THE EFFECTS OF ELEVATED WATER TEMPERATURE ON ADULT PACIFIC SALMON GENE EXPRESSION, BLOOD PHYSIOLOGY AND MORTALITY

by

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ABSTRACT

Many populations of Pacific salmon (*Oncorhynchus* spp.) now encounter warmer rivers during their once-in-a-lifetime spawning migrations, which can result in premature mortality and may have contributed to some of the population declines observed in recent decades. One objective of this thesis was to determine the effects of high water temperatures on wild caught adult sockeye (*O. nerka*) and pink (*O. gorbuscha*) salmon at the level of the individual, tissue and cell through a series of temperature exposure studies. Fish held at 19°C consistently had higher mortality compared with fish held at a cooler temperature (13°C or 14°C) for both species with sockeye salmon demonstrating sex-specific mortality patterns. Sockeye salmon had higher plasma chloride levels when held at 19°C and both sockeye and pink salmon had reduced levels of plasma sex steroids at 19°C compared with fish at 13°C, which suggests an osmoregulatory disturbance and a potential reproductive consequence, respectively. Sockeye and pink salmon held at 19°C showed evidence of a common cellular stress response in their gill transcriptome characterized by the upregulation of genes associated with heat shock, immune and oxidative stress responses, variable regulation of genes involved in protein biosynthesis and the downregulation of genes involved in ion transport.

The second objective of this thesis was to characterize some of the physiological effects of senescence and mortality on Pacific salmon. I found dramatic declines in plasma osmolality, chloride and sodium levels that occur days in advance of a fish's death and determined that plasma chloride levels are strong predictors of longevity in maturing sockeye salmon. I also examined the effects of mortality on the transcriptome of sockeye salmon for the first time. Dying fish were characterized by an upregulation of several transcription factors associated with apoptosis and the downregulation of genes involved in immune function and antioxidant activity, consistent with immunosuppression.

Many of these results, especially the gene expression results, are novel for Pacific salmon. This thesis greatly improves our knowledge of the physiological effects of high river water temperatures and senescence on adult Pacific salmon. Management implications of these results and future research directions are discussed.

PREFACE

Chapter 2: Temporal changes in blood variables during final maturation and senescence in male sockeye salmon *Oncorhynchus nerka*: reduced osmoregulatory ability can predict mortality.
Authors: KM Jeffries, SG Hinch, MR Donaldson, MK Gale, JM Burt, LA Thompson, AP Farrell, DA Patterson, KM Miller.
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Chapter 3: Sex and proximity to reproductive maturity influence the survival, final maturation, and blood physiology of Pacific salmon when exposed to high temperature during a simulated migration.
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Chapter 4: Consequences of high temperatures and premature mortality on the transcriptome and blood physiology of wild adult sockeye salmon (*Oncorhynchus nerka*).
Authors: KM Jeffries, SG Hinch, T Sierocinski, TD Clark, EJ Eliason, MR Donaldson, S Li, P Pavlidis, KM Miller.
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DEDICATION

This thesis is dedicated to my Mom and Dad - thanks for everything.

Chapter 1: Introduction

1.1 Background – climate change and Pacific salmon

Temperature has a major influence on an ectotherm's biology and is often a primary factor in determining a species' distributional limits. Global climate change will have dramatic impacts on local biodiversity and species distributions due to predicted increases in temperature (Graham and Harrod 2009). Determining the consequences of global climate change has been described as a "grand challenge" in ecology (Thuiller 2007). Mean global temperatures could increase by as much as 4°C by 2100, which will result in many organisms experiencing an overall increase in ambient temperature (IPCC 2007). The magnitude of change is predicted to differ depending on the geographic region considered and these spatially heterogeneous changes may be the most relevant in terms of the ecological impacts of climate change (Walther et al. 2002).

Global temperature changes are predicted to influence many hydrological processes, which include precipitation patterns, the timing and magnitude of snowmelt run-off, and other general mechanisms of global water transport (Ferrari et al. 2007). However, a major result of global climate change will be an increase in mean surface water temperatures in both marine and freshwater environments, which can affect individual organisms, alter population size and structure, and change community species composition (Portner and Knust 2007). It can be assumed that warming temperatures will affect species differently depending on the relative thermal tolerances of the species considered (Graham and Harrod 2009). Although some eurythermal fishes may be able to simply tolerate some changes in temperature, it has been suggested that fishes living in moderately variable environments will be the most adaptable to warming water (Tomanek 2010). Stenotherms and species that are already near their species-specific thermal limits will likely be the most affected by increases in ambient temperature (Tomanek 2010). Because of the increases in temperature that many organisms will face due to climate warming, understanding how they will physiologically respond to temperature has become a fundamental research focus in conservation biology.

Climate warming will likely adversely affect anadromous species such as Pacific salmon (*Oncorhynchus spp*.) as both marine and freshwater environments will be impacted. The Pacific Ocean has undergone a net warming in recent decades and this trend is expected to continue (Levitus et al. 2000; IPCC 2007). Temperatures in the Northeast Pacific Ocean are predicted to increase 2-4°C in the next 50-100 years (Rand et al. 2006). Further increases in sea surface temperatures may compress the ocean distributions for Pacific salmon or force species to adjust their distribution patterns to maintain more favourable conditions (Welch et al. 1998). This is already occurring in Pacific salmon as some species are expanding their natural range in the Arctic Ocean (Babaluk et al. 2000). This along with a concomitant reduction in food production could increase competition, leading to lower growth rates and smaller adult Pacific salmon returning to freshwater streams (Hinch et al. 1995). In freshwater systems, climate change could result in higher peak summer temperatures directly due to heating, and indirectly due to reduced flows occurring during the hottest parts of the year (Ferrari et al. 2007; Morrison et al. 2002). For example, in the Fraser River, British Columbia, Canada, peak summer temperatures have increased approximately 1.5°C since the 1940's and 13 of the past 20 summers have been the warmest on record (Patterson et al. 2007; eWatch 2011). Global climate models predict that the Fraser River summer water temperatures will increase approximately 0.12°C each decade from 2000 to 2100 (Ferrari et al. 2007). Peak flows in the Fraser River are also expected to occur earlier in the year (Morrison et al. 2002), which can result in warmer temperatures after the river subsides post-freshet. Although these types of predicted changes could have profound effects on future behaviour and survival of all life stages of Pacific salmon, adult migrating Pacific salmon may be particularly affected as many populations are already encountering temperatures that are near or exceed their thermal maximum for aerobic performance (Eliason et al. 2011).

1.2 Life history overview of sockeye and pink salmon

To understand the potential effects of elevated water temperature on Pacific salmon, it is important to briefly introduce their life histories and physiologically challenging up-river spawning migrations. Pacific salmon have very diverse life histories that are variable both among and within species (i.e., among populations). There is no single life-history strategy for all species of Pacific salmon (Groot and Margolis 1991). However, there are general patterns that

exist that can be exemplified by sockeye (*O. nerka*) and pink (*O. gorbuscha*) salmon, which are the species that are the focus of this thesis. *O. nerka* have both anadromous ecotypes referred to as sockeye salmon, and a derived freshwater-resident ecotype called kokanee (Wood et al. 2008). Sockeye salmon undergo substantial migrations at multiple stages of their life histories. Sockeye salmon eggs hatch and the alevins stay under gravel until their yolk-sac is absorbed, then they emerge as fry and move from natal streams (or lakes). Most fry reside in nursery lakes to grow for 1-2 yrs, after which they prepare for saltwater through the process of smoltification, and migrate to the North Pacific Ocean (Burgner 1991). However, there is also a 'sea-type' ecotype of sockeye salmon that migrate to the ocean immediately after emergence (Wood et al. 2008). Most sockeye salmon start to mature after two to three years in the North Pacific Ocean (Burgner 1991) and then begin to migrate back along coastal waters towards their natal streams (Cooke et al. 2006). Migrating Pacific salmon then transition from saltwater into freshwater and complete their spawning migration back to natal spawning grounds (Groot and Margolis 1991). Sockeye salmon generally demonstrate high fidelity to their natal streams and there is little genetic evidence of straying (Gustafson and Winans 1999; Withler et al. 2000).

In contrast to sockeye salmon, pink salmon life histories are similar among populations (Heard 1991). Anadromous pink salmon also undergo substantial migrations throughout their life cycle and many of the physiological changes that occur may be similar to those of sockeye salmon. The typical pink salmon life history is more similar to 'sea-type' sockeye salmon than any other sockeye salmon ecotype. Rather than reside in a nursery lake for any period of time, pink salmon immediately migrate to the ocean, feed for approximately eighteen months and return to their natal stream to spawn (Heard 1991). Because pink salmon may spawn in tidally-influenced reaches of a river (Heard 1991), they may already possess the physiological ability to tolerate saltwater upon emergence from the gravel (e.g., Grant et al. 2009). Pink salmon exhibit more straying behaviour (Wood et al. 2008) and show fewer molecular/biochemical differences among spawning populations (Wild Salmon Policy 2005) than sockeye salmon.

Adult Pacific salmon cease feeding prior to river entry and consequently complete their upriver migrations using endogenous energy stores (Hinch et al. 2006). In addition to fuelling an energetically demanding migration, adult Pacific salmon use finite energy stores for developing gonads and secondary sex characteristics during their upriver migration (Groot and Margolis 1991). Upon arrival at spawning grounds, spawning occurs at redd sites that are dug by females

that spawn one to several times within the redd (Groot and Margolis 1991). Because of their semelparous life history, death occurs shortly after spawning in Pacific salmon (Groot and Margolis 1991). Interestingly, the ultimate cause of death is still unknown, but disease and parasite progression (Gilhousen 1990; Bradford et al. 2010), osmoregulatory dysfunction (Shrimpton et al. 2005), energy exhaustion (Dickhoff 1989) and organ deterioration (Finch 1990) have all been suggested as causal factors. Pacific salmon senescence and mortality will be reviewed further below.

1.3 Senescence and Pacific salmon

The interpretation of physiological responses of wild adult Pacific salmon to different migration temperatures can be complicated by the potential interactions and confounding processes of starvation, the energetic demands of an upriver migration and sexual maturation. A further complicating issue is senescence - the process of aging. There may be a variety of potential causes (or effects) of senescence in Pacific salmon, however it has been characterized by a loss of osmoregulatory ability (Shrimpton et al. 2005; Hruska et al. 2010), immunosuppression, energy exhaustion and organ deterioration (reviewed in Dickhoff 1989). Much of the Pacific salmon senescence research has addressed the period of rapid deterioration of health and eventual death that occurs post-spawning (Carlson et al. 2004; Hendry et al. 2004; Morbey et al. 2005). Recent work has monitored changes in blood properties for an individual sockeye salmon upon arrival at spawning grounds and as those same fish became moribund (Hruska et al. 2010). This study showed that moribund fish were characterized by reduced levels of plasma ions along with increased plasma cortisol and lactate compared with values upon arrival at the spawning grounds (Hruska et al. 2010). This added an important temporal component to the study of senescence in Pacific salmon; however, any potential changes in physiological processes prior to arrival on spawning grounds remain unknown. Some studies have examined changes in blood and enzyme activity (Shrimpton et al. 2005), along with changes in gene expression (Flores et al. 2012) at various points along the migration. These studies suggest an overall loss of osmoregulatory ability throughout the freshwater portion of the spawning migration. However, these studies were performed on different fish from the same population and therefore represent a snapshot of the physiological processes occurring at that

stage of the migration. Any effects of senescence inferred by those studies are confounded by responses to other environmental and capture stressors that have influenced those fish prior to sample collection.

It is possible that the process of senescence begins as early as during the rapid reproductive development that occurs throughout upriver migrations, the transition from saltwater to freshwater, or even when the fish cease feeding during their coastal migration. The degeneration of Pacific salmon health may be related to elevated sex steroid and cortisol levels associated with sexual development and migration (Dickhoff 1989). Plasma sex steroid and cortisol levels increase upon return to freshwater, and plasma levels peak sometimes several hundred kilometres before reaching natal spawning grounds (Williams et al. 1986; Hinch et al. 2006). The interaction between sexual development and senescence was identified by castrating immature kokanee and the castrated fish survived one to four years longer than non-castrated kokanee (Robertson and Wexler 1962). Interestingly, the level of organ degeneration in moribund castrated kokanee was similar those in naturally spawning kokanee, which indicates that organ degeneration may be characteristic of Pacific salmon mortality (Robertson and Wexler 1962).

Elevated cortisol during reproductive development comes at a cost as it may contribute to the degeneration of the kidney, liver and spleen, overall immunosuppression and an inability to maintain homeostasis observed in senescing Pacific salmon (Dickhoff 1989; Finch 1990). The cortisol increase during final maturation is likely genetically programmed in Pacific salmon because kokanee also show elevated cortisol levels despite not undertaking a long anadromous migration (Carruth et al. 2000). However, plasma cortisol may be required for the olfaction necessary to recognize and complete the migration to the natal stream (Carruth et al. 2002). If senescence is initiated through the elevation of plasma sex steroids and cortisol, it is possible that Pacific salmon senescence gradually occurs throughout the final upriver migration, followed by the rapid senescence that occurs post-spawning. It has also been suggested that oxidative stress contributes to mortality in senescing fish because of the activation of an oxidative stress response in Pacific salmon on spawning grounds (Sawada et al. 1993; Miller et al. 2009). It is conceivable that information on the plasma sex steroids and cortisol levels of an individual fish, along with indices associated with an oxidative stress response might provide insight into the stage of senescence for a particular fish.

Information is sparse on long-term physiological changes in an individual fish during natural senescence. It is virtually impossible to fully tease apart the interactions between processes associated with starvation, migration, maturation, environmental conditions and senescence in field-based studies. Therefore laboratory-based studies that characterize senescence patterns in Pacific salmon may help disentangle physiological processes associated with senescence and other environmental stressors. It is possible that because water temperature will alter an individual's metabolic rate, this could potentially influence rates of senescence, or the physiological processes related to senescence (Morbey et al. 2005), although this has not been shown experimentally. It is currently unknown whether temperature influences rates of senescence or whether natural senescence influences the thermal stress response of Pacific salmon.

1.4 Thermal challenges and adult Pacific salmon migrations

Most fishes are ectotherms and consequently experience variable body temperatures at diurnal, seasonal and annual timescales. Therefore, many fishes must physiologically or behaviourally respond to these variable temperature regimes in order to maintain necessary cellular functions. As ectotherms, the internal body temperature of fish is generally the same (or slightly higher) than that of the water the fish inhabits, therefore the optimal environmental temperature for a fish is the temperature best suited for all the fish's physiological and biochemical processes (Brett 1956). The biochemical and enzymatic processes have adapted to perform optimally at species-specific temperatures and it is these biochemical processes that may dictate natural distributional limits for aquatic ectotherms (Somero and Hofmann 1997). Temperatures that go beyond the species-specific thermal optimum, particularly those that go above the optimum, can cause stress to a fish. Temperature stressors may vary in severity (i.e., mild to severe) and be acute (i.e., minutes to hours) or chronic (days to weeks) in duration. These factors dictate the degree of response by an organism to the temperature stressor and factor into the ability of the organism to tolerate the stress.

Exposure to high temperatures by adult Pacific salmon during freshwater spawning migrations can cause a myriad of physiological and behavioural effects, which can have survival implications. High temperature exposure during the adult spawning migration is associated with

increased rates of mortality in migrating (termed *en route* mortality) sockeye (Macdonald et al. 2000; Keefer et al. 2008; Macdonald et al. 2010; Martins et al. 2011), pink (reviewed in Heard 1991), and Chinook salmon (*O. tshawytscha*; Keefer et al. 2010). There can also be a 'delayed' mortality effect in years of high temperature as adult Pacific salmon may survive thermally stressful upriver migrations, yet still suffer mortality on spawning grounds prior to spawning (termed prespawn mortality; Gilhousen 1990; Quinn et al. 2007; Taylor 2008). There have been only a handful of laboratory-based studies which have confirmed that high temperatures cause elevated mortality in adult sockeye (Servizi and Jensen 1977; Crossin et al. 2008; Gale et al. 2011) and pink salmon (Jensen et al. 2004). Absent from these studies was a quantitative estimate of the differences in mortality that occur due to exposure to different temperature treatments. High-temperature exposure studies are relatively rare, likely due to logistical difficulties associated with holding senescing, maturing adult Pacific salmon in captivity (e.g., diseases and infrastructure limitations).

Nowhere has the high temperature migration issue been more pronounced than with Fraser River Late-run sockeye salmon (Hinch et al. 2012). Since 1996, segments of all Late-run populations (and segments of pink salmon and Chinook salmon runs) have begun upriver spawning migrations 2-6 weeks earlier than their historical normal time. These early migrating sockeye salmon may experience river temperatures up to 5-6°C warmer than normal timed migrants; temperatures often exceed 20°C for early migrating sockeye salmon, which is considered highly stressful for sockeye salmon (Macdonald et al. 2000). Associated with the early migration is extremely high premature (*en route* and prespawn) mortality ranging from ~50-95% (Cooke et al. 2004; Hinch et al. 2012). Although the causes of the earlier upriver migration are still unknown, exposure to high water temperature is the overarching cause of the high premature mortality (Hinch et al. 2012). It is not known whether adult Fraser River pink and Chinook salmon exhibit increased levels of *en route* mortality associated with earlier migrations, although incidences of high premature mortality associated with elevated water temperatures have been reported in other systems (Heard 1991; Keefer et al. 2010).

Several recent studies have suggested that thermal-based mortality in adult Pacific salmon could be sex-specific. Specifically, migrating female sockeye salmon suffered much higher mortality than males at high temperatures (Martins et al. 2012). Other studies on sockeye and Chinook salmon have also observed higher rates of mortality in females compared with

males that was associated with temperature (Crossin et al. 2008; Keefer et al. 2010; Gale et al. 2011). The trend of sex-specific mortality has also been demonstrated in sockeye salmon exposed to other stress events such as variable flow conditions (Patterson et al. 2004; Nadeau et al. 2010), and the ascension of a fishway (Roscoe et al. 2011). In general, when migration conditions or laboratory treatments become highly stressful, females tend to have disproportionately higher mortality rates. Female sockeye salmon have higher routine heart rates than males when confined, which may reduce their scope in heart rate and their ability to tolerate additional stressors (Sandblom et al. 2009). Cortisol levels are naturally elevated in maturing female sockeye salmon compared with males (Macdonald et al. 2000; Patterson et al. 2004; Crossin et al. 2008; Sandblom et al. 2009; Clark et al. 2010). This may indicate an already heightened basal cortisol level that reduces the ability of females to cope with additional stressors, such as capture and confinement. Elevated cortisol in adult female sockeye salmon may serve to mobilize energy stores to fuel the migration and relatively greater reproductive investment of females compared with males (Mommsen et al. 1999). Presently, the exact physiological mechanisms responsible for the higher mortality in female Pacific salmon remain unknown, although higher basal cortisol levels and susceptibility to cumulative stresses may play a role.

Many studies that have examined the effects of elevated water temperatures on fishes have measured the temperature-mediated effects on metabolic performance. Because fishes are ectothermic, there is a temperature-induced increase in their resting/routine metabolic rate (e.g., Brett 1971). The difference between routine and maximum oxygen consumption during aerobic activity, aerobic scope (Fry 1947), is influenced by temperature, and aerobic scope has been recommended as a tool in determining the thermosensitivity of fishes (Portner 2002; Portner and Knust 2007). The aerobic scope concept suggests that there is an optimal temperature for aerobic activity. Temperatures that exceed the optimal temperature result in reduced aerobic performance and an eventual collapse in aerobic capabilities (termed the critical temperature) where fish switch to unsustainable anaerobic respiration (Portner 2002). The reduced aerobic scope is due to the inability of the fish's circulatory and ventilatory systems to match oxygen demand in the tissues (Portner and Knust 2007), which impacts an individual's ability to tolerate increased temperatures (Farrell et al. 2008). This approach has also been used to determine the thermosensitivity of Fraser River Pacific salmon populations (Lee et al. 2003; Clark et al. 2011;

Eliason et al. 2011). These studies have demonstrated that there may be species- and population-specific thermosensitivities in aerobic performance. However, these studies have utilized acute exposures to high water temperatures, therefore the physiological effects of chronic exposure to sub-lethal water temperatures for Pacific salmon still remain relatively unknown and descriptions of possible effects have been largely speculative.

