing factors leading to early mortality of held fish based on information from plasma stress indices, gill pathogen burdens and immune gene expression, as well as influences of high temperature and gillnet treatment. To condense information describing stress, maturation and immunity at T1, we conducted principal components analysis (PCA) of parameters measured in blood and another of immune gene expression in gill, and then used Monte Carlo randomization tests (permutation of raw data to assess the significance of eigenvalues at $P < 0.05$; McCune *et al.,* 2002; McGarigal 2015) to identify 'significant' components to include in the GLM. If necessary, parameters were log-transformed prior to PCA to meet assumptions of normality. Pathogen burdens in gill were condensed into RIB for inclusion in the GLM. Coefficient estimates (β) and standard errors of significant parameters ($P < 0.05$) identified by the GLM are reported. Linear regression was then used to describe relationships between RIB and condensed gene expression and blood property data (i.e., significant PCA axes identified by GLM). Blood and gene expression data from fish that survived to T2 were also incorporated into PCAs and significant components were used as response variables in linear regressions with RIB (gill or pooled tissues), temperature and gillnet treatment (with an interaction term) to assess the extent to which infection burden correlated with host condition and immune activity

following exposure to stressors and their individual and combined influence on host physiology.

For the telemetry study, we aimed to test whether fisheries capture and/or infectious agents (determined from non-lethal gill samples) were associated with longevity, migratory success, and migration time. Longevity was defined as the number of days surviving in the Chilliwack River following release, migratory success was defined as detection at the Tamihi Rapids radio receiver (rkm 28, downstream extent of suitable spawning habitat), and migration time was the difference between the time of the first detection at rkm 28 and the time of release. These response variables were tested by comparing models containing infectious agent indices that were identified in the holding study as being associated with mortality: richness, RIB, and loads of *Flavobacterium psychrophilum*, *Ceratonova shasta,* and '*Candidatus* Branchiomonas cysticola'. Treatment (gillnet vs. control) was included in all models, and sex and body size (fork length) were included where applicable. Five candidate models, varied by the inclusion of an infectious agent variable, were compared using the information theoretic approach by AICc values to determine the best fit for each modeling objective. For longevity, all accelerated failure time (AFT) models included treatment and sex. We fit the longevity data with the Weibull, Gaussian, exponential, log logistic, log normal, and logistic distributions and compared both AICc values and plots of the negative log of the Cox-Snell residuals over time. The Weibull distribution was selected as the best fit for the longevity data. Due to its skewed distribution, RIB was log-transformed for this and following analyses. Analysis was performed with the *survival* package. For modeling migration time, AFT modeling was again employed but in this case the log logistic

distribution was determined to fit the data best. Only males could be included in the migration time analysis since only one female arrived at the spawning grounds. We included both treatment and body size in these models. Body size was included to account for differences in energy use during swimming and ability to navigate hydraulic challenges (Hinch and Rand 1998; Crossin *et al.*, 2004). Similar to the AFT approach, we created five GLMs to fit the migratory success (binomial) data and compared them by AICc. Again, we included both treatment and body size in these models.

4.4 Results

4.4.1 Holding study

In cool water (9 ˚C), males that died prior to T1 (<4 days after collection, n = 18) had higher RIB (χ^2 = 10.8, P = 0.001) and infectious agent richness (χ^2 = 16.7, P < 0.001), and higher loads of *C. shasta* (χ^2 = 14.8, P < 0.001), *F. psychrophilum* (χ^2 = 7.6, P = 0.006) and *Ca.* B. cysticola (χ^2 = 7.8, P = 0.005) in gill relative to those that survived (biopsied controls, n = 15; Fig. 4.2). Loads of *Ichthyophthiriis multifiliis* (χ^2 = 1.1, P = 0.298) did not significantly differ between early mortalities and survivors.

Even though the duration of the holding study was brief, survival was not consistent across groups (Table 4.1a). Low sample sizes for females precluded any statistical analysis of sex-specific impacts on survival, and percent mortality reported here for held females should be interpreted with caution. Pre-treatment mortality was approximately 30% for both sexes and temperatures. For fish that survived to T1, percent mortality was higher for females than males in cool (86%) and warm (14 ˚C; 100%) control groups but similar between sexes following gillnet treatment at both temperatures. Among males, percent mortality was higher in warm water than cool, similar for gillnetted and control

fish in warm water (approx. 70%), and slightly higher for gillnetted fish in cool water (control = 37%, gillnetted = 46%). Survival analysis (males only) identified a significant effect of temperature on survival where fish held in warm water experienced 1.2 times the hazard of mortality (standard error = 3.3) as those held in cool water ($P = 0.019$; Fig 4.3*a*). Neither gillnet treatment nor the interaction between high temperature and gillnetting significantly impacted survival during the holding period.

Infectious agent prevalence for held fish is shown in Table 4.2. For linear mixed effects models (Fig. 4.4), a random intercept was best applied for richness (ΔAICc = 0.22, $P = 0.385$; $\sigma^2 = 0.60$) and *Ca.* B. cysticola loads (ΔAICc = 0.49, $P = 0.075$; $\sigma^2 = 0.21$), while random slope and intercept models were better suited to *I. multifiliis* (ΔAICc = 2.97, $P = 0.013$; intercept $\sigma^2 = 2.85$, slope $\sigma^2 = 3.86$, cor = -0.88), *C. shasta* (ΔAICc = 1.49, $P = 0.041$; intercept $\sigma^2 = 0.79$, slope $\sigma^2 = 0.85$, cor = 0.78), *F. psychrophilum* (ΔAICc = 7.25, $P = 0.003$; intercept $\sigma^2 = 0.46$, slope $\sigma^2 = 0.25$, cor = -0.82) and RIB (ΔAICc = 10.19, $P < 0.001$; intercept $\sigma^2 = 0.64$, slope $\sigma^2 = 0.51$, cor = -0.84). Fixed effects impacting richness included gillnet treatment ($\beta = 0.54 \pm 0.28$, $P = 0.050$), temperature ($\beta = -1.53 \pm 0.70$), time ($\beta = 1.60 \pm 0.28$), and an interaction between temperature and time ($\beta = 1.29 \pm 0.49$, $P = 0.009$, ΔAICc=6.63); no *P*-values are presented for the independent effects of temperature and time due to the significance of their interaction, which prevented further reduction of the model. *F. psychrophilum* was influenced by gillnet treatment ($\beta = 0.35 \pm 0.12$, $P = 0.004$) and time ($\beta = 1.01 \pm 0.08$, $P < 0.001$, ΔAICc=7.25). RIB was also significantly associated with gillnet treatment ($\beta = 0.27 \pm 0.11$, $P = 0.012$) and time ($\beta = 0.69 \pm 0.07$, $P < 0.001$, ΔAICc=5.28). Time was the only significant fixed effect describing *C. shasta* ($\beta = 0.66 \pm 0.15$, $P < 0.001$, ΔAICc=6.58), *I. multifiliis* ($\beta =$

0.81±0.38, $P = 0.036$, ΔAICc=10.19), and *Ca.* B. cysticola ($\beta = 0.45 \pm 0.11$, $P < 0.001$, ΔAICc=3.18).

A primary factor explaining the variation in blood properties of males at T1 was fate (i.e., survival to study termination). For fish that would die within 4 days, cortisol ($F = 27.5$, $P < 0.001$), glucose ($F = 4.8$, $P = 0.032$) and lactate ($F = 27.6$, $P < 0.001$) were elevated at T1, while testosterone ($F = 13.7$, $P = 0.001$), osmolality ($F = 14.8$, $P < 0.001$), sodium ($F = 11.5$, $P = 0.001$) and chloride ($F = 20.6$, $P < 0.001$) were depressed. For both survivors and mortalities at T1, lactate ($F = 17.8$, $P < 0.001$), potassium ($F = 17.8$, $P < 0.001$), and estradiol ($F = 9.5$, $P = 0.003$) were increased at 14˚C, while osmolality ($F = 7.2$, $P = 0.009$), sodium ($F = 6.5$, $P = 0.014$) and testosterone ($F = 8.2$, $P = 0.006$) were increased immediately following gillnet treatment. However, some aspects of the stress response differed depending on fate. At T1, only survivors showed an increase in cortisol (interaction: $F = 6.6$, $P = 0.013$) following gillnet treatment, and a greater increase in estradiol at high temperature (interaction: $F = 5.7$, $P = 0.021$) relative to mortalities. Among survivors at T2, high temperature was the only factor significantly contributing to the variation in blood properties, with increased potassium ($F = 25.2$, $P < 0.001$), lactate ($F = 8.6$, $P = 0.006$), cortisol ($F = 5.2$, $P = 0.030$) and estradiol ($F = 7.2$, $P = 0.011$), but no significant impact of gillnet stress (P-values > 0.05).

Patterns in immune gene expression, plasma stress indices and temperature were associated with short-term survival of males (<4 days after T1; binomial GLM: null deviance = 88.5, df = 63; residual deviance = 40.0, df = 55; AICc = 61.33). Twelve infectious agents were positively detected in the gill of 64 adult male Chinook salmon sampled at T1; RIB values ranged between 0.001 and 2.643 with a mean of 0.289. Data

from blood properties measured at T1 were incorporated into the GLM as the first two components of the PCA (variation explained: PC1: 35%, PC2: 20%; $P < 0.001$; Fig. 4.5); gene expression data at T1 were also represented in the GLM by the first two components of the PCA (PC1: 24%, PC2: 22%; $P < 0.001$). At T1, low plasma ions and testosterone as well as high lactate, cortisol, and glucose characterized fish that died within four days (plasma PC1: $\beta = -1.09 \pm 0.38$, $P = 0.004$). Higher relative expression of genes associated with wound healing (MMP13, IL11), as well as complement (C7) and iron metabolism (TF), but decreased relative expression of cell-mediated immunity (MHCI, MHCII, CD4) and IL15 (gene expression PC2: $\beta = 0.79 \pm 0.33$, $P = 0.016$) were characteristic of fish that would die within four days. High temperature was associated with early mortality ($\beta = 4.05 \pm 2.00$, $P = 0.042$) but gillnet treatment and the interaction between stressors were not significant factors in the model. RIB in gill was marginally but non-significantly associated with mortality ($\beta = 1.81 \pm 1.03$, $P = 0.081$). Linear regression of gill RIB with PC1 of blood properties showed a negative relationship ($\beta = -1.55 \pm 0.33$, adjusted $r^2 = 0.25$, $P < 0.001$), where heavy infections corresponded to the characteristics identified by the GLM as associated with premature mortality. The relationship of gill RIB to immune gene expression data (PC2) was also highly significant and associated with characteristics of early mortality ($\beta = 1.54 \pm 0.33$, adjusted $r^2 = 0.25$, $P < 0.001$).

Blood properties and gene expression measured at the termination of the study (T2) showed similar grouping of variables in PCAs to those identified at T1 (Fig. 4.6). Blood properties (PC1: 46%, PC2: 20%) and immune gene expression (PC1: 21%, PC2: 20%) were both adequately described by two components ($P < 0.001$); note that HCT was not calculated at death and so was not included in the T2 analysis. RIB in gill was negatively

associated with PC2 of gill gene expression data (β = -2.01±0.80, P = 0.017, adjusted r^2 = 0.18) with similar gene associations as observed at T1, and no significant effect of gillnetting or temperature. Gillnet treatment and high temperature were negatively associated with PC2 of plasma data (gillnet: β = -0.97±0.44, P = 0.037; temperature: β = -2.11±0.58, P = 0.001; adjusted r^2 = 0.35), but no interaction or association with RIB was detected. Testosterone, estradiol, potassium, and stress metabolites loaded negatively on PC2, showing association of these characteristics with fish held in warm water and/or exposed to gillnet treatment.

4.4.2 Telemetry results

A total of 118 fish were tagged and released for the telemetry component of our study, equally divided between gillnet treatment and control groups (Table 4.1b). Two of the control and one gillnet-treated fish entered and remained in Sweltzer Creek (Fig. 4.1) and were therefore removed from analyses. A single control fish exited the Chilliwack River and swam up the Fraser River to the Harrison River and was also removed. Seven gillnet-treated and two control fish were captured and reported by anglers. Female fish were detected at lower proportions at the receivers upstream of the release location compared to males; only one female arrived at the spawning habitat and none returned to the hatchery (Fig. 4.7). Fifty-five percent of released Chinook salmon moved downstream after tagging (fell back) and 68% of these fish were subsequently detected upstream. Gillnet-treated and control fish moved downstream at similar proportions (55 and 56%, respectively) but a greater percentage of control fish were detected at the start of the spawning grounds and hatchery (48% control, 31% gillnet-treated), with this difference more pronounced among males (males only: 76% Control, 38% gillnet-treated).

Including censored individuals (39% of population), the median post-release longevity for the population (*n* = 97) was 6.18 days (5.57 – 7.30, 95% C.I.; Fig. 4.3b). AICc comparison of models including different indices of infectious agents identified a model with the bacteria, *F. psychrophilum*, as the best fit of the longevity data (Table 4.3). For each log unit increase in copy number of *F. psychrophilum*, longevity decreased by a factor of 0.16 (0.04 – 0.29, 95% C.I.; Table 4.4, Fig. 4.8). Neither treatment nor sex were associated with longevity in any models (Table 4.4). There was not a single generalized linear model that stood out from the others in terms of AICc (Table 4.3) when determining the factors associated with the probability of male Chinook salmon (*n* = 67) arriving on the spawning grounds. However, in all models, treatment was a significant predictor of success, with control fish being 1.4 (0.29 – 2.50, 95% C.I.) times more likely to arrive at the spawning grounds than gillnetted fish (Table 4.4). The time required for male Chinook salmon (*n* = 67, 26% censored) to arrive at spawning grounds (median = 2.92 days, 2.49 – 3.41 95% C.I.) was best fit by a model including RIB, although a competing model including infectious agent richness instead was within 1.5 AICc (Table 4.3). In the top model, both elevated RIB and the control treatment were associated with more rapid migration (Table 4.4). For each log unit increase in RIB, migration time decreased by a factor of 0.09 (0.02 – 0.17, 95% C.I.) and control fish required a factor of 0.31 (0.07 – 0.56, 95% C.I.) less time to migrate (Fig. 4.9).

4.5 Discussion

Our paired study design used laboratory holding and telemetry to demonstrate impacts of multiple stressors on infection development and host physiology and linked infection burdens to longevity, migration rate and migration success of adult Chinook salmon in

fresh water. Thermal stress was associated with early mortality of held fish while simulated gillnet capture resulted in slower migration rates of tagged fish and reduced distance traveled upriver. Both stressors elicited immediate physiological changes in blood properties, while only thermal stress was apparent after 4 days of holding. Correlations between physiological stress parameters and infectious disease agents support previous findings (Bass *et al.*, 2017), which were predictive of survival and temperature-dependent. Among held males, early mortality prior to treatment was characterized by higher composite scores of multiple infection intensities (RIB), infectious agent richness, and individual agent loads. We detected distinct immune responses correlated with RIB that were largely driven by load and prevalence of the bacteria *F. psychrophilum*; similar patterns have been described in sockeye salmon (*O. nerka*) exposed to capture stress (Teffer *et al.*, 2017; this thesis, Chapters 2 & 5), with upregulation of genes associated with tissue damage and bacterial defense. RIB and infection intensity of *F. psychrophilum* increased at a faster rate in fish exposed to experimental gillnetting and, when measured at release, *F. psychrophilum* load was negatively associated with longevity of tagged fish in the river while RIB was associated with faster migrations. Increased agent richness likely indicates enhanced transmission among stressed fish, especially after 4 days of holding, suggesting that less prevalent agents were introduced to new hosts more frequently under stressful conditions (gillnetting and high temperature) or possibly via gillnets directly. This increase in agent diversity would be expected to influence host responses and increase immune surveillance. The correlation of RIB with immune activity in surviving males after 4 days of holding could suggest that fish were responding to new infections and/or addressing

current ones (e.g. *F. psychrophilum*). We will argue in the discussion that these results support a role of infectious disease development in the migratory behaviour and early mortality of adult Chinook salmon in fresh water that is dependent on thermal and fishery stressors as well as host physiological responses.

Telemetry data demonstrated lower proportions of females compared to males successfully arriving at the hatchery. Females and males were observed to be close to or fully mature at the time of release. Because tagged fish were transported downstream prior to release, the choice to repeat this migration or find suitable spawning habitat in other portions of the river may favor the latter for females investing finite energy stores into egg development, nest digging and defense (Crossin *et al.,* 2004; Kiessling *et al.,* 2004; Esteve, 2005), but cannot specifically be defined as migration failure. Females carry higher loads of several infectious agents (Bass *et al.,* 2017) and have demonstrated greater susceptibility to mortality during stressful migrations (Martins *et al.,* 2012b; Donaldson *et al.,* 2014), possibly due to sex-specific responses to stressors (e.g., Kubokawa *et al.,* 2001; Jeffries *et al.,* 2012b; Donaldson *et al.,* 2014) that influence infection development (Teffer *et al.,* 2017; this thesis, chapter 2). These sex-specific differences in infectious loads and migration behaviours warrant further investigation before conclusions can be drawn as to their relationship.

Although survival of held males showed no effect of gillnet treatment, gillnet-treated males in the river were less likely to return to the hatchery and took longer to do so. These results suggest that at current autumn temperatures, acute fisheries stress can still slow migration rates and reduce migration success of adult male Chinook salmon but is unlikely to significantly impact longevity. The inherent stressors of river migration, such

as hydraulic challenges and predator/fishery avoidance, would not have been captured by the holding component of our study but likely influenced migration behaviour in the river, possibly compounding gillnet stress (Miller *et al.*, 2014). Slower migrations and augmented *F. psychrophilum* infection development following gillnet capture and release could increase bacterial loads at spawning grounds and indirectly amplify other infections by extending freshwater residence (Hinch *et al.*, 2012; Benda *et al.*, 2015; Chiaramonte *et al.*, 2016). Richness, RIB and *F. psychrophilum* loads were significantly increased by gillnet treatment in held fish measured four days after treatment, coinciding with when tagged fish would be arriving at the hatchery. Dermal abrasions, scale and mucus loss from the net and handling likely enhance opportunistic infections (Svendsen and Bøgwald 1997; Baker and Schindler 2009; Teffer *et al.*, 2017); even minor injuries have been shown to reduce migration success of adult Chinook salmon in the Willamette River basin and are hypothesized to be the result of secondary infections (Keefer *et al.*, 2017). *F. psychrophilum* infection has also been associated with suppressed humoral immunity (Siwicki *et al.*, 2004), which may facilitate infections by other organisms to enhance richness. These factors may contribute a higher likelihood of prespawn mortality (after arrival at spawning grounds) and warrant further study with a longer time course of observation, beginning shortly after freshwater entry and continuing throughout the spawning period. Efforts to reduce handling and injury during gillnet capture or use of alternative gears such as tangle nets may help to reduce impacts on released fish (Vander Haegen *et al.*, 2004), especially during periods of high temperature.

All infectious agents and composite metrics measured in held fish increased with time. Immune suppression due to high cortisol levels associated with senescence and

maturation (Maule *et al.*, 1996; Dolan *et al.*, 2016) as well as confinement stress (Donaldson *et al.*, 2011) likely reduce defenses of adult salmon against these agents, in addition to experimentally applied stressors. Among held fish, initially distinct and sparse infectious agent communities in gill became more similar over time, likely via transmission, driving enhanced agent richness after 4 days, especially among stressed fish. Although holding also enhances the likelihood of transmission, possibly contributing to these temporal increases, conditions experienced at the hatchery where returning fish crowd into raceways as they mature, are similar and perhaps more intimate than those in our holding experiment. Behaviour also likely factors into disease development, as held males demonstrated frequent dominance displays (e.g., chasing, biting), causing injuries that likely altered hormone levels (Hruska *et al.*, 2010) and influenced immune competence (Slater and Schreck, 1993). Such behaviours are known to occur in the wild (Hruska *et al.*, 2010) and were observed at the hatchery, adding another dimension to the disease dynamics of Pacific salmon. Among survivors at the termination of the study, stressors were more strongly correlated with blood properties than localized gill immune activity, which was instead associated with gill infection burden. Early mortality likely removed individuals with greater physiological impairment associated with infections (i.e., with greater disease development), especially among stressed fish. Physiological responses to stressors (primarily thermal), directed toward maintaining homeostasis rather than managing infections, may overshadow relationships of blood properties with RIB in surviving males at spawning grounds.

In the river, higher RIB was associated with faster return of males to the hatchery. Time spent in fresh water correlates with decreased immune defenses (Dolan *et al.*,

2016), increased infection intensity (Teffer *et al.,* 2017, this thesis, chapters 2-3) and advanced maturity (Fitzpatrick *et al.,* 1986); hence, well-developed infections are likely characteristic of mature fish, which have been shown to migrate more quickly through the river (Crossin *et al.,* 2008). Damage to osmoregulatory function caused by pathogens like *F. psychrophilum* (Barnes and Brown, 2011), *P. minibicornis* (Bradford *et al.,* 2010a) and *I. multifiliis* (Ewing *et al.,* 1994) could signal other physiological changes that influence migration rate. For example, (Donaldson *et al.,* 2010) found a negative correlation between osmolality and migration rate of Adams-Shuswap sockeye salmon (though not for Chilko sockeye). Low osmolality was also characteristic of summer run sockeye salmon held in a net pen prior to release and associated with a temporary increase in the migration rate and decreased overall survival and migration success (Donaldson *et al.,* 2011). Those authors point to stress as a potential cause, but Cook *et al.,* (2014) observed no influence of acute stress (elevated cortisol) on migration rate. It is possible that these relationships observed by Donaldson and colleagues are related to infectious disease processes, whereby damage to epithelial tissues caused by fishing gear and handling facilitate infections by agents like *Saprolegnia* fungus, *F. psychrophilum* and others (Barnes and Brown 2011; Keefer *et al.,* 2017; Teffer *et al.,* 2017, this thesis), causing a decrease in osmolality (Bradford *et al.,* 2010a). From a transcriptome perspective, a genomic signature indicative of poor health has also been linked to faster migrations and reduced migration success of sockeye salmon tagged and biopsied in the marine environment (Miller *et al.,* 2011). Migration distance and difficulty as well as infection severity likely dictate the extent to which heavy pathogen loads and poor health are associated with faster migrations, but further evaluation of this relationship is warranted.