High temperatures that lead to increases in adult Pacific salmon metabolic rates may lead to exhaustion of finite energy reserves faster than at cooler temperatures. In years of extremely high temperatures, energy reserve depletion is a possible cause of *en route* mortality for Fraser River sockeye salmon (Rand et al. 2006). Moreover, temperatures in extreme years may reach levels under which aerobic metabolic performance is impossible and this can prevent or delay successful migration (Farrell et al. 2008). Delays in migration can increase the duration of exposure to high water temperatures and extend freshwater residency times, two factors that can elevate the risk of premature mortality in Pacific salmon (Hinch and Martins 2011).

Pacific salmon are exposed to a suite of diseases and parasites after transitioning from saltwater to freshwater during spawning migrations (Rucker et al. 1954), many which have temperature-dependent progressions [e.g., *Flexibactor columnaris*, *Parvicapsula minibicornis* and *Saprolegnia* spp. (Servizi and Jensen 1977; Gilhousen 1990; Crossin et al. 2008; Bradford et al. 2010)]. Water temperature can have a large effect on the immune system of ectotherms. The two main immune responses of fishes are the rapid innate (non-specific) immune response (recognizes non-self and 'danger' signals), and the subsequent slower acquired/adaptive (specific) immune response (Magnadottir 2006) involved in resistance to particular pathogens. Cold water is believed to be immunosuppressive for the specific immune response in teleost fishes (Bly and Clem 1992). This immunosuppression may be counteracted by the temperature-independent innate immune system (Le Morvan et al. 1998; Magnadottir 2006). Warm water on the other hand may be immunostimulatory for the specific immune response (Perez-Casanova et al. 2008). Due to the exposure to temperature-dependent diseases upon returning to freshwater combined with the immunostimulatory nature of warm water temperatures, it could be expected that Pacific salmon that migrate during warm water periods would show evidence of an activated adaptive immune system.

Migrating adult Pacific salmon have several ways they can respond to increasing river temperatures in order to slow parasite or disease progression, reduce their overall metabolic rate

and energy expenditure, and increase survival. Pacific salmon may attempt to avoid stressful high temperatures by entering freshwater before or after the peak summer river temperatures (Hodgson and Quinn 2002); although temperature may not inhibit migration for pink salmon (Heard 1991). For a phenological response like altered migration timing, spawn-timing constraints will limit how broadly such a behavioural change could occur across populations (Reed et al. 2011). Alternatively, they may actively seek out cool water refuges in lakes or tributaries during migrations (Hodgson and Quinn 2002; Goniea et al. 2006; Keefer et al. 2008; Mathes et al. 2010; Roscoe et al. 2010). The physiological consequences of these forms of behavioural thermoregulation for Pacific salmon are still unknown; however, utilizing thermal refuges has been linked with improved survival. For example, in 2004, early-entry Weaver Creek sockeye salmon in British Columbia experienced Fraser River temperatures of ≥20°C and all perished unless they occupied the cool hypolimnion (~6.5°C) of nearby Harrison Lake, British Columbia, during the final stages of maturation (Mathes et al. 2010). Over 80% of the time in Harrison Lake was spent in deep water at temperatures of ~6.5°C (Farrell et al. 2008), which suggests that these fish were preferentially selecting cooler water. Because most sockeye salmon populations utilize lakes as natal rearing areas, adults can transit through or reside in lakes while maturing so they have the potential to readily utilize a lake hypolimnion as a thermal refuge. A final way Pacific salmon could respond to warming temperatures is to physiologically adapt to them. While there is no strong evidence that physiological adaptation has occurred in response to recent warming, population level variability in thermal optima for aerobic performance exists (Eliason et al. 2011), which suggests that physiological adaptation may be possible.

Species-specific differences in adult thermal tolerances are believed to have evolved in Pacific salmon (Crozier et al. 2008). For example, laboratory studies have found that pink salmon have higher survival at high temperatures (Jensen et al. 2004) compared with sockeye salmon (Crossin et al. 2008). Pink salmon are also known to spawn across a wider range of temperatures than sockeye salmon (Heard 1991). Swim tunnel respirometry indicates that pink salmon have higher critical temperatures for aerobic scope than sockeye salmon (Clark et al. 2011). Collectively, these studies suggest a higher thermal tolerance in pink salmon compared with sockeye salmon. Fishes are generally adapted to the water temperatures that they routinely experience (e.g., Beitinger et al. 2000; Fangue et al. 2006; Portner and Knust 2007), and the possibility that populations within a species may also differ in thermal tolerance was recently

explored. Swim tunnel respirometry studies found considerable variability in thermal tolerance among populations of Fraser River sockeye salmon with peak swim performance and aerobic capabilities that correlate with historically encountered temperatures (Lee et al. 2003; Eliason et al. 2011). Therefore, conclusions about species-specific thermal tolerance likely require a population level rather than just a species level perspective.

1.5 The generalized stress response

Below, I will review some of the effects that warm water temperatures have on the physiology of fish with a focus on salmonids, but to aid in that discussion, I will first provide a brief review of the general 'stress response' in fish. It is important to note that there are many different adaptations to, and therefore different responses to, environmental stressors in fishes and the following descriptions can be viewed as an overview of some of the common whole-organism, tissue and cellular responses to stress. This is not an exhaustive description of all the specialized responses that may occur in different fishes. Despite the knowledge that many environmental stress responses converge to cellular responses associated with low oxygen and oxidative stress conditions (Kassahn et al. 2009), each environmental stressor has the potential to induce a unique response that may or may not be species-specific. This is likely reflective of the tolerance to the stressor of the fish considered. For example, high-stress responders of rainbow trout (*Oncorhynchus mykiss*) will exhibit different cellular responses to a confinement stressor than low-stress responders (Pemmasani et al. 2011). There can also be variable cellular responses to acute temperature stress in eurythermal fish versus a stenothermal fish species [e.g., the longjaw mudsucker (*Gillichthys mirabilis*; Logan and Somero 2010) compared with Arctic charr (*Salvelinus alpinus*; Quinn et al. 2011a)]. However, there are some common responses to certain stressors across taxa (Kultz 2005). Additionally, the context of a study can be important as there can be a greater stress response in wild individuals compared with laboratory-confined individuals (Somero and Hofmann 1997).

A stressor may be loosely defined as a stimulus that perturbs an organism's homeostasis, and initiates a coordinated behavioural and physiological response that enables the organism to overcome the stress event (Wendelaar Bonga 1997). A behavioural response, in its simplest form, is for the organism to move or avoid the stressor, such as when migrating Pacific salmon actively

seek cooler thermal refuges during periods of warm water temperatures. It is the physiological responses to stress that commonly manifest at multiple levels of biological organization and can potentially affect behaviour, feeding, and reproduction. The physiological stress response in fishes can be broken down into three main components. The primary stress response occurs with the release of catecholamines and corticosteroids into the blood, which mainly drives the 'fight or flight' response in organisms (Wendelaar Bonga 1997). The secondary stress response is the tissue level response to the increase in catecholamines and corticosteroids in the blood, where the tertiary stress response is the effect of the stressor at the level of the individual (Wendelaar Bonga 1997).

The corticosteroid cortisol is considered the main stress hormone in fishes and increases in the blood plasma after a stress event. Cortisol also influences many physiological processes in fishes, which include metabolic, osmoregulatory, growth and reproductive processes (Wendelaar Bonga 1997) and therefore increases in blood plasma cortisol have many effects on a fish. In general, the stress response of an organism is adaptive; however, during periods of chronic or extreme stress, prolonged activation of the stress response can become detrimental to fish health (Wendelaar Bonga 1997; Schreck 2010), which could partly be due to the high levels of plasma cortisol. For example, prolonged activation of a stress response and high plasma cortisol levels can be immunosuppressive, which results in increased susceptibility to diseases and parasites (Schreck et al. 2001). Interpretations of cortisol as purely a stress hormone in salmon research may be confounded by the fact that cortisol has a major influence on reproductive processes and migration in Pacific salmon (Carruth et al. 2002; Milla et al. 2009). Although cortisol is primarily considered a stress hormone, its myriad of other physiological roles in fish suggests that a stress-induced change in plasma cortisol may have implications beyond simply the recovery from a stress event.

1.6 The cellular stress response

There are many general cellular stress responses (CSR), which may or may not be induced by extracellular signals, that can be activated by a wide variety of environmental stresses (Kassahn et al. 2009). A physiological stress response often occurs at the level of the cell in the form of a relatively rapid response followed by a slower cellular homeostasis response (Kultz

2005). The CSR is remarkably conserved across taxa, with commonalities occurring even between humans and bacteria in regards to how cells respond to certain stress events (Kultz 2005). An example of this is the heat shock response, which involves the induction of heat shock proteins (HSPs) and is ubiquitous in cells exposed to temperature stress (Feder and Hofmann 1999). Broadly, HSPs are molecular chaperones that are involved in protein folding associated with many cellular functions and levels may increase during periods of cellular stresses (Feder and Hofmann 1999; Iwama et al. 1999). Temperature stress may lead to the unfolding of proteins and stress-inducible HSPs (e.g., HSP70) are often activated as a response to the unfolded proteins (Somero and Hofmann 1997; Iwama et al. 2004). The magnitude of the HSP response in fish may be specific to the tissue sampled, the acclimation temperature of the fish and the HSP measured (Currie et al. 2000). A limitation to measuring the HSP response is that it does not provide an indication of how much of the unfolded protein is reversible versus irreversible, which can have major implications on the overall cellular energy budget (Somero and Hofmann 1997). Many HSPs are involved in physiological processes other than responding to unfolded proteins. For example, HSPs are involved in supporting components of enzymes, steroid hormone receptors and the cytoskeleton (e.g., HSP90), preventing protein aggregations (e.g., low-molecular-mass HSPs; Iwama et al. 2004) and collagen binding during development (HSP47; Krone et al. 1997). In general, HSPs are important in protein and cell homeostasis in a CSR and can indicate exposure to water temperatures that are stressful to a fish. The heat shock response is therefore an important indicator of the degree of temperature stress experienced by organisms and can be used to predict the ability of organisms to adapt to changing thermal regimes (Tomanek 2010).

Intense stressors can also result in increased production of reactive oxygen species (ROS) that can initiate an oxidative stress response (Martinez-Alvarez et al. 2005). Exposure to high water temperatures that raises aerobic respiration due to temperature-induced increases in metabolic rate can cause the production of ROS (Portner 2002), which can cause oxidative damage to lipids, proteins and DNA within tissues (Lesser 2006). Fish exposed to extreme water temperatures must then activate their antioxidant defences to protect their cells from damage caused by oxidative stress (Martinez-Alvarez et al. 2005). Failure to properly respond to oxidative stress may result in irreversible damage to cell structure and function (Lesser 2006). Severe oxidative stress can cause an influx of Ca^{2+} into the cytoplasm from the extracellular

environment and from the cell Ca^{2+} storage compartment, the endoplasmic reticulum (Ermak and Davies 2002). Cellular Ca^{2+} is used in a wide variety of cell signalling pathways, which include pathways involved in cell proliferation and apoptosis (Ermak and Davies 2002). Therefore, cellular Ca^{2+} and ROS levels may be indicative of intense cellular stress.

The endoplasmic reticulum is a key organelle involved in major stress-induced cellular responses. Acute and chronic cellular stress can lead to a build up of unfolded proteins in the endoplasmic reticulum, which activates the unfolded protein response (Rutkowski and Kaufman 2004). The unfolded protein response, like other cellular responses, is characterized by an activation phase, followed by an acute response, then either an adaptive response or an apoptotic response (Rutkowski and Kaufman 2007). During periods of extreme cellular stress, cells may undergo programmed cell death (Kultz 2005) by a variety of cell-signalling pathways that ultimately activate effector/executioner caspases during the final stages of apoptosis (dos Santos et al. 2008; Krumschnabel and Podrabsky 2009; Eimon and Ashkenazi 2010). In a temperature context, apoptosis may occur when organisms experience temperatures outside their thermal tolerance window.

During periods of sub-lethal chronic stress, cells may induce the cellular homeostasis response, which can be a permanent response to environmental change that remains until the environmental conditions are changed again (Kultz 2005). This could be considered in the same context as cellular acclimation. Ultimately, the magnitude of all CSRs is largely dependent on the severity and the duration of the stress event (Rutkowski and Kaufman 2007). Major physiological problems or even death may result if a stress event overwhelms the individual's cellular ability to cope with the stressor (Iwama et al. 2004). However, it is important to note that the significance of CSRs is often contentious as the link between cellular responses and individual level responses, such as growth, reproduction and survival, is not always clear (Kassahn et al. 2009).

To adapt to a warming climate, some species may be required to adapt at the cellular level to the changing thermal regime or risk extirpation or extinction. Over evolutionary timescales, changes in gene expression and physiological function can result in what has been described as biochemical adaptation (Hochachka and Somero 2002). Because of the importance of temperature on biological function, species have evolved species-specific thermal optima (Beitinger et al. 2000), which can limit the potential environments that an organism can inhabit

(Podrabsky and Somero 2004). Temperatures above a species-specific threshold can alter gene expression, which affects protein synthesis and protein function. Alterations in gene expression may lead to changes in the amount of protein produced (quantitative changes) or to changes in the type of protein produced (qualitative changes; Hochachka 1967; Schulte 2004). Quantitative changes in gene expression are often important in thermal acclimation (Schulte 2004), as the constituent levels of certain proteins and the onset temperature of thermally responsive proteins are crucial in determining a species' thermal tolerance (Tomanek 2010; Logan and Somero 2011). Because sometimes only small changes in protein sequence are required to cause a protein to become adapted to a new temperature range, it has been suggested that climate-induced protein evolution could be rapid and may result from relatively small increases in temperature (Somero and Hofmann 1997). Qualitative changes may also be involved in thermal acclimation as a response to temperature can result in utilizing one protein over another, however, this might influence the biochemical kinetics of what is functionally the same biochemical pathway (Hochachka 1967; Schulte 2004). Alterations in biochemical kinetics are believed to be partly responsible for differences in thermal tolerances in congeneric species (Somero and Hofmann 1997). Qualitative changes can manifest in the form of alternate isoforms of a protein from different loci or alternate splicing or promoter use that generate different isoforms from the same locus (Schulte 2004). Alternate isoforms from different loci have been suggested to be relatively more common in organisms that have undergone genome duplication events, such as salmonids, and expression levels of different isoforms that have become specialized for a particular environment are altered when an organism is exposed to a change in environmental conditions (Schulte 2004). An example of this is the different isoforms of Na^+/K^+-ATPase (the α sub-unit) in migratory salmonids (Shrimpton et al. 2005; Bystriansky et al. 2006; Bystriansky and Schulte 2011). These studies demonstrate that the α1a isoform is more dominant in the freshwater environment whereas the α1b isoform is more highly expressed in saltwater. It is possible that qualitative changes can contribute to a population's biochemical adaptation to a changing environment at evolutionary timescales. It is conceivable that the relatively recent genome duplication in salmonids has enabled some of the local adaptation to environmental conditions that has been observed in Pacific salmon species and populations.

1.7 Microarrays - a method to study cellular stress responses in fish

Alterations in gene expression levels are one of the earliest responses to cellular stress that can be relatively easily measured and therefore provide valuable information regarding cellular responses to stress events. Genomics technology has greatly improved our ability to detect large-scale changes in gene expression to facilitate our understanding of the complex CSR to abiotic environmental stresses. Microarrays are a technique for simultaneously screening the expression of hundreds to thousands of genes, which can be compared with conventional methods [e.g., Northern blots, quantitative real-time polymerase chain reaction (qRT-PCR)] that examine the expression of one to several gene(s) at a time. Major advantages of using microarray technology include the potential to identify groups of differentially expressed functionally similar genes that indicate pathways associated with a biological response (i.e., functional analysis), or the ability to identify strong biomarkers of exposure to environmental stressors that were previously not known to be associated with a response to that particular stressor. Additionally, by measuring these transcript changes at a genome-wide scale, it is possible to begin unravelling the true complexity of the CSR. Because of the large number of pathways screened by microarrays, they are a valuable explorative methodology that has been used to study genome-wide responses to various biotic and abiotic stresses in fishes and other organisms. It is important to acknowledge that there are limitations to microarray studies to go along with the many benefits. Studies that measure mRNA transcript levels are limited because mRNA transcript levels do not necessarily indicate relative protein levels, do not assess protein turnover rates in the cell, and cannot indicate the type and amount of post-translational modification and epigenetic interaction that occurs, which are all major processes involved in cell function (Cossins et al. 2006; Prunet et al. 2008).

Although the stress response in fishes has been well studied at various levels of biological organization, the molecular mechanisms that underlie the physiological response to stress are less understood (Prunet et al. 2008). The use of microarray technology to understand ecologically relevant responses to environmental stressors in fishes has increased in recent years, with the majority of studies examining responses to hypoxia, salinity changes or extreme temperatures (cold or hot). In fishes, there are many examples of temperature-response microarray studies that have applied acute (e.g., Buckley et al. 2006; Kassahn et al. 2007; Healy

et al. 2010; Hori et al. 2010; Lewis et al. 2010; Logan and Somero 2011; Quinn et al. 2011b) and chronic exposures (e.g., Gracey et al. 2004; Malek et al. 2004; Podrabsky and Somero 2004; Castilho et al. 2009; Logan and Somero 2010; Quinn et al. 2011a). The duration of temperature exposure will influence the type of cellular response detected, either a rapid CSR or a slower homeostasis response depending on whether it was an acute or chronic exposure, respectively. However, it has been argued that both chronic and acute exposures need to be examined, along with variability of exposure, to gain the most comprehensive understanding of a species' thermal tolerance (Schulte et al. 2011). Additionally, due to the contentious link between the CSR and whole-organism responses to stressors, it is still important to attempt to scale across levels of biological organization from the cellular response to the individual's fitness to be able to determine the true ecological relevance of a response to environmental stressors.

The development of salmonid microarrays through the Genomics Research on Atlantic Salmon Project (GRASP; von Schalburg et al. 2005) and Consortium for Genomic Research on All Salmon Project (cGRASP; Jantzen et al. 2011), along with salmonid genomics research in Europe, have made gene expression profiling more common in salmonid research. These salmonid microarrays were developed primarily using Atlantic salmon (*Salmo salar*) and rainbow trout gene sequences; however, they have been shown to be useful in genomics studies with other species of salmonid (Miller et al. 2009; Miller et al. 2011; Quinn et al. 2011a; Quinn et al. 2011b). The salmonid microarrays have been used to provide genome-wide responses to exposures to various diseases in Atlantic salmon (Miller et al. 2007; Roberge et al. 2007; Young et al. 2008) and rainbow trout (Baerwald et al. 2008), along with toxicological responses in juvenile rainbow trout exposed to diesel (Mos et al. 2008). Salmonid microarray techniques have also been used to evaluate the molecular underpinnings of ecologically relevant concepts such as the molecular basis of different reproductive behaviours (Aubin-Horth et al. 2005), identifying population structure of endangered Atlantic salmon populations for conservation purposes (Tymchuk et al. 2010), comparisons between different ecotypes of sockeye salmon (Pavey et al. 2011), and understanding complex physiological responses to migratory challenges (Miller et al. 2009; Evans et al. 2011; Miller et al. 2011). These studies demonstrate that the salmonid microarray can be successfully used for wild salmon gene expression profiling and can be a valuable tool in determining physiological changes that occur during periods of stress, such as exposure to elevated water temperatures.

1.8 Thesis objectives and thesis chapter overview

The main objective of my thesis was to determine the effect of elevated water temperature on survival, blood properties and gene expression of wild-caught adult sockeye and pink salmon. This was addressed though a series of temperature exposure studies conducted in three consecutive years (2007-2009). The broad *a priori* hypotheses and some general predictions for this objective are presented below:

1: High water temperature will increase sockeye and pink salmon mortality with pink salmon having the greater thermal tolerance between the species and females of both species will have a reduced thermal tolerance when compared with males.

-This hypothesis was tested by comparing survival patterns in a late-run sockeye salmon population and Lower Fraser River pink salmon in chapter 3. Using parametric survival analysis, quantitative differences in survival between temperature treatments and sexes were assessed. Controlled laboratory studies have not previously explicitly quantified the effects of water temperature on the sexes of two species of Pacific salmon. Details of mortality patterns were also presented in chapters 4 and 5.

2: High water temperature will elicit primary and secondary stress responses as revealed by increases in plasma concentrations of cortisol and glucose, decreases in sex steroid levels, and alterations in plasma ions and osmolality.

-This hypothesis was tested by comparing blood properties that may be indicative of stress or metabolic processes (e.g., cortisol, glucose and lactate), osmoregulatory ability (e.g., osmolality, chloride and sodium) and reproductive status (e.g., testosterone and 17β-estradiol) between groups of fish held at different temperatures in chapters 3 and 4. Very few data exist that demonstrate the potential effects of elevated water temperature on blood properties in Pacific salmon.

3: High water temperature treatments will elicit a CSR in sockeye and pink salmon characterized by an induction of a heat shock response (e.g., HSPs), alterations in expression of genes involved

in protein biosynthesis and metabolism, and an increase in the expression of genes associated with an immune response.

-This hypothesis was tested by using state-of-the-art microarray technology on non-lethally sampled gill tissue from summer-run and late-run sockeye salmon, and Lower Fraser River pink salmon populations in chapters 4 (using 16K gene cDNA microarrays) and 5 (using 44K gene oligonucleotide microarrays). This characterized the transcriptomic response to exposure to high water temperature in Pacific salmon for the first time. It was also the first comparison of thermal stress responses between different run-timings of sockeye salmon and pink salmon using molecular techniques.

My broad approach was to collect wild migrating Pacific salmon and expose them to ecologically relevant 'cool' (13°C or 14°C depending on the population studied) and 'warm' (19°C) temperature treatments for what would represent a significant portion of their freshwater migration. The 'cool' temperature treatment closely resembled the historical thermal conditions experienced in the Fraser River by Pacific salmon in September and early October. In contrast, the 'warm' temperature treatment represented an extreme situation that has frequently occurred in the Fraser River in recent years. After a specific period of time, I used rapid, non-lethal biopsy techniques (e.g., Cooke et al. 2005) to sample gill and blood tissue to examine the effects of water temperature on the gill transcriptome and blood properties. Because Pacific salmon migrating upstream often experience the warmest water temperatures for days to weeks depending on the population (English et al. 2005), I chose to sample the fish after 5-7 days of exposure to the temperature treatments to examine the chronic effects of high water temperature on the gill transcriptome and blood properties. I attempted to set up my experiments to examine the effects of water temperature at multiple levels of biological organization, from the cellular (transcriptome), tissue (blood), and the individual (survival) levels. Because of the relatively sparse data that exist, especially in terms of the molecular responses to water temperature in Pacific salmon, the microarray components of my thesis were exploratory in nature and present some of the first data on the subject. This limited my ability to formulate detailed predictions for the transcriptomic response to water temperature in advance of conducting the experiments; however some broad predictions based on common temperature-induced CSR in fishes were

possible. I attempt to connect these exploratory components of my thesis with the more hypothesis-driven experiments in the concluding chapter of this thesis.

To fully understand the effects of water temperature on Pacific salmon biology, it is first important to attempt to separate the potential effects of senescence on Pacific salmon physiology from the temperature-induced changes that may occur. Therefore, the second objective of my thesis was to examine the effect of senescence on blood properties and gene expression of wild-caught adult sockeye salmon. The broad *a priori* hypotheses and some general predictions for this objective are presented below:

4: As individual fish mature, there will be decreases in plasma ions and osmolality along with declining sex steroid levels that are characteristic of senescence in semelparous Pacific salmon.

> -To address this hypothesis in chapter 2, I sampled the same individual sockeye salmon multiple times over a period of six weeks to characterize the changes in blood properties during final maturation and senescence. This was the longest study that I am aware of that continuously monitored the physiological changes due to final maturation and senescence in Pacific salmon. It is also significant because these fish were collected prior to what would have been their 'arrival' on spawning grounds and therefore before many of the physiological changes that occur during final maturation.

5: Moribund Pacific salmon will have altered expression of genes involved in cellular processes associated with cell death (e.g., apoptosis, oxidative stress) that are detectable in the transcriptome of dying salmon. Additionally, there will be dramatic decreases in plasma ions and osmolality, and increases in plasma lactate and cortisol as these fish become moribund.

> -This hypothesis was addressed in chapter 4 by sampling sockeye salmon that became moribund for gill tissue and used microarray technology (16K gene cDNA microarrays) to examine the changes in the transcriptome. To my knowledge, this is the first study to examine the changes in gene expression associated with mortality in Pacific salmon. Chapter 2 and 4, characterized the dramatic changes in blood properties that occur as sockeye salmon become moribund.

As mentioned above, there is a paucity of data that demonstrate the effects of water temperature on Pacific salmon. However, there are even fewer data that describe the physiological effects of senescence and mortality on Pacific salmon blood properties and gene expression. Ultimately, the cause(s) of mortality in wild semelparous Pacific salmon remain(s) unknown; however, in this thesis, I attempted to elucidate some of the physiological processes associated with senescence and mortality in Pacific salmon. Where appropriate, I discuss the potential connection between processes involved in senescence and mortality, and how they might influence a salmon's ability to successfully respond to exposure to high water temperatures.

Chapter 2: Temporal changes in blood variables during final maturation and senescence in male sockeye salmon *Oncorhynchus nerka*: Reduced osmoregulatory ability can predict mortality

2.1 Synopsis

This study is the first to characterize temporal changes in blood chemistry of individuals from one population of male sockeye salmon during the final 6 weeks of sexual maturation and senescence in the freshwater stage of their spawning migration. Fish that died before the start of their historic mean spawning period (~ November 5) were characterized by a 20–40% decrease in plasma osmolality, chloride and sodium, probably representing a complete loss of osmoregulatory ability. As fish became moribund, they were further characterized by elevated levels of plasma cortisol, lactate and potassium. Regressions between time to death and plasma chloride (October 8: $P < 0.001$; October 15: $P < 0.001$) indicate that plasma chloride was a strong predictor of longevity in sockeye salmon. That major plasma ion levels started to decline 2–10 days (mean of 6 days) before fish became moribund, and before other stress, metabolic or reproductive hormone variables started to change, suggests that a dysfunctional osmoregulatory system may initiate rapid senescence and influence other physiological changes (i.e., elevated stress and collapsed reproductive hormones) which occur as sockeye salmon die on spawning grounds.

2.2 Introduction

When adult sockeye salmon migrate up-river to their natal spawning area, they have already ceased feeding, are rapidly maturing and are undergoing dramatic morphological changes in preparation for a single spawning event. Because sockeye salmon are semelparous, they die shortly after spawning. The rapid senescence that occurs post-spawning in adult Pacific salmon is characterized by immunosuppression and organ deterioration (Dickhoff 1989; Finch 1990). If however the fishes die before spawning, either in river (termed *en route* mortality) or

on the spawning grounds (termed prespawn mortality), lifetime fitness is zero. Prespawn mortality rates are highly variable but can reach 90% in some years (mean rates of 3.3–23.7%; Gilhousen 1990). There are many factors that potentially contribute to these mortalities [e.g., high water temperature (Crossin et al. 2008; Farrell et al. 2008), high discharge (Rand et al. 2006), parasites and disease (Gilhousen 1990; Jones et al. 2003), and possibly increased rates of senescence], but at present there are no reliable indicators to predict whether an individual arriving at a spawning area will in fact survive to spawn.

Rapid biosampling (Cooke et al. 2005) offers a snapshot of the physiological status of migrating Pacific salmon which includes characteristics associated with *en route* mortality (Cooke et al. 2006; Crossin et al. 2009). There is a decrease in plasma osmolality and chloride levels as adult sockeye salmon migrate in fresh water (Shrimpton et al. 2005), which suggests a progressively reduced osmoregulatory ability during freshwater spawning migrations. There also appears to be an increase in indices of stress during spawning ground residence (Hruska et al. 2010). The present study tested the hypothesis that individual fish would show signs of physiological impairment as they senesce. It was predicted that fish nearing death would show signs of reduced osmoregulatory ability, have elevated indices of stress and decreased sex steroid levels when compared with fish that survived to the mean historic spawning period. To test this hypothesis, the same individuals were repeatedly sampled for blood to characterize changes in blood chemistry associated with mortality and to quantify temporal patterns in blood chemistry during final maturation and senescence in adult male sockeye salmon.

2.3 Materials and methods

2.3.1 Fish collection and handling

Male sockeye salmon from the Harrison Rapids population were collected from the Harrison River, British Columbia, which is a major tributary of the Fraser River. Seventy-eight fish were collected on Chehalis First Nation's land downstream of Harrison Lake by beach seine from September 15-18, 2008 (Figure 2.1). Harrison Rapids sockeye salmon may enter the Fraser River 6–11 weeks prior to spawning in the Harrison River (Lapointe 2009) and hold in the

Harrison River or in Harrison Lake until spawning immediately downstream of the collection site. Fish were transported live to the Fisheries and Oceans Canada Cultus Lake Laboratory (~45 min by vehicle) and upon arrival, were PIT-tagged and an adipose fin clip was taken for DNA stock identification (Beacham et al. 2005). The PIT-tagged fish were held in a large ~20 000 l stock tank at ~10.5°C. Harrison River water temperature during collection ranged between 14 and 18°C. Eleven of the fish collected, used as controls for potential effects of repeated handling, were not PIT-tagged or immediately sampled for DNA and placed in separate tanks after being transported to the Cultus Lake Laboratory. On September 22–23, 2008, fish were removed by dip-net from the large stock tank and immediately sampled for blood. Fish were then placed into smaller ~8000 l tanks for ease of repeat sampling. The same individual fish were re-sampled on October 8, October 15 and November 5, 2008. Fish were not anaesthetized prior to blood sampling to attempt to reduce the handling time required to obtain the blood samples as many blood properties may change rapidly post-handling. Fish that no longer maintained equilibrium, but were still ventilating, were terminally sampled throughout the experiment and designated 'moribund'. Little is known about the migration behaviour of Harrison Rapids sockeye salmon, but co-migrating Weaver Creek sockeye salmon, which spawn in a tributary of the Harrison River near the capture site (Figure 2.1), are known to migrate into and occupy the cool hypolimnion of nearby Harrison Lake (~6 km upstream of capture site). Telemetry tracking studies have revealed that the use of cool water as a thermal refuge enhances chances of survival to spawning, especially for fish that encounter high river temperatures during migration (Farrell et al. 2008; Mathes et al. 2010). To mimic fish utilizing a thermal refuge, such as those available in Harrison Lake, water temperature was decreased to ~7°C on October 5. On November 5, 2008, which is approximately the beginning of the natural historic spawning period for Harrison Rapids sockeye salmon (peak spawning occurred between November 11 and 13, 2008), all fish, including control fish, were terminally sampled. Terminal sampling included a blood sample, as well as measurement of postorbital–hypural bone (POH) length (±0.1 cm), body mass (wet mass, ±10 g), body depth (±0.1 cm), liver and gonad mass (wet mass, ±1 g), and kype length (±0.1 cm). Kype length can increase throughout sexual maturation in male sockeye salmon and is considered a secondary sexual characteristic (Hendry and Berg 1999).

Fish were categorized into groups based on longevity during the holding period (Table 2.1). Group 1 died within a week of October 8, group 2 died after two rounds of sampling

between October 8 and 15, group 3 died after three rounds of sampling between October 15 and November 5 and group 4 survived until November 5, when they were terminally sampled. Only fish that survived a minimum of 2 days after being sampled were included in the survival group comparisons to minimize the potential for handling to have contributed to the subsequent mortality. Fish were only included in the survival group analysis if there was a moribund or terminal blood sample matched with other live samples for an individual fish. All fish were used to calculate regressions between time to death and plasma chloride.

2.3.2 Blood sampling

Blood samples (~3 ml) were taken from fish by caudal puncture using a 21 gauge needle and a heparinized vacutainer. Whole-blood samples were centrifuged for 6–7 min and plasma samples were placed into three 0.5 ml tubes for storage at −86°C until subsequent analysis. Plasma osmolality, ions (sodium, chloride, potassium), glucose and lactate were measured using the procedures outlined in Farrell et al. (2001). Plasma cortisol, testosterone and 17β-estradiol were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Neogen Corporation; www.neogen.com). Testosterone and 17β-estradiol samples were extracted in ethyl ether according to manufacturer's protocols. Cortisol, testosterone and 17β-estradiol samples were all run in duplicate at appropriate dilutions.

2.3.3 Statistical analysis

All statistical tests were performed using SAS software version 9.1 (SAS Institute; www.sas.com). Analysis of variance (ANOVA) was used to test for differences between groups for mass, body length and kype length. In all cases, homogeneity of variances was assessed by Bartlett's test and normality was tested using Kolmogorov–Smirnov tests (Sokal and Rohlf, 1995). Data were log10-transformed if the assumption of homogeneity of variances could not be met. Where the assumptions of normality could not be met (body depth only), the non-parametric Kruskal–Wallis test was used (Sokal and Rohlf, 1995). Mass and length relationships between groups were compared using analysis of covariance (ANCOVA) with length as the covariate.

Relative liver and gonad mass (as a percentage of mass) were compared using ANCOVA with adjusted mass (mass − organ mass) as the covariate.

Plasma variables were analysed using a repeated measures split-plot ANOVA, with the sample time within a group treated as the subplot and sample groups as the whole plot. Multiple pair-wise comparisons were made using the Bonferroni adjustment method (Zar 1999). There were 26 possible comparisons (within groups and between groups at a single sample time), which made a highly conservative critical $\alpha = 0.0019$ after the Bonferroni correction. When appropriate, significant differences before Bonferroni correction are discussed. Samples taken from fish that became moribund were only compared within their group. Comparisons between the survivors and the control fish on the day that the experiment was terminated (November 5, 2008) were assessed using *t* -tests. Data were log10-transformed if the assumption of homogeneity of variances was not met. If the assumption of normality could not be met, non-parametric Wilcoxon tests were used (Sokal and Rohlf, 1995). Relationships between plasma chloride levels and time to death post-sampling for each sampling date were assessed using linear regressions on log10-transformed data.

2.4 Results

2.4.1 Morphology

Among survival groups, there were no differences between length (ANOVA, $P > 0.05$), mass (ANOVA, $P > 0.05$) and kype length (ANOVA, $P > 0.05$; Table 2.1). When comparing mass and length relationships between treatment groups, the individuals from the control group were heavier for a given length than group 2 individuals (ANCOVA, $P < 0.001$). Individuals from group 4 (survivors) and control groups had greater body depth than individuals that died before November 5 (Kruskal–Wallis, $P < 0.001$). The slopes of the liver mass to adjusted mass relationships were different between individuals from the survivors and the group 2 individuals (ANCOVA, $P < 0.05$). Individuals from the control group had heavier gonads for a given mass than group 3 individuals (ANCOVA, $P < 0.001$).

2.4.2 Blood variables

There was an overall decrease in plasma osmolality (split-plot ANOVA, within group: $P < 0.001$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.001$), chloride ions (split-plot ANOVA, within group: $P < 0.001$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.001$) and sodium ions (split-plot ANOVA, within group: $P < 0.001$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.001$) with values decreasing sharply in moribund fish (Figure 2.2). The difference in osmolality on October 15 between group 4 (survivors) and group 3 was significant at $P < 0.05$, but not after Bonferroni correction (Figure 2.2A). Plasma chloride levels were significantly lower before a fish died when compared to group 4 (survivors) and were the best predictor of survival (Figure 2.2B). The difference in sodium ion means on October 15 between the group 4 (survivors) and group 3 individuals was significant at the $P < 0.05$ level, but not after Bonferroni correction (Figure 2.2C)]. For all three blood plasma variables, there were no significant differences between the group 4 (survivors) and the control fish on November 5. Because two of the 11 control fish died during the holding period, there were only nine control fish that were terminally sampled on November 5.

Because chloride was the best predictor of longevity according to the split-plot ANOVAs, only regressions between chloride and number of days until death are presented. There was a significant relationship between plasma chloride levels and time to death (Figure 2.3) for samples taken on October 8 (regression, $P < 0.001$, $r^2 = 0.830$) and October 15 (regression, $P < 0.001$, $r^2 = 0.751$). There was no significant relationship, however, between plasma chloride levels and time to death for samples taken on September 22–23 (regression, $P > 0.05$, $r^2 = 0.009$).

Within a sampling group, 17β-estradiol levels decreased when compared to initial samples taken from September 22 to 23, and moribund fish had the lowest levels of 17β-estradiol (Figure 2.4A; split-plot ANOVA; within group: $P < 0.01$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.01$). Testosterone levels generally increased with time within a sampling group compared to initial samples and decreased in moribund fish (Figure 2.4B; split-plot ANOVA; within group: $P < 0.01$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.01$). For both hormones, there were no significant differences between group 4 (survivors) and control fish at the termination of the experiment.

Cortisol levels decreased over time, but increased dramatically in moribund fish (Figure 2.5A; split-plot ANOVA, within group: $P < 0.001$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.001$). Conversely, glucose levels and the variability in the glucose values generally increased with time (Figure 2.5B; split-plot ANOVA, within group: $P < 0.01$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.001$). Plasma lactate, like cortisol, increased dramatically in moribund fish (Figure 2.5C; split-plot ANOVA, within group: $P < 0.001$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.001$). Plasma potassium levels increased in moribund fish (Figure 2.5D), however, the increase was not always significant within the sample groups (split-plot ANOVA, within group: $P < 0.001$; between sampling times: $P < 0.001$; group–time interaction: $P < 0.001$). For these four blood plasma variables, there were no significant differences between group 4 (survivors) and the control fish on November 5.

2.5 Discussion

Depletion of energy reserves, physiological stress caused by metabolic or disease issues and impaired osmoregulatory functions are all proposed mechanisms to account for the rapid senescence and mortality of Pacific salmon at the spawning ground (Shrimpton et al. 2005; Hruska et al. 2010). Understanding the mechanisms of the rapid senescence has been hampered by the fact that there have been no studies on wild Pacific salmon that quantify temporal changes in blood chemistry of individuals in one population during their final weeks of life. The present study found that plasma osmolality, chloride and sodium levels decreased during the final weeks of life in adult male sockeye salmon, followed by a further decrease (~20–40%) as fish became moribund. This final decrease in plasma ions probably represents a complete loss of osmoregulatory ability. The relationship between plasma chloride levels and time to death became stronger as fish approached the historic peak spawning period, which suggests that the ability of plasma ion levels to predict mortality is probably more applicable to sockeye salmon on or approaching the spawning grounds. Major plasma ion levels started to decline 2–10 days (mean of 6) before fish became moribund, and before other stress, metabolic or reproductive variables started to change, which suggests that osmoregulatory dysfunction may represent the initiation of rapid senescence. Osmoregulatory dysfunction may be a precursor to other

physiological changes (i.e., elevated stress and collapsed reproductive hormones) that the present study and others have found in moribund sockeye salmon (Hruska et al. 2010).