Our holding study findings support previous work demonstrating the negative impact of warm river temperatures on the physiology and survival of adult salmon (e.g., Crossin *et al.,* 2008; Keefer *et al.,* 2008; Martins *et al.,* 2011; Jeffries *et al.,* 2012b), with an associated 20% increased risk of mortality for fish held in warm water for four days. From a host physiological and aerobic standpoint, thermal tolerance varies among species and populations according to the historic conditions of migrations (Pörtner and Knust 2007; Eliason *et al.,* 2011). Current and projected temperatures for the Fraser River watershed and other salmon bearing rivers demonstrate clear impacts of climate change and a need to comprehend how alterations to the historic thermal experiences of adult spawners will impact population productivities (Patterson *et al.,* 2007, Ferrari *et al.,* 2007; Macdonald *et al.,* 2010; McDaniels *et al.,* 2010; Reed *et al.,* 2011). Our results support previous findings from thermal stress studies on pink (*O. gorbuscha*) and sockeye salmon (Jeffries *et al.,* 2012a), but also show increased infectious agent richness in individuals exposed to thermal stress for only a few days. The interaction between high temperature and time for increased richness suggests delayed impacts of chronic thermal stress that likely accelerated transmission among hosts. High temperature can enhance the development of many infections including *I. multifiliis, P. minibicornis* and *C. shasta* (Ewing *et al.,* 1986; Crossin *et al.,* 2008; Ray *et al.,* 2012) and can also externally influence infection severity by accelerating the life cycle of parasites like *C. shasta*, increasing infective spore densities released by intermediate hosts into the migration corridor (Stocking *et al.,* 2006). Environmental effects such as this would not be captured by our holding study where fish were held in a relatively pathogen-free environment (source water is UV-sterilized and filtered) but may be more prominent for tagged fish

migrating in the wild. Despite its designation as the agent of bacterial coldwater disease, *F. psychrophilum* can also cause severe disease and mortality of salmonids within the range of temperatures we evaluated (Nematollahi *et al.*, 2003; Barnes and Brown, 2011). Testosterone, which has demonstrated immunosuppressive effects (Slater and Schreck, 1993), was higher in survivors held in warm water at the close of the study and may have contributed to increased pathogen richness. This finding is contrary to studies describing either a decrease (Manning and Kime, 1985) or no change (Jeffries *et al.*, 2012b) in sex steroids by males in response to stress. Because testosterone levels decreased in cool water fish during holding and most were spawning-ready even at collection, the peak in testosterone characteristic of maturing Pacific salmon may have occurred prior to our experiment (Hruska *et al.*, 2010). Further research that includes assessment of ripeness is needed to clarify this relationship before impacts on maturity can be concluded. The physiological limits of both salmon and infectious agents, including impacts of thermal stress on host immune capacity (Jeffries *et al.*, 2012a; Hori *et al.*, 2013) and cumulative effects of high temperature and infections on swimming stamina (Kocan *et al.*, 2009), will dictate the resilience of salmon populations to climate-driven changes in river temperatures (Crozier *et al.*, 2008; Altizer *et al.*, 2013).

Our predictive analysis of held fish demonstrated that, in addition to high temperature exposure, heavier infection intensities and extracellular immune responses in gill as well as stress metabolites and indices of osmoregulatory impairment in blood were characteristic of fish that would die within four days. Whether fate-associated differences in blood properties at the start of the holding study indicated advanced senescence (Hruska *et al.*, 2010; Jeffries *et al.*, 2011) or stress accrued prior to collection (Wendelaar

Bonga, 1997) is unknown. Because stronger physiological responses (cortisol and estradiol regulation) to stressors were observed in survivors, their condition likely affected their capacity to respond to stressors and their likelihood of survival. Recent studies of Chinook (Bass *et al.*, 2017), sockeye (Teffer *et al.*, 2017; this thesis, chapter 2) and coho salmon (chapter 3) have demonstrated similar associations between infection status and physiological indices. Relationships between infection burdens, blood properties and immune gene expression point to disease-associated interactions occurring locally and systemically that influence longevity and migration behaviour of Chinook salmon in fresh water. The fitness consequences of early mortality are therefore tied to infectious agents, host condition, and stress and immune responses.

Major sources of uncertainty in current and historic Chinook salmon productivity estimates (Riddell *et al.*, 2013) emphasize the importance of understanding the mechanisms of adult mortality to provide reliable data for predictive modeling of *en route* losses. Here, we provide information to be used toward this purpose and add another dimension of data describing the disease ecology of Chinook salmon in southwestern British Columbia. Collectively, our results support an influence of infectious disease development on early mortality and migratory behaviour of adult Chinook salmon during freshwater residence. This role is modulated by thermal and fishery stressors as well as host physiology during and after exposure. Our findings add to growing knowledge of the disease dynamics of wild Pacific salmon and improve our predictive capability regarding how multiple stressors can reduce migration success and longevity in the river.

Table 4.1 A) Sample sizes and percent mortality of held Chinook salmon by treatment and sex, B) Sample size and number arriving at spawning grounds, by sex and treatment group, for radio tagged Chinook salmon released into the Chilliwack River, BC.

A)	Female		Male	
Treatment	*n*	% Mortality	*n*	% Mortality
Cool*	14	36	75	37
Warm *	15	27	46	28
Cool Control	7	86	19	37
Cool Gillnet	2	50	28	46
Warm Control	4	100	17	71
Warm Gillnet	7	71	16	69

*Mortality prior to T1 treatment

B)	Female		Male	
Treatment	*n*	*n* arriving at spawning (%)	*n*	*n* arriving at spawning (%)
Gillnet	14	1 (7)	44	17 (39)
Biopsy	20	0 (0)	34	26 (76)

Table 4.2 Positive detections and prevalence of pathogens measured in the gill of adult fall run Chilliwack Chinook salmon that were tagged and released or held (T1: study start; T2: after 4 d).

		Male		Female	
Infectious agent	Group	Positive detections	% prevalence	Positive detections	% prevalence
Flavobacterium psychrophilum	Tagged	70	100	32	100
	Held T1	73	100	20	100
	Held T2	40	100	8	100
Ca. Branchiomonas cysticola	Tagged	70	100	32	100
	Held T1	73	100	20	100
	Held T2	40	100	8	100
Ceratonova shasta	Tagged	70	100	32	100
	Held T1	65	89	19	95
	Held T2	38	95	8	100
Ichthyophthirius multifiliis	Tagged	37	53	26	81
	Held T1	32	44	12	60
	Held T2	39	98	7	88
Rickettsia-like organism	Tagged	7	10	1	3
	Held T1	11	15	4	20
	Held T2	29	73	4	50
Aeromonas salmonicida	Tagged	6	9	1	3
	Held T1	12	16	7	35
	Held T2	19	48	4	50
Parvicapsula minibicornis	Tagged	19	27	5	16
	Held T1	16	22	5	25
	Held T2	12	30	2	25
Cryptobia salmocitica	Tagged	13	19	8	25
	Held T1	9	12	3	15
	Held T2	12	30	3	38
Kudoa thyrsites	Tagged	12	17	6	19
	Held T1	9	12	5	25
	Held T2	7	18	3	38
Dermocystidium salmonis	Tagged	9	13	3	9
	Held T1	3	4	2	10
	Held T2	8	20	1	13
Viral erythrocytic necrosis virus	Tagged	11	16	8	25
	Held T1	7	10	0	0
	Held T2	2	5	0	0
Loma salmonae	Tagged	2	3	7	22
	Held T1	2	3	0	0
	Held T2	1	3	0	0
Tetracapsuloides bryosalmonae	Tagged	4	6	3	9
	Held T1	1	1	0	0
	Held T2	0	0	0	0
Piscine Orthoreovirus	Tagged	1	1	0	0
	Held T1	0	0	1	5
	Held T2	0	0	1	13
Sphaerothecum destruens	Tagged	2	3	1	3
	Held T1	0	0	2	10
	Held T2	0	0	0	0

Table 4.3 Model results based on radio tagged Chinook salmon released in the Chilliwack River, BC following experimental manipulation, and biopsy. AIC comparison of candidate models is presented for each response variable including: longevity (modeled using accelerated failure time, aka AFT), migratory success (general linear models), and time to arrival at spawning (AFT).

Response	Common variables	Infectious agent variable	Param	AICc	ΔAICc	AICc weight
Longevity	Sex + treatment	*F. psychrophilum*	5	366.1	0.0	0.80
		RIB	5	371.2	5.1	0.10
		Richness	5	372.3	6.2	0.04
		Ca. B. cysticola	5	372.5	6.4	0.03
		C. shasta	5	372.6	6.5	0.03
Migratory Success	Treatment + body size	Richness	4	92.7	0.0	0.30
		C. shasta	4	93.4	0.6	0.20
		Ca. B. cysticola	4	93.4	0.7	0.20
		F. psychrophilum	4	93.5	0.7	0.20
		RIB	4	93.5	0.8	0.20
Time to Arrival at spawning	Treatment + body size	RIB	4	135.3	0.0	0.60
		Richness	4	136.7	1.5	0.30
		C. shasta	4	139.4	4.1	0.10
		Ca. B. cysticola	4	140.8	5.6	0.04
		F. psychrophilum	4	141.2	6.0	0.03

Table 4.4 Model statistics for the top model for each model comparison. Variables significant at ($P < 0.05$) are indicated in bold.

Response	Method	Distribution	Parameter	β	SE	Z stat	*P*-value
Longevity	Accelerated	Weibull	**Log *F. psychrophilum***	-0.16	0.06	-2.53	**0.01**
	Failure		Treatment (gillnet)	0.25	0.19	1.29	0.20
	Time		Sex (Male)	0.21	0.21	1.02	0.31
Migratory success	Generalized Linear model	Binomial	Richness	0.18	0.21	0.90	0.37
			Treatment (control)	1.35	0.56	2.41	**0.02**
			Fork length (cm)	-0.04	0.03	-1.43	0.15
Time to Arrival at spawning	Accelerated Failure Time	Log logistic	**Log RIB**	-0.09	0.04	-2.44	**0.01**
			Treatment (control)	-0.31	0.13	-2.48	**0.01**
			Fork length (cm)	0.001	0.01	0.20	0.84

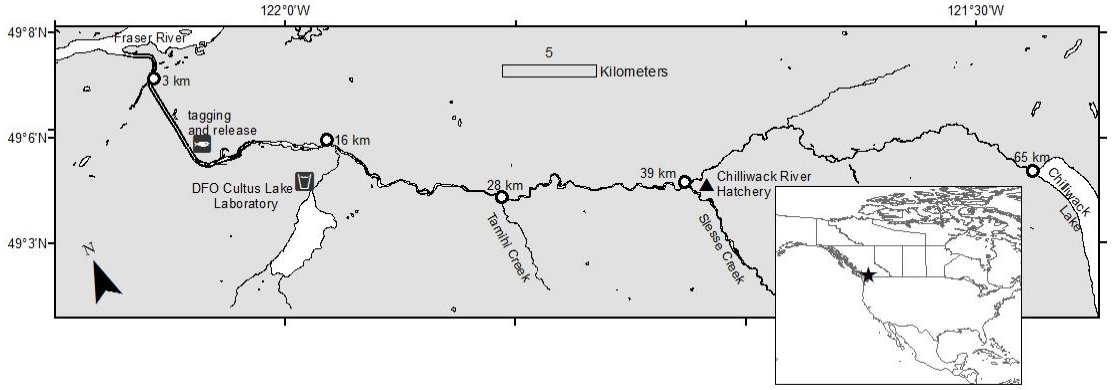

Figure 4.1 Map of Chilliwack River including Chilliwack River Hatchery (collection site), DFO Cultus Lake Laboratory (holding facility), tagging and release location for tagged fish, and telemetry receiver locations.

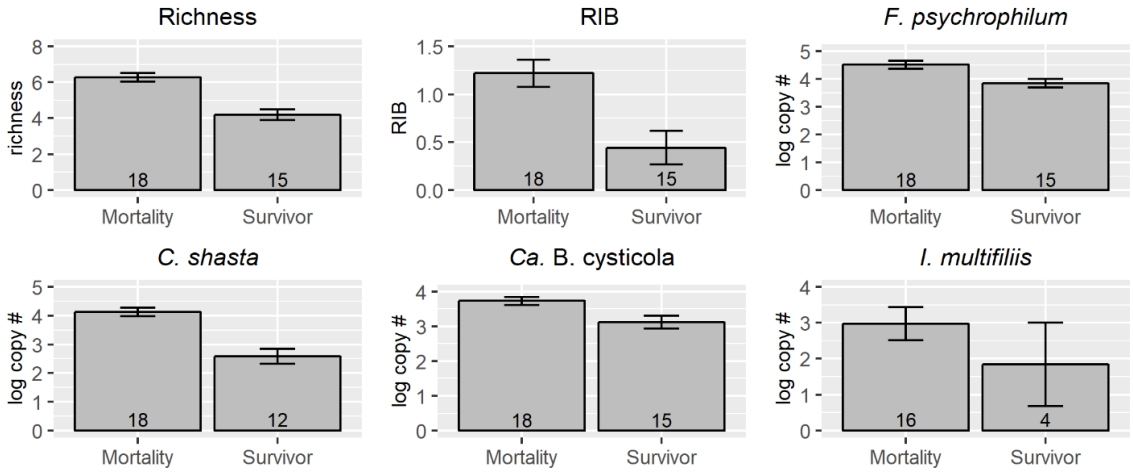

Figure 4.2 Differences in average pathogen richness, relative infection burden (RIB), and relative loads (log RNA copy number) of prevalent pathogens of male Chinook salmon that died <4 days after collection (mortality; $n = 21$) or survived to T1 (survivor [biopsied controls]; $n = 22$). Error bars represent standard errors; all differences were significant at $P < 0.05$ except for *I. multifiliis* ($P = 0.298$).

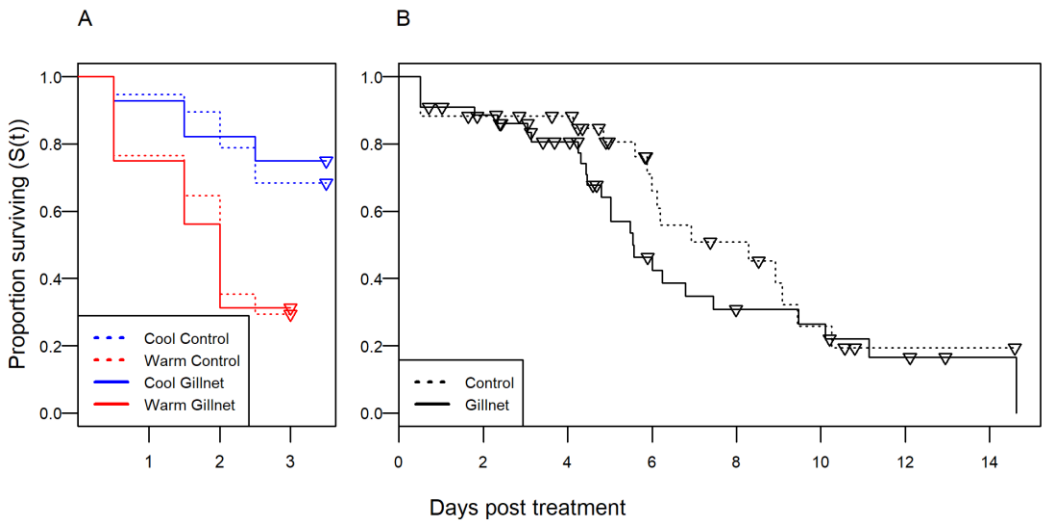

Figure 4.3 A) Kaplan Meier curve of survival of male Chilliwack River Chinook salmon following experimental temperature manipulation and gillnet entanglement treatment. Color indicates holding temperature (9 ˚C = blue; 14 ˚C = red) and treatment is indicated by line type (20 s gillnet entanglement and 1 min air exposure = solid, control = dashed). Sample sizes can be found in Table 4.1. B) Kaplan Meier curve of the longevity of radio tagged Chinook salmon receiving either a gillnet (solid) or control (dashed) treatment. Censored individuals are indicated by triangles.

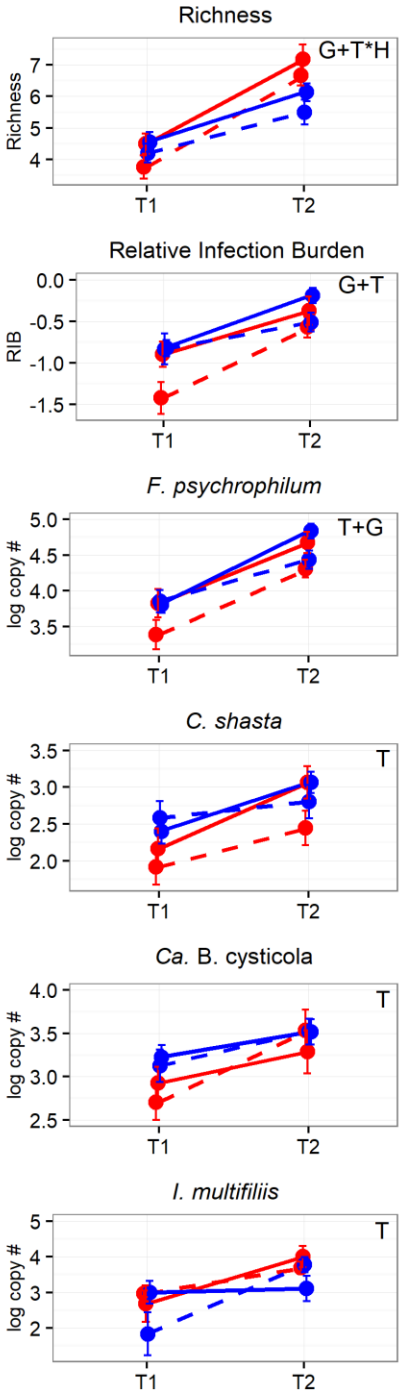

Figure 4.4 Pathogen richness, relative infection burden (RIB), and loads (log RNA copy number) of prevalent pathogen measures in the gill of male adult fall run Chilliwack Chinook salmon on two occasions (T1: study start; T2: 4 days later). Color indicates temperature during holding (9 ˚C = blue; 14 ˚C = red) and treatment is indicated by line type (20 s gillnet entanglement and 1 min air exposure=solid, control=dashed). Significant effects of time (T), gillnet treatment (G), and high temperature (H) on each metric are indicated in the upper right corner of each plot with additive effects (+) or interactions between terms (*).

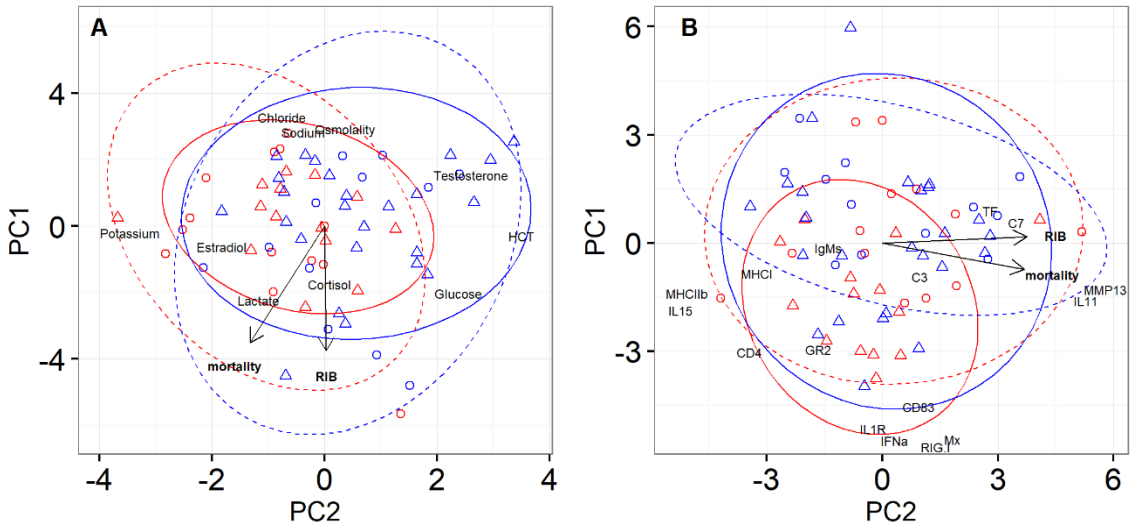

Figure 4.5 Principal components analysis of A) blood properties and B) immune gene expression in gill of adult male Chilliwack River Chinook salmon collected soon after river entry and held in freshwater tanks. Blood and gill tissue sampling and treatment took place approx. 4 days after arrival at the holding facility (T1). Point color corresponds to water temperature during holding (blue: 9 ˚C, $n = 37$; red: 14 ˚C, $n = 27$) and shape designates treatment group (▲= 20 s gillnet entanglement and 1 min air exposure, $n = 39$; ● = control, $n = 25$). Relationships with early mortality and relative infection burden (RIB) in gill are shown by vectors.

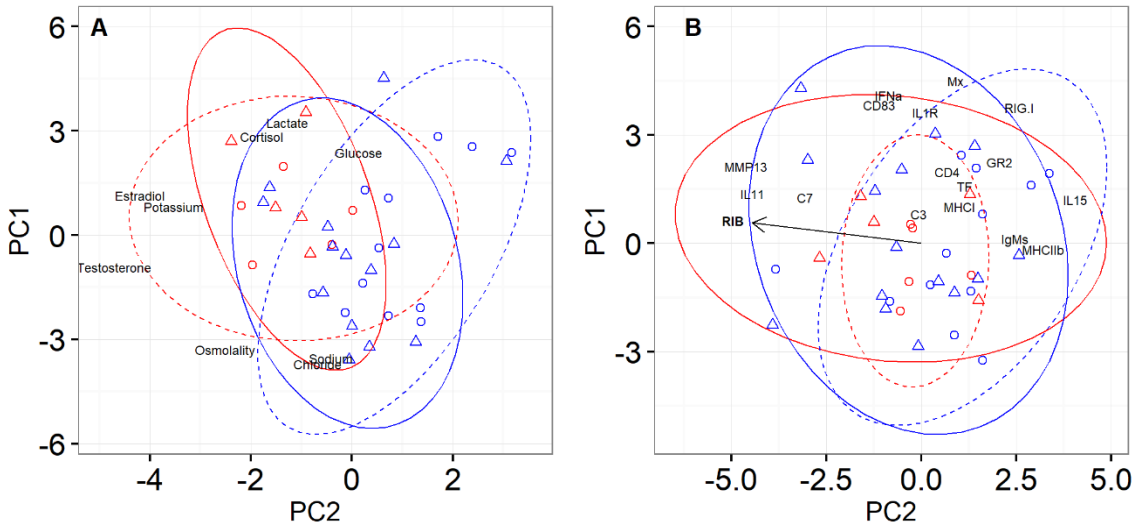

Figure 4.6 Principal components analysis of A) blood properties and B) immune gene expression in gill of surviving adult male Chilliwack River Chinook salmon four days after treatment and holding in freshwater tanks (T2). Point color corresponds to water temperature during holding (blue: 9 ˚C, *n* = 37; red: 14 ˚C, *n* = 27) and shape designates treatment group (▲= 20 s gillnet entanglement and 1 min air exposure, *n* = 39; ● = control, *n* = 25). Ellipses show 95% confidence intervals of each treatment and temperature group (dashed = control, solid = gillnet). Relationships with relative infection burden (RIB) in gill are shown by a black vector.

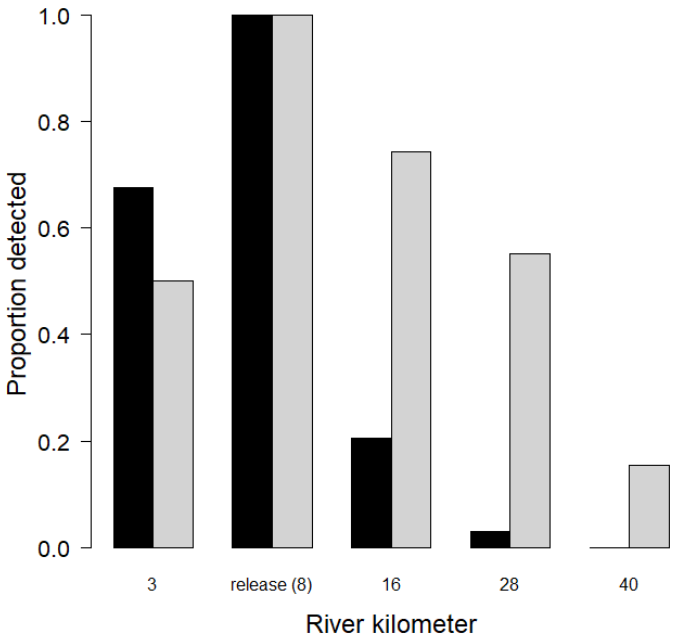

Figure 4.7 Proportions of radio tagged female (dark bars) and male (light bars) Chinook salmon detected at fixed receiver stations along the Chilliwack River, BC.

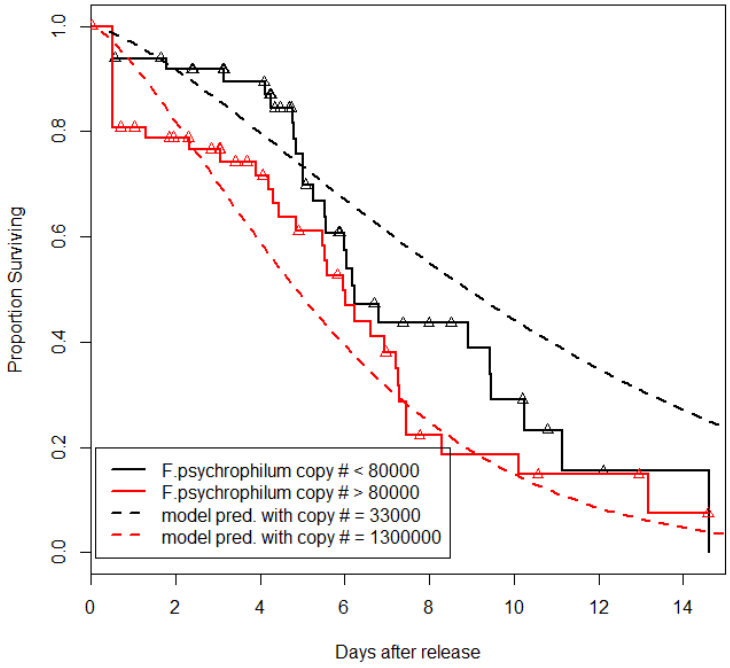

Figure 4.8 Kaplan Meier curves for longevity of Chinook salmon based on stationary radio receivers in the Chilliwack River, BC. The raw data used to generate the curves were split at the median value for *F. psycrophilum* copy number in the population (80,000). The predicted curves overlaid (dashed line) are based on the top AFT model for longevity (Longevity ~ log (*F. psychrophilum*) + Sex + Treatment). Survival was predicted based on the mean *F. psychrophilum* copy number for each group in the plot.

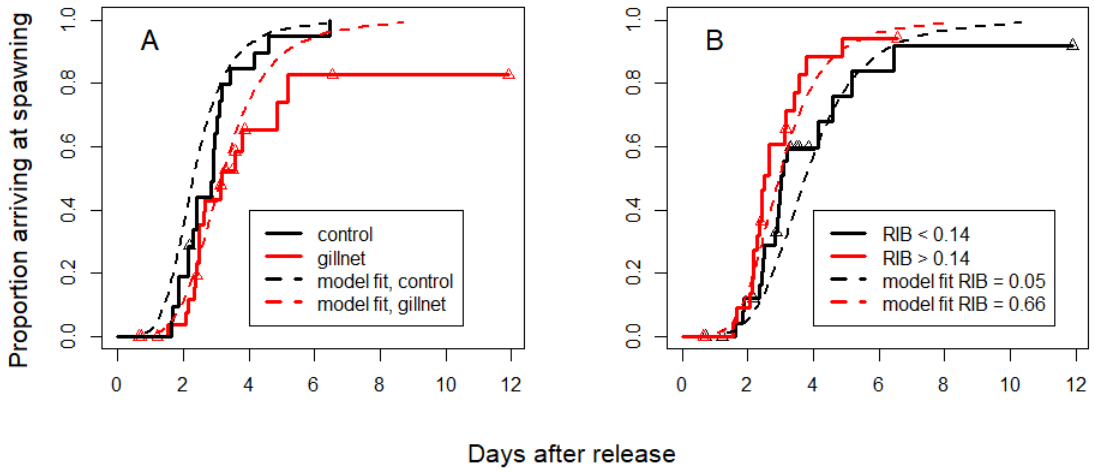

Figure 4.9 Kaplan Meier curves of migration time from release to spawning grounds for Chinook salmon released into the Chilliwack River, BC. Both A) treatment and B) RIB were significant predictors of migration time in the model (Time to arrival at spawning ~ RIB + treatment + fork length). In panel a, color designates treatment (red = 20 s gillnet entanglement and 1 min air exposure, black = control); in panel b, color represents infection burden (red = high RIB, black = low RIB). The predicted curves overlaid (dashed line) show the model fit for each group plotted. The median value for RIB (panel B) was 0.14 and predicted curves were created for the mean RIB value for each group.

Chapter 5 - Infections accumulated after river entry influence survival and health of sockeye salmon (*Oncorhynchus nerka*) exposed to multiple stressors

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5.1 Abstract

Infectious disease is often assumed to be the ultimate cause of mortality of adult salmon that die before they reach spawning grounds. The barrage of infectious agents that salmon encounter after leaving the marine environment likely play a major role in their

survival, especially in the presence of migratory stressors. To quantify the role river-acquired infections play in host survival, we collected returning adult sockeye salmon prior to and after river entry and held them in filtered and UV-treated water for the duration of freshwater residence (4 weeks). Marine-sourced fish were hypothesized to have lower infections than river-sourced fish. Fish were held at either optimal (14 °C) or high (18 °C) temperature, with a subset of each temperature group exposed to a gillnet bycatch simulation. Molecular analysis of weekly biopsied gill revealed distinct infection and host response trajectories depending on river-exposure and stressors. At 14 °C, river-exposed fish lived fewer days and had greater initial infections and infection development than marine-sourced fish. All fish held at 18 °C died within 4 weeks unless not handled or river-exposed. Genomic stress and immune responses of river-exposed fish were focused on infections, while profiles of marine-sourced fish associated with stressors (primarily thermal). Our findings suggest that in cool water, river-derived pathogens reduce survival, especially following handling/gillnetting, while mortality in warm water is independent of initial infections and likely caused by accelerated infection development and impaired physiological resilience. River-derived infections are therefore supported as causal factors contributing to the early mortality of adult Pacific salmon exposed to thermal or fisheries stressors, while cumulative stressors are detrimental regardless of initial infection status.

5.2 Introduction

Environmental and anthropogenic stressors are increasingly affecting wild animals and their pathogens, with disease and survival outcomes that likely depend as much on host responses and recovery as they do on pathogen community dynamics (Altizer *et al.*,

2013; Mitchell *et al.,* 2005). As opposed to cultured animals, life history aspects of wild animals such as migration, which is known to modulate disease development at individual and population scales (Altizer *et al.,* 2011), can amplify or focus the effects of these stressors (Lennox *et al.,* 2016). Infectious agents are an integral component of the ecology of wild animals and are commonly associated with co-infections that produce variable impacts on host survival and fitness (i.e., virulence; Johnson *et al.,* 2015; Sofonea *et al.,* 2017). Indeed, the virulence of one infectious agent may be diminished or enhanced by the presence of another, which can influence proximal virulence (e.g., current host survival and reproductive success) as well as the evolution of virulence factors (Sofonea *et al.,* 2015, 2017). Our knowledge of how multiple infections and cumulative stressors affect wild animal population dynamics is currently limited, especially within the context of their life history requirements. Quantitative data describing these interactions are needed to improve the precision of population estimates and enhance management effectiveness. To address this complexity, an experimental approach that incorporates multiple infections and cumulative stressors with adequate controls can be applied using a model species.

Pacific salmon (*Oncorhynchus* spp) are ideal for this purpose given their complex life histories (Groot and Margolis, 1991) and the multiple stressors and infections they currently encounter (Bass *et al.,* 2017; Miller *et al.,* 2014). Pacific salmon begin their lives as eggs in fresh water, then migrate as juveniles to the marine environment to feed and grow, and finally return to natal freshwater spawning grounds to spawn and then die (Groot and Margolis, 1991). High frequency of *en route* and prespawn mortality of migrating adults can have population level consequences with economic, ecological and

cultural repercussions, including lost individual fitness and reduced spawning biomass (Hinch *et al.,* 2012; Jacob *et al.,* 2010; Spromberg and Scholz, 2011; Willson and Halupka, 1995). As adult Pacific salmon cease feeding prior to river entry, individuals exhaust endogenous resources during freshwater residence, culminating in mortality after spawning (Miller *et al.,* 2009; Rand *et al.,* 2006). Early mortality of adult Pacific salmon is generally hypothesized to be disease-associated, especially under stressful migratory conditions (e.g., Gilhousen, 1990). Recent work has demonstrated temporal increases in infection development and decreases in immune defenses, with alterations to these trajectories induced by stressors and associated with early mortality of adult Pacific salmon (e.g., Miller *et al.*, 2014; Dolan *et al.,* 2016; Teffer *et al.,* 2017; Bass *et al.* in review; this thesis). Causal linkages, however, have yet to be established between infection severity and mortality of wild salmon, especially in the context of cumulative stressors and multiple infections (Miller *et al.,* 2014).

Pacific salmon physiology and disease ecology has been well studied in the Fraser River watershed, British Columbia (BC), Canada. Pathogen richness and loads have been shown to spike after adult salmon enter the Fraser River (Bass *et al.,* 2017; unpublished data) and includes freshwater pathogens like *Parvicapsula minibicornis* and *Ceratonova shasta*, which have been shown to cause thermally-mediated disease in wild salmon (Bradford *et al.,* 2010a; Fujiwara *et al.,* 2011). Infection intensities of bacterial (e.g., *Flavobacterium psychropihilum*) and parasitic (e.g., *Ichthyophthirius multifiliis*) agents generally increase with time spent and distance traveled in the river (Bass *et al.,* 2017; Miller *et al.,* 2014; Teffer *et al.,* 2017; this thesis, chapter 2). New data have also

demonstrated viral response indices expressed by wild BC salmon (Miller *et al.,* 2017) that led to the discovery of several novel viruses (K. Miller, unpublished data).

Importantly, the Fraser River has experienced climate-driven warming in recent decades (Patterson *et al.,* 2007) and hosts Canada's largest salmon fishery comprising commercial, recreational and subsistence users. Different salmon species co-migrate during fishery openings, so non-target species are frequently caught and released if they do not first escape fishing gear. Fishery non-retention can have drastic delayed impacts on the health, maturity and survival of released and escaped catch, especially if rivers are warm (Baker *et al.,* 2013; Patterson *et al.,* 2017; Raby *et al.,* 2015; this thesis, chapters 2-4). The cumulative effects of these stressors can vary depending on the species but are generally associated with infection development and early mortality (chapters 3-4). As disease development is a function of the environment, host and pathogens, application of relevant thermal and fisheries stressors and measurement of infection and host response trajectories can characterize how cumulative stressors contribute to disease-associated mortality of Fraser salmon.

To test the hypothesis that freshwater pathogens increase the likelihood of mortality of returning adult sockeye salmon, we conducted a river exposure "challenge" experiment, where returning adults were captured in either the marine environment or the lower Fraser River and then held in filtered, UV-treated fresh water. Adult salmon infection burdens prior to river entry are generally mild (Bass *et al.,* 2017; Miller *et al.,* 2014), whereas fish that enter the river acquire a "dose" of river-derived infections. Collection dates were aligned with projected migration rates for the dominant sockeye salmon stock (Adams-Shuswap) so that marine- and river-sourced fish were matched in their

maturation trajectories. Both source groups were held for four weeks in either cool (14 °C) or warm (18 °C) water characteristic of current or projected conditions, respectively, and exposed to either mild (seine) or more severe (gillnet) fishery capture/treatment. Under optimal migratory conditions (cool water, minimal capture stress), differences in survival depending on source location would provide strong evidence for freshwater infections as causal factors contributing to the early mortality of adult Pacific salmon in the absence of stressors. Furthermore, differences in survival of fish exposed to thermal and fishery stressors provides insight into how freshwater infections affect the resilience of adult salmon to cumulative stressors and the role of infectious disease in early mortality. Finally, differences in host responses depending on river exposure, stressors and survival would reveal the effectiveness of coping strategies depending on infection burdens and migratory conditions.

Two hypotheses provided the basis for our objectives: H_1) Infection burdens will be lower and develop more slowly in fish that bypass the river (marine-sourced) relative to those exposed to the river (river-sourced), H_2) Survival will be better for fish that bypass the river under optimal (cool water, no fishery stress) and suboptimal (thermal and/or fishery stress) conditions, but both sources will show reduced survival under independent and cumulative stressors. Our objectives were then to: 1) compare survival of source groups under optimal conditions and with individual and cumulative stressors, 2) identify differences in the infection trajectories of fish depending on source, stressors, sex and survival, and 3) characterize differences in host stress and immune responses based on source, stressors, sex, and survival. The findings of this study will improve our predictive capability regarding how changes to the environment due to climate change and

anthropogenic activities can exacerbate infections to cause disease and mortality of wild animals and potentially affect virulence evolution.

5.3 Methods

5.3.1 Fish collection

We focused fishing effort during the "late run" sockeye salmon migration in the Fraser River, which was projected to be dominated by the Adams-Shuswap stock complex during our collection period. On September 11-12, 2014, 153 sockeye salmon were collected using a commercial purse seiner in the Strait of Georgia (49.232 N, 123.271 W; Fig 5.1). Fish were transported in live-wells filled with seawater to a dock at the DFO West Vancouver Laboratory, West Vancouver, BC (40 min transport), where they were transferred using dipnets to truck-mounted tanks filled with cold (~10 °C), filtered, UV-treated water for transport to the DFO Cultus Lake Salmon Research Laboratory, Cultus Lake, BC (1.25 h transport). Tanks were fitted with air stones and continually monitored for temperature and dissolved oxygen during transport. At the Cultus Lake lab, fish were sequentially distributed among 12 holding tanks filled with sand-filtered, UV-treated water from the neighboring Cultus Lake at equal temperature to the lower Fraser River during collection (14 °C). Densities within holding tanks depended on tank size, which included large (8000-10000 L), medium (4000 L) and small (1400 L) tanks; large tanks held ≤22 fish, while medium tanks held ≤13 fish, and small tanks ≤5 fish. All tanks were covered and fitted with air stones and a submersible pump (large tanks only) that produced a slow current around the tank periphery, encouraging fish to swim in place during holding (approximately 1 body length sec^{-1}). The velocity of water entering small tanks was sufficient to produce the same current, with no need for a pump. Tank

replicates included one large and one small or medium tank per temperature-treatment group. Fish were left undisturbed for one week to allow recovery from transport and to simulate the approximate migration time from the collection location to the lower Fraser River. No marine-sourced fish died during the first week of holding. Beginning on 17-Sep, the temperature was incrementally increased over 48 h from 14 °C to 18 °C in half (six) of the tanks, producing two temperature treatment groups with either a cool (14 °C) or warm (18 °C) thermal experience; 18 °C is an ecologically relevant thermal extreme affecting late run salmon with increasing frequency (Morrison *et al.*, 2002; Patterson *et al.*, 2007).

One week after collection (19 Sep), a third of the marine-sourced fish from each temperature group was exposed to a gillnet treatment that simulated capture and release from a gillnet fishery. The treatment proceeded as follows: the fish was removed from its holding tank using a dipnet and immediately submerged in a small (1400 L) treatment tank within the bag of the dipnet. The opening of the dipnet faced a taught monofilament gillnet (mesh size: 5.25-inch, 13.3 cm) mounted in a wide frame. Upon exiting the dipnet, the fish was "caught" in the gillnet and entanglement was maintained for 20 s. If the fish escaped, the timer was stopped until entanglement had been achieved. After 20 s of sustained entanglement, the fish and gillnet were pulled from the water and placed into a dipnet for 1 min of air exposure while the fish was detangled from the gillnet (simulating bycatch release by fishers). The fish was then submerged in a foam-lined, flow-through sampling trough (water flowing over gills and body) where a small amount of gill tissue (2-3 filament tips, ~0.5 mg) was taken using sterile end clippers (sample preservation details below), 2 mL of blood was extracted from the caudal vasculature (21-gauge

needle with lithium heparinized Vacutainer®, Becton-Dickson, NJ; data not shown), a Floy® "spaghetti" style tag (Seattle, WA) was secured in the dorsal musculature, and a brief assessment of external injuries and condition was recorded. The fish was then placed into a recovery tank (3000 L) for up to 30 min before being returned to its holding tank. Water temperature throughout the treatment, biopsy and recovery were consistent with that of the fish's holding tank. The remaining marine-sourced fish were divided into two control groups: one biopsied and one left undisturbed until the termination of the study. Biopsied controls followed the same tissue and blood sampling protocol described for gillnet-treated fish but proceeded directly from holding tanks to the sampling trough (no gillnet or air treatment). The biopsy procedure took <2 min overall and included <10 sec of total air exposure.

During 24-26 Sep, 183 sockeye salmon were collected from the lower Fraser River near Fort Langley, approximately 50 river kilometers (rkm) from the mouth of the Fraser River. River-sourced fish were not gillnet-treated in the lab but instead collected with either a gillnet (treatment; n = 125) or a beach seine (control; n = 58). Beach seines have been previously demonstrated as a less invasive fishing gear, contributing to high survival of released catch relative to other gear types (Bass *et al.*, 2018; Donaldson *et al.*, 2012; Raby *et al.*, 2015). Disparity in sample sizes between gear types was due to river conditions at the time of collection that were more favorable to gillnet capture. Beach seines were deployed from shore, encircling and corralling fish into shallow (0.5–1 m depth) water without beaching them. Gillnets were deployed in deeper water near the middle of the river for <20 min sets. Gillnet- and seine-collected fish were removed from nets following best fishery practices (e.g., quick removal of fish from gillnets by fishers

"picking" the net by boat, dip-net removal of fish from the seine) and placed into net pens anchored in the river until biopsy and/or transfer to truck-mounted tanks. Subsets of gillnet-collected (n = 70) and seine-collected (n = 25) fish were biopsied riverside for gill and blood and tagged following the same protocols described for marine-sourced fish (sampling trough supplied with fresh river water) prior to transport to the Cultus Lake lab. The remaining fish were not biopsied, serving as non-handled controls; however, to identify "treatment" (gear type), the adipose fin was clipped from gillnet-collected controls using scissors in a cylindrical recovery bag submerged in water (duration ≤10 s, no air exposure). Truck-transport conditions were identical to those described for marine-collected fish, but transit time was approximately 40 min. Upon arrival at the lab, fish were sequentially distributed among 12 holding tanks of equal temperature to the river during collection (14 °C), separate from marine-sourced fish. Transport mortalities (n = 16) were immediately biopsied for gill and blood, examined for gross pathology (lesions, organ discoloration, macroparasites), and morphometrics recorded including length, total weight and organ weights as well as tag ID if applicable and gear type. An operculum punch was preserved in 90% EtOH for stock identification using microsatellite analysis (Beacham *et al.*, 2004).

Tank temperatures were held relatively constant, allowing for some diurnal variation (±1.5 °C). However, to simulate behavioral thermoregulation of adult Pacific salmon during freshwater migration, whereby individuals reside near the thermocline of corridor lakes *en route* to spawning grounds (Newell and Quinn, 2005), all tank temperatures were decreased to 10 °C for 48 h beginning approximately 10 d after treatment (i.e., 10 d after gillnet entanglement for marine-sourced, 10 d after collection for river-sourced).

Nonlethal biopsy of all initially biopsied fish (gillnet-treated and biopsied marine fish, initially biopsied river fish) was repeated weekly until study termination (16–18 Oct), producing 4 weeks of nonlethal biopsies for marine-sourced fish and 3 weeks for river-sourced fish, plus a terminal biopsy at death. For all sampling occasions, after each tank was processed (treatment and/or biopsy), sampling troughs and recovery tanks were sanitized (1% Virkon™ solution, Wilmington, DE, USA), rinsed, and dried to prevent transmission of infectious agents among tanks. Throughout the experiment, tanks were monitored for water quality, temperature and morbidity at ≤4 h intervals from 0800-2400 h. Fish that became moribund (gulping, loss of equilibrium) during the study and all surviving fish at the termination of the study (16–18 Oct, marking beginning of the spawning period for the Adams stock complex) were sacrificed using cerebral concussion and cervical dislocation and sampled according to the protocol described for transport mortalities. Gill samples were immediately submerged in 1.5 mL RNA*later*® solution (Ambion, Inc., Austin, TX, USA) and stored at 4 °C for 24 h, then -20 °C for up to two months, and then -80 °C for three months until analysis.

5.3.2 Laboratory analyses

Gill samples were processed at the DFO Pacific Biological Station in Nanaimo, BC using high-throughput quantitative polymerase chain reaction (HT-qPCR) on the Fluidigm Biomark Dynamic Array microfluidics platform™ (Fluidigm, San Francisco, CA, USA). This technology allows for the simultaneous quantification of 96 molecular assays (i.e., targeting either host or infectious agent genes) on 96 tissue samples; the platform has been analytically validated for its use in infectious agent screening (Miller *et al.*, 2016), applied in multiple field surveys of wild salmon populations (Bass *et al.*,2017;

unpublished data) and paired with evaluations of host gene expression (Jeffries *et al.*, 2014a; Miller *et al.*, 2014; Teffer *et al.*, 2017; this thesis). Here, we used this tool to characterize the development of multiple infections in gill during a 5-week period, in concert with the expression of a suite of host stress and immune genes, to describe how differences in infection burdens, infection development and host responses in gill contribute to the early mortality of Pacific salmon. A suite of 17 infectious agents were evaluated in gill based on the findings of Bass and colleagues (unpublished data), which screened for 45 infectious agents in multi-tissue pools of wild sockeye salmon throughout their migration to spawning grounds (Table 5.1). The most prevalent and potentially pathogenic of those organisms that were positively detected were included in our analysis. Biomarkers of host stress and immunity (n = 27 genes) comprised aspects of osmotic stress, heat shock, innate and adaptive immunity, tissue repair and others, which were evaluated simultaneously with two reference genes and the 17 infectious agents (Table 5.1). Our approach quantifies RNA rather than DNA of infectious agents to measure variation in productivity of active infections based on expression of the target gene. As target gene types differed depending on the agent (i.e., surface protein, ribosomal, etc.; see Miller *et al.*,2016), relative loads can only be compared within agents, not across.