The causes of a precursory reduction in major plasma ions are unclear but may involve an inability to resorb ions at the kidney, a sequestering of ions in the body, a loss of ions across body surfaces and an inability to take ions up at the gill. Because immune functions become impaired in sockeye salmon on the spawning ground (Miller et al. 2009), naturally occurring diseases, particularly those which affect kidney or gill function (Wagner et al. 2005; Bradford et al. 2010*a*), may be partially responsible for such osmoregulatory problems. Reductions in plasma ions as fishes approach historic spawning periods also appear to occur in adult coho salmon (*O. kisutch*; Donaldson et al. 2010) and Chinook salmon (T.D. Clark, unpublished data), which suggests that osmoregulatory dysfunction may be a common phenomenon in all senescing semelparous Pacific salmon species.

Finch (1990) hypothesized that rapid senescence in adult Pacific salmon post-spawning may be related to elevated cortisol levels that lead to the deterioration of the kidney, liver and spleen and to overall immunosuppression. The observed decline in plasma cortisol levels over the 6 week interval of the present study has been observed previously (Fagerlund 1967; Hinch et al. 2006). The decline was followed by a rapid four to nine-fold increase as fish became moribund. Such an increase may be caused by decreased cortisol clearance by the kidney and liver and is potentially related to organ degeneration in Pacific salmon (Finch 1990; Carruth et al. 2000). Elevated cortisol levels have also been detected in diseased sockeye salmon days before they died, possibly as a response to fungal or bacterial infections (Fagerlund 1967). As fish in the present study were not treated with antibiotics, a disease-induced increase in cortisol levels is plausible, as increased incidence of disease is commonly observed in Pacific salmon held in captivity. Additionally, fishes may become immunocompromised when stressed (Schreck et al. 2001); these stressors may include confinement and sampling stress. An impaired immune response due to sampling and confinement stress may have made some of the sampled fish more susceptible to disease, fungal and parasite infection and contributed to the elevated cortisol levels near death and the overall increased mortality in the sampled group compared to the control group. Both direction and magnitude of the increases in plasma cortisol levels in the present study, however, were similar to those observed in moribund sockeye salmon from spawning grounds at Weaver Creek, a population which spawns near the Harrison Rapids population

(Figure 1; Hruska et al. 2010). Therefore, it is difficult to distinguish between the possibility that the elevated cortisol levels are associated with general disease progression and immunosuppression, which commonly occurs in senescing fishes, versus senescence alone. Regardless, it can be concluded that elevated cortisol is characteristic of mortality in Pacific salmon.

Cortisol plays a large role in osmoregulation and metabolism in fishes as it has both mineralcorticoid and glucocorticoid roles (Mommsen et al. 1999). Mineralcorticoids function mainly in the regulation of hydromineral balance (Milla et al. 2009). Shrimpton et al. (2005) showed that Na^+/K^+-ATPase activity, which may be regulated by cortisol, increases in spawning sockeye salmon and that the increased Na^+/K^+-ATPase activity on the spawning grounds is an attempt to compensate for an osmotic perturbation during spawning. Degeneration of the kidney due to prolonged elevation of cortisol and resulting loss of kidney function could result in the dramatic loss of osmoregulatory ability detected in the present study. Previous work has shown that severe parasite-induced kidney damage in adult sockeye salmon is correlated with a decrease in plasma osmolality, indicative of reduced osmoregulatory ability (Bradford et al. 2010*b*).

There was an increase in plasma glucose levels during the final 6 weeks of maturation and senescence. Cortisol has a metabolic role in fishes, which includes enhancing the rate of gluconeogenesis in the liver (Mommsen et al. 1999). Although the evidence is not always consistent, an increase in cortisol may eventually result in elevated plasma glucose levels in fishes (Mommsen et al. 1999). In male sockeye salmon caught throughout the reproductive season, elevated plasma glucose levels generally occurred when cortisol levels were also high (Kubokawa et al. 1999). The increase in plasma glucose levels in the present study did not necessarily correspond to elevated cortisol levels. Glucose levels often increase when a fish is confined (Portz et al. 2006). Plasma glucose, however, is known to increase during the period of final maturation in sockeye salmon (Kubokawa et al. 1999). Patterson et al. (2004) found no difference in glucose levels between sockeye salmon captured during migration and held in captivity and fish from the same population collected from natal spawning grounds. Therefore, the general increase in plasma glucose levels in the present study may be due to natural processes associated with final maturation and senescence, rather than a response to confinement stress. Furthermore, there was higher among individual variability in plasma glucose during later sampling periods, as fish became more mature, suggesting that there may be multiple

physiological mechanisms that affect individual plasma glucose levels during final maturation and senescence in Pacific salmon, an interpretation also supported by other research on sockeye salmon at spawning grounds (Hruska et al. 2010).

Blood-circulating sex steroids, such as testosterone and 17β-estradiol, increase during reproductive maturation and peak in males well before spawning (sometimes several hundred kilometres before reaching natal spawning grounds and several weeks before final maturation), then diminish and continue to decline prior to death in sockeye (Kubokawa et al. 1999; Hinch et al. 2006), pink (Dye et al. 1986; Williams et al. 1986), coho (Fitzpatrick et al. 1986) and chum (*O. keta*; Onuma et al. 2009) salmon. Despite an extended holding period that should have spanned the timing of previously observed peaks in sex steroids, there was only a moderate increase in testosterone during the experiment, and no increase in 17β-estradiol. Acute confinement stress (up to 30 min) has been shown to reduce blood-circulating sex steroid levels in sockeye salmon (Kubokawa et al. 1999), probably due to negative feedback control mechanisms by the hypothalamic–pituitary–interrenal axis during periods of stress (Wendelaar Bonga 1997). While it is possible that confinement stress may have been a factor in the present study, Patterson et al. (2004) found that sex steroid levels in sockeye salmon held for 4 weeks were comparable to levels detected in fish collected from natal spawning sites, which indicates captivity may not necessarily result in reduced blood-circulating sex steroid levels. Alternatively, the peak in 17β-estradiol and testosterone may have already occurred in the Harrison Rapids population before the September collection date. Elevated cortisol, as observed in fish that became moribund in the present study, has been shown to inhibit the reproductive cycle in male fish and has been associated with reductions in blood-circulating sex steroid levels in migrating sockeye salmon during periods of acute stress (Hinch et al. 2006) and in post-spawned death in sockeye salmon held in captivity (Kubokawa et al. 1999). The low levels of sex steroids detected near death in the present study may be associated with these dramatically increased plasma cortisol levels that occur as sockeye salmon become moribund. Decreases in 17β-estradiol and testosterone levels occurred earlier in fish that did not survive to the mean historic spawning period, which may suggest a disconnect between maturation and mean spawning time in fish that suffer prespawn mortality. Because fish that died prematurely had smaller gonad mass and body depth (a secondary sexual characteristic), it is probable that these fish died before they became fully mature, in contrast to the gonad mass and body depth in fish in the survivors and control

groups. It is unclear whether senescence occurs because of reproductive state or whether the advanced reproductive state is because the fish are further along a senescence trajectory.

Plasma lactate and potassium increased dramatically in fish that became moribund. These changes, along with plasma cortisol, appear to be primarily associated with mortality rather than changing because of senescence alone. Plasma lactate, a by-product of anaerobic metabolism, increases post-exercise and may increase post-stress in fishes (Barton et al. 2002). Following periods of exercise, potassium levels increase in blood plasma due to a loss of potassium from muscle cells (Sejersted and Sjogaard 2000). Plasma potassium can also increase during periods of hypoxia, possibly indicative of cell damage or muscle depolarization (Matey et al. 2008). In rainbow trout (*Oncorhynchus mykiss*) hepatocytes, potassium effluxes accompany cellular apoptosis and may be a factor in initiating apoptosis (Krumschnabel et al. 2007). Therefore, the increase in plasma potassium levels and lactate is probably indicative of activity, stress and cellular degradation, which may be associated with final senescence in Pacific salmon.

Sampling and confinement stress could potentially have influenced the patterns in blood properties in the sampled fish. Adult Pacific salmon exhibit transient increases in plasma cortisol, glucose and lactate after a single stress event, which then return to pre-stressor levels (Donaldson et al. 2010). When exposed to a chronic stressor, these plasma variables may return to baseline values after a period of acclimation (Trenzado et al. 2003; Cairns et al. 2008). Because the magnitude and direction of change in the blood variables was consistent with previous studies on spawning ground fish (Hruska et al. 2010) and with control fish that were not previously sampled, the patterns described in the present study are probably characteristic of the final weeks of final maturation and senescence in sockeye salmon.

In conclusion, the present study characterized the temporal changes in blood properties and documented the initial osmoregulatory failure of male sockeye salmon during the freshwater stage of their spawning migration, and subsequent elevated stress and depressed reproductive hormone levels which characterize final maturation and senescence. Because rapid declines in osmoregulatory ability preceded death by 2–10 days, specific levels of major plasma ions might be useful indicators for predicting mortality. This information could be useful for fisheries management because there were no differences in the external morphological measurements between the survival groups in the present study, which suggests that, morphologically, there were no visible differences between individuals that survived or died early. The incorporation

into fisheries science and management of rapid bioassay approaches, and use of field instruments which can provide immediate osmoregulatory and stress metabolite information from plasma samples, have recently been advocated (Cooke et al. 2008; Hasler et al. 2009). Prespawning mortality can be substantial within sockeye salmon populations in some years (Gilhousen 1990), and *en route* migration mortality has been at 50–90% recently in some populations of Fraser River sockeye salmon (Cooke et al. 2004). Advanced warning of the extent or proportion of a population that may suffer prespawning or *en route* mortality could help managers in determining whether to open or close in-river fisheries or in deciding how many fish to allow onto artificial spawning grounds.

Table 2.1. Means ± S.D. and sample sizes (n) of morphological characteristics for the three mortality groups, survivors and controls of male sockeye salmon (*Oncorhynchus nerka*).

Sample Group	n	POH Length (cm) *	Mass (g) *	Body Depth (cm)	Kype Length (cm)	Liver Mass (g) **	Gonad Mass (g)
Died before Oct. 8	13	52.04 ± 1.30^{A}	3012.31 ± 325.17^{A}	14.38 ± 0.96^{A}	7.26 ± 0.71^{A}	67.55 ± 10.53^{A}	90.16 ± 24.70^{AB}
Died after 2 sampling rounds	13	52.36 ± 2.40^{A}	3115.85 ± 534.17^{A}	14.93 ± 1.38^{A}	7.57 ± 0.86^{A}	72.17 ± 14.97^{A}	96.18 ± 32.71^{AB}
Died after 3 sampling rounds	18	53.04 ± 3.38^{A}	3009.22 ± 453.44^{A}	14.78 ± 1.02^{A}	7.62 ± 0.75^{A}	70.26 ± 14.93^{A}	76.08 ± 21.53^{A}
Survivors	11	53.55 ± 2.60^{A}	3312.91 ± 573.50^{A}	16.64 ± 1.58^{B}	8.21± 1.17^{A}	71.30 ± 6.57^{B}	89.94 ± 10.42^{AB}
Control	9	50.70 ± 2.10^{A}	3064.63 ± 369.08^{A}	16.44 ± 0.92^{B}	7.64 ± 0.77^{A}	65.97 ± 7.35^{A}	100.48 ± 22.22^{B}

Different letters within a column indicate statistical differences.
**n* for the control group for POH length and the mass-length ANCOVA = 8.
***the slopes were significantly different between the survivors and the fish that died after two rounds and therefore the survivors were removed from the ANCOVA.

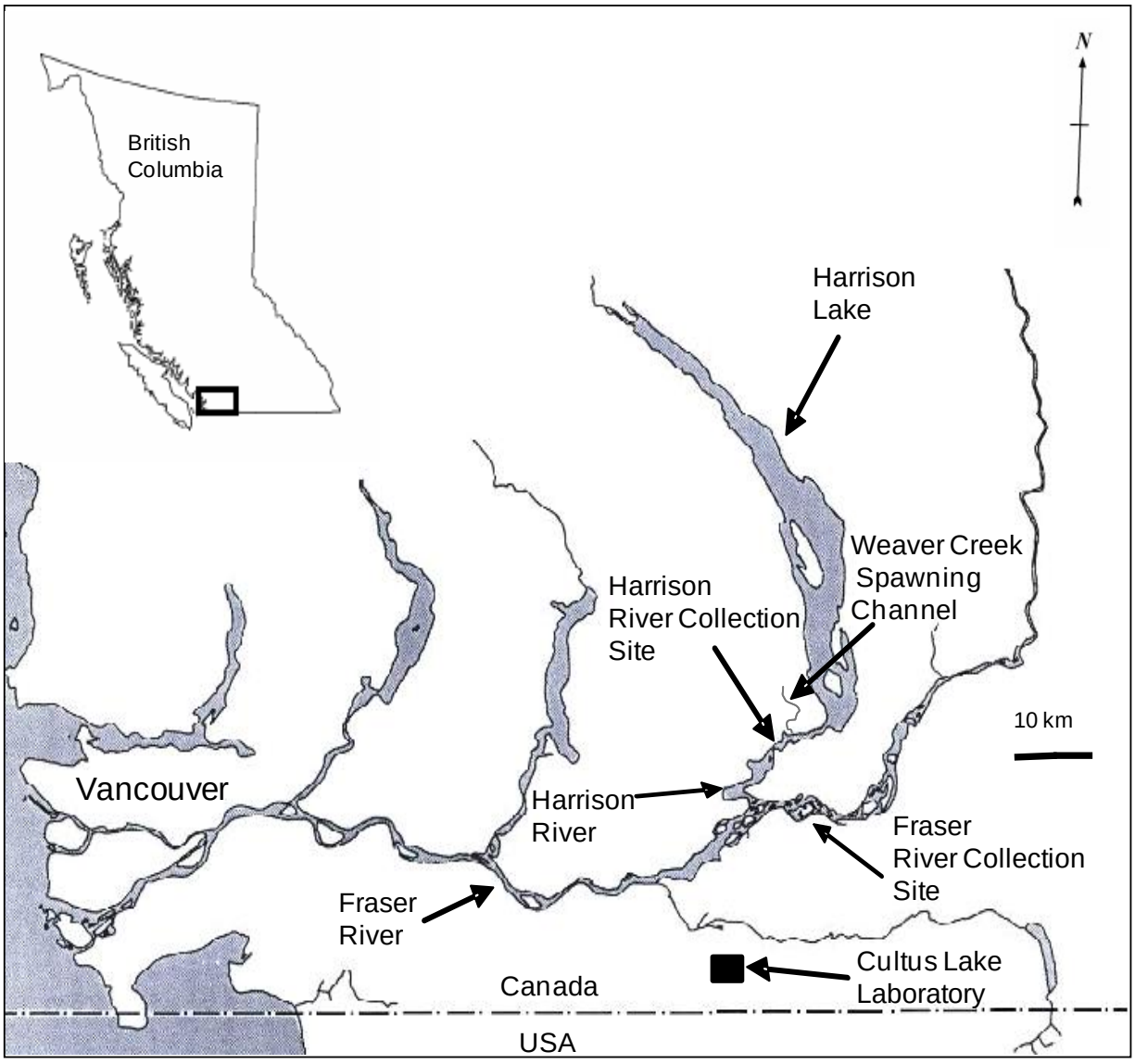


Figure 2.1. Map of the lower Fraser River, British Columbia, Canada, with the locations of the Harrison River and Fraser River mainstem collection sites pertinent to this thesis and the Fisheries and Oceans Canada Cultus Lake Laboratory.

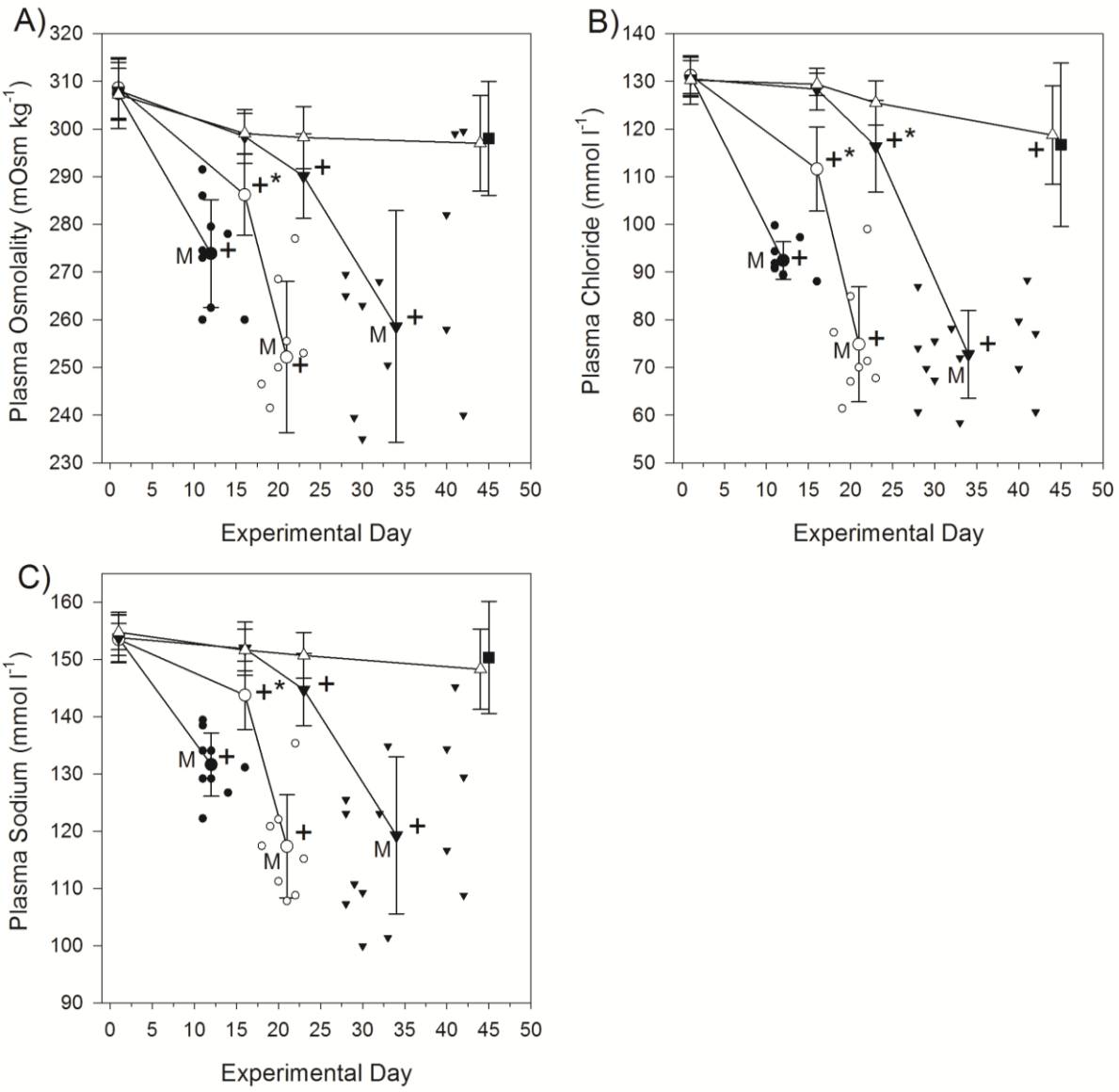


Figure 2.2. Plasma levels of A) osmolality, B) chloride, and C) sodium sampled from male Harrison Rapids sockeye salmon over the final 6 weeks of maturation and senescence in 2008 [● = Died before October 8 (Group 1); ○ = Died after 2 rounds (Group 2); ▼ = Died after 3 rounds (Group 3); Δ = Survivors (Group 4); ■ = Controls]. Data are presented as means ± S.D. Statistical differences from the September 22/23, 2008 (experimental day 1), sampling date within groups are indicated by (+) and differences between groups and the survivors group at a given time are indicated by (*). The October 8, October 15 and November 5, 2008, samplings occurred on experimental day 16, 23 and 44, respectively. Mean experiment day and value of samples taken including raw data (smaller data points) as fish became moribund are indicated by (M). Control fish were sampled on experimental day 44.