Tissue samples were trimmed in the lab for size uniformity and then homogenized in sterile microtubes with stainless steel beads using 600 µL TRI-reagent^{TM} 148 (Ambion Inc., Austin, TX, USA), 75 µL 1-bromo-3-chloropropane and a MM301 mixer mill (Restch Inc., Newtown, PA, USA). Centrifugation (6.5 min) separated the aqueous phase of samples, which was aliquoted into 96-well plates for RNA purification. The “spin

method" for Magmax™-96 for Microarrays Kits (Ambion Inc.) was used to purify RNA following manufacturer's instructions, using a Biomek FXP liquid handler (Beckman-Coulter, Indianapolis, IN, USA) and including a DNase treatment after the first wash. RNA quality and quantity were assessed using spectrophotometry (A_{260}, $A_{260/280}$) and samples were normalized to 1 µg RNA prior to cDNA synthesis. Low RNA samples were removed for gene expression analyses. Invitrogen™ SuperScript™ VILO™ (Carlsbad, CA, USA) cDNA Synthesis Kit synthesized cDNA under cycling conditions 25°C for 10 min, 42 °C for 60 min and 85 °C for 5 min. As per manufacturer's recommendations (Biomark™), pre-amplification of cDNA was completed in a multiplex PCR including all primers to be evaluated by qPCR (200 nM primer mix, TaqMan Preamp Master Mix, Applied Biosystems, Foster City, CA, USA). Due to the nanofluidic properties of the Fluidigm Biomark™, pre-amplification is necessary to achieve adequate sensitivity. Cycling conditions for the pre-amplification were 95 °C for 10 min then 15 cycles of 95 °C for 10 s and 60 °C for 4 min, which was followed by ExoSap-it® Product Clean-up (Affymetrix Inc., Santa Clara, CA, USA) cycled at 37 °C for 15 min then 80 °C for 15 min, and then a 5-fold dilution (TEKnova suspension buffer, Hollister, CA, USA). A pool of gill samples from n = 20 fish collected with river-sourced fish and sacrificed riverside was included on all chips as a positive control prior to and following pre-amplification; negative controls were also included at each step in the protocol. A serial dilution of artificial positive constructs (APC clones) matching the primer-probe sequence for each infectious agent under evaluation was included just prior to qPCR and tagged with a secondary probe (NED™ reporter dye) to identify potential contamination of samples. Samples (TaqMan Universal Master-Mix, Life Technologies; GE Sample Loading

Reagent, Fluidigm, pre-amplified cDNA) and assays (in duplicate; 10 µM primers, 3µM probes for Taqman assays) were loaded onto dynamic arrays using the integrated fluidics controller HX (Fluidigm) and qPCR was completed following 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

The BioMark Real-Time PCR analysis software was used to manually score output, following protocols described in Miller *et al.*,(2016). Infectious agents that were not positive in duplicate were failed and quantification cycles (Cq) for duplicates were averaged for host genes and infectious agents. Host biomarkers were normalized to the average of the two reference genes and are reported as relative expression following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Infectious agent Cq was subtracted from 40 (maximum Cq), producing what is referred to herein as "relative load," and is therefore a representation of RNA expression. Further information regarding the protocols described herein can be found in chapter 2 and details regarding the Biomark™ platform's applications in infectious agent screening and validation can be found in Miller *et al.*,(2016).

5.3.3 Statistical analyses

Longevity was calculated as the total days surviving after treatment (marine) or collection (river); note that the holding period was shorter for river-sourced fish due to the lag between collection dates. Differences in survival between capture locations (source), sexes, treatments (gillnet-treated/captured, biopsied controls, non-biopsied controls) and temperatures were identified using survival analysis (Cox proportional hazards, *survival* package) and linear models (LM) in the R statistical software (R Core Team, 2015; Therneau, 2014). For Cox regressions including both sources, days

surviving was censored at 21 d post-treatment to avoid the bias of the longer holding period for low temperature marine-sourced fish (i.e., earlier capture and longer survival), while models including only one source used all days surviving. Because non-handled controls were included among both gillnet- and seine-captured fish, repeated biopsy was included as an additional factor in the survival analyses for river-sourced fish. For marine-sourced fish, survival analyses were performed relative to non-handled and handled controls to identify potential impacts of biopsy on survival. Overall effects of sex were first identified in a model that stratified treatments, source and temperature. Source effects were then evaluated stratifying treatments and temperature. Treatment, biopsy and temperature effects were then evaluated within each source group. For Cox proportional hazards analyses, hazard ratios (exponents of coefficients) for significant effects are presented, which correspond to the daily hazard of mortality, as well as model r^2 and likelihood ratio tests for significance. Where assumptions of the Cox regressions could not be met, coefficients ($\beta \pm$ standard error) are presented from LMs for significant parameters and interactions.

To assess the relative influences of source, temperature, gillnetting (treatment or collection gear), sex and time, we used linear mixed effects (LME) models with a random intercept that accounted for re-sampling of individuals over time (i.e., fish ID as a random effect for repeated measures). Interactions of time (week post-treatment) with source, temperature and gillnetting tested for differences in infection development depending on each factor (i.e., interactions with time). Interactions of source with temperature and treatment also elucidated source-dependent differences in infection development. A top-down approach for model selection was used to identify significant

interactions and factors associated with relative infections burden (RIB, a composite metric of infectious loads and richness, see Bass *et al.,* in review), richness, and infectious loads of highly prevalent agents (Zuur *et al.,* 2009). A $P < 0.05$ *a priori* cut-off was applied for likelihood ratio tests comparing models including and excluding each variable and interaction term, starting with a full model that included all possible factors and interactions and then removing those with low t-values and high p-values in a step-wise fashion. Significance therefore pertains to the importance of each variable or interaction to describing the infection metric data within the final model. The final model included only significant interaction terms and factors, as well as main effects that served as components of significant interactions.

Permutational multivariate analysis of variance (PERMANOVA) was used to assess contributions of source, sex, RIB, temperature, and gillnetting (including a temperature by gillnet interaction term) to overall variation in gene expression data. Twenty-two biomarkers of stress and immunity comprised aspects of heat shock responses, osmotic imbalance, innate and adaptive immunity and tissue repair (Table 5.1). Relative expression of all biomarkers was used as the response matrix for the PERMANOVA during each week of the study. Data from samples taken at morbidity were included in the analysis within the week that the animal died, and non-handled marine controls and river seine-collected fish were excluded from the analysis. Non-biopsied gillnet-collected fish showed similar survival patterns to biopsied fish and were therefore included in the analysis. Morbidity data from gillnet-collected fish from the river were included in gene expression analyses as these fish had similar survival to biopsied fish and improved our power to detect thermal and sex-specific differences. Unsupervised principal component

analysis (PCA) was used to relate individual biomarker expression to each factor using linear models with component axes (PC) as response variables and factors used in PERMANOVAs as predictors, including a temperature-treatment interaction where sample sizes permitted. PCs were included that explained >10% of the variance (i.e. eigenvalues) and contributed to significant ($P < 0.05$) linear models. Significant factors in linear models are discussed with respect to biomarkers most positively and negatively loaded (i.e., eigenvectors) on the corresponding PC axis. Only fish identified as part of the Adams stock complex were included in analyses of infection metrics and gene expression to avoid potential stock biases.

5.4 Results

5.4.1 Survival

Survival was highest at 14 °C for both sources, with marine-collected controls (with and without biopsy) and gillnet-treated males surviving 100%, while gillnet-treated females survived 92% (Fig. 5.2, Table 5.2). River-collected, seine-captured males and females survived 100% at 14 °C if not biopsied, while biopsy reduced survival of females to 0% and males to 75%. Low sample sizes for seine-collected fish warrant caution in sex-specific comparisons of survival (Table 5.2). Gillnet-captured fish held at 14 °C survived better if not biopsied (F = 55%, M = 82%) than those biopsied (F = 44%, M = 50%). At 18 °C, gillnetting and biopsy reduced survival of marine-sourced males and females to 0%, while non-biopsied controls survived relatively well (F = 73%, M = 100%). Similarly, river-sourced fish held at 18 °C survived 0% if biopsied, regardless of gear type, and few non-biopsied gillnet-captured (F = 8%, M = 0%) or non-biopsied seine-captured fish (F = 8%, M = 17%) survived to study termination.

No significant effect of sex was identified overall ($P = 0.255$) or within source groups ($P > 0.400$), so sexes were pooled in subsequent survival models. Survival analysis identified a significant effect of source, where river-sourced fish were 3.3 times ($P < 0.001$) more likely to die (each day) than marine-sourced fish (model $r^2 = 0.14$, $P < 0.001$). Among river-sourced fish, a significant interaction between temperature and biopsy ($e^{\beta} = 0.26$, $P = 0.015$) suggested that the negative effect of biopsy ($e^{\beta}=5.16$, $P = 0.012$) on survival was reduced at high temperature ($e^{\beta} = 47.98$, $P < 0.001$; model $r^2 = 0.48$, $P < 0.001$). Gillnetting increased the daily risk of mortality by 5.3-times that of seine-captured fish ($P = 0.041$), with no significant interaction with temperature ($P = 0.098$). Among marine-sourced fish, excellent survival at 14 °C and extremely poor survival at 18 °C restricted our analysis due to model assumptions. Survival among marine-sourced treatments and sexes was similar at 14 °C (only one female held at 14 °C died prior to study termination). At 18 °C, violation of proportional hazards assumptions prompted the used of linear models to characterize survival of marine-sourced fish. A model including both temperature groups of marine sourced fish identified significant interactions of temperature with gillnetting ($\beta = -11.99 \pm 1.15$, $P < 0.001$) and biopsy ($\beta = -13.39 \pm 1.23$, $P < 0.001$), suggesting different responses to handling stress at high temperature. Longevity of marine-sourced fish was also independently reduced by high temperature ($\beta = -1.75 \pm 0.84$, $P = 0.041$). At 14 °C, no impact of either biopsy or gillnetting was apparent ($P > 0.05$), but at 18 °C, biopsy ($\beta = -12.54 \pm 1.07$, $P < 0.001$) and gillnetting ($\beta = -12.05 \pm 1.03$, $P < 0.001$) reduced survival relative to non-handled controls, but gillnetting had no effect beyond biopsy-associated handling ($P = 0.587$).

5.4.2 Infection metrics in gill

Richness, *F. psychrophilum*, *I. multifiliis*, and *Ca.* B. cysticola loads increased at a faster rate in river fish (i.e., significant interaction of source with time; Fig. 5.3, model coefficients in Table 5.3). RIB and *Ca.* B. cysticola increased at a faster rate in warm water, while *I. multifiliis* and RLO were consistently higher in warm water (no interaction). *Ca.* B. cysticola increased at a faster rate in gillnetted fish, while positive effects of gillnetting on RIB were only observed in river-sourced fish. *F. psychrophilum* was higher in females than males. *C. shasta* and *P. minibicornis* loads were only increased by time (tested in LR fish only). Low intraclass correlation coefficients were apparent for *I. multifiliis*, RLO, *C. shasta* and *P. minibicornis*, suggesting high variability of repeated samples within individuals.

5.4.3 Host stress and immune responses

For fish collected from marine waters, high temperature was the primary factor contributing to variation in gene expression during weeks 0-3 (r^2 range: 0.24–0.42), increasing in importance over time (Table 5.4, Fig. 5.4). The effect of gillnet treatment was temperature dependent during weeks 0-1 (interactions $P \leq 0.022$), but after only cool temperature fish remained (weeks 3-4), its independent effect increased ($r^2 = 0.08$–0.19). RIB was only significantly associated with gene expression of marine-sourced fish during week 2 ($r^2 = 0.17$, $P < 0.001$).

The temperature-gillnetting interaction term at week 0 was negatively associated with PC1, while high temperature negatively associated with PC2, demonstrating association of thermally-stressed fish with cellular and osmotic stress (HSP90, GR2, NKA_a1b), iron regulation (TF) and tissue repair (MMP13). Thermally-stressed gillnetted fish also

associated with the expression of JUN, HSC70, and all other biomarkers, which negatively loaded on PC1. Gillnetting was positively associated with PC3 along with stress (HSC70, JUN) and intracellular immunity (b2m) and antiviral (RIG1) activity, while females were negatively associated with PC3, correlating with the expression of cytokines (IL15, IL11, IL1R, IL8), immune receptors (CD83), and antiviral genes (IFNa, Mx). By the end of week 1, thermally stressed fish were negatively associated with PC1 and positively associated with PC2, which corresponded to the expression of HSP90, MMP13 and C7, while cool temperature fish were more strongly associated with antiviral indicators (IFNa, RIG1, Mx), cellular receptors (MHCIIb, b2m, CD83) and cellular energy generation (ATP5G3C). At week 2, thermally stressed fish were positively associated with PC1 and PC2, corresponding to gene loadings indicating tissue repair (MMP13), cellular stress (HSP90, JUN, GR2), cytokine and chemokine activity (IL11, IL8, IL1R, CXCR4), iron regulation (TF) and complement (C7). RIB was also positively associated with PC1, but negatively with PC2, suggesting greater energy needs (ATP5G3C) and osmotic stress (NKA_a1b) in addition to the associations described for thermally stressed fish. Weeks 3 and 4 included only cool temperature fish due to mortality at high temperature, and week 4 included survivors sacrificed at the start of the spawning period (study termination). Gillnetted fish were positively loaded on PC2 at week 3 indicating tissue repair and inflammation (IL8, MMP13), iron regulation (TF), complement (C7), and increased cellular stress and energy needs (GR2, ATP5G3C). At week 4, gillnetted fish loaded negatively on PC1 with indices of inflammation (IL8), cellular stress (JUN, HSC70) and some cellular receptors (CD4, b2m), and loaded opposite to the expression of Mx and other antiviral components.

For river-collected fish, temperature was not evaluated at week 0 (no thermal application at capture) but gained importance in weeks 1 and 2 (r^2 = 0.06 & 0.22, respectively). Gillnetting was marginally associated with gene expression ($r^2 \leq 0.06$) only at week 0 and 1 and with no interaction with temperature. RIB was the primary factor associated with gene expression of river-collected fish, increasing the amount of variation explained with time (r^2 range: 0.04-0.51).

For river-exposed fish at collection (treatment), gillnetted fish were negatively associated with PC2 and PC3, indicating expression profiles consistent with cellular stress (GR2, JUN, HSC70), osmotic imbalance (NKA_a1b), iron regulation (TF), antiviral activity (RIG1) and extracellular receptor (CD4) genes. During week 1, RIB was positively associated with PC1, indicating inflammation (IL11, IL8, CXCR4), iron regulation (TF), tissue repair (MMP13) and complement (C7) as characteristics of fish with high RIB, while cellular immunity (b2m, MHCIIb), RIG1 and protein repair (HSC70) were associated with low RIB. At week 2, RIB was again strongly positively associated with PC1, demonstrating a similar profile to that described for week 1. Thermally stressed fish and females were both positively associated with PC2, suggesting that these fish were recruiting aspects of cellular stress response (JUN, HSP90), inflammation (IL11, IL8), tissue repair (MMP13), and iron regulation (TF), while neglecting most cellular immune aspects. In the final week of holding, including only cool water fish and survivors sacrificed at study termination, RIB was strongly negatively associated with PC1, reflecting the same gene set correlations as previous weeks.

5.5 Discussion

This study used river entry of wild adult Pacific salmon as an ecological infection challenge with controlled application of thermal and fishery stressors to evaluate how infections, host responses and multiple stressors influence survival before spawning. Consistent with previous studies (Bass *et al*. 2017; unpublished data) and our first hypothesis, fish collected from the lower Fraser River carried heavier infections in their gills than fish collected from the Strait of Georgia. This finding is striking in that such a short migration distance (approx. 100 km, 5-7 d lag in sampling) could produce such profound differences in infection burdens. Consistent with our second hypothesis, fish that bypassed the lower river had excellent survival in cool water, while river-exposed fish survived poorly (approximately 50%) and died sooner unless they were collected and held under the most benign conditions (seine-captured and not biopsied = 100% survival). Decreased survival of river-exposed fish under optimal collection and holding conditions supports an influence of river-derived infections in contributing to *en route* mortality of hosts following ideal capture-and-release conditions.

Our survival results support those described by Martins *et al*., (2011), where at 14 °C, model-averaged survival for Adams sockeye salmon was 90-100% (± standard error range) in seawater and 60-100% in the river. However, at 18 °C, Martins and colleagues estimated 80-90% survival in seawater and 15-25% in the river, which more closely resembles survival rates of non-biopsied controls in the present study, suggesting that holding at high temperature may have further reduced the resilience of adult salmon to handling in this study. Regardless of capture location, handled fish held at an ecologically relevant high temperature (18 °C) did not survive to the spawning period of their

population (≥4 weeks). Handling effects are emphasized in these findings because survival of fish exposed to gillnetting and air exposure showed no difference from that of biopsied controls, further emphasizing detrimental effects of any level of handling (chapters 2-4). Handling effects on survival and gene expression were temperature- and source-dependent, with marine-sourced fish showing cumulative effects of multiple stressors (handling effects only apparent in warm water) while river-exposed fish showed independent stressor effects. River exposure increased mortality associated with handling in cool water and among non-handled fish in warm water (i.e., independent stressors).

These findings have drastic implications for the survival of released sockeye bycatch in the lower Fraser River when temperatures are high, even with minimal handling. Findings from marine-sourced fish support previous correlative studies describing cumulative effects of multiple stressors on adult salmon survival, infection development and health (Gale *et al*. 2011, 2013, Teffer *et al*. 2017; this thesis, chapter 3), but also demonstrate causal influences of river-derived infections on host responses and survival during freshwater migration. Results suggest that the mechanisms of early mortality of wild sockeye salmon are driven by river-derived pathogens, with differences in immune and infection trajectories that are dependent on pathogen exposure as well as migratory conditions that alter infection development.

Temperature has been coined the "master" factor (Fry, 1971) due to its strong influence on fish physiology and behavior (Pacific salmon: e.g., Jain and Farrell 2003, MacNutt *et al.* 2004, Kocan *et al.* 2009, Jeffries *et al.* 2012a, 2014b), including the potential to exacerbate fishery impacts (Gale *et al*., 2013). High river temperatures equal to those applied in this study are already impacting sockeye salmon populations during

freshwater migrations (Patterson *et al*., 2007) and have been shown here and previously to accelerate infection development (Bradford *et al*., 2010b; Miller *et al*., 2014; Wagner *et al*., 2005; this thesis, chapters 3-4), modulate immune gene expression (Jeffries *et al*., 2012a), and are consistent with increased mortality of wild adult Pacific salmon during freshwater migration (Hinch *et al*., 2012; Keefer *et al*., 2008; Martins *et al*., 2012). Thermal stress accelerated the development of multiple infections, including bacterial and parasitic agents that either maintained high loads over time or showed greater discrepancy with cool water fish at later time points. Given that thermal impacts on infection development (RIB) were not source-dependent but gillnetting responses were, heavy initial infections likely exacerbate infection development following fishery capture and release at optimal temperature, while thermally-driven infection amplification is independent of initial infection status. Survival curves of handled river- and marine-sourced fish at high temperature were almost identical, supporting a minor role of initial infection status on mortality when cumulative stressors are present. However, in the absence of handling, river-exposed fish survived more poorly than marine-sourced fish in warm water, suggesting that river-derived infections do reduce the survival of migrating adults at high temperature (e.g., natural mortality).

Mortality of thermally-stressed fish was associated with enhanced infection *development*, which is a common response of infectious agents to increased temperature (e.g., Mitchell *et al.* 2005, Bettge *et al.* 2009, Kocan *et al.* 2009), in combination with cellular stress responses. Thermally-stressed fish were more positively associated with stress indices and immune aspects such as complement, iron metabolism and inflammatory responses rather than adaptive immunity and antiviral responses. Our

survival results suggest that these responses are inadequate to prevent mortality at high temperature, given that all thermally-stressed fish died early. Chronic stress is known to be immunosuppressive, including negative impacts of high cortisol on antibody production and inflammatory responses through glucocorticoid receptor suppression (Zwollo, 2018). Indeed, thermally stressed marine-sourced fish showed an initial positive association with GR2, but then little correlation in following weeks when inflammatory biomarkers were more prominently featured. An acute stress response, as would follow handling, has been shown to divert immunity toward innate responses in mammals (Zwollo, 2018). Regarding the mechanisms of cumulative stressor effects, acute stress responses may be maladaptive in already immune-compromised adult salmon exposed to chronic high temperature (Jeffries *et al*., 2012a).

Our results show that river-sourced fish, which were initially compromised by heavy infections, demonstrated divergence from marine-sourced fish in stress and immune gene expression, with profiles associating with infections rather than stressors in subsequent weeks. Conversely, gene expression profiles of marine-sourced fish were more strongly influenced by temperature, which also modulated their responses to fishery and handling stress. Overall, this divergence in expression profiles depending on river-exposure supports alternate host response tactics to stressors that influence longevity and are contingent on infection burdens. Because marine-sourced fish were treated to fishery simulations at high temperature, while river-sourced fish were "treated" in cool water and then held in warm water, the effect of thermal stress was delayed for river-sourced fish but immediate for marine-sourced. Furthermore, infections accumulated during the capture process may have contributed to gill loads of river-sourced fish, while marine-

collected fish were gillnet-treated in the lab in filtered and UV-treated water. It is possible that these methodological constraints contributed to differences in response profiles between sources and potentially reduced our ability to detect cumulative stressor effects in river-exposed fish.

Gillnetting enhanced infection burdens (RIB) primarily in river-sourced fish, suggesting that exposure to riverine pathogens and subsequently heavier infections reduce the ability of hosts to maintain infectious loads following acute fishery stress. The mechanisms of this predisposal may be associated with already heightened demands on the immune system, as RIB was a primary contributor to variation in immune gene expression of river-collected fish, but not marine fish. Gillnetting enhanced *Ca***.** B. cysticola infections and RIB, suggesting that defenses against this bacterial agent may be reduced by gillnetting or the net may act as a transmission vector, thereby producing bacterially-driven increases in RIB. Better survival of fish captured and released in the ocean versus river (Martins *et al*., 2011) may therefore be partially explained by infection burdens, in addition to differences in salinity and temperature of the recovery environment, predation, fishing pressure, gear type, etc. (Raby *et al*., 2015). Indeed, more fish died in the first 24 h following collection from the river than from the marine environment. This result points to proximal causes of mortality, such as cardiac collapse or anaerobiosis in river-exposed fish (Eliason *et al*., 2011; Fenkes *et al*., 2016; Raby *et al*., 2015), in addition to the delayed mortality that followed. It is possible that infections accumulated in the river prior to collection may have predisposed river-collected fish to mortality if osmoregulatory or aerobic capacity were compromised by infections (Bradford *et al*., 2010b; Ewing *et al*., 1994; Nematollahi *et al*., 2003). Temperatures

during marine collection (15 °C surface temperature) were also slightly lower than those during river collection (15-17 °C), which may have influenced stress resilience.

The life history of infectious agents also influences infection dynamics within hosts. As has been observed previously (Bass *et al.* 2017, unpublished data), freshwater myxozoans *C. shasta* and *P. minibicornis* were prevalent only in fish collected in the river, but not entirely absent in marine-collected fish, suggesting either resilience of spores in the Fraser River plume within the Strait of Georgia or retention of myxozoan infections from juvenile life stages (likely the former; Mahony *et al*., 2017). These agents both require an intermediate freshwater polychaete worm host, which releases infective myxozoan spores into the river during salmon migrations (Bartholomew *et al*., 1997, 2006). The greater prevalence of these agents in river-exposed fish contributed to higher overall infection burdens and richness; if fish were not transported to the laboratory, continued river exposure would further increase infective dosage (Ray *et al*., 2012). As not only infectious loads but community composition differed between source locations, we cannot specifically assign mortality to severe infections of these individuals agents, but rather a combination of infection severity and enhanced agent richness. New infections may elicit stronger responses directed toward novel infections, as was observed in river-exposed fish. This predisposal potentially detracted from their ability to successfully respond to thermal and fishery stress, decreasing their stress tolerance and resulting in early mortality.