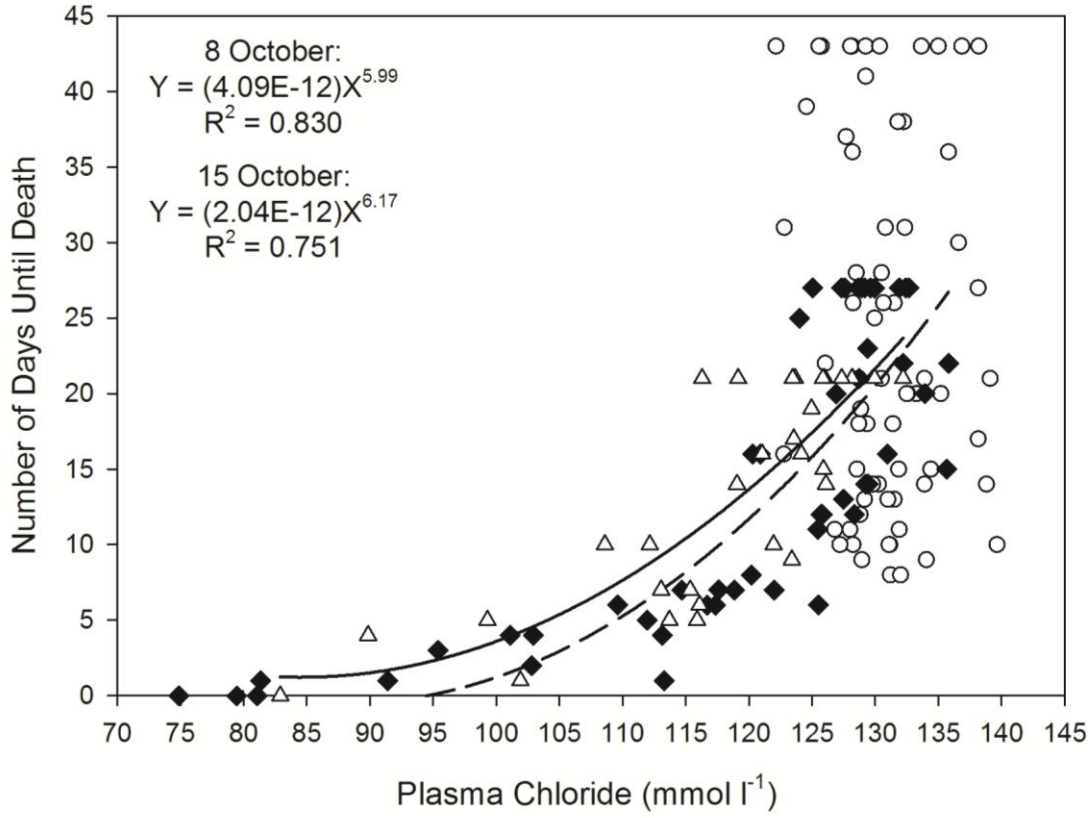


Figure 2.3. Relationship between the number of days to death (post-sampling) and plasma chloride levels in male Harrison Rapids sockeye salmon sampled on September 22/23 [○], October 8 [♦, dashed trend line], and October 15 [Δ, solid trend line] 2008. Trend lines and corresponding equations are only presented for the statistically significant relationships. Equations are derived from the linear regression estimates of the slope and intercept for $\log_{10}$-transformed data to fit a power function.

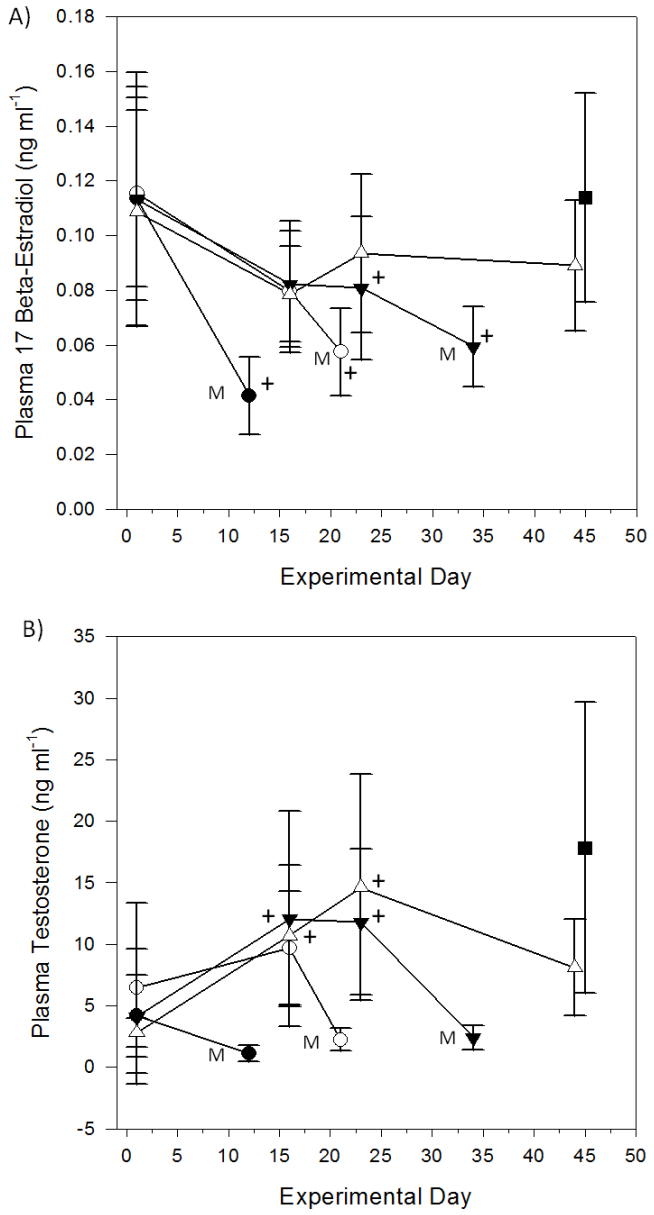


Figure 2.4. Plasma levels of A) 17β-estradiol and B) testosterone sampled from male Harrison Rapids sockeye salmon over the final 6 weeks of maturation and senescence in 2008 [● = Died before October 8 (Group 1); ○ = Died after 2 rounds (Group 2); ▼ = Died after 3 rounds (Group 3); Δ = Survivors (Group 4); ■ = Controls]. Data are presented as means ± S.D. Statistical differences from the September 22/23, 2008 (experimental day 1), sampling date within groups are indicated by (+). One sample from the October 8 sample from the after 3 rounds of sampling group was below the detection limits for the testosterone assay and therefore was not included in the mean calculation. The October 8, October 15 and November 5, 2008, samplings occurred on experimental day 16, 23 and 44, respectively. Mean experiment day and value of samples taken as fish became moribund are indicated by (M). Control fish were sampled on experimental day 44.

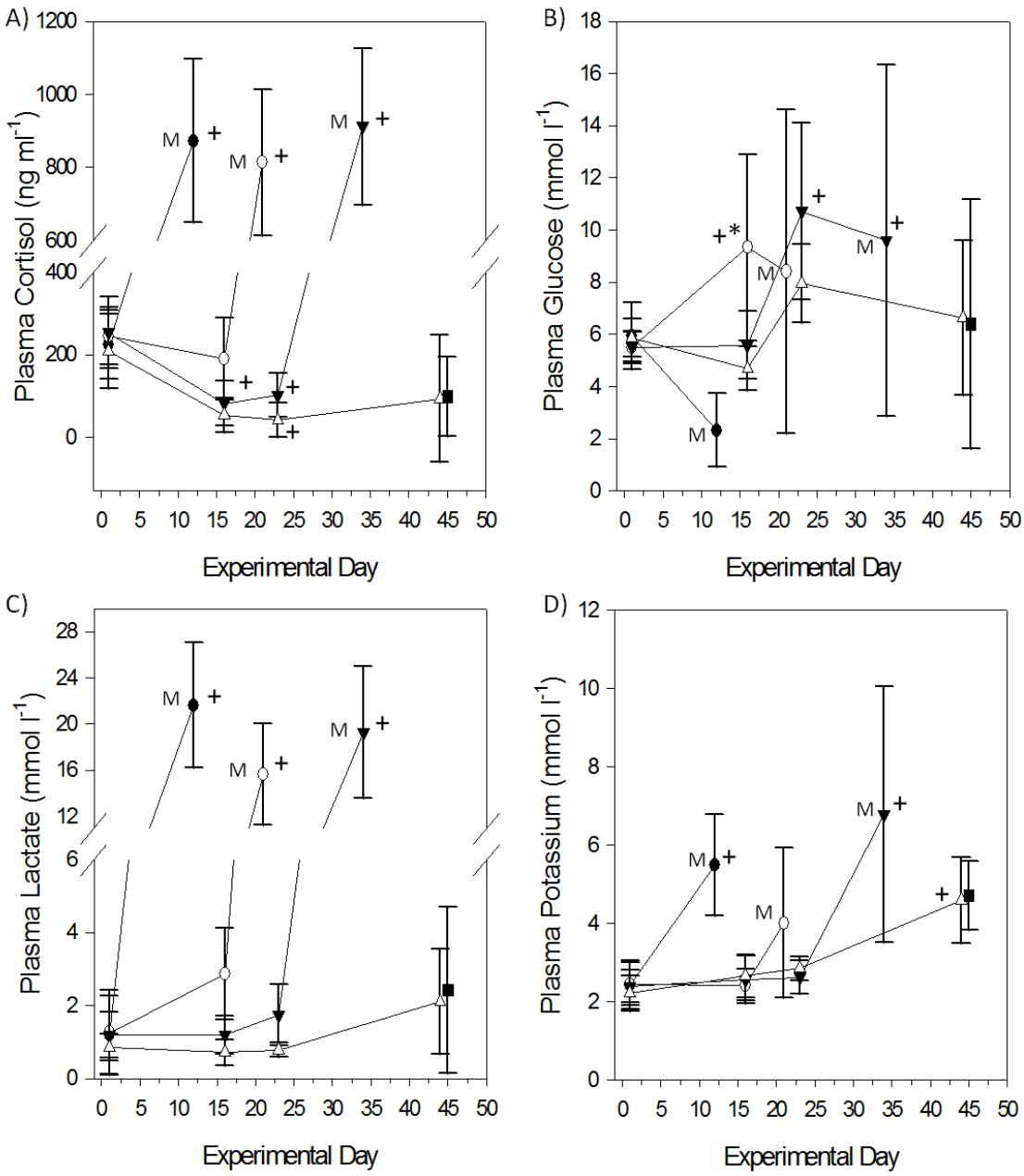


Figure 2.5. Plasma levels of A) cortisol, B) glucose, C) lactate, and D) potassium sampled from male Harrison Rapids sockeye salmon over the final 6 weeks of maturation and senescence in 2008 [● = Died before October 8 (Group 1); ○ = Died after 2 rounds (Group 2); ▼ = Died after 3 rounds (Group 3); Δ = Survivors (Group 4); ■ = Controls]. Data are presented as means ± S.D. Statistical differences from the September 22/23, 2008 (experimental day 1), sampling date within groups are indicated by (+) and differences between groups and the survivors group at a given time are indicated by (*). The October 8, October 15 and November 5, 2008, samplings occurred on experimental day 16, 23 and 44, respectively. Mean experiment day and value of samples taken as fish became moribund are indicated by (M). Control fish were sampled on experimental day 44.

Chapter 3: Sex and proximity to reproductive maturity influence the survival, final maturation, and blood physiology of Pacific salmon when exposed to high temperature during a simulated migration

3.1 Synopsis

Some Pacific salmon populations have been experiencing increasingly warmer river temperatures during their once-in-a lifetime spawning migration, which has been associated with *en route* and prespawn mortality. The mechanisms underlying such temperature-mediated mortality are poorly understood. Wild adult pink and sockeye salmon were used in this study. The objectives were to investigate the effects of elevated water temperature on mortality, final maturation, and blood properties under controlled conditions that simulated a 'cool' (13°C) and 'warm' (19°C) freshwater spawning migration. After 10 d at 13°C, observed mortality was 50%–80% in all groups, which suggested that there was likely some mortality associated with handling and confinement. Observed mortality after 10 d at 19°C was higher, reaching ≥98% in male pink salmon and female pink and sockeye salmon. Thus, male sockeye salmon were the most thermally tolerant (54% observed mortality). Model selection supported the temperature- and sex-specific mortality patterns. The pink salmon were closer to reproductive maturation and farther along the senescence trajectory than sockeye salmon, which likely influenced their survival and physiological responses throughout the experiment. Females of both species held at 19°C had reduced plasma sex steroids compared with those held at 13°C, and female pink salmon were less likely to become fully mature at 19°C than at 13°C. Male and female sockeye salmon held at 19°C had higher plasma chloride and osmolality than those held at 13°C, indicative of a thermally-related stress response. These findings suggest that sex differences and proximity to reproductive maturity must be considered when predicting thermal tolerance and the magnitude of *en route* and prespawn mortality for Pacific salmon.

3.2 Introduction

Many populations of Pacific salmon (*Oncorhynchus* spp.) now encounter warmer rivers during their spawning migration than at any time since records have been kept. For example, average summer water temperatures experienced by migratory salmon in one of Canada's largest rivers, the Fraser River in British Columbia, has increased by >1.8°C over the past ~60 yr, with 13 of the warmest years on record occurring over the past 20 summers (Patterson et al. 2007; eWatch 2011). Similarly, Pacific salmon in the Columbia River in the United States now migrate through waters that can be ~2.5°C above historical levels (Quinn and Adams 1996). Spawning migrations during high water temperature episodes are associated with increased levels of *en route* mortality (mortality during migration; Macdonald et al. 2010; Martins et al. 2011). Because the semelparous life history of Pacific salmon allows for only one opportunity to complete their spawning migration and reproduce, *en route* mortality results in a lifetime fitness of zero for those individuals. Even the survivors of migrations during warm-water episodes may experience profound negative consequences, such as delayed final maturation − a common response to elevated water temperature in salmonids (Pankhurst and King 2010) − or elevated levels of premature mortality on spawning grounds (termed 'prespawn mortality'; Gilhousen 1990). Climate models predict a continued warming of approximately 0.12°C–0.14°C per decade over the next century for summer water temperatures in the Fraser River (Ferrari et al. 2007). Thus, temperature-related mortality in Pacific salmon is predicted to occur more frequently and will likely result in a reduced number of salmon reaching spawning grounds and successfully reproducing in the future (Morrison et al. 2002; Mantua et al. 2010; Hague et al. 2011; Martins et al. 2011); temperature-related mortality is also likely to alter suitable habitat availability in the southern periphery of these species' distributions (Eaton and Scheller 1996; Beechie et al. 2006).

Several field-based studies have shown a relationship between water temperature and *en route* mortality in various Pacific salmon species throughout their natural range (Keefer et al. 2008, 2010; Taylor 2008; Martins et al. 2011). There are several proposed mechanisms to explain why elevated water temperatures may influence Pacific salmon survival, which include (1) more rapid depletion of endogenous energy reserves (Rand et al. 2006), which provide the sole fuel source for in-river migration and gonadal development because Pacific salmon cease feeding in the ocean; (2) a collapse in aerobic scope (Farrell et al. 2008) and impairment of

cardiorespiratory function (Clark et al. 2008); and (3) a temperature-dependent increase in disease and parasite progression (Servizi and Jensen 1977; Gilhousen 1990; Crossin et al. 2008; Bradford et al. 2010). These mechanisms may act solely or synergistically to ultimately affect spawning migration success. Regardless, the physiological mechanisms underlying the mortality remain speculative, and there are few controlled laboratory-based studies that have examined the effects of water temperature on wild adult Pacific salmon. Of those that do exist, elevated water temperature has been shown to increase mortality (Jensen et al. 2004; Crossin et al. 2008), but physiological analyses have been sparse and physiological disturbances due to temperature treatments have not been detected after prolonged thermal exposure (i.e., 24 d in Crossin et al. 2008).

In light of these knowledge gaps, this study sought to quantify the physiological and survival consequences of continuous exposure to elevated water temperature in Pacific salmon. I held wild-caught adult pink and sockeye salmon under thermal conditions that simulated a 'cool' (13°C) or 'warm' (19°C) freshwater migration. I hypothesized that if the 19°C water treatment induced chronic stress, it would result in increased mortality, elevated indices of stress in the blood plasma, and delayed or inhibited final maturation. Because sex-based differences in survival have been reported previously for Pacific salmon (Patterson et al. 2004; Crossin et al. 2008; Keefer et al. 2010), I also contrasted thermally driven mortality patterns and blood plasma responses between males and females. Furthermore, because Fraser River pink salmon appear to be more thermally tolerant (Clark et al. 2011) than sockeye salmon populations (Macdonald et al. 2010; Eliason et al. 2011), I expected pink salmon to be less affected by the warm-temperature treatment.

3.3 Materials and methods

Adult sockeye salmon (n = 128) were collected from the Harrison River (a major tributary of the Fraser River), British Columbia, Canada, from September 15 to 18, 2008 (see Figure 2.1). DNA stock identification (Beacham et al. 2005) confirmed that all sockeye salmon were from the Harrison Rapids population (hereafter referred to as Harrison sockeye salmon). Pink salmon (n = 156) were collected from the same location from September 22 to 24, 2009. Fraser River pink salmon are generally divided into Lower and Upper Fraser River populations,

which can be distinguished on the basis of migration timing and capture location (Groot and Margolis 1991; Crossin et al. 2003) but not by DNA identification (T. D. Beacham, personal communication). The pink salmon that spawn in the Harrison River (used in this study) belong to the Lower Fraser River stock complex. Harrison sockeye salmon typically spawn from early to mid-November in the Harrison River, while Lower Fraser River pink salmon in the Harrison River system typically spawn from early to mid-October.

In 2008 and 2009, fish were collected by beach seine from the Harrison River (water temperatures ranged from 15°C to 18°C in 2008 and from 13°C to 16°C in 2009) near Agassiz, British Columbia, and transported in aerated ~12°C water monitored continuously for dissolved oxygen levels to the Fisheries and Oceans Canada Cultus Lake Laboratory, where they were held in large tanks containing 10°C–11°C water that had been sand filtered and UV sterilized. Fish recovered from transport for 2–5 d before the experimental temperature treatment period. At this time, all fish appeared vigorous, and there were no external signs of disease. A pre-treatment blood sample was obtained from the caudal vasculature using a heparinized vacutainer to determine initial values for plasma variables (all sockeye salmon in 2008: n = 22 males and 20 females destined for the 13°C treatment, 46 males and 40 females destined for the 19°C treatment; a subset of pink salmon in 2009: n = 16 males and 16 females destined for the 13°C treatment, 31 males and 33 females destined for the 19°C treatment). Immediately afterward, fish were randomly distributed among six 8,000-L aerated tanks at 10°C–11°C using equal fish densities and sex ratios. Each tank contained a submersible pump that created a water flow of approximately 0.3 m s^{-1} in which the fish were able to orient and maintain position by continuously swimming. The tank water temperatures were then raised at a rate of ~3°C per day until the test temperatures of 13°C and 19°C were reached (four tanks for the 19°C treatment and two tanks for the 13°C treatment in each year). The 60-yr average river water temperature encountered by Harrison sockeye salmon and Lower Fraser River pink salmon during late September and early October is approximately 13°C–14°C (Patterson et al. 2007). Thus, the 13°C temperature treatment closely resembled the historical thermal conditions experienced in the river. In contrast, the 19°C temperature treatment represented an extreme situation encountered in the Fraser River by pink salmon and early-entry late-run sockeye salmon (see below) in five of 15 years from 1992 to 2006 (Patterson et al. 2007) and in 2008 and 2009 (eWatch 2011). Since 1995, some late-run sockeye salmon have entered the Fraser River 2–8 wk

earlier than their historical norm, exposing them to warmer than normal water temperatures and for longer periods of time, leading to high incidences of *en route* mortality in these early migrants (exceeding 90% mortality in some instances; Cooke et al. 2004).

After 5 d of a stable test temperature for the 19°C treatment (termed 'experimental day 5,' but 8 d after the pre-treatment samples were taken), blood was resampled from surviving fish in both temperature groups (temperature treatment samples) to determine the effect of water temperature on plasma properties (sockeye salmon in 2008: n = 11 males and 7 females exposed to 13°C, 16 males and 4 females exposed to 19°C; pink salmon in 2009: n = 23 males and 21 females exposed to 13°C, 21 males and 18 females exposed to 19°C). During the 10-d temperature treatment, dead fish were promptly removed from tanks, and the mortality data were used in the survival analysis. A 10-d temperature exposure period was considered ecologically relevant because this length of time represents a significant portion of the freshwater migration of many stocks of Fraser River Pacific salmon (English et al. 2005). On experimental day 10 in 2009, all female pink salmon in the 13°C treatment were killed to determine maturation status. Surviving female sockeye salmon from the 13°C treatment in 2008 were not killed on experimental day 10 because they were still approximately 1month from their historical spawning period and reproductive maturity. Instead, female sockeye salmon were assessed for maturation status on an individual basis as they were found dead in the tanks. Only female fish were examined for maturation status and were considered mature ('ripe') when eggs were released upon firmly squeezing the fish along the lateral lines or when eggs were loose within the body cavity during dissection. Female gonads were weighed (±0.1 g) to compare gonad masses between treatment groups. Gonadosomatic indices were calculated as gonad mass/(body mass-gonad mass)x100.

3.3.1 Plasma analyses

Blood samples (~3 mL) were immediately centrifuged for 7 min, and plasma was stored at -80°C before analyses. Plasma osmolality, chloride, glucose, and lactate were measured in duplicate or triplicate using the procedures outlined in Farrell et al. (2001). Plasma cortisol, testosterone, and 17β-estradiol were measured using commercial ELISA kits (Neogen

Corporation, http://www.neogen.com, catalog nos. 402710, 402110, 402510). Testosterone and 17β-estradiol samples were extracted in ethyl ether according to manufacturer's protocols. Cortisol, testosterone, and 17β-estradiol samples were run in duplicate at appropriate dilutions.

3.3.2 Survival analysis

The effects of sex and temperature on cumulative mortality were assessed using parametric survival analysis (Harrell 2001). Survival models for pink and sockeye salmon assuming no effects, temperature effects (temperature), or sex effects (sex), as well as models including a combination of these effects (temperature + sex) and their interaction (temperature X sex), were fitted to the data. Model selection was carried out using Akaike Information Criterion corrected for small sample sizes (AIC_c; Burnham and Anderson 2002). According to this criterion, the model with the lowest AIC_c value is the most parsimonious one describing the data and other models differing from this one in <2 units (Δ_i) are regarded as also having substantial support from the data. To account for model selection uncertainty, model-averaged cumulative mortality and associated 95% confidence intervals were computed using the weight AIC_c (w_i) of the models included in a 95% confidence set for the best model (Burnham and Anderson 2002). Ratios between the model-averaged estimates were used as a measure of the effect size of the temperature treatments on cumulative mortality (presented as percentages). The parametric distribution used for the survival data analysis was log logistic for pink salmon and Weibull for sockeye salmon. The adequacy of these distributions and the fit of the models were assessed graphically as described by Harrell (2001). Model fitting and selection were performed using R-2.13.1 (R Development Core Team 2008).

3.3.3 Statistical analysis

It should be noted that logistical constraints and access to salmon prevented further tank/treatment replication. However, I conducted some preliminary analyses and found no strong tank effects; therefore, I pooled results across tanks and used individual fish as the replicates in the statistical analyses. Nevertheless, the samples sizes and inclusion of some replicate tanks in

this study were considered exceptional in light of previous studies of large wild adult salmon. Statistical differences between groups for blood plasma variables were determined using a two-factor ANOVA with temperature and sex as factors (SAS, ver. 9.1; SAS Institute) unless specified otherwise. Species were analyzed separately. Tukey-Kramer pairwise comparisons were made *a posteriori* (Zar 1999). In all cases, homogeneity of variances was assessed by F_{max} tests, and normality was tested using Kolmogorov-Smirnov tests (Sokal and Rohlf 1995). Data were log10-transformed if the assumption of homogeneity of variances could not be met. Where the assumption of normality could not be met, a Freidman nonparametric two-factor ANOVA was used with temperature and sex as factors (Zar 1999). Sexes were analyzed separately for plasma sex steroid levels using either *t*-tests or Wilcoxon two-sample tests if the assumption of normality could not be met. Differences in the number of pink salmon females that became ripe were compared between temperature treatments using a χ^2 contingency table. Length-adjusted gonad masses were compared between temperature treatments using ANCOVA with postorbital fork length as the covariate.

3.4 Results

3.4.1 Mortality patterns and maturation

Observed mortality was greater at 19°C than at 13°C at experimental days 5 and 10 for both species and sexes (Figure 3.1). Accordingly, the most parsimonious model describing the cumulative mortality data showed that temperature alone best described the mortality patterns for pink salmon (Table 3.1). Model-averaged mortality estimates indicate that the 19°C treatment mortality patterns for male and female pink salmon are on average 30% and 32%, respectively, higher compared with those of the 13°C treatment (Figure 3.1). Sex-specific differences in mortality patterns existed for sockeye salmon because females had greater observed mortality than males, a pattern not observed in pink salmon. Model selection supported this because the most parsimonious model describing the cumulative mortality data for sockeye salmon had an interaction between temperature and sex (Table 3.1). Indeed, the 10-d model-averaged mortality estimates indicate that female sockeye salmon suffer on average 92% (at 13°C) and 62% (at

19°C) higher mortality than male sockeye salmon (Figure 3.1). Observed mortality of female sockeye salmon did not vary substantially by temperature, and model-averaged estimates indicate that their mortality is on average only 10% higher at 19°C than at 13°C (Figure 3.1). Conversely, model-averaged estimates indicate that the 19°C treatment mortality in male sockeye salmon is on average 34% higher than at 13°C (Figure 3.1). Male sockeye salmon had the lowest mortality of all the groups, suggesting that male sockeye salmon were the most thermally tolerant.

There was evidence that exposure to the high-temperature treatment delayed or inhibited final maturation (Table 3.2). While there were no differences in length-adjusted gonad mass between female pink salmon held at 13°C and 19°C (ANCOVA, $P > 0.05$), more female pink salmon held at 13°C [19 out of 30 (63.3%)] became ripe compared with females held at 19°C [11 out of 48 (22.9%); $\chi^2 = 11.09$, $P < 0.001$]. Within the 19°C treatment, female pink salmon that became ripe had greater length-adjusted gonad mass than females that did not become ripe (ANCOVA, $F = 17.73$, $P < 0.0001$), although this difference was not detected within the females held at 13°C ($P = 0.09$). Only one female sockeye salmon (held at 13°C) that died during the 10-d holding period was ripe.

3.4.2 Plasma variables

The effect of the holding temperature on blood plasma physiology was limited to effects on osmoregulatory and reproductive indices. Sockeye salmon held at 19°C had higher plasma osmolality (F =19.37, $P < 0.0005$) and chloride ($F = 19.32$, $P < 0.0005$) than those held at 13°C on experimental day 5 (Figure 3.2). Females held at 19°C had lower testosterone than fish held at 13°C in sockeye (Wilcoxon two-sample test, $T = 10.00$, $P < 0.05$) and pink (Wilcoxon two-sample test, $T = 217.00$, $P < 0.001$) salmon, and female sockeye salmon held at 19°C had lower 17β-estradiol than those held at 13°C (t-test, $t = 2.53$, $P < 0.05$; Figure 3.3).

Female pink salmon had higher pre-treatment chloride than males (Freidman ANOVA, $F = 5.63$, $P < 0.02$), and this difference was maintained throughout the temperature treatments (Freidman ANOVA, F =15.47, $P < 0.0005$). Similarly, female pink ($F = 35.16$, $P < 0.0001$) and sockeye ($F = 23.60$, $P < 0.0001$) salmon had higher pre-treatment cortisol than males, yet this

was maintained throughout the temperature treatments for sockeye salmon only ($F = 17.58$, $P < 0.0005$; Figure 3.4). Female sockeye salmon had higher pre-treatment ($F = 14.13$, $P < 0.0005$) and treatment ($F = 5.38$, $P < 0.05$) glucose and pre-treatment (Freidman ANOVA, $F = 10.43$, $P < 0.005$) and treatment ($F = 8.66$, $P < 0.01$) lactate compared with males. Conversely, male pink salmon had higher treatment lactate levels compared with females (Freidman ANOVA, $F = 21.06$, $P < 0.0001$).

3.5 Discussion

Strong temperature- and sex-specific differences in mortality were detected in this study. After 10 d at 19°C, nearly all female sockeye salmon and both sexes of pink salmon had died, while mortality was lower in pink salmon held at 13°C and male sockeye salmon held at both temperatures. Male sockeye salmon were the most thermally tolerant group in this experiment, contrary to my prediction that pink salmon would be less affected by the temperature treatments. The pink salmon mortality patterns may have been influenced by the fact that these fish were closer to final maturation than the sockeye salmon at time of capture and the start of the experiment. It is important to note that there may be considerable interannual variation in mortality patterns in Pacific salmon populations that are often related to in-river migration conditions (Gilhousen 1990; Macdonald et al. 2010). There were also additional stressors in this study, such as capture, transport, handling, and laboratory holding, which likely enhanced rates of mortality from those experienced naturally. Therefore, it is difficult to generalize the details of these results (e.g., actual levels of mortality) to other migration years and systems, especially because of the relatively high mortality in the 13°C treatment. However, the effect of the elevated water temperature treatment was clear in that both sexes of both species suffered greater mortality at 19°C than at 13°C.

The measured blood plasma properties did not provide direct evidence for a physiological mechanism for the higher mortality observed at 19°C. Because fish were held at 19°C for a relatively short period of time (≤10 d), mortality was unlikely to be due to energy exhaustion, especially for the female sockeye salmon that were approximately 1 mo from spawning. Therefore, the most likely cause of death may be an increase in disease and parasite progression (e.g., Servizi and Jensen 1977; Crossin et al. 2008) and/or impaired cardiorespiratory function

leading to insufficient oxygen transport capacity (e.g., Clark et al. 2008; Farrell et al. 2008). Because some populations of Pacific salmon can tolerate temperatures much greater than 19°C for short periods without lethally impacting cardiorespiratory performance (Steinhausen et al. 2008; Clark et al. 2011) and cardiovascular impairments would likely be accompanied by changes in blood properties (e.g., lactate linked with hypoxemia), temperature-dependent disease and parasite progression (e.g., *Parvicapsula minibicornis*, *Saprolegnia* spp., and *Flexibactor columnaris*) were likely large contributors to the higher mortality observed at 19°C in this study. However, this is speculative as these diseases and parasites progressions were not assessed. The relatively high mortality in fish held at 13°C (50%–80% after 10 d) potentially suggests that disease and parasite progressions may have been under way before collection and exposure to the temperature treatments. Indeed, there were *en route* losses of approximately 80% for Harrison sockeye salmon in the wild in 2008, and these fish would have experienced extended freshwater residence time as a result of their early entry into the Fraser River (http://www.psc.org), increasing potential exposure to diseases and pathogens and allowing more time for infections to become virulent.

The experiments revealed the higher and more rapid mortality of female sockeye salmon compared with males, independent of temperature treatment. A similar pattern has been reported previously for experimentally held and freely migrating Fraser River sockeye salmon (Patterson et al. 2004; Crossin et al. 2008). A key regulator of Pacific salmon population productivity is considered to be the total number of eggs deposited during the spawning period (Quinn 2005) because the number of eggs from females is limited while males are capable of spawning with several females (Mehranvar et al. 2004). Consequently, a higher level of premature mortality in females could have a proportionally greater negative impact on the abundance of Pacific salmon populations.

Insight into the mechanisms causing higher mortality in female sockeye salmon, and their apparent increased sensitivity to stress, comes from an examination of their physiology. Female salmonids respond to stress differently at the transcriptional level compared with males, emphasizing the importance of accounting for sex when studying physiological responses to stress (Momoda et al. 2007). Previous work has demonstrated that female sockeye salmon have higher routine heart rates than males when confined, which may reduce their scope in heart rate and their ability to tolerate additional stressors (Sandblom et al. 2009). It is well established that

cortisol levels are naturally elevated in maturing female sockeye salmon compared with males (Macdonald et al. 2000; Patterson et al. 2004; Crossin et al. 2008; Sandblom et al. 2009; Clark et al. 2010). Consistent with this, female sockeye salmon in my study had higher pre-treatment plasma cortisol, along with higher glucose and lactate levels, than males. The elevated cortisol in female sockeye salmon, which was maintained through the temperature treatments, is perhaps to mobilize energy stores to fuel the migration and the greater relative reproductive investment of females (Mommsen et al. 1999). Nevertheless, cortisol is an important biomarker for stress. Macdonald et al. (2000) previously observed higher cortisol levels in compromised (external fungus, skin lesions, and lethargic behavior) female versus 'healthy' male and female sockeye salmon. Thus, the higher pre-treatment cortisol (and glucose and lactate) levels of female sockeye salmon in my study may also suggest that they were less healthy or more stressed before the temperature exposure period, consistent with the rapid mortality that occurred. Indeed, prespawn mortality of female sockeye salmon in 2008 was high throughout the Fraser River watershed (~34% averaged across all stocks), which led to lower numbers of successful spawners (K. Benner, Fisheries and Oceans Canada, personal communication) and suggests that 2008 was an anomalous year. While the exact physiological mechanisms responsible for the higher mortality in female sockeye salmon remain unknown, higher basal stress levels and susceptibility to stress and disease may have played a role.

Temperature and handling/confinement stress, along with proximity to reproductive maturation, likely contributed to the higher mortality of pink salmon held at 19°C compared with those held at 13°C. These are ecologically important results because Lower Fraser River pink salmon spawn in the mainstem of the Fraser River and its major tributaries (e.g., Harrison River) and therefore may experience high water temperatures during final maturation, which may lead to high rates of prespawn mortality. However, because some female pink salmon became ripe in the 19°C treatment, it is likely that some successful spawning still occurs at elevated water temperatures in natural conditions. Captive pink salmon in this study were observed attempting to dig redds, eliciting courtship behaviors, and interacting aggressively with conspecifics, similar to the behavior observed in another study that held pink salmon in captivity (Williams and Brett 1987). In contrast, sockeye salmon were observed to school and swim into the current. Additionally, pink salmon sex steroid levels in my study were comparable to levels detected in migrating pink salmon on arrival at spawning grounds (Williams et al. 1986). These behavioral

observations and sex steroid patterns suggest that the pink salmon used in my study were close to final maturation. The relative increases in plasma cortisol, glucose, and lactate in pink salmon from pre-treatment levels to temperature treatment levels likely reflect an elevated stress response associated with proximity to final maturation. Enhanced senescence may have also been a factor, as plasma cortisol and lactate have been reported to increase in moribund Pacific salmon (Hruska et al. 2010; Chapter 2). Mortality of pink salmon associated with natural senescence may help to explain the mortality patterns observed at both temperatures in my study.

In addition to the implications for survival, exposure to elevated water temperature may also delay or inhibit final maturation in salmonids through its effects on steroid biosynthesis and the inhibition of the pre-ovulatory shift to maturational hormone production (Pankhurst and King 2010). Chronic exposure to 19°C water resulted in a reduction in plasma testosterone and 17β-estradiol levels in female pink and sockeye salmon after 5 d. These reduced sex steroid levels are consistent with those observed by Macdonald et al. (2000), who suggested that the temperature threshold for sex steroid production is between 15°C and 19°C for Fraser River sockeye salmon. Decreased sex steroid biosynthesis may lead to reduced 17α,20β-dihydroxy-4-pregen-3-one (hormone required for final maturation) production, which decreases the likelihood of females reaching maturity and having viable eggs (Macdonald et al. 2000). Indeed, in comparison with fish at 13°C, female pink salmon held at 19°C were less likely to become ripe, which may be linked with the reduced plasma sex steroid levels and demonstrates a potential reproductive consequence to exposure to elevated water temperatures during final maturation. In contrast, temperature effects on final maturation were not measurable in female sockeye salmon because the temperature treatments occurred over a month before these fish historically reach maturity. The mechanisms for decreased steroid biosynthesis and the possible link to delayed or inhibited maturation in temperature-challenged migrating Pacific salmon warrant further investigation.

Water temperature appeared to affect ion balance, with plasma chloride and osmolality being higher in adult sockeye salmon held at 19°C compared with fish held at 13°C (the higher plasma chloride likely contributed to the higher osmolality), indicative of an osmoregulatory disturbance. Plasma chloride has been shown to increase with temperature in rainbow trout (Smit et al. 1981; Wagner et al. 1997), in an African cichlid (*Tilapia mossambica*; Allanson et al. 1971), and even in the nonteleost Adriatic sturgeon (*Acipenser naccarii*; Cataldi et al. 1998). Branchial chloride uptake increases at higher temperatures in Arctic grayling (*Thymallus arcticus*), and this

increase may be required for a blood buffer readjustment at the higher temperature to maintain acid-base equilibrium (Cameron 1976). Fish typically show a decrease in plasma pH with an increase in temperature (Reeves 1977), and chloride ions can be used to counter the increase in plasma H^+ to maintain ionic balance. Therefore, the higher plasma chloride detected in 19°C-held sockeye salmon may be associated with maintaining blood acid-base equilibrium at warm water temperatures and is indicative of a thermal stress response.

In summary, a 10-d exposure to an ecologically relevant high temperature (19°C) resulted in higher mortality in both pink and sockeye salmon, with evidence for sex-specific mortality patterns in sockeye salmon. This suggests that during years with extreme water temperatures, the magnitude of temperature-associated *en route* and prespawn mortality will vary by species and sex in Pacific salmon. It must be noted that the qualitative differences in mortality patterns detected in my study may have been influenced by the fact that the pink salmon were closer to final maturation than the sockeye salmon. While fisheries management models have evolved to include information on river temperature, my data suggest that the models would be improved by including sex and proximity to final maturation as additional factors. Additionally, my study demonstrated that there are reproductive consequences to exposure to high water temperature during final maturation, a response to temperature with profound ecological implications. If Pacific salmon populations are unable to adapt to the changing thermal regime in natal rivers, populations at the southern periphery of their species' distribution may continue to decline or possibly become extirpated.

Table 3.1. Model selection statistics summary for models describing survival for pink and sockeye salmon held at 13°C and 19°C. Models are ranked by increasing order of their AIC_c value and models included in the 95% confidence set for the best models are shown in bold. See text for description of AIC_c, Δ_i, w_i.

Pink Salmon

Model	AIC_c	Δ_i	w_i	# of parameters
Temperature	609.53	0.00	0.59	3
Temperature + Sex	611.01	1.48	0.28	4
Temperature x Sex	612.45	2.92	0.14	5
No Effects	670.81	61.28	0.00	2
Sex	672.75	63.22	0.00	3

Sockeye Salmon

Model	AIC_c	Δ_i	w_i	# of parameters
Temperature x Sex	339.19	0.00	0.71	5
Temperature + Sex	340.98	1.79	0.29	4
Sex	349.39	10.19	0.00	3
Temperature	372.17	32.97	0.00	3
No Effects	373.70	34.51	0.00	2

Table 3.2. Maturation status and gonadosomatic indices [means (S.D.)] of female sockeye and pink salmon that died during the 10 day treatment period. All pink salmon were sacrificed immediately after the experiment and were included in the analysis; any surviving female sockeye salmon were kept alive after the temperature holding period. Superscript letters that are different within a row indicate statistical significance at $P < 0.05$.