This study provides valuable information regarding the mechanisms of mortality of wild salmon from a major salmon-producing river, including the impacts of climate change and rising demand for fisheries resources. The relationships identified here can be

applied to develop hypotheses and experiments for other systems and species. We identified significant differences in the survival and infectious loads of sockeye salmon based on river exposure. Multiple infections responded to thermal stress regardless of river exposure with increases in the loads of most agents, whereas gillnetting only increased infections among river-exposed fish. These findings combined with low survival at high temperature, especially among river-exposed and handled fish, suggest that resilience to thermal stress is reduced by heavy infections as well as handling. Mortality at high temperature is likely driven by enhanced infection development and host physiological impairment. Cumulative effects of stressors were only observed in marine-sourced fish, where handling reduced survival only at high temperature, suggesting that the threshold for cumulative stress is greater prior to river entry. Given that all fish that were handled and held at an ecologically relevant high temperature died prior to this population's spawning period, managers should continue to reduce or eliminate fishery practices while rivers are warm.

Infections are a natural component of ecosystems and can drive the evolutionary basis of wild animal migrations, but anthropogenic changes to these conditions may alter the effectiveness of life history strategies (Altizer *et al*., 2011). As climate change alters weather patterns and hydrology, driving increases in temperatures across aquatic ecosystems and shifts in host-pathogen relationships and home ranges, management of salmon productivity and maintenance of indigenous, commercial and recreational fisheries will prove more difficult (Altizer *et al*., 2013; Jacob *et al*., 2010; McDaniels *et al*., 2010; Reed *et al*., 2011). The findings of this chapter offer insight as to the mechanisms of early mortality of adult sockeye salmon, suggesting that strategic fishing

prior to river entry or only when rivers are cool will be needed to minimize *en route* losses. Management models can incorporate these results to potentially improve predictions of early mortality and estimates of spawning stock biomass by comprising the influence of infections on stressor resilience in fresh water.

Table 5.1 Assay information for host biomarkers of stress and immunity, reference genes and infectious agents evaluated using qPCR, including gene functions, EST/Accession numbers, primer and probe sequences, and sources. Assays referenced as "In house" refer to assays developed at the molecular genetics lab, Pacific Biological Station, Nanaimo, BC.

Assay name	Assay type	Gene information	EST/Accession#	Forward primer	Reverse primer	Probe	source
b2m	Acquired immunity	Cell receptor	AF180490	F - TTTACAGCGCGGTGGAGTC	R - TGCCAGGGTTACGGCTGTAC	P - AAAGAATCTCCCCCCAAGGTGCAGG	Haugland *et al.* 2005
CD83	Acquired immunity	Cell receptor	AY263794	F - GATGCACCCCTTGAGAAGAA	R - GAACCCTGTCTCGACCAGTT	P - AATGTTGATTTACACTCTGGGGCCA	(Raida *et al.*, 2011)
MHCIIb	Acquired immunity	Major histocompatibility complex IIβ	AF115533	F - TGCCATGCTGATGTGCAG	R - GTCCCTCAGCCAGGTCACT	P - CGCCTATGACTTCTACCCCAAACAAAT	(Raida and Buchmann, 2008)
Mx	Antiviral	Antiviral protein		F - AGATGATGCTGCACCTCAAGTC	R - CTGCAGCTGGGAAGCAAAC	P - ATTCCCATGGTGATCCGCTACCTGG	(Eder *et al.*, 2009)
RIGI	Antiviral	Retinoic acid inducible gene I	NM_001163699	F - ACAGCTGTTACACAGACGACATCA	R - TTTAGGGTGAGGTTCTGTCCGA	P - TCGTGTTGGACCCCACTCTGTTCTCTC	(Larsen *et al.*, 2012)
CD4	Aquired immunity	Cell receptor	AY973028	F - CATTAGCCTGGGTGGTCAAT	R - CCCTTTCTTTGACAGGGAGA	P - CAGAAGAGAGAGCTGGATGTCTCCG	(Raida and Buchmann, 2008)
ATP5G3C	Cellular energy	ATP synthase lipid-binding protein	CB493164	F - GGAACGCCACCATGAGACA	R - CGCCATCCTGGGCTTTG	P - AGCCCCATTGCCTC	In house
CXCR4	Immune regulation	chemokine receptor	CA054133	F - GGAGATCACATTGAGCAACATCA	R - GCTGCTGGCTGCCATACTG	P - TCCACGAAGATCCCCA	In house
IFNa	Immune regulation	Interferon-α	AY216595	F - CGTCATCTGCAAAGATTGGA	R - GGGCGTAGCTTCTGAAATGA	P – TGCAGCACAGATGTACTGATCATCCA	(Ingerslev *et al.*, 2009)
IL11	Immune regulation	Cytokine	AJ535687	F - GCAATCTCTTGCCTCCACTC	R - TTGTCACGTGCTCCAGTTTC	P - TCGCGGAGTGTGAAAGGCAGA	(Raida and Buchmann, 2008)
IL15	Immune regulation	Cytokine	AJ555868.1	F - TTGGATTTTGCCCTAACTGC	R - CTGCGCTCCAATAAACGAAT	P - CGAACAACGCTGATGACAGGTTTTT	(Raida *et al.*, 2011)
IL1R	Immune regulation	Cytokine	AJ295296	F - ATCATCCTGTCAGCCCAGAG	R - TCTGGTGCAGTGGTAACTGG	P - TGCATCCCCTCTACACCCCAAA	(Raida *et al.*, 2011)
IL8	Immune regulation	Cytokine	AJ279069	F - AGAATGTCAGCCAGCCTTGT	R - TCTCAGACTCATCCCCTCAGT	P - TTGTGCTCCTGGCCCTCCTGA	Raida and Buchmann 2008
C7	Innate immunity	Complement factor	CA052045	F - ACCTCTGTCCAGCTCTGTGTC	R - GATGCTGACCACATCAAACTGC	P - AACTACCAGACAGTGCTG	In house
IgMs	Innate immunity	Immunoglobulin	S63348, AB044939	F - CTTGGCTTGTTGACGATGAG	R - GGCTAGTGGTGTTGAATTGG	P - TGGAGAGAACGAGCAGTTCAGCA	(Raida *et al.*, 2011)
NKA_a1b	Ion regulation	Sodium potassium ATPase subunit	CK879688	F - GCTACATCTCAACCAACAACATTACAC	R - TGCAGCTGAGTGCACCAT	P - ACCATTACATCCAATGAACACT	Nilson et al. 2007
TF	Iron regulation	Transferrin	D89083	F - TTCACTGCTGGAAAATGTGG	R - GCTGCACTGAACTGCATCAT	P - TGGTCCCTGTCATGGTGGAGCA	(Raida and Buchmann, 2009)
GR-2	Stress	Glucocorticoid receptor		F - TCCAGCAGCTATGCCAGTTCT	R - TTGCCCTGGGTTGTACATGA	P - AAGCTTGGTGGTGGCGCTG	(Yada *et al.*, 2007)
HSC70	Stress	Heat shock cognate 70	CA052185	F - GGGTCACACAGAAGCCAAAAG	R - GCGCTCTATAGCGTTGATTGGT	P - AGACCAAGCCTAAACTA	In house
HSP90	Stress	Heat shock protein 90	CB493960, CB503707	F - TGGGCTACATGGCTGCCAAG	R - TCCAAGGTGAACCCAGAGGAC	P - AGCACCTGGAGATCAA	In house
JUN	Stress	Transcription factor	CA056351	F - TTGTTGCTGGTGAGAAAACTCAGT	R - CCTGTTGCCCTATGAATTGTCTAGT	P - AGACTTGGGCTATTTAC	In house
MMP13	Wound healing	Matrix metalloproteinase	213514499	F - GCCAGCGGAGCAGGAA	R - AGTCACCTGGAGGCCAAAGA	P - TCAGCGAGATGCAAAG	(Tadiso *et al.*, 2011)
78d16.1	Reference gene		CA056739	F - GTCAAGACTGGAGGCTCAGAG	R - GATCAAGCCCCAGAAGTGTTTG	P - AAGGTGATTCCCTCGCCGTCCGA	In house
COIL-P84-2	Reference gene		CA053789	F - GCTCATTTGAGGAGAAGGAGGATG	R - CTGGCGATGCTGTTCCTGAG	P - TTATCAAGCAGCAAGCC	In house
ae_hyd	Bacterium	*Aeromonas hydrophila*		F - ACCGCTGCTCATTACTCTGATG	R - CCAACCCAGACGGGAAGAA	P - TGATGGTGAGCTGGTTG	(Lee *et al.*, 2006)
ae_sal	Bacterium	*Aeromonas salmonicida*		F - TAAAGCACTGTCTGTTACC	R - GCTACTTCACCCTGATTGG	P - ACATCAGCAGGCTTCAGAGTCACTG	(Keeling *et al.*, 2013)
c_b_cys	Bacterium	*Candidatus Branchiomonas cysticola*		F - AATACATCGGAACGTGTCTAGTG	R - GCCATCAGCCGCTCATGTG	P - CTCGGTCCCAGGCTTTCCTCTCCCA	(Mitchell *et al.*, 2013)
fl_psy	Bacterium	*Flavobacterium psychrophilum*		F - GATCCTTATTCTCACAGTACCGTCAA	R - TGTAAACTGCTTTTGCACAGGAA	P - AAACACTCGGTCGTGACC	(Duesund *et al.*, 2010)
rlo	Bacterium	*Rickettsia-like organism*		F - GGCTCAACCCAAGAACTGCTT	R - GTGCAACAGCGTCAGTGACT	P - CCCAGATAACCGCCTTCGCCTCCG	(Lloyd *et al.*, 2011)
ce_sha	Parasite	*Ceratonova shasta*		F - CCAGCTTGAGATTAGCTCGGTAA	R - CCCCGGAACCCGAAAG	P - CGAGCCAAGTTGGTCTCTCCGTGAAAAC	(Hallett and Bartholomew, 2006)
cr_sal	Parasite	*Cryptobia salmositica*		F - TCAGTGCCTTTCAGGACATC	R - GAGGCATCCACTCCAATAGAC	P - AGGAGGACATGGCAGCCTTTGTAT	In house
de_sal	Parasite	*Dermocystidium salmonis*		F - CAGCCAATCCTTTCGCTTCT	R - GACGGACGCACACCACAGT	P - AAGCGGCGTGTGCC	In house
ic_mul	Parasite	*Ichthyophthirius multifiliis*		F - AAATGGGCATACGTTTGCAAA	R - AACCTGCCTGAAACACTCTAATTTTT	P - ACTCGGCCTTCACTGGTTCGACTTGG	In house
lo_sal	Parasite	*Loma salmonae*		F - GGAGTCGCAGCGAAGATAGC	R - CTTTTCCTCCCTTTACTCATATGCTT	P - TGCCTGAAATCACGAGAGTGAGACTACCC	In house
my_arc	Parasite	*Myxobolus arcticus*		F - TGGTAGATACTGAATATCCGGGTTT	R - AACTGCGCGGTCAAAGTTG	P - CGTTGATTGTGAGGTTGG	In house
pa_min	Parasite	*Parvicapsula minibicornis*		F - AATAGTTGTTTGTCGTGCACTCTGT	R - CCGATAGGCTATCCAGTACCTAGTAAG	P - TGTCCACCTAGTAAGGC	(Hallett and Bartholomew, 2009)
pa_pse	Parasite	*Parvicapsula pseudobranchicola*		F - CAGCTCCAGTAGTGTATTTCA	R - TTGAGCACTCTGCTTTATTCAA	P - CGTATTGCTGTCTTTGACATGCAGT	(Jørgensen *et al.*, 2011)
pa_ther	Parasite	*Paranucleospora theridion*		F - CGGACAGGGAGCATGGTATAG	R - GGTCCAGGTTGGGTCTTGAG	P - TTGGCGAAGAATGAAA	(Nylund *et al.*, 2010)
sp_des	Parasite	*Sphaerothecum destruens*		F - GCCGCGAGGTGTTTGC	R - CTCGACGCACACTCAATTAAGC	P - CGAGGGTATCCTTCCTCTCGAAATTGGC	In house
pspv	Virus	Pacific salmon parvovirus		F - CCCTCAGGCTCCGATTTTTAT	R - CGAAGACAACATGGAGGTGACA	P - CAATTGGAGGCAACTGTA	In house
ven	Virus	Viral erythrocytic necrosis virus		F - CGTAGGGCCCCAATAGTTTCT	R - GGAGGAAATGCAGACAAGATTTG	P - TCTTGCCGTTATTTCCAGCACCCG	James Winton, pers. comm.

Table 5.2 Sample sizes, longevity (mean ± standard deviation) and length (post-orbital hypural, cm) by sex for adult Adams-Shuswap sockeye salmon collected from either marine or riverine waters, held at cool or warm temperature for up to 4 weeks. Gillnet treatment included entanglement and air exposure in the lab (marine) or as the means of collection (river); biopsy refers to weekly gill biopsy from group subsets.

Source	Temperature	Treatment	Sex	N	Longevity (d)	Length (cm)	Survival (%)
Marine	14°C	Gillnet	F	13	27.7±2.5	49.2±2.0	92
			M	5	28.6±0.9	50.8±0.7	100
		Control					
		Biopsy	F	7	29.0±0.0	49.5±2.2	100
			M	6	28.7±0.5	50.5±2.5	100
		No biopsy	F	15	28.0±0.0	50.4±3.3	100
			M	2	28.0±0.0	51.3±0.1	100
	18°C	Gillnet	F	14	14.3±1.3	48.7±1.5	0
			M	6	14.0±0.9	49.1±1.8	0
		Control					
		Biopsy	F	12	13.2±3.4	49.4±1.5	0
			M	5	15±4.8	49.2±2.6	0
		No biopsy	F	11	25.5±4.4	49.1±2.3	73
			M	5	28.0±0.0	50.3±1.6	100
River	14°C	Gillnet					
		Biopsy	F	21	13.9±7.0	49.8±1.2	44
			M	2	20.5±2.1	49.5±1.0	50
		No biopsy	F	11	16.1±8.1	50.2±6.8	55
			M	11	20.3±3.6	49.7±1.9	82
		Seine					
		Biopsy	F	3	9.3±4.0	51.1±5.7	0
			M	4	18.3±3.5	51.1±2.8	75
		No biopsy	F	8	21.5±0.5	52.3±7.5	100
			M	2	22.0±0.0	51.3±0.4	100
	18°C	Gillnet					
		Biopsy	F	18	8.9±4.1	49.3±1.9	0
			M	9	13.0±2.4	50.3±2.1	0
		No biopsy	F	12	8.8±6.1	49.9±6.3	8
			M	10	8.7±3.6	48.8±1.5	0
		Seine					
		Biopsy	F	6	10.3±4.5	49.8±1.1	0
			M	3	8.0±3.6	50.6±0.5	0
		No biopsy	F	12	10.0±4.8	49.7±2.5	8
			M	6	11.7±4.8	52.2±1.3	17

Table 5.3 Parameters (β ± s.e.m.) of significant ($P < 0.05$) factors associated with infection metrics measured in adult sockeye salmon during five weeks of freshwater residence. Factors evaluated included source (R; river vs marine), high temperature (H; 18 °C vs 14 °C), gillnet entanglement (G; entanglement and air exposure), sex (S) and time (T; weeks), with significant interactions. ICC is the intraclass correlation coefficient of the model.

Metric	R	R:T	R:G	H	H:T	G	G:T	S	T	ΔAICc	ICC
RIB*	0.21±0.07		0.18±0.09, P=0.043	-0.10±0.05	0.08±0.03, P=0.003	-0.06±0.06	0.06±0.02, P=0.025		0.04±0.02	5.12	0.27
Richness	-0.02±0.21	1.09±0.12, P<0.001							0.25±0.06	1.39	0.22
I. multifiliis	-5.95±1.79	3.70±1.02, P<0.001		3.35±0.87, P<0.001					2.68±0.38	2.82	<0.001
RLO				2.29±0.63, P<0.001					2.17±0.26, P<0.001	3.80	<0.001
F. psychrophilum	1.05±0.71	3.13±0.43, P<0.001						1.12±0.56, P=0.043	0.11±0.23	8.44	0.33
Ca. B. cysticola	-1.14±0.54	0.62±0.26, P=0.019		1.09±0.53	1.36±0.25, P<0.001	-0.47±0.54	0.84±0.21, P<0.001		1.07±0.17	3.38	0.50
C. shasta ‡									1.43±0.53, P=0.007	3.57	<0.001
P. minibicornis ‡									2.70±0.44, P=0.001	3.65	<0.001

*log transformed

‡ River fish only

Table 5.4 Results from A) permutational multivariate analysis of variance (PerMANOVA) and B) principal component analysis (PCA) of the expression of 22 stress and immune gene biomarkers (Table 5.1) in adult sockeye salmon from marine or riverine waters. Linear models (LM) were used to identify factors contributing to the variation in each PC axis (V= % variance explained by each PC). Models describe weekly variation in gene expression in association with stressors (high temperature [H], gillnet entanglement [G], and their interaction (H:G]), relative infection burden in gill (RIB), and sex (S). Non-significant ($P > 0.05$) models and factor parameters ($\beta \pm$ s.e.m.) are not shown, or in grey if components of significant interactions in LMs.

A

	PerMANOVA			
	H	G	H:G	RIB
Marine				
Wk 0	r^2=0.24, P<0.001	r^2=0.07, P<0.001	r^2=0.06, P<0.001	
Wk 1	r^2=0.29, P<0.001	r^2=0.02, P=0.040	r^2=0.03, P=0.022	
Wk 2	r^2=0.42, P<0.001			r^2=0.17, P<0.001
Wk 3*	NA	r^2=0.19, P<0.001	NA	
Wk 4*‡	NA	r^2=0.08, P=0.028	NA	
River				
Wk 0	NA	r^2=0.06, P=0.003	NA	r^2=0.04, P=0.048
Wk 1	r^2=0.06, P=0.006	r^2=0.05, P=0.032		r^2=0.23, P<0.001
Wk 2	r^2=0.22, P<0.001			r^2=0.32, P<0.001
Wk 3*‡				r^2=0.51, P=0.001

B

	PC1						PC2						PC3			
	V	LM	H	G	H:G	RIB	V	LM	H	G	RIB	S	V	LM	G	S
Marine																
Wk 0	25	r^2=0.14, P=0.019	1.23±0.84, P=0.151	0.83±0.91, P=0.365	-3.14±1.15, P=0.009		14	r^2=0.70, P<0.001	-3.39±0.38, P<0.001				12	r^2=0.39, P<0.001	1.21±0.54, P=0.030	-1.17±0.36, P=0.002
Wk 1	35	r^2=0.44, P<0.001	-3.88±0.77, P<0.001	-1.62±0.76, P=0.037			17	r^2=0.11, P=0.037	1.73±0.67, P=0.012				10	r^2=0.06, P=0.132		
Wk 2	41	r^2=0.70, P<0.001	3.27±0.56, P<0.001			4.40±0.89, P<0.001	20	r^2=0.16, P=0.035	1.68±0.66, P=0.015		-2.21±1.04, P=0.040		10	r^2=0.01, P=0.362		
Wk 3*	28	r^2=0.04, P=0.287					17	r^2=0.30, P=0.017	NA	2.49±0.71, P=0.002			16	r^2=0.18, P=0.073		
Wk 4*‡	26	r^2=0.34, P=0.004	NA	-2.47±0.75, P=0.003	NA		15	r^2=0.10, P=0.131					13	r^2=0.00, P=0.952		
River																
Wk 0	26	r^2=0.09, P=0.062					15	r^2=0.18, P=0.008	NA	-1.82±0.53, P=0.002			10	r^2=0.21, P=0.003	-1.44±0.41, P=0.001	
Wk 1	31	r^2=0.59, P<0.001				5.14±0.82, P<0.001	22	r^2=0.07, P=0.145					11	r^2=0.02, P=0.328		
Wk 2	36	r^2=0.66, P<0.001				8.29±1.27, P<0.001	27	r^2=0.49, P<0.001	3.53±0.69, P<0.001			1.71±0.71, P=0.025	8	r^2=0.01, P=0.389		
Wk 3*‡	41	r^2=0.69, P<0.001				-10.46±1.85, P<0.001	21	r^2=0.09, P=0.252					15	r^2=0.05, P=0.322		

*cool water only

‡includes survivors

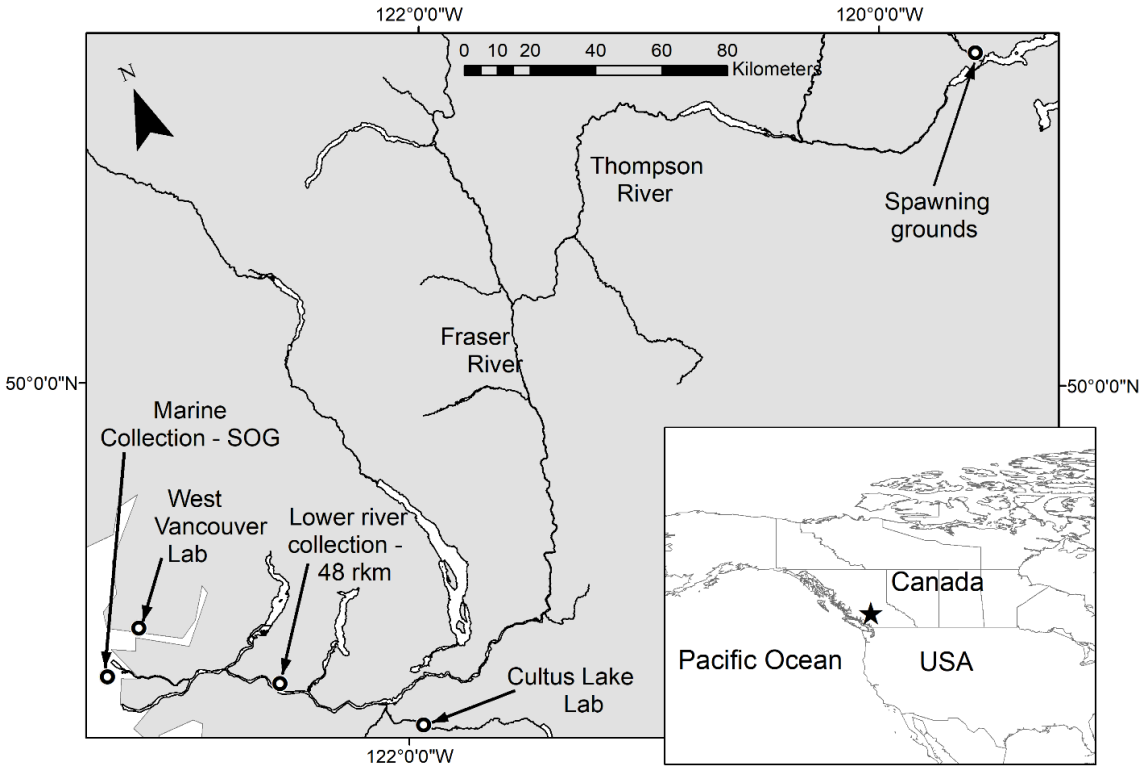

Figure 5.1 The southern portion of the Fraser River watershed, BC, Canada, showing collection locations in the Strait of Georgia and lower Fraser River (48 river km), transfer location for marine-sourced fish from boat to truck tanks (West Vancouver Lab), Cultus Lake Lab holding facility and spawning grounds for the Adams-Shuswap sockeye salmon population under study.