			Ripe		Not Ripe	
Species	Holding Temperature	Total Females (#)	n	GSI	n	GSI
Sockeye Salmon	13°C	16	1	17.4	15	13.8 (2.3)
	19°C	19	0	N/A	19	15.3 (5.4)
Pink Salmon	13°C	30	19	21.1 (3.1)^{A}	11	18.2 (4.0)^{A}
	19°C	48	11	22.2 (2.0)^{A}	37	18.1 (2.4)^{B}

GSI = Gonadosomatic index

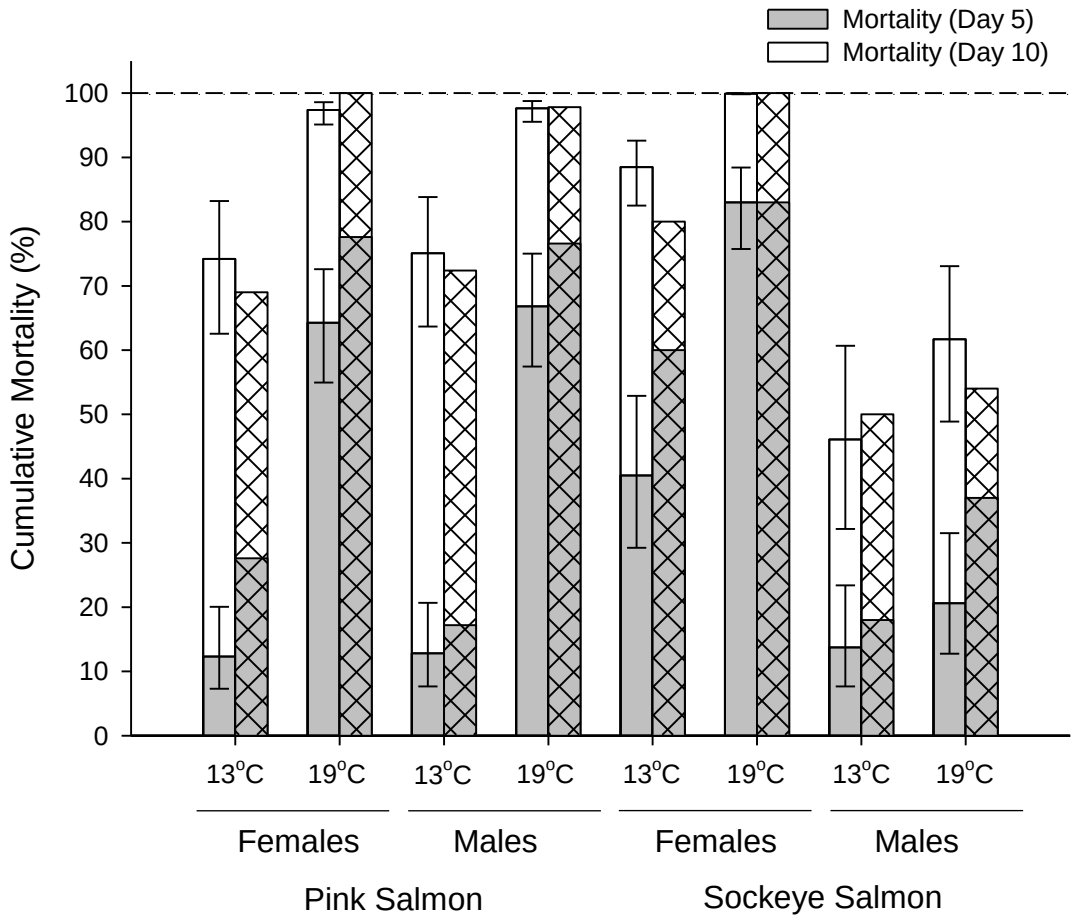


Figure 3.1. Model-average estimated (open bars) and observed (hatched bars) cumulative mortality at experimental days 5 and 10 for female and male pink and sockeye salmon held for 10 days at the treatment temperatures of 13°C and 19°C. Error bars denote 95% confidence intervals for the model-averaged estimates.

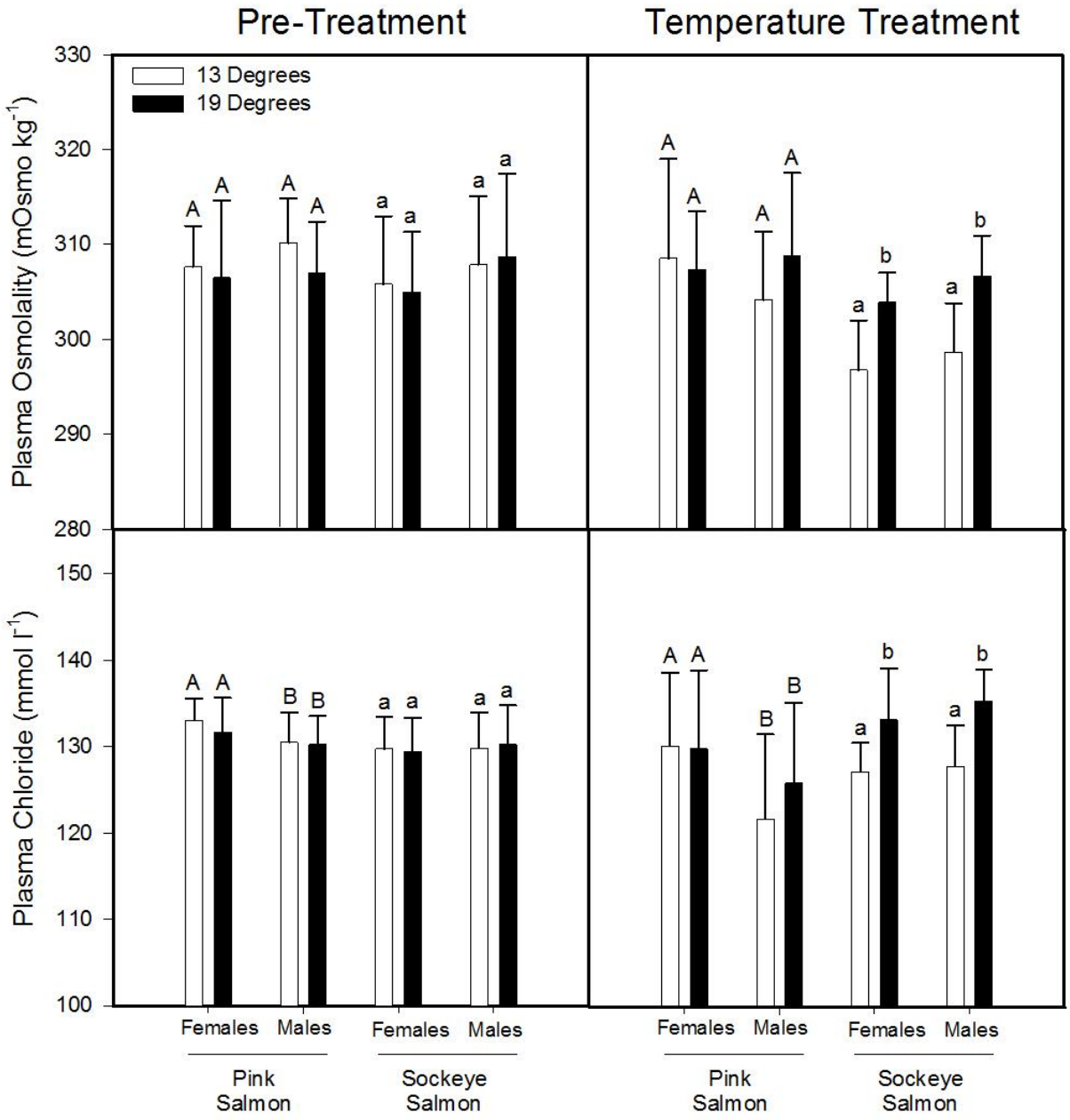


Figure 3.2. Pre-treatment and treatment (experimental day 5) plasma osmolality and chloride for female and male pink and sockeye salmon held at 13°C and 19°C (mean ± S.D.). Upper case letters indicate statistical differences among pink salmon groups and lower case letters indicate statistical differences among sockeye salmon groups.

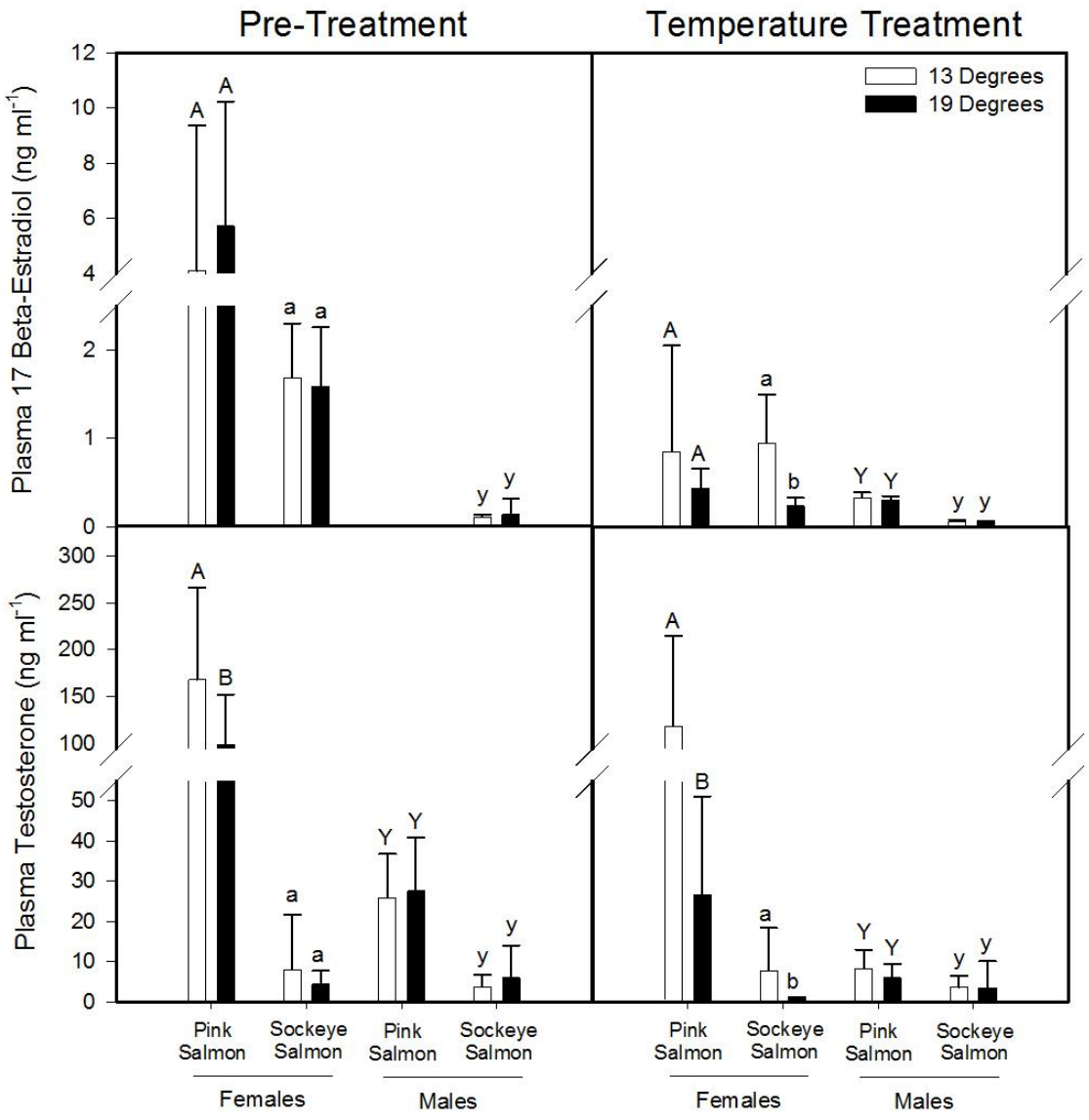


Figure 3.3. Pre-treatment and treatment (experimental day 5) plasma 17β-estradiol and testosterone for female and male pink and sockeye salmon held at 13°C and 19°C (mean ± S.D.). Upper case letters indicate statistical differences among pink salmon groups and lower case letters indicate statistical differences among sockeye salmon groups. There were no direct sex comparisons for plasma 17β-estradiol and testosterone.

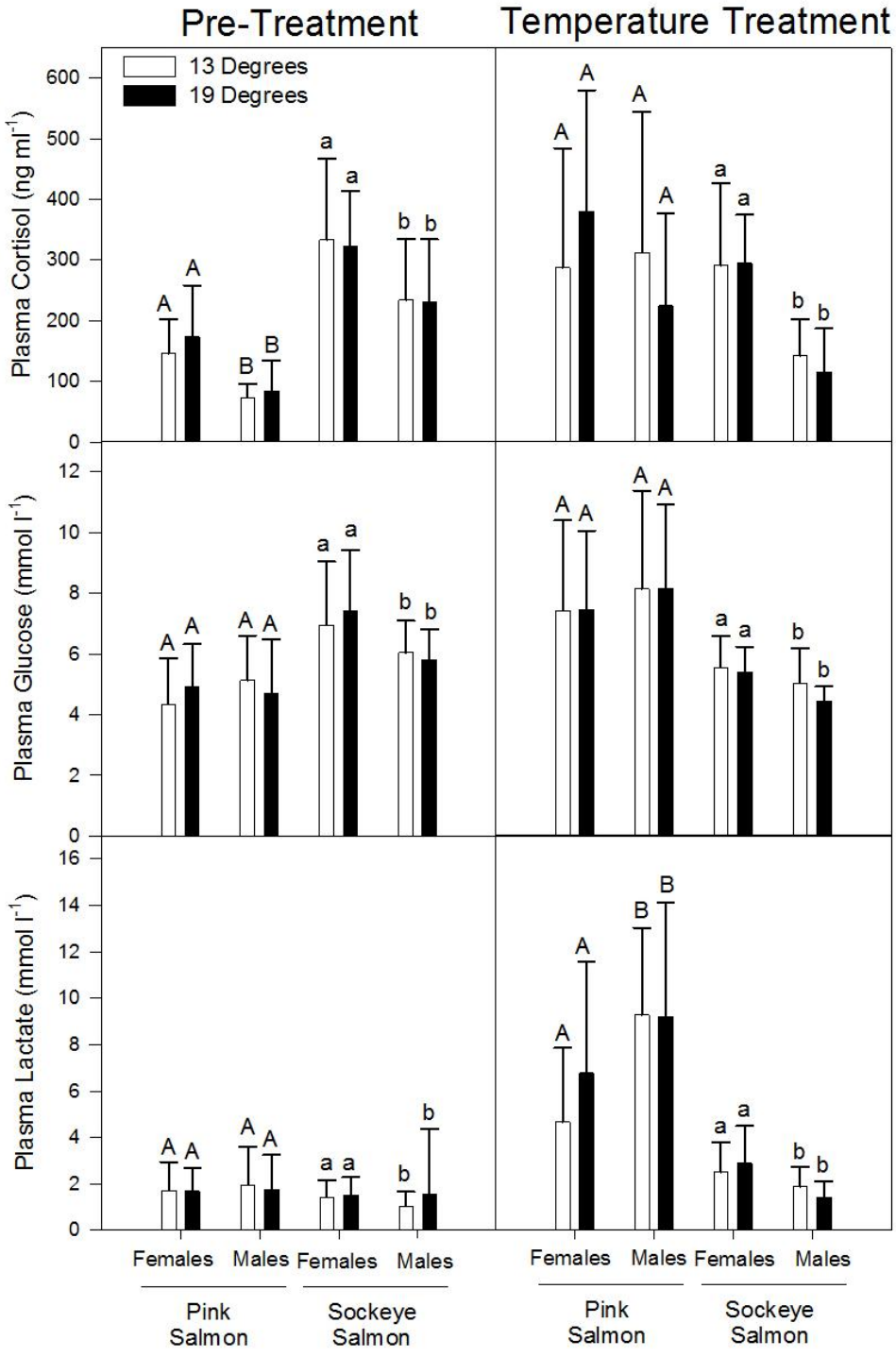


Figure 3.4. Pre-treatment and treatment (experimental day 5) plasma cortisol, glucose and lactate for female and male pink and sockeye salmon held at 13°C and 19°C (mean ± S.D.). Upper case letters indicate statistical differences among pink salmon groups and lower case letters indicate statistical differences among sockeye salmon groups.

Chapter 4: Consequences of high temperatures and premature mortality on the transcriptome and blood physiology of wild adult sockeye salmon (*Oncorhynchus nerka*)

4.1 Synopsis

Elevated river water temperature in the Fraser River, British Columbia, Canada, has been associated with enhanced mortality of adult sockeye salmon (*Oncorhynchus nerka*) during their up-river migration to spawning grounds. I undertook a study to assess the effects of elevated water temperatures on the gill transcriptome and blood plasma variables in wild-caught sockeye salmon. Naturally migrating sockeye salmon returning to the Fraser River were collected and held at ecologically relevant temperatures of 14°C and 19°C for seven days, a period representing a significant portion of their upstream migration. After seven days, sockeye salmon held at 19°C stimulated heat shock response genes as well as many genes associated with an immune response when compared with fish held at 14°C. Additionally, fish at 19°C had elevated plasma chloride and lactate, suggestive of a disturbance in osmoregulatory homeostasis and a stress response detectable in the blood plasma. Fish that died prematurely over the course of the holding study were compared with time-matched surviving fish; the former fish were characterized by an upregulation of several transcription factors associated with apoptosis and downregulation of genes involved in immune function and antioxidant activity. Ornithine decarboxylase (ODC1) was the most significantly upregulated gene in dying salmon, which suggests an association with cellular apoptosis. I hypothesize that the observed decrease in plasma ions and increases in plasma cortisol that occur in dying fish may be linked to the increase in ODC1. By highlighting these underlying physiological mechanisms, this study enhances our understanding of the processes involved in premature mortality and temperature stress in Pacific salmon during migration to spawning grounds.

4.2 Introduction

As a consequence of climate change, many populations of sockeye salmon (*Oncorhynchus nerka*) now experience significantly warmer river conditions during their once-in-a-lifetime spawning migration from the Pacific Ocean to freshwater spawning grounds. Spawning migrations during warm water periods are associated with increased levels of *en route* mortality (premature mortality during migration) and prespawn mortality (premature mortality on spawning grounds) in sockeye salmon (Gilhousen 1990; Macdonald et al. 2010; reviewed in Hinch and Martins 2011). Unless sockeye salmon are able to adapt to these climatic shifts, continued river warming will likely result in a reduced number of individuals reaching spawning grounds in the future for some populations (Hague et al. 2011; Martins et al. 2011). To assist with understanding the pervasive role of temperature in determining spawning success, my aim herein is to identify some of the physiological and molecular mechanisms involved in a temperature-induced stress response in sockeye salmon.

Interpretations of temperature-induced physiological changes during spawning migrations are complicated by the fact that sockeye salmon are simultaneously maturing, senescing, and starving (having ceased feeding prior to river entry). The fish also undergo dramatic physiological and morphological restructuring in preparation for spawning (Groot and Margolis 1991). This is followed by a period of rapid senescence, which typically occurs within days post-spawning and is accompanied by immunosuppression and organ deterioration (Dickhoff 1989; Finch 1990). The exact factors that cause post-spawning mortality remain contentious, but may include parasites and disease (Servizi and Jensen 1977; Gilhousen 1990; Bradford et al. 2010) and reduced osmoregulatory ability (Shrimpton et al. 2005; Chapter 2). Currently, it is unclear whether premature mortality associated with chronically elevated river temperatures results from acceleration of natural senescence processes, elevated virulence of disease, cardiovascular impairment, or some other factor. However, as yet, the molecular mechanisms involved in premature mortality in Pacific salmon have not been examined experimentally.