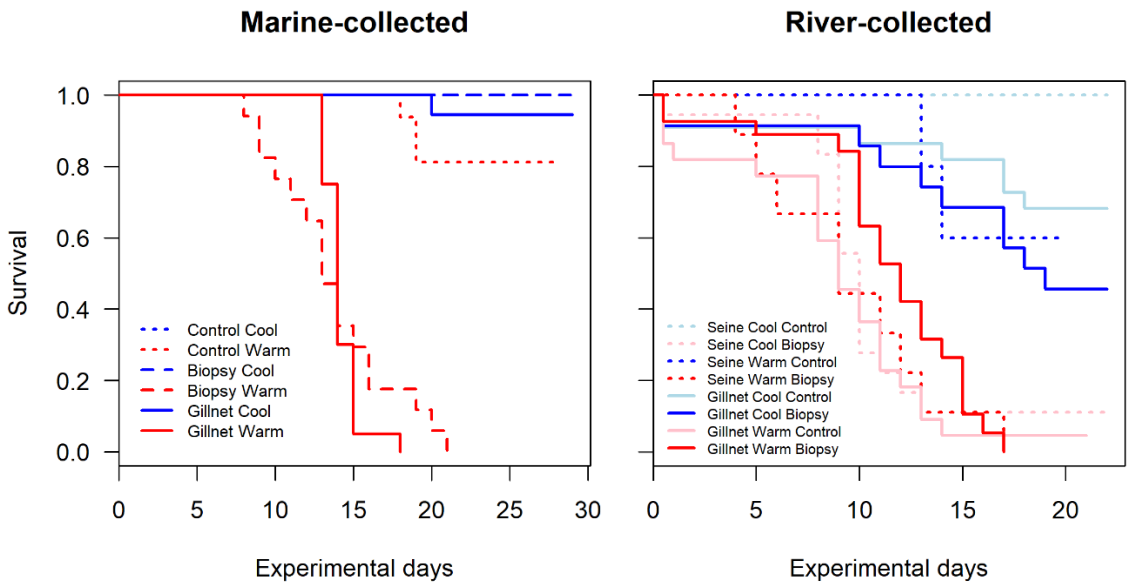

Figure 5.2 Kaplan Meier curves describing the survival of adult sockeye salmon held in fresh water at 14 °C (blue) or 18 °C (red) for up to four weeks. Line type denotes treatment (left plot: solid = gillnetted and air exposed, dashed = biopsied control, dotted = non-biopsied control; right plot: solid = gillnet-collected, dashed = seine-collected, lighter colors = non-biopsied, darker colors = biopsied). The left plot shows survival of fish collected in the Strait of Georgia, while the right plot shows survival of fish collected from the lower Fraser River.

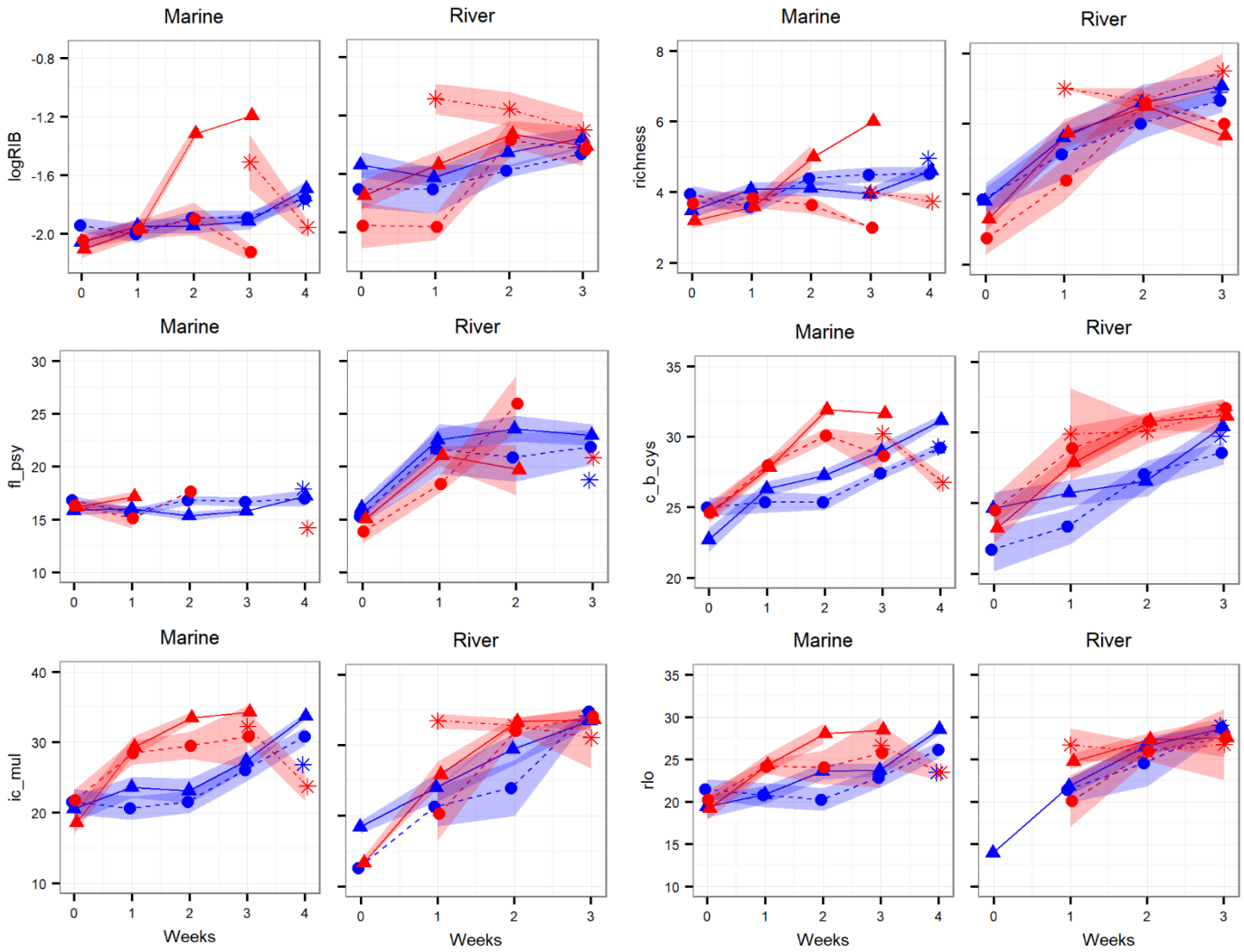

Figure 5.3 Relative infection burden (RIB), infectious agent richness, and relative loads of four prevalent agents (fl_psy = *F. psychrophilum*, c_b_cys = *Ca.* B. cysticola, ic_mul = *I. multifiliis*, rlo = *Rickettsia*-like organism) in adult sockeye salmon during four weeks of freshwater holding after collection from marine or riverine environments. Infectious agents were measured in nonlethally sampled gill using qPCR. Colors indicate water temperature (blue = 14 °C, red = 18 °C), while lines and symbols indicate treatment (▲, solid = gillnet, air exposed; ●, dashed = biopsied controls; *, dot-dashed = controls sampled only at death. Agent loads correspond to the inverse of qPCR-derived quantification thresholds (Cq).

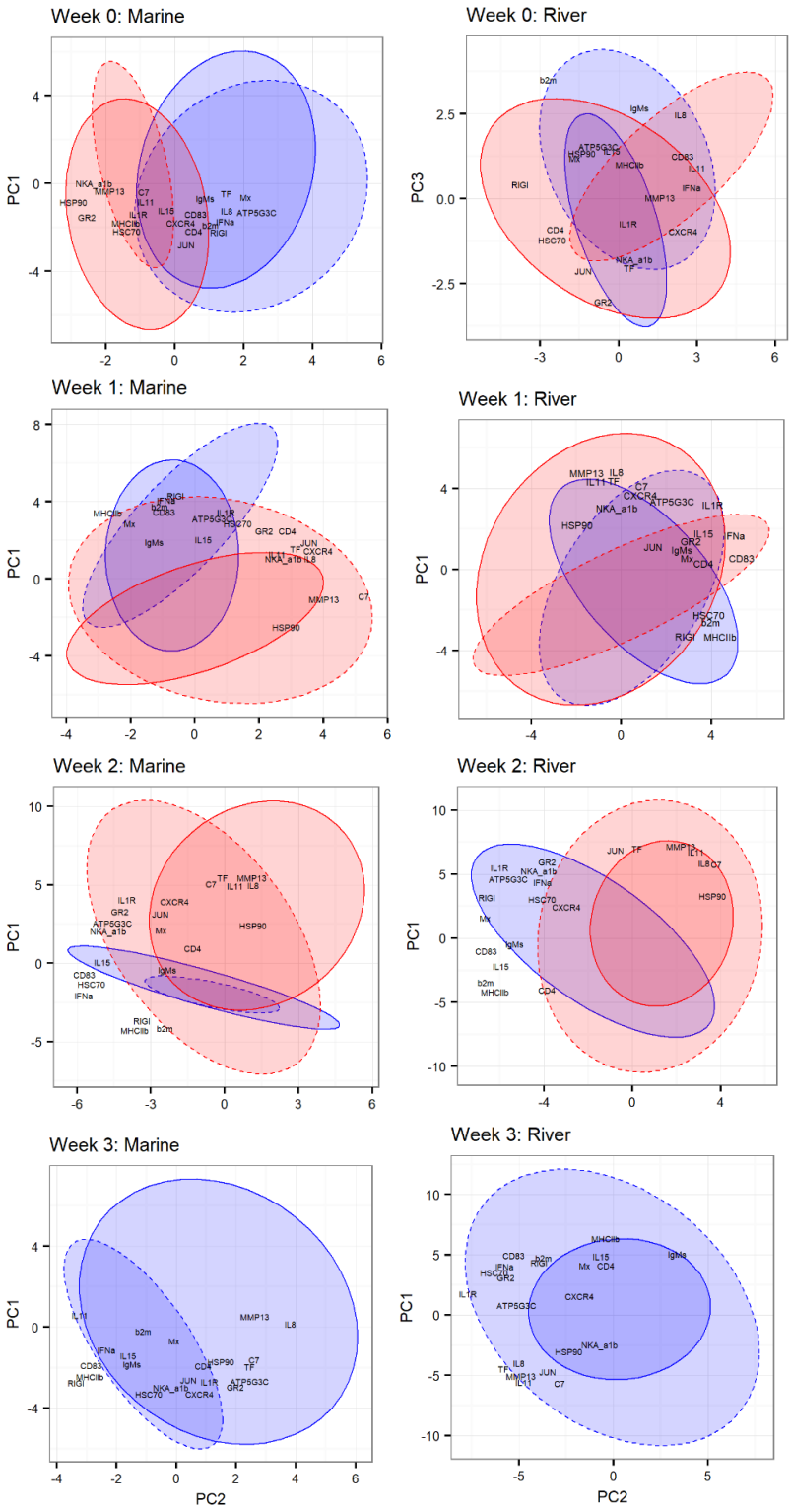

Figure 5.4 Principal component analysis (PCA) of stress and immune gene expression in adult sockeye salmon gill at 14 °C (blue) and 18 °C (red) for controls (dashed) or gillnetted and air exposed (solid) fish. 95% confidence intervals.

Chapter 6 – Discussion and synthesis

Stress is a common component of the lives of all fish, which have evolved responses to improve survival under adverse circumstances (Barton, 2002). However, if these responses cannot be resolved due to chronic stress or other factors that inhibit the clearance of metabolites such as infections (chapter 5), they can have detrimental effects on host health that include facilitation of infection development through suppressed immunity (Tort, 2011). The complex, multi-pathogen infection dynamics of wild fish introduce variability regarding the intersection between host, environment and infectious agents that contribute to disease development (Sofonea *et al.*, 2015, 2017). This thesis has demonstrated how methodical evaluation of temporal shifts in multiple infection burdens under controlled stressor application can improve our knowledge of the disease ecology of wild fish populations. The four data chapters describe infection trajectories of wild adult Pacific salmon exposed and unexposed to individual or cumulative thermal and fishery stressors during their freshwater residence period prior to spawning. Relationships among multiple infections and host physiological, immune and behavioral responses highlight sex-, species- and location-specific differences in the relative impacts of individual and cumulative stressors and host survival outcomes.

6.1 Stressors and infections of adult Pacific salmon in the Fraser River

This dissertation has confirmed a prominent role of temperature in affecting the survival and physiology of wild adult Pacific salmon during freshwater residence (Fry, 1971, Eliason *et al.*, 2013; Jeffries *et al.*, 2012b; Martins *et al.*, 2011, 2012b), but has built upon this knowledge by linking thermal stress to enhanced infection development.

In the introduction, I hypothesized that temperature would increase infection rates and mortality of hosts (chapters 3-5), which was generally supported, though variable among host species and infections in the magnitude of this response. Given that the infection "challenge" study (chapter 5) found survival differences among non-handled fish at high temperature based on Fraser River exposure, this suggests infections can contribute to early mortality at high temperature as well as impaired physiological and immune function (Dittmar *et al.*, 2014). However, not all agents increased infection intensities at high temperature (e.g., *C. shasta, F. psychrophilum*) and agent responses to thermal stress also showed variability among studies (and host species) as well as tissue types (see below). For example, gillnetting increased *F. psychrophilum* loads in Chinook salmon (chapter 4) but instead increased *Ca*. B. cysticola in river-exposed sockeye salmon (chapter 5). Such inconsistencies may be due to host-specific differences in initial loads of these agents, as *F. psychrophilum* was carried at much higher levels in Chinook salmon, or possibly associated with the treatment environment (lab for Chinook, river for sockeye) or response profiles (gillnetting associated with greater immediate response among sockeye). As these studies were conducted in different years and months, annual and seasonal variation in infections within the river may also contribute to differences in infection responses across studies. Future research that tracks interannual and seasonal variation in infection densities within host populations of the same river system would be beneficial to characterize temporal sources of variation.

Multiple infections were a common trait of all species examined by this thesis and consistent with the findings of Bass and colleagues (2017; unpublished data). The responses of these agents to stress were not only stressor-dependent but also agent-

specific. For example, *C. shasta* showed disparity between gill and the multi-tissue pool in how loads changed over time and under stress: pooled tissues of coho salmon demonstrated increased infection development under thermal stress, while gill did not (chapter 3). These findings suggest that infection development in the gill of several Pacific salmon species can inform changes in infectious agent community structure, particularly for some parasites and other tissue-specific agents, but are not necessarily representative of other tissues in the body. Analysis of each tissue type included in the multi-tissue pool will be needed to further clarify this trend; such a study could be complimented by an analysis (genomic, histopathological) of host immune responses within each tissue type to characterize where and how disease development occurs.

Tissue tropism is an important aspect of disease development and the results of chapter 3 demonstrated that the location of infection for *P. minibicornis* was important for both infection development as well as impacts on survival (i.e., gill infection was more relevant to survival than multi-tissue pool loads). Bradford *et al.*, (2010) proposed that gill infections likely influenced physiology by impairing respiratory capacity, while kidney infections are more characteristically linked to osmoregulatory disfunction via ruptured glomeruli. Infections were shown in chapter 4 to modulate the behavior of Chinook salmon, increasing migration rates (heavy overall infections) but decreasing longevity in the river (heavy *F. psychrophilum* infections). The mechanisms driving these relationships warrant further study of how bacterial damage to gill and skin epithelium may reduce osmolality, which has been previously linked to migration rate (Donaldson *et al.*, 2011). This question could be addressed by testing divergent migration behaviors in a laboratory setting (e.g., flume trials) paired with a genomic analysis of host brain, whole

body infection burdens, and osmoregulatory impairment to identify linkages between swim speeds or attempt rates (e.g., hydraulic challenge) with disease development and brain signaling. Alternatively, advanced infections and low osmolality may correlate with days in fresh water and maturation, driving increased migration rates through hormonal changes (Munakata *et al.*, 2001); this assumption could be tested by by comparing anadromous salmon populations with resident freshwater salmon populations (e.g., kokanee sockeye) with hypothetically minimal infection community shifts.

One of the most fruitful areas of continued research will be the interactions among infectious agents and how those community shifts affect survival and health of their hosts. The results presented within this thesis reflected the natural patterns of infections carried by wild salmon. However, these data could be further explored to understand how temporal changes in dominance hierarchies and new infections (e.g., transmission dynamics) also affect individual host responses. Additionally, challenge studies could be undertaken to identify relative effects of individual agents or by isolating a subset of agents to reduce complexity and obtain virulence information pertaining to specific co-infections (Sofonea *et al.*, 2017). Alternatively, data within this thesis could be mined to examine how directional changes in infection intensities of each agent relate to those of others over time. Temporal increases in the loads of most agents is known, but these rates may differ depending on co-infections.

The results of this thesis point to cumulative impacts of multiple agents given significant associations of relative infection burdens and infectious agent richness with host survival; this information could be refined or broadened to tissues beyond gills. In addition to the correlative results of chapters 2-4 and enhanced rates of infection in early

mortalities and thermally of (in some cases) fishery stressed fish, the ecological "challenge" study (chapter 5) provided strong evidence for a role of freshwater-derived infections in early mortality of adult salmon, especially when handled or exposed to high temperature. Collectively, this information gives a mechanistic basis for the assumption that infections kill fish *en route* to spawning grounds and infection-associated mortality is exacerbated by migratory stressors.

6.2 Host responses to stressors and infections

I hypothesized that host resilience would be compromised at high temperature (chapters 3-5), likely due to immune suppression resulting from chronic stress (Tort, 2011). Overall immune suppression was not apparent in genomic profiles at high temperature, but rather a consistent modulation of immune components that suggested an innate response to bacterial or parasitic agents. A subset of immune genes showed consistent positive association with thermal stress across host species and studies, such as complement (C7), transferrin (TF), and inflammatory messengers (IL11). Infections accelerated in gill by thermal stress (e.g. *Ca.* B. cysticola, *I. multifiliis*) may elicit enhanced expression of such components. However, this modulation of gene expression toward a subset of immune components suggests that immune responses are focused on extracellular pathogens at high temperature, possibly diverting limited resources away from intracellular (i.e., viral) agents or those that can avoid innate defenses (e.g., immune evasion; Alvarez-Pellitero, 2008). It should be noted, however, that transcriptomic data do not comprise proteomic shifts, such as post-translational modifications of immune proteins that may occur in response to infections and cellular stress. If incorporated in

future studies, proteomic data may alter interpretation of the results presented in this thesis.

Overall, cellular components of immunity including aspects of the major histocompatibility complex and cell receptors linked to adaptive immunity were negatively associated with high temperature, infection severity and early mortality. Fish at cool temperature were still recruiting cellular and anti-viral components of the immune response late in freshwater residence (though less so than at collection), suggesting that these components may be important for longevity and/or indicate the presence of a viral agent. Although we did not detect many viruses in fish overall, several novel viruses have been identified in BC salmon (K. Miller, unpublished data) and host response profiles can provide reliable indicators of the presence of viral agents (Miller *et al.*, 2017).

Generally, a viral response would only be detected in fish carrying a virus; host responses at high temperature in this thesis therefore do not indicate viral infection, but rather the absence of one. If an unidentified virus was present and survivors demonstrated a viral response profile while early mortalities did not, it is possible that mortality among thermally stressed fish could be due to reduced viral defenses at high temperature. Given that studies have previously shown enhanced viral responses in inoculated Atlantic cod (*Gadus morhua*) at high temperature (Hori *et al.*, 2013), but survival of Pacific salmon at high temperature is potentially contingent upon enhanced immune activation (Jeffries *et al.*, 2012a), the absence of a response may contribute to early mortality if a viral agent is indeed present. Future research could use challenge studies with temperature modulation to identify whether viral response gene regulation is suppressed in adult Pacific salmon that die prematurely relative to survivors at high temperature. Archived samples could

also be screened for these novel viruses (K. Miller, unpublished data) to test this hypothesis.

Importantly, plasma physiology of Chinook, sockeye and coho salmon demonstrated hormonal shifts that suggested delayed or suppressed maturation under chronic thermal stress and cumulative stressors. Impaired maturation has been described previously in response to thermal (Jeffries *et al.*, 2012b) and fishery (Baker *et al.*, 2013) stressors. This consistent finding has implications for sublethal impacts of multiple stressors on population productivity if animals are unable to spawn before death occurs. Further, correlation of delayed maturation with heavy infections warrants exploration of the linkages between these factors, where infections may contribute to an inability to resolve stress and continue reproductive investment. Given the ticking clock of infection development during freshwater residence associated with senescence processes, stressors that alter the maturation trajectory by modulating hormone levels as part of the stress response may contribute to prespawn mortality by derailing reproductive processes, which are physiologically costly to restore (Wendalaar Bonga, 1997; Baker *et al.*, 2013). Infections may be too severe by the time the stress is resolved to allow sufficient time and energy resources for maturation and spawning, but further work is needed that comprises other maturation indices such as egg size and ovulation.

Consistent with my hypotheses for chapter 2, stressor severity matters to survival and physiological responses. Survival was significantly reduced by handling stress in all chapters, even among biopsied controls, emphasizing that any level of handling is detrimental to adult salmon, especially after freshwater entry. Shorter durations of capture and handling in chapter 2 (20 s gillnet entanglement and biopsied controls vs. 20

min gillnet entanglement) improved survival and caused less physiological disturbance. Small differences in plasma lactate levels were predictive of survival following gillnet capture and release simulation in chapter 2, suggesting that any effort toward minimizing the duration of capture benefits post-release recovery and the odds of post-release survival. Consistent with two aspects of the behavioral hypotheses for chapter 4, release from gillnetting decreased migration rate and success of Chinook salmon, with many gillnetted fish failing to arrive at spawning grounds. Therefore, survival and behavioral consequences are associated with capture and release from gillnets in the river and as chapter 5 demonstrated, infectious agents accumulated in the river can decrease host resiliency to handling.

Contrary to my hypothesis that heavy infections at capture would be predictive of early mortality of sockeye salmon released from gillnets (chapter 2), it was an interaction of metabolic stress (lactate) and antiviral (Mx) biomarker expression that influenced fate. This finding points to a predisposal of individuals to post-release mortality if anti-viral responses are activated at the time of capture. Anti-viral activity has been demonstrated to be predictive of juvenile salmon mortality *en route* to the ocean (Jeffries *et al.,* 2014a) and adult salmon migration success in the marine environment and river and longevity at spawning grounds (Miller *et al.,* 2011). As Mx gene expression was predictive of survival in chapter 2, but also showed increased expression with days in holding, especially in cool water (chapters 2-5), this biomarker may also be linked to senescence processes (see discussion in chapter 2). Additionally, upregulation of this gene was generally associated with cool temperature and control fish after one or more weeks of holding, suggesting that thermal and fishery stress suppress its transcription. It is possible that immune

profiles of adult Pacific salmon instinctively shift toward agents of the highest concern (e.g., viruses) when spawning approaches to ensure survival and reproductive success. These correlations warrant further exploration to identify the role of Mx and other anti-viral aspects in senescence, maturation and stress responses. Studies that manipulate the expression of Mx (e.g., gene knockout) could identify whether senescence processes are altered in its absence. Comparison of this gene's activity in adult Pacific salmon with an iteroparous species (e.g., steelhead trout, *O. mykiss*) during freshwater spawning migration may also help to elucidate its function. Given the relationship of Mx expression and anaerobic metabolism with survival in chapter 2 and the consistent downregulation of this gene at high temperature, this gene may serve as a key indicator of adult Pacific salmon resilience that is predictive of spawning success.

6.3 Sex-specific effects

Although I did not include hypotheses for sex-specific effects, differences in the responses and survival of males and females was revealed as an important aspect of this thesis. Females were generally more severely impacted than males with respect to stressor effects and infection burdens, thought his was variable across studies (sex-specific survival differences in chapters 2-3, not measurable in chapter 4, no effect in chapter 5). Females have previously been shown to be more drastically affected by stressors than males (Jeffries *et al.*, 2012b; Martins *et al.*, 2012b) and as females generally have higher resting cortisol levels (Kubokawa *et al.*, 2001), this is often hypothesized to place them at greater risk of disease development. Sex-specific differences in immune gene regulation were noted in chapter 3 but were not consistent across studies and species. Physiological impairment was more strongly associated with

sex (chapters 2-4), and female coho (chapter 3) were less able to resolve stress relative to males. Lower survival (chapters 2-3) and alternate behavior (chapter 4) of females suggest that sex-specific differences in physiology and infection burdens may contribute to fitness differences between male and female Pacific salmon during spawning migration. Further research on energy allocation pertaining to stress recovery, maturation, metabolic demands of swimming and immune defenses would provide further insight as to the interconnectedness of these sex-specific correlations and the potential for enhanced susceptibility of females to stress- or infection-induced mortality.

6.4 Management implications and conclusions

I am hopeful that the findings and approaches described in this thesis can inform managers regarding the temporal and spatial scope of fisheries to reduce bycatch mortality. Furthermore, management strategies could potentially be modernized by incorporating genomic tools used in this thesis to improve biological data used in management models of *en route* mortality. This thesis used HT-qPCR to nonlethally measure infections and host response signatures of migrating adults. Infection and health status could be analyzed in small amounts of tissue collected by test fisheries in the lower river, similar to procedures for stock identification (Beacham *et al.*, 2004). This information could be incorporated into management models to improve the precision of management adjustments that predict the number of fish arriving at spawning grounds based on total fish entering the river by gauging the resilience of migrating stocks to anticipated stressors (e.g., river temperature, fishing intensity, flow). Future work should experiment with inclusion of disease-associated data (i.e., stressors, infections, host

health indices) within management models to identify the degree of variability that can be accounted for by these variables.

The results of Chapter 5 implied that river-derived infections reduced the resiliency of fish to capture, which argues for fishing effort to be focused outside of the lower river, as fish are simultaneously acclimating to fresh water (Shrimpton *et al.,* 2005) while fighting an array of new infections (this thesis). The marine environment provides a more saline recovery setting and access to thermal refugia (e.g., deep water) when sea surface temperatures are high, whereas in-river fisheries are generally forced to close when rivers are warm due to low survival of released catch (Martins *et al.*, 2011, this thesis, chapters 2-5). However, careful thought must be given to management decisions regarding where and when to fish given the socio-political impacts of altering fishing locations. For example, focusing fisheries in the marine environment would drastically increase costs of fishing for groups currently equipped for in-river fishing (e.g., First Nations beach seine and gillnet fisheries) and may thereby restrict access among these groups. Furthermore, marine fisheries potentially put marine species other than salmon at risk for bycatch (e.g., fish, mammals, turtles, birds), for which the effects and magnitude may be severe (Davies *et al.,* 2009). Terminal freshwater fisheries (i.e., close to spawning grounds) would likely reduce bycatch due to the high likelihood of catching target species and stocks and would provide economic benefits to First Nations communities (Johnsen 2009; Nesbitt and Moore, 2016), but flesh quality and market value decrease with time in fresh water (Johnston et al., 2006) and access may be restricted based on land rights and ownership. Decisions regarding where and when to fish for Pacific salmon must be reached through collaborative partnerships and open discussions that include social and political as well as

scientific perspectives. The findings of this thesis are one component of a complex set of data and insight that can guide decision-making to ensure a thriving and sustainable fishery.

This thesis highlights the impacts of capture and release from fisheries on infection development, behavior and survival, with effects that are dependent on physiological and infection status at the time of capture as well as the conditions (water temperature) of the capture event and recovery. The experiments herein provide several rare examples of how cumulative stressors impact infection development, physiology and survival of wild fish. Furthermore, the inclusion and emphasis of infection development in response to stressors under ecologically relevant conditions offers some of the first insight into how the health of wild salmon influences their likelihood of surviving to spawn. Mortality in most cases was generally delayed by more than a week after acute stress or the onset of chronic thermal stress, supporting a role of infectious disease (i.e., enhanced infection development and impaired host resilience) rather than proximal aerobic impairment as the cause. Chapters 3-5 demonstrate the detrimental effects of thermal stress on wild salmon and interplay between enhanced infections (though variable among agents) and decreased physiological resilience that promote mortality. Chapter 3 identified sex-specific differences in both stress responses and infection development, with female coho salmon unable to resolve stress or suppress infection development. Infection dynamics were generally similar among host species, with divergence primarily dependent on run timing or collection location, suggesting that the environment is a primary driver of infection dynamics in Fraser River salmon populations. The information provided in this thesis highlights the impacts of cumulative stressors in the disease ecology of wild

Pacific salmon in the Fraser River watershed, but also offers fruitful areas of continued research where knowledge gaps remain.

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Appendix

7.1 Table S3.1

Infectious agent assay information. Assays referenced as "MGL" were developed at the Molecular Genetics Lab, DFO Pacific Biological Station, Nanaimo, BC. For efficiencies marked with "*", artificial constructs were not available to quantify assay efficiency.

Pathogen	Abbreviation	Type	Primer F	Primer R	Probe	Efficiency	Reference
Aeromonas hydrophila	ae_hyd	Bacterium	ACCGCTGCTCATTACTCTGATG	CCAACCCAGACGGGAAGAA	TGATGGTGAGCTGGTTG	*	Lee et al. 2006
Aeromonas salmonicida	ae_sal	Bacterium	TAAAGCACTGTCTGTTACC	GCTACTTCACCCTGATTGG	ACATCAGCAGGCTTCAGAGTCACTG	*	Keeling et al 2013 (modified)
Renibacterium salmoninarum	re_sal	Bacterium	CAACAGGGTGGTTATTCTGCTTTC	CTATAAGAGCCACCAGCTGCAA	CTCCAGCGCCGCAGGAGGAC	*	Powell et al. 2005
Flavobacterium psychrophilum	fl_psy	Bacterium	GATCCTTATTCTCACAGTACCGTCAA	TGTAAACTGCTTTTGCACAGGAA	AAACACTCGGTCGTGACC	0.52	Duesund et al. 2010
Piscichlamydia salmonis	pch_sal	Bacterium	TCACCCCCAGGCTGCTT	GAATTCCATTTCCCCCTCTTG	CAAAACTGCTAGACTAGAGT	0.74	Nylund et al. 2008
Piscirickettsia salmonis	pisck_sal	Bacterium	TCTGGGAAGTGTGGCGATAGA	TCCCGACCTACTCTTGTTTCATC	TGATAGCCCCGTACACGAAACGGCATA	*	Corbeil et al. 2003
Rickettsia-like organism	rlo	Bacterium	GGCTCAACCCAAGAACTGCTT	GTGCAACAGCGTCAGTGACT	CCCAGATAACCGCCTTCGCCTCCG	*	Lloyd et al. 2011
Gill chlamydia (Sch)	sch	Bacterium	GGGTAGCCCGATATCTTCAAAGT	CCCATGAGCCGCTCTCTCT	TCCTTCGGGACCTTAC	0.89	Duesund et al. 2010
Vibrio anguillarum	vi_ang	Bacterium	CCGTCATGCTATCTAGAGATGTATTTGA	CCATACGCAGCCAAAAATCA	TCATTTCGACGAGCGTCTTGTTCAGC	*	MGL
Atlantic salmon paramyxovirus	aspv	Virus	CCCATATTAGCAAATGAGCTCTATCTT	CGTTAAGGAACTCATCATTGAGCTT	AGCCCTTTTGTTCTGC	0.92	Nylund et al. 2008
Infectious hematopoietic necrosis virus	ihnv	Virus	AGAGCCAAGGCACTGTGCG	TTCTTTGCGGCTTGGTTGA	TGAGACTGAGCGGGACA	0.84	Purcell et al. 2013
Piscine reovirus (HSMI, CMS)	prv	Virus	TGCTAACACTCCAGGAGTCATTG	TGAATCCGCTGCAGATGAGTA	CGCCGGTAGCTCT	0.90	Wiik-Nielson et al. 2011
Pacific salmon parvovirus	pspv	Virus	CCCTCAGGCTCCGATTTTTAT	CGAAGACAACATGGAGGTGACA	CAATTGGAGGCAACTGTA	0.90	MGL
Piscine totivirus (CMS)	cms	Virus	TTCCAAACAATTCGAGAAGCG	ACCTGCCATTTTCCCCTCTT	CCGGGTAAAGTATTTGCGTC	0.92	Løvoll et al. 2010
Salmon alphavirus 1, 2, and 3 (PD/SD/HSS)	sav	Virus	CCGGCCCTGAACCAGTT	GTAGCCAAGTGGGAGAAAGCT	TCGAAGTGGTGGCCAG	0.84	Andersen et al. 2007
Viral encephalopathy and retinopathy virus	ver	Virus	TTCCAGCGATACGCTGTTGA	CACCGCCCGTGTTTGC	AAATTCAGCCAATGTGCCCC	0.90	Korsnes et al. 2005
Viral hemorrhagic septicemia virus	vhsv	Virus	ATGAGGCAGGTGTCGGAGG	TGTAGTAGGACTCTCCCAGCATCC	TACGCCATCATGATGAGT	0.85	Garver et al. 2011
Ceratonova shasta	ce_sha	Myxozoan	CCAGCTTGAGATTAGCTCGGTAA	CCCCGGAACCCGAAAG	CGAGCCAAGTTGGTCTCTCCGTGAAAAC	*	Hallett and Bartholomew 2006
Kudoa thyrsites	ku_thy	Myxozoan	TGGCGGCCAAATCTAGGTT	GACCGCACACAAGAAGTTAATCC	TATCGCGAGAGCCGC	*	Funk et al. 2007
Myxobolus arcticus	my_arc	Myxozoan	TGGTAGATACTGAATATCCGGGTTT	AACTGCGCGGTCAAAGTTG	CGTTGATTGTGAGGTTGG	0.93	MGL
Myxobolus cerebralis	my_cer	Myxozoan	GCCATTGAATTTGACTTTGGATTA	ACCATTCATGTAAGCCCGAACT	TCGAAGCCTTGACCATCTTTTGGCC	*	Kelley et al. 2004
Parvicapsula pseudobranchicola	pa_pse	Myxozoan	CAGCTCCAGTAGTGTATTTCA	TTGAGCACTCTGCTTTATTCAA	CGTATTGCTGTCTTTGACATGCAGT	0.83	Jørgensen et al. 2011
Tetracapsuloides bryosalmonae	te_bry	Myxozoan	GCGAGATTTGTTGCATTTAAAAAG	GCACATGCAGTGTCCAATCG	CAAAATTGTGGAACCGTCCGACTACGA	*	Bettge et al. 2009
Parvicapsula minibicornis	pa_min	Myxozoan	AATAGTTGTTTGTCGTGCACTCTGT	CCGATAGGCTATCCAGTACCTAGTAAG	TGTCCACCTAGTAAGGC	0.91	Hallett and Bartholomew 2009
Parvicapsula kabatai	pa_kab	Myxozoan	GTCGGATGATAAGTGCATCTGATT	ACACCACAACTCTGCCTTCCA	TGCGACCATCTGCACGGTACTGC	0.94	MGL
Facilispora margolisi	fa_mar	Microsporidian	AGGAAGGAGCACGCAAGAAC	CGCGTGCAGCCCAGTAC	TCAGTGATGCCCTCAGA	0.79	MGL
Nucleospora salmonis	nu_sal	Microsporidian	GCCGCAGATCATTACTAAAAACCT	CGATCGCCGCATCTAAACA	CCCCGCGCATCCAGAAATACGC	1.01	Foltz et al. 2009
Paranucleospora theridion	pa_ther	Microsporidian	CGGACAGGGAGCATGGTATAG	GGTCCAGGTTGGGTCTTGAG	TTGGCGAAGAATGAAA	0.81	Nylund et al. 2010
Heterosigma akashiwo	he_aka	Flagellate	GCTTCTGCATTGCCGTTTCT	CCCAACTTCCTTCGGTTAGTCA	CTTCTTAGAGGGACTTTC	*	MGL
Spironucleus salmonicida	sp_sal	Flagellate	AACCGGTTATTCGTGGGAAAG	TTAACTGCAGCAACACAATAGAATACTC	TGCCAGCAGCCGCGGTAATTC	0.97	MGL
Nanophyetus salmincola	na_sal	Fluke	CGATCTGCATTTGGTTCTGTAACA	CCAACGCCACAATGATAGCTATAC	TGAGGCGTGTTTTATG	0.88	MGL
Gyrodactylus salaris	gy_sal	Fluke	CGATCGTCACTCGGAATCG	GGTGGCGCACCTATTCTACA	TCTTATTAACCAGTTCTGC	*	Collins et al. 2010
Ichthyophonus hoferi	ic_hof	Protozoan	AGTGGGTGCTCTTAATTGAGTGTCT	GCCTGCTTTGAACACTCTAATTTTC	CTGAGTTCGGGACTTT	0.88	MGL
Sphaerothecum destruens	sp_des	Protozoan	GCCGCGAGGTGTTTGC	CTCGACGCACACTCAATTAAGC	CGAGGGTATCCTTCCTCTCGAAATTGGC	0.84	MGL
Ichthyophthirius multifiliis	ic_mul	Ciliate	AAATGGGCATACGTTTGCAAA	AACCTGCCTGAAACACTCTAATTTTT	ACTCGGCCTTCACTGGTTCGACTTGG	0.92	MGL

*No artificial constructs developed at this time to accurately characterize efficiency. See Miller et al. 2016 for assay efficiency and platform information.

7.2 **Table S3.2**

Immune gene biomarker assay information. Biomarkers referenced as "MGL" were developed at the Molecular Genetics Lab, DFO Pacific Biological Station, Nanaimo, BC.

Assay	Gene information	Assay type	EST/Accession#	Primer and probe sequences	Efficiency	Reference
b2m	Beta 2-microglobulin	Immune	AF180490	F - TTTACAGCGCGGTGGAGTC	0.87	(Haugland *et al.*, 2005)
				R - TGCCAGGGTTACGGCTGTAC		
				P - AAAGAATCTCCCCCCAAGGTGCAGG		
C3	Complement factor	Immune	U61753, AF271080	F - ATTGGCCTGTCCAAAACACA	1.01	(Raida and Buchmann, 2009)
				R - AGCTTCAGATCAAGGAAGAAGTTC		
				P - TGGAATCTGTGTGTCTGAACCCC		
CD4	Cell receptor	Immune	AY973028	F - CATTAGCCTGGGTGGTCAAT	0.94	(Raida and Buchmann, 2008)
				R - CCCTTTCTTTGACAGGGAGA		
				P - CAGAAGAGAGAGCTGGATGTCTCCG		
CD83	Cell receptor	Immune	AY263794	F - GATGCACCCCTTGAGAAGAA	0.95	(Raida *et al.*, 2011)
				R - GAACCCTGTCTCGACCAGTT		
				P - AATGTTGATTTACACTCTGGGGCCA		
Hep	Hepcidin	Immune	AF281354.1	F - GAGGAGGTTGGAAGCATTGA	0.96	(Raida and Buchmann, 2009)
				R - TGACGCTTGAACCTGAAATG		
				P - AGTCCAGTTGGGGAACATCAACAG		
IFNa	Interferon-α	Immune	AY216595	F - CGTCATCTGCAAAGATTGGA	0.94	(Ingerslev *et al.*, 2009)
				R - GGGCGTAGCTTCTGAAATGA		
				P – TGCAGCACAGATGTACTGATCATCCA		
IgMs	Immunoglobulin	Immune	S63348, AB044939	F - CTTGGCTTGTTGACGATGAG	1.00	(Raida *et al.*, 2011)
				R - GGCTAGTGGTGTTGAATTGG		
				P - TGGAGAGAACGAGCAGTTCAGCA		
IL-11	Cytokine	Immune	AJ535687	F - GCAATCTCTTGCCTCCACTC	0.97	(Raida and Buchmann, 2008)
				R - TTGTCACGTGCTCCAGTTTC		
				P - TCGCGGAGTGTGAAAGGCAGA		
IL-15	Cytokine	Immune	AJ555868.1	F - TTGGATTTTGCCCTAACTGC	1.06	(Raida *et al.*, 2011)
				R - CTGCGCTCCAATAAACGAAT		
				P - CGAACAACGCTGATGACAGGTTTTT		
IL-1R	Cytokine	Immune	AJ295296	F - ATCATCCTGTCAGCCCAGAG	0.94	(Raida *et al.*, 2011)
				R - TCTGGTGCAGTGGTAACTGG		
				P - TGCATCCCCTCTACACCCCAAA		
IRF1	Interferon regulatory factor 1	Immune	CB511515	F - CAAACCGCAAGAGTTCCTCATT	0.85	MGL
				R - AGTTTGGTTGTGTTTTTGCATGTAG		
				P - CTGGCGCAGCAGATA		
MHCI	Major histocompatibility complex I	Immune		F - GCGACAGGTTTCTACCCCAGT	1.00	(Ingerslev *et al.*, 2009)
				R - TGTCAGGTGGGAGCTTTTCTG		
				P - TGGTGTCCTGGCAGAAAGACGG		
MHCIIb	Major histocompatibility complex IIβ	Immune	AF115533	F - TGCCATGCTGATGTGCAG	0.95	(Raida and Buchmann, 2008)
				R - GTCCCTCAGCCAGGTCACT		
				P - CGCCTATGACTTCTACCCCAAACAAAT		
MMP13	Matrix metalloproteinase	Immune	213514499	F - GCCAGCGGAGCAGGAA	0.86	(Tadiso *et al.*, 2011)
				R - AGTCACCTGGAGGCCAAAGA		
				P - TCAGCGAGATGCAAAG		
Mx	Antiviral protein	Immune		F - AGATGATGCTGCACCTCAAGTC	0.94	(Eder *et al.*, 2009)
				R - CTGCAGCTGGGAAGCAAAC		
				P - ATTCCCATGGTGATCCGCTACCTGG		
RIG-I	Retinoic acid inducible gene I	Immune	NM_001163699	F - ACAGCTGTTACACAGACGACATCA	0.96	(Larsen *et al.*, 2012)
				R - TTTAGGGTGAGGTTCTGTCCGA		
				P - TCGTGTTGGACCCCACTCTGTTCTCTC		
TF	Transferin	Immune	D89083	F - TTCACTGCTGGAAAATGTGG	0.94	(Raida and Buchmann, 2009)
				R - GCTGCACTGAACTGCATCAT		
				P - TGGTCCCTGTCATGGTGGAGCA		
78d16.1		Reference	CA056739	F - GTCAAGACTGGAGGCTCAGAG	0.85	MGL
				R - GATCAAGCCCCAGAAGTGTTTG		
				P - AAGGTGATTCCCTCGCCGTCCGA		
COIL-P84-2		Reference	CA053789	F - GCTCATTTGAGGAGAAGGAGGATG	0.81	MGL
				R - CTGGCGATGCTGTTCCTGAG		
				P - TTATCAAGCAGCAAGCC		

7.3 **Table S4.1**

Primer and probe sequences corresponding to host immunity biomarkers, two reference genes, and infectious agents of evaluated via qPCR on adult Chinook salmon. References and qPCR efficiencies are provided with citations; in house designs were completed at the Molecular Genetics Laboratory at the Pacific Biological Station, Nanaimo, BC (continued below).

Assay name	Assay type	Gene information or infectious agent name	EST/Accession#	Primer and probe sequences	Efficiency	Source
C3	Host biomarker	Complement factor	U61753, AF271080	F - ATTGGCCTGTCCAAAACACA	0.96	(Raida and Buchmann, 20
				R - AGCTTCAGATCAAGGAAGAAGTTC		
				P - TGGAATCTGTGTGTCTGAACCCC		
CD4	Host biomarker	Cell receptor	AY973028	F - CATTAGCCTGGGTGGTCAAT	0.82	(Raida and Buchmann, 20
				R - CCCTTTCTTTGACAGGGAGA		
				P - CAGAAGAGAGAGCTGGATGTCTCCG		
CD83	Host biomarker	Cell receptor	AY263794	F - GATGCACCCCTTGAGAAGAA	0.82	(Raida *et al.*, 2011)
				R - GAACCCTGTCTCGACCAGTT		
				P - AATGTTGATTTACACTCTGGGGCCA		
IFNa	Host biomarker	Interferon-α	AY216595	F - CGTCATCTGCAAAGATTGGA	0.89	(Ingerslev *et al.*, 2009)
				R - GGGCGTAGCTTCTGAAATGA		
				P – TGCAGCACAGATGTACTGATCATCCA		
IgMs	Host biomarker	Immunoglobulin	S63348, AB044939	F - CTTGGCTTGTTGACGATGAG	0.80	(Raida *et al.*, 2011)
				R - GGCTAGTGGTGTTGAATTGG		
				P - TGGAGAGAACGAGCAGTTCAGCA		
IL11	Host biomarker	Cytokine	AJ535687	F - GCAATCTCTTGCCTCCACTC	0.84	(Raida and Buchmann, 20
				R - TTGTCACGTGCTCCAGTTTC		
				P - TCGCGGAGTGTGAAAGGCAGA		
IL15	Host biomarker	Cytokine	AJ555868.1	F - TTGGATTTTGCCCTAACTGC	0.89	(Raida *et al.*, 2011)
				R - CTGCGCTCCAATAAACGAAT		
				P - CGAACAACGCTGATGACAGGTTTTT		
IL1R	Host biomarker	Cytokine	AJ295296	F - ATCATCCTGTCAGCCCAGAG	0.83	(Raida *et al.*, 2011)
				R - TCTGGTGCAGTGGTAACTGG		
				P - TGCATCCCCTCTACACCCCAAA		
MHCI	Host biomarker	Major histocompatibility complex I		F - GCGACAGGTTTCTACCCCAGT	0.88	(Ingerslev *et al.*, 2009)
				R - TGTCAGGTGGGAGCTTTTCTG		
				P - TGGTGTCCTGGCAGAAAGACGG		

Table S4.1 (continued)

Assay name	Assay type	Gene information or infectious agent name	EST/Accession#	Primer and probe sequences	Efficiency	Source
MHCIIb	Host biomarker	Major histocompatibility complex IIβ	AF115533	F - TGCCATGCTGATGTGCAG	0.83	(Raida and Buchmann, 20
				R - GTCCCTCAGCCAGGTCACT		
				P - CGCCTATGACTTCTACCCCAAACAAAT		
MMP13	Host biomarker	Matrix metalloproteinase	213514499	F - GCCAGCGGAGCAGGAA	0.82	(Tadiso *et al.*, 2011)
				R - AGTCACCTGGAGGCCAAAGA		
				P - TCAGCGAGATGCAAAG		
Mx	Host biomarker	Antiviral protein		F - AGATGATGCTGCACCTCAAGTC	0.82	(Eder *et al.*, 2009)
				R - CTGCAGCTGGGAAGCAAAC		
				P - ATTCCCATGGTGATCCGCTACCTGG		
RIG.I	Host biomarker	Retinoic acid inducible gene I	NM_001163699	F - ACAGCTGTTACACAGACGACATCA	0.81	(Larsen *et al.*, 2012)
				R - TTTAGGGTGAGGTTCTGTCCGA		
				P - TCGTGTTGGACCCCACTCTGTTCTCTC		
TF	Host biomarker	Transferrin	D89083	F - TTCACTGCTGGAAAATGTGG	0.87	(Raida and Buchmann, 20
				R - GCTGCACTGAACTGCATCAT		
				P - TGGTCCCTGTCATGGTGGAGCA		
C7	Host biomarker	Complement factor	CA052045	F - ACCTCTGTCCAGCTCTGTGTC	0.82	In house
				R - GATGCTGACCACATCAAACTGC		
				P - AACTACCAGACAGTGCTG		
GR-2	Host biomarker	Glucocorticoid receptor		F - TCCAGCAGCTATGCCAGTTCT	0.85	(Yada *et al.*, 2007)
				R - TTGCCCTGGGTTGTACATGA		
				P - AAGCTTGGTGGTGGCGCTG		
78d16.1	Reference gene		CA056739	F - GTCAAGACTGGAGGCTCAGAG	0.82	In house
				R - GATCAAGCCCCAGAAGTGTTTG		
				P - AAGGTGATTCCCTCGCCGTCCGA		
COIL-P84-2	Reference gene		CA053789	F - GCTCATTTGAGGAGAAGGAGGATG	0.73	In house
				R - CTGGCGATGCTGTTCCTGAG		
				P - TTATCAAGCAGCAAGCC		

Table S4.1 (continued)

Assay name	Assay type	Gene information or infectious agent name	EST/Accession#	Primer and probe sequences	Efficiency	Source
ae_hyd	Bacterium	*Aeromonas hydrophila*		F - ACCGCTGCTCATTACTCTGATG	0.87	(Lee *et al*., 2006)
				R - CCAACCCAGACGGGAAGAA		
				P - TGATGGTGAGCTGGTTG		
ae_sal	Bacterium	*Aeromonas salmonicida*		F - TAAAGCACTGTCTGTTACC	0.91	(Keeling *et al.*, 2013)
				R - GCTACTTCACCCTGATTGG		
				P - ACATCAGCAGGCTTCAGAGTCACTG		
re_sal	Bacterium	*Renibacterium salmoninarum*		F - CAACAGGGTGGTTATTCTGCTTTC	0.92	(Powell *et al.*, 2005)
				R - CTATAAGAGCCACCAGCTGCAA		
				P - CTCCAGCGCCGCAGGAGGAC		
c_b_cys	Bacterium	*Candidatus Branchiomonas cysticola*		F - AATACATCGGAACGTGTCTAGTG	0.83	(Mitchell *et al.*, 2013)
				R - GCCATCAGCCGCTCATGTG		
				P - CTCGGTCCCAGGCTTTCCTCTCCCA		
ye_ruc	Bacterium	*Yersinia ruckeri*		F - TGCCGCGTGTGTGAAGAA	0.93	(Glenn *et al.*, 2011)
				R - ACGGAGTTAGCCGGTGCTT		
				P - AATAGCACTGAACATTGAC		
fl_psy	Bacterium	*Flavobacterium psychrophilum*		F - GATCCTTATTCTCACAGTACCGTCAA	0.76	(Duesund *et al.*, 2010)
				R - TGTAAACTGCTTTTGCACAGGAA		
				P - AAACACTCGGTCGTGACC		
pch_sal	Bacterium	*Piscichlamydia salmonis*		F - TCACCCCCAGGCTGCTT	0.79	(Nylund *et al.*, 2008)
				R - GAATTCCATTTCCCCCTCTTG		
				P - CAAAACTGCTAGACTAGAGT		
pisck_sal	Bacterium	*Piscirickettsia salmonis*		F - TCTGGGAAGTGTGGCGATAGA	0.93	(Corbeil *et al.*, 2003)
				R - TCCCGACCTACTCTTGTTTCATC		
				P - TGATAGCCCCGTACACGAAACGGCATA		
rlo	Bacterium	*Rickettsia-like organism*		F - GGCTCAACCCAAGAACTGCTT	0.90	(Lloyd *et al*., 2011)
				R - GTGCAACAGCGTCAGTGACT		
				P - CCCAGATAACCGCCTTCGCCTCCG		

Table S4.1 (continued)

Assay name	Assay type	Gene information or infectious agent name	EST/Accession#	Primer and probe sequences	Efficiency	Source
sch	Bacterium	*gill chlamydia*		F - GGGTAGCCCGATATCTTCAAAGT	0.79	(Duesund *et al.*, 2010)
				R - CCCATGAGCCGCTCTCTCT		
				P - TCCTTCGGGACCTTAC		
vi_ang	Bacterium	*Vibrio anguillarum*		F - CCGTCATGCTATCTAGAGATGTATTTGA	0.86	In house
				R - CCATACGCAGCCAAAAATCA		
				P - TCATTTCGACGAGCGTCTTGTTCAGC		
vi_sal	Bacterium	*Vibrio salmonicida*		F - GTGTGATGACCGTTCCATATTT	0.85	In house
				R - GCTATTGTCATCACTCTGTTTCTT		
				P - TCGCTTCATGTTGTGTAATTAGGAGCGA		
aspv	Virus	Atlantic salmon paramyxovirus		F - CCCATATTAGCAAATGAGCTCTATCTT	0.87	(Nylund *et al.*, 2008)
				R - CGTTAAGGAACTCATCATTGAGCTT		
				P - AGCCCTTTTGTTCTGC		
pmcv	Virus	Piscine totivirus (CMS)		F - TTCCAAACAATTCGAGAAGCG	0.88	(Løvoll *et al.*, 2010)
				R - ACCTGCCATTTTCCCCTCTT		
				P - CCGGGTAAAGTATTTGCGTC		
ver	Virus	Viral encephalopathy and retinopathy virus		F - TTCCAGCGATACGCTGTTGA	0.86	(Korsnes *et al.*, 2005)
				R - CACCGCCCGTGTTTGC		
				P - AAATTCAGCCAATGTGCCCC		
vhsv	Virus	Viral hemorrhagic septicemia virus		F - ATGAGGCAGGTGTCGGAGG	0.84	(Garver *et al.*, 2011)
				R - TGTAGTAGGACTCTCCCAGCATCC		
				P - TACGCCATCATGATGAGT		
omv	Virus	Salmonid herpesvirus		F - GCCTGGACCACAATCTCAATG	0.87	In house
				R - CGAGACAGTGTGGCAAGACAAC		
				P - CCAACAGGATGGTCATTA		
sav	Virus	Salmon alphavirus		F - CCGGCCCTGAACCAGTT	0.85	(Andersen *et al.*, 2007)
				R - GTAGCCAAGTGGGAGAAAGCT		
				P - TCGAAGTGGTGGCCAG		

Table S4.1 (continued)

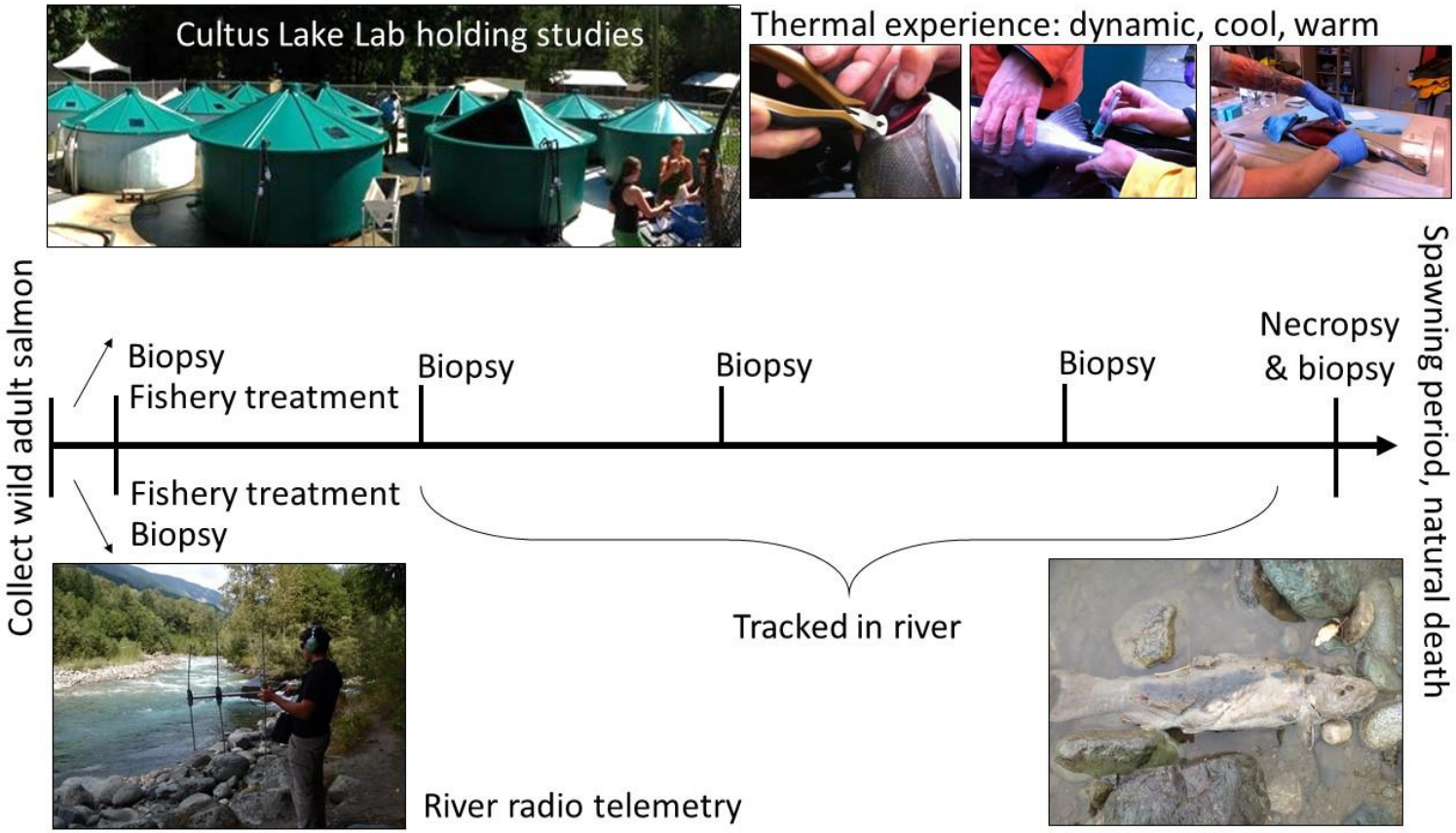
Assay name	Assay type	Gene information or infectious agent name	EST/Accession#	Primer and probe sequences	Efficiency	Source
ven	Virus	Viral erythrocytic necrosis virus		F - CGTAGGGCCCCAATAGTTTCT	0.90	James Winton, pers. comm
				R - GGAGGAAATGCAGACAAGATTTG		
				P - TCTTGCCGTTATTTCCAGCACCCG		
pspv	Virus	Pacific salmon parvovirus		F - CCCTCAGGCTCCGATTTTTAT	NA	In house
				R - CGAAGACAACATGGAGGTGACA		
				P - CAATTGGAGGCAACTGTA		
prv	Virus	Piscine reovirus (HSMI, CMS)		F - TGCTAACACTCCAGGAGTCATTG	0.91	(Wiik-Nielsen *et al.*, 2012
				R - TGAATCCGCTGCAGATGAGTA		
				P - CGCCGGTAGCTCT		
ihnv	Virus	Infectious haematopoietic necrosis virus		F - AGAGCCAAGGCACTGTGCG	0.83	(Purcell *et al.*, 2013)
				R - TTCTTTGCGGCTTGGTTGA		
				P - TGAGACTGAGCGGGACA		
cr_sal	Parasite	*Cryptobia salmositica*		F - TCAGTGCCTTTCAGGACATC	0.86	In house
				R - GAGGCATCCACTCCAATAGAC		
				P - AGGAGGACATGGCAGCCTTTGTAT		
ce_sha	Parasite	*Ceratonova shasta*		F - CCAGCTTGAGATTAGCTCGGTAA	0.92	(Hallett and Bartholomew
		(formerly *Ceratomyxa shasta*)		R - CCCCGGAACCCGAAAG		
				P - CGAGCCAAGTTGGTCTCTCCGTGAAAAC		
de_sal	Parasite	*Dermocystidium salmonis*		F - CAGCCAATCCTTTCGCTTCT	0.88	In house
				R - GACGGACGCACACCACAGT		
				P - AAGCGGCGTGTGCC		
fa_mar	Parasite	*Facilispora margolisi*		F - AGGAAGGAGCACGCAAGAAC	0.85	In house
				R - CGCGTGCAGCCCAGTAC		
				P - TCAGTGATGCCCTCAGA		
gy_sal	Parasite	*Gyrodactylus salaris*		F - CGATCGTCACTCGGAATCG	0.82	(Collins *et al.*, 2010)
				R - GGTGGCGCACCTATTCTACA		
				P - TCTTATTAACCAGTTCTGC		

Table S4.1 (continued)

Assay name	Assay type	Gene information or infectious agent name	EST/Accession#	Primer and probe sequences	Efficiency	Source
ic_mul	Parasite	*Ichthyophthirius multifiliis*		F - AAATGGGCATACGTTTGCAAA	0.91	In house
				R - AACCTGCCTGAAACACTCTAATTTTT		
				P - ACTCGGCCTTCACTGGTTCGACTTGG		
ku_thy	Parasite	*Kudoa thyrsites*		F - TGGCGGCCAAATCTAGGTT	0.89	(Funk *et al.*, 2007)
				R - GACCGCACACAAGAAGTTAATCC		
				P - TATCGCGAGAGCCGC		
lo_sal	Parasite	*Loma salmonae*		F - GGAGTCGCAGCGAAGATAGC	0.86	In house
				R - CTTTTCCTCCCTTTACTCATATGCTT		
				P - TGCCTGAAATCACGAGAGTGAGACTACCC		
my_arc	Parasite	*Myxobolus arcticus*		F - TGGTAGATACTGAATATCCGGGTTT	0.84	In house
				R - AACTGCGCGGTCAAAGTTG		
				P - CGTTGATTGTGAGGTTGG		
my_ins	Parasite	*Myxobolus insidiosus*		F - CCAATTTGGGAGCGTCAAA	0.84	In house
				R - CGATCGGCAAAGTTATCTAGATTCA		
				P - CTCTCAAGGCATTTAT		
my_cer	Parasite	*Myxobolus cerebralis*		F - GCCATTGAATTTGACTTTGGATTA	0.94	(Kelley *et al.*, 2004)
				R - ACCATTCATGTAAGCCCGAACT		
				P - TCGAAGCCTTGACCATCTTTTGGCC		
ne_per	Parasite	*Neoparamoeba perurans*		F - GTTCTTTCGGGAGCTGGGAG	0.87	(Fringuelli *et al.*, 2012)
				R - GAACTATCGCCGGCACAAAAG		
				P - CAATGCCATTCTTTTCGGA		
nu_sal	Parasite	*Nucleospora salmonis*		F - GCCGCAGATCATTACTAAAAACCT	0.96	(Foltz *et al.*, 2009)
				R - CGATCGCCGCATCTAAACA		
				P - CCCCGCGCATCCAGAAATACGC		
pa_ther	Parasite	*Paranucleospora theridion*		F - CGGACAGGGAGCATGGTATAG	0.80	(Nylund *et al.*, 2010)
				R - GGTCCAGGTTGGGTCTTGAG		
				P - TTGGCGAAGAATGAAA		

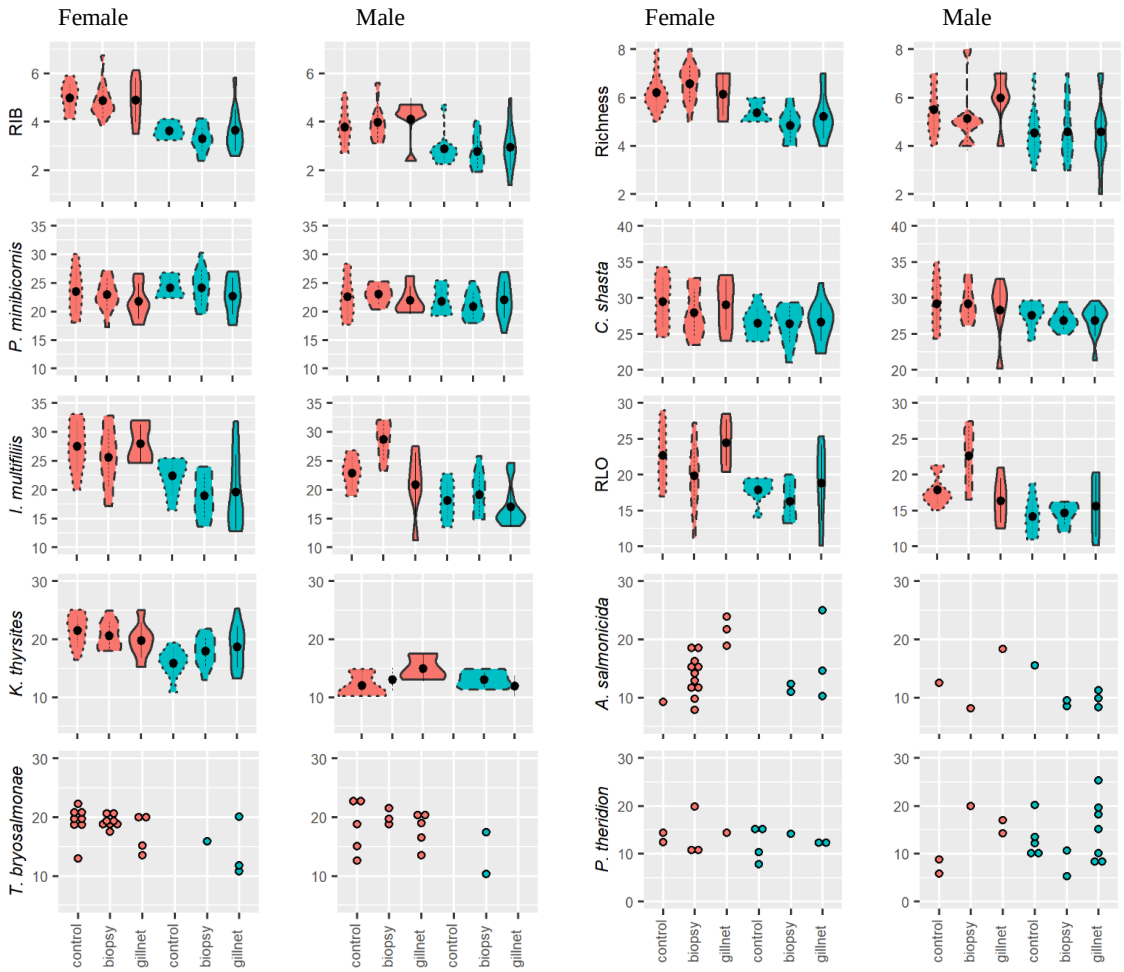
Table S4.1 (continued)

Assay name	Assay type	Gene information or infectious agent name	EST/Accession#	Primer and probe sequences	Efficiency	Source
pa_pse	Parasite	*Parvicapsula pseudobranchicola*		F - CAGCTCCAGTAGTGTATTTCA	0.82	(Jørgensen *et al.*, 2011)
				R - TTGAGCACTCTGCTTTATTCAA		
				P - CGTATTGCTGTCTTTGACATGCAGT		
pa_kab	Parasite	*Parvicapsula kabatai*		F - GTCGGATGATAAGTGCATCTGATT	0.93	In house
				R - ACACCACAACTCTGCCTTCCA		
				P - TGCGACCATCTGCACGGTACTGC		
te_bry	Parasite	*Tetracapsuloides bryosalmonae*		F - GCGAGATTTGTTGCATTTAAAAAG	0.93	(Bettge *et al.*, 2009)
				R - GCACATGCAGTGTCCAATCG		
				P - CAAAATTGTGGAACCGTCCGACTACGA		
pa_min	Parasite	*Parvicapsula minibicornis*		F - AATAGTTGTTTGTCGTGCACTCTGT	0.87	(Hallett and Bartholomew
				R - CCGATAGGCTATCCAGTACCTAGTAAG		
				P - TGTCCACCTAGTAAGGC		
sp_des	Parasite	*Sphaerothecum destruens*		F - GCCGCGAGGTGTTTGC	0.80	In house
				R - CTCGACGCACACTCAATTAAGC		
				P - CGAGGGTATCCTTCCTCTCGAAATTGGC		
sp_sal	Parasite	*Spironucleus salmonicida*		F - AACCGGTTATTCGTGGGAAAG	0.92	In house
				R - TTAACTGCAGCAACACAATAGAATACTC		
				P - TGCCAGCAGCCGCGGTAATTC		
ic_hof	Parasite	*Ichthyophonus hoferi*		F - GTCTGTACTGGTACGGCAGTTTC	0.85	(White *et al.*, 2013)
				R - TCCCGAACTCAGTAGACACTCAA		
				P - TAAGAGCACCCACTGCCTTCGAGAAGA		
na_sal	Fluke	*Nanophyetus salmincola*		F - CGATCTGCATTTGGTTCTGTAACA	0.91	In house
				R - CCAACGCCACAATGATAGCTATAC		
				P - TGAGGCGTGTTTTATG		



7.4 **Fig. S1.1**

Conceptual diagram of general experimental approach and timeline, which pairs long-terms holding (weeks-months) and telemetry studies to characterize impacts of thermal and fishery stressors and infection development on survival, health and behavior of adult Pacific salmon. Held fish were biopsied for gill and blood weekly, while tagged fish were biopsied before release and then tracked in the river until the spawning period or death (radio telemetry, stationary receivers and mobile tracking). Both study types applied a fishery stressor (bycatch simulation: gillnet entanglement, air exposure); held fish were held at optimal (historic), warm (climate change, projected increases), or dynamic (thermal experience of successful migrant in river) temperatures.



7.5 **Fig. S3.1**

Pathogen richness, relative infection burden (RIB), and relative loads of infectious agents detected using qPCR in a pool of organ tissues from female and male Chilliwack River coho salmon. Lines designate treatments (dotted = control, dashed = biopsied control, solid = gillnet-treated) and color denotes temperature (red = 15 ˚C, blue = 10 ˚C). Dot plots are used to show prevalence differences. Only surviving fish are included.