The development of a 16K gene salmonid cDNA microarray through GRASP (von Schalburg et al. 2005) has facilitated a rapid growth in salmonid genomics research. This salmonid microarray has been used to determine genomic responses to a variety of infectious

diseases negatively affecting the aquaculture industry, including infectious hematopoietic necrosis (IHN) virus (Miller et al. 2007), amoebic gill disease (Young et al. 2008), and saprolegniasis infection (Roberge et al. 2007). This microarray has also been used to characterize the heat stress response in rainbow trout (Lewis et al. 2010) and killifish (*Fundulus heteroclitus*; Healy et al. 2010). It has been applied to wild fish to examine molecular processes associated with intricate reproductive behaviors (Aubin-Horth et al. 2005), and to understand complex physiological processes in wild-caught adult sockeye salmon during spawning migrations (Miller et al. 2009, 2011; Evans et al. 2011), demonstrating the utility of this tool in understanding pertinent aspects of the biology of wild salmon.

Here, I utilize the GRASP 16K gene salmonid cDNA microarray to examine, for the first time, the physiological and molecular mechanisms associated with temperature stress and premature mortality in wild-caught adult sockeye salmon from two populations held under controlled conditions. I compare gene expression profiles of sockeye salmon held at ecologically relevant migration temperatures (14°C and 19°C) to determine the common effect of elevated water temperature on cellular processes between populations in non-lethally sampled gill tissue. I also contrast gene expression profiles of moribund fish with time-matched surviving fish to examine cellular processes associated with premature mortality. The gene expression profiles are complemented by blood plasma analyses to provide the most comprehensive examination to date of the mechanisms underlying temperature stress and premature mortality in adult sockeye salmon.

4.3 Materials and methods

4.3.1 Fish collection and thermal holding

Adult sockeye salmon (n = 130) from multiple populations were collected by beach seine from the Fraser River, British Columbia, Canada, 5–7 September 2007 (see Figure 2.1). Water temperatures during collection ranged from 15°C to 17°C. Fish were transported 45 min by road in aerated approximately 12°C water to the Fisheries and Oceans Canada Cultus Lake Laboratory, near Chilliwack, British Columbia, Canada, where they were randomly distributed

among four 8000-L aerated tanks at 12°C and at equal fish densities (sand-filtered and ultraviolet-sterilized water). Each tank contained a submersible pump that created a water flow of approximately 0.3 m s^{-1} into which the fish were able to orient and maintain position by continuous, gentle swimming. Fish were given three to six days to recover from transport, at which time all fish appeared vigorous and there were no external signs of disease. The tank water temperatures were subsequently raised at a rate of 2–2.5°C day^{-1} until the test temperatures of 14°C and 19°C were reached (two tanks at each temperature).

4.3.2 Tissue collection

DNA identification (Beacham et al. 2005) confirmed that the sockeye salmon sampled for blood and gill were from the Lower Adams and Chilko River populations. Any other populations collected were not used in the present study. After seven days of a stable test temperature (termed 'experimental day 7'), gill tissue was non-lethally sampled from surviving fish in both temperature groups (termed 'survivor' samples; 19°C treatment: n = 3 Lower Adams, n = 8 Chilko River; 14°C treatment: n = 6 Chilko River) to determine the effect of water temperature on gene expression. A time point of seven days was chosen because it approximates the period of time that these populations spend moving through the Lower Fraser River and the Fraser River Canyon (English et al. 2005); the sections of the Fraser River where these populations often experience the highest water temperatures. Fish were considered 'survivors' if they were swimming and maintaining position in the current on experimental day 7. There was higher mortality in the 19°C treatment (50% mortality after seven days compared with 25% mortality after seven days at 14°C). Survival patterns were continuously monitored in these fish for an additional 13 days. Fish that no longer maintained equilibrium around experimental day 7 (mean of 6.26 ± 0.46 days), but were still ventilating, were removed from the tanks, sampled for gill tissue and sacrificed (termed 'moribund' samples; 19°C treatment: n = 5 Lower Adams, n = 8 Chilko River; 14°C treatment: n = 4 Lower Adams, n = 6 Chilko River). Individuals from the survivor and moribund groups were mutually exclusive. Gill samples were immediately flash frozen in liquid nitrogen and stored at –80°C until analysis.

Blood samples obtained from the caudal vasculature using a heparinized vacutainer were collected at the same time as the gill samples for survivors and moribund fish to detect changes in osmoregulatory and stress indices. Blood samples (~3 mL) were immediately centrifuged for 7 min at 7000 × *g* and plasma was stored at –80°C prior to analyses. Plasma variables were measured using the procedures outlined in Farrell et al. (2001).

4.3.3 RNA extraction, amplification, labeling, and hybridization

The pre-hybridization protocols used in the present study were similar to those used in Miller et al. (2009, 2011). Briefly, total RNA from gill samples was purified from individual fish gill using Magmax^{TM}-96 for Microarrays Kits (Ambion Inc., Austin, TX) with a Biomek FXP (Beckman-Coulter, Mississauga, ON) automated liquid-handling instrument. Approximately 0.5 mg of gill tissue per fish was homogenized with stainless steel beads in 400 µL of TRI-reagent (Ambion Inc.) on a MM301 mixer mill (Retsch Inc., Newtown, PA). Aliquots of 100 µL of homogenate were pipetted into 96-well plates and extractions were carried out using the 'no-spin procedure' according to the manufacturer's instructions. In the final step, RNA was eluted and RNA yield was determined by measuring the A260 using a DTX 880 Multimode Detector (Molecular Devices, Sunnyvale, CA). Purity was assessed by measuring the A260/A280 ratio. Solutions of RNA were stored at –80°C until used for the microarray experiment or for qRT-PCR.

Total RNA (500 ng–5 µg) was amplified using a MessageAmp^{TM}II-96 kit (Ambion Inc.) according to manufacturer's instructions. Five µg of aRNA were reverse transcribed into cDNA, purified using Zymo-25 Clean-Up columns (Zymo Research, Irvine, CA) and labelled with Alexa dyes using the Invitrogen Indirect Labelling Kit (Invitrogen, Carlsbad, CA) following manufacturer's instructions. Treatment samples were labelled with Alexa 555 and reference samples, comprised of a pool of RNA from all the fish used in the study, were labelled with Alexa 647. After 1 h at room temperature, 50 µL of DNA binding buffer was added to each Alexa tube and sample and references for each slide were combined and purified in Zymo-25 Clean-Up columns (Zymo Research). Labeled cDNA was washed three times with DNA wash buffer (Zymo Research) and eluted in 9 µL of 1× TE buffer. Two µL of poly dA were added to

the labeled cDNA, followed by 10-min denaturation at 80◦C and the addition of 125 μL of prewarmed (65°C) SlideHybe3 buffer (Ambion Inc.). Samples were loaded into hybridization chambers in a Tecan-HS4800 Pro Hybridization Station (Tecan Trading AG, Männedorf, Switzerland).

4.3.4 Hybridization, normalization, and quality control

Each individual in the array study was hybridized on a single slide against a reference control. All steps from washing, hybridization, denaturation, and slide drying were carried out automatically on the Tecan-HS4800 Pro Hybridization Station. Fluorescent images were scanned using a Perkin Elmer ScanArray Express (Perkin Elmer, Boston, MA) and the signal-to-noise ratio was adjusted for optimized visualization of each image. The images were quantified using the program Imagene (BioDiscovery, El Segundo, CA). Each slide was normalized in BASE using the print-tip LOESS method. A detailed description of how slide quality and outliers were assessed can be found in supplemental material from Miller et al. (2011). The microarray data have been deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) with the accession number GSE33586.

4.3.5 Quantitative RT-PCR

Five genes known to be associated with a temperature response or with premature mortality were analyzed using qRT-PCR to validate microarray results (Table 4.1). Primers for four of the genes were designed in-house, with primers developed to equally match contigs of rainbow trout and Atlantic salmon. Transcription factor Jun B (JUNB) primers were published in Momoda et al. (2007). cDNA was synthesized from total RNA (1 μg) using an iScriptTM cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA) following manufacturer's instructions. A 1:2.5 dilution of cDNA was used as a template in the qRT-PCR assays. The relative quantification (RQ) assays were performed on an ABI 7900HT Fast real-time PCR system (Applied Biosystems, Carlsbad, CA) in 384-well plates using 20 μL reaction volumes that included 10 μL Kapa SYBR fast QPCR Master Mix (2x) (Kapa Biosystems, Inc., Woburn, MA),

0.4 μL of a mixture of 0.2 μM forward and reverse primers, 2 μL of diluted cDNA, and 7.6 μL of RNase/DNasefree water. The cycling conditions were 95°C for 3 min followed by 40 cycles of 95°C for 3 sec and 60°C for 30 sec, and a dissociation stage was added at each RQ run to ensure the presence of only a single amplicon. All samples were run in duplicate and with non-template controls included. Relative expression of target genes was determined using the comparative Ct method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). Target gene expression was normalized to two housekeeping genes (78d16.1 and BMP4); these housekeeping genes were not responsive to the experimental factors (M. R. Donaldson and S. Li, unpublished data).

4.3.6 Data analysis

I utilized both supervised and unsupervised analysis approaches on the microarray data. Unsupervised principal component analysis (PCA), conducted to identify the major transcriptional trajectories in the data, was computed using R as detailed in Alter et al. (2000). Imputed values were used when values were missing for the PCA. This method provides a ranking of genes characterizing their contribution (in terms of variance) with respect to the principal component (PC) of interest (i.e., the PC loading). Both positive and negative loadings were considered. The correlation of each PC axis with the blood plasma variables (continuous variables) was assessed using Spearman rank correlations. The relationship between each PC and the treatment groups (binary categorical variables) was assessed using Mann–Whitney U (MWU) tests. Supervised analyses were used to compare between treatment groups using MWU tests. Genes were considered significantly different between treatment groups at $q < 0.05$ [the false discovery rate (FDR) corrected *P*-value]. However, genes significant at $P < 0.001$, consistent with previous work on sockeye salmon (Miller et al. 2009), were also considered. It is important to note that multiple contigs of the same gene may reflect different expressed sequence tags (ESTs) of the same gene or duplicated copies of that gene; hence, I included all ESTs for each gene that was significant for my analyses.

Functional analysis was performed with ErmineJ (Lee et al. 2005) using the receiver operator characteristic (ROC) scoring method. The ROC method is a non-threshold method

performed on all gene rankings, which may be more robust than using raw gene scores (Lee et al. 2005). All three categories of the GO hierarchy (Biological Process, Molecular Function, and Cellular Component) were considered, limited to groups with 5–100 genes. However, only the categories Biological Process and Molecular Function are presented. The 'best-scoring replicate' method was used in ErmineJ to handle repeated measurements of the same gene. Gene sets in the ErmineJ analyses were considered significant at Benjamini–Hochberg FDR < 0.1 (e.g., consistent with Lockwood and Somero 2011).

All statistical tests on qRT-PCR and blood data were performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC) or SigmaPlot version 11.0 (SYSTAT Software, Inc., Chicago, IL). Differences in blood properties between temperature treatments or survival versus moribund status were assessed using *t*-tests with a Bonferroni adjusted critical alpha of 0.016 (Zar 1999) for the blood osmoregulatory (plasma osmolality, chloride, and sodium) and stress (plasma glucose, lactate, and potassium) indices. Potassium was included as a stress variable on the basis that it increases concurrently with lactate in stressed and moribund Pacific salmon (Chapter 2). If the assumption of normality could not be met as determined by Kolmogorov–Smirnov tests, nonparametric MWU tests were used (Sokal and Rohlf 1995). Statistical differences in qRT-PCR results were analyzed by two-factor analyses of variance (ANOVAs) with temperature and survival as factors. Tukey-Kramer pairwise comparisons were made *a posteriori* using the Bonferroni adjustment method with an adjusted critical alpha of 0.01 for the five genes used for qRT-PCR analysis. For the two-factor ANOVAs, homogeneity of variances was assessed by Bartlett's tests and normality was tested using Kolmogorov–Smirnov tests. All data were log10-transformed if the assumption of homogeneity of variances could not be met.

4.4 Results

4.4.1 Temperature effects on survivors

The temperature treatments were most strongly associated with PC3 (MWU, $P < 0.02$) in fish from the survivors group. Fish in the 19°C treatment were generally on the positive end of

the PC3 axis (Figure 4.1). Interestingly, the three 19°C treatment fish that were grouped on the negative end of the PC3 axis, with the 14°C fish, died one to two days after the experimental day 7 sampling. ErmineJ ROC analysis indicated that functional categories involved in protein biosynthesis, oxidative phosphorylation, immune response, and protein targeting and transport were significantly affected by the temperature treatments (Table 4.2). Plasma chloride ($\rho = 0.74$), sodium ($\rho = 0.57$), and osmolality ($\rho = 0.57$) were positively correlated with PC3 and therefore were generally higher in individuals in the 19°C treatment. In all cases, the population from which each fish belonged (Lower Adams or Chilko River) was not significantly associated with any of the PCs (MWU, $P > 0.05$); hence, populations were pooled for all the analyses. However, as there were only three surviving Lower Adams fish, the power to detect differences among populations was low. Pooling the populations for subsequent analyses allowed for the detection of a more general response to temperature that is common between these populations.

Relatively few genes were significantly different between the temperature treatment groups among survivors when supervised approaches were used, potentially due to small sample sizes. Of the 32 genes that had different levels of expression at $P < 0.001$ (MWU tests; Figure 4.2), only one gene was significantly upregulated in the 19°C treatment at $q < 0.05$ (heat shock protein 90; HSP90AB1). However, several of the 32 genes are known to be temperature responsive [e.g., HSP90AB1, serpin peptidase inhibitor (SERPINH1; also called heat shock protein 47), and cold-inducible RNA binding protein (CIRBP)] or are biologically relevant to the temperature exposure (see Discussion). Surprisingly, only two of the 32 genes [mitochondrial ribosomal protein (VAR1) and eukaryotic translation elongation factor 2 (EEF2)] were loaded highly on the PC3 axis. Moreover, as observed with PC3, survivors held at 19°C had higher plasma chloride (*t*-test, $P = 0.014$), but also possessed higher plasma lactate (*t*-test, $P < 0.001$) and potassium (*t*-test, $P < 0.005$) than survivors held at 14°C (Figure 4.3). Survivors held at 19°C had higher plasma osmolality (*t*-test, $P = 0.037$) and glucose (MWU, $P = 0.039$) than survivors held at 14°C; however, they were not significantly different after Bonferroni correction.

4.4.2 Temperature effects on moribund fish

The temperature treatment was nearly significantly associated with PC1 (MWU, $P = 0.06$) and marginally significantly associated with PC2 (MWU, $P = 0.049$) in the fish that became moribund. Only plasma glucose was strongly correlated with PC1 ($\rho = 0.81$). ErmineJ ROC analysis on PC2 indicated that functional categories associated with protein biosynthesis, cell structural components, and protein transport and binding were significantly affected by the temperature treatments in fish that became moribund (Table 4.2). When the moribund fish were grouped by temperature treatment and directly contrasted, 80 genes had different levels of expression at $P < 0.001$ (MWU tests; Figure 4.2) with nine genes differentially expressed at $q < 0.05$ [Upregulated at 19°C: protein disulfide isomerase family A, member 4 (PDIA4), cyclin B1 (CCNB1), HSP90AB1; Downregulated at 19°C: hyperosmotic glycine rich protein and splicing factor, arginine/serine-rich 2 (no gene symbols available), CIRBP, peroxisomal proliferator-activated receptor A interacting complex 285 (PRIC285), matrix metallopeptidase 2 (MMP2), and cytokine-induced protein 29 KDA (CIP29)]. The genes F9 (coagulation factor IX), SERPINH1, CIRBP and splicing factor, arginine/serine-rich 2 (Figure 4.4), and copies of HSP90AB1 and EEF2 (different contigs between the two groups) had the same transcriptional response due to the temperature treatments as fish in the survivors group. Moribund fish held at 19°C had higher plasma glucose (MWU, $P = 0.021$) and lower plasma potassium (MWU, $P = 0.039$) than moribund fish held at 14°C, but they were not significantly different after Bonferroni correction (Figure 4.3).

4.4.3 Moribund versus survivor patterns

When all samples were combined into a single PCA, PC1 was strongly associated with differences between fish that survived versus those that became moribund (MWU, $P < 1 \times 10^{-7}$), but did not differentiate fish on the basis of temperature (MWU, $P > 0.05$). In fact, temperature was not significantly associated with the first five PCs, which explained much (25.9%) of the variance in the data. Survivors were generally located on the positive end of the PC1 axis (Figure 4.1). The two survivor fish that were grouped with the moribund fish died one to two days after

the experimental day 7 sampling. ErmineJ ROC analysis indicated that functional categories involved in immune response, protein biosynthesis, antioxidant activity, and protein catalytic activity were significantly affected in fish that became moribund (Table 4.2). Plasma chloride (ρ = 0.75), sodium (ρ = 0.70), and glucose (ρ = 0.67) were positively correlated with PC1 (and therefore survivorship), while plasma lactate (ρ = –0.75) was negatively correlated with PC1. Because plasma chloride levels are the strongest predictor of longevity in adult sockeye salmon (Chapter 2) and were highly correlated with PC1, I assessed the 46 genes most correlated ($P < 1 \times 10^{-7}$) with plasma chloride levels (Figure 4.5). Many contigs of the immune response genes, MHC class II associated invariant chain (CD74), histocompatibility complex class II (H2-EB1, also called MHC II), and invariant chain-like protein 2 (ICLP2) along with ornithine decarboxylase 1 (ODC1), were among the 46 genes most correlated with plasma chloride levels.

Because the temperature treatments were not a significant factor in the PCA when survivors and moribund fish were pooled for analysis, both temperature treatment groups were pooled for the subsequent analysis. When survivors and moribund fish were contrasted directly, 751 genes had different levels of expression at $P < 0.001$ (MWU tests), with 1503 genes differentially expressed at $q < 0.05$ (Figure 4.6). There was little overlap between these 751 genes and the significant gene lists associated with the temperature treatments (Figure 4.4). The most significantly upregulated gene in the moribund fish was ODC1 (MWU, $P < 1.0 \times 10^{-13}$). Of the 136 genes that had different levels of expression at $P < 1.0 \times 10^{-6}$, there was significant overlap in the transcriptional response with the genes most correlated with the PC1 axis (Figure 4.1). Survivors had significantly higher plasma chloride (MWU, $P < 0.001$), sodium (MWU, $P < 0.001$), and glucose (*t*-test, $P < 0.005$), and significantly lower plasma lactate (MWU, $P < 0.001$) and potassium (MWU, $P < 0.001$) than fish that became moribund (Figure 4.2).

4.4.4 qRT-PCR results

The five genes used to validate the microarray results showed the same directional change as determined by the microarrays (Figure 4.7). For both survivors and moribund fish, HSP90AB1 ($F = 120.7$, $P < 0.0001$) and SERPINH1 ($F = 269.3$, $P < 0.0001$) had significantly higher mean normalized expression in fish held at 19°C compared with fish held at 14°C, and

CIRBP ($F = 71.3$, $P < 0.0001$) had lower mean normalized expression in fish held at 19°C. These data provide some validation that the $P < 0.001$ array results were providing true positives, despite the poor level of *q*-value support. HSP90AB1 ($F = 36.2$, $P < 0.0001$) and CIRBP ($F = 36.3$, $P < 0.0001$) were also significantly affected by survival versus moribund status, as both were expressed at a higher level in moribund fish than survivors in the qRT-PCR analysis; however, these genes were not among the 751 most differentially expressed genes in the array analysis. Cytochrome C [cyt c (≈Cycs); $F = 92.9$, $P < 0.0001$] and JUNB ($F = 128.6$, $P < 0.0001$) also had significantly higher mean normalized expression in the moribund group compared with survivors, as predicted from the microarray data.

4.5 Discussion

4.5.1 Temperature patterns

River temperatures reaching 19°C become stressful for migrating adult Fraser River sockeye salmon (Macdonald et al. 2000), but this temperature has not been historically considered acutely lethal (e.g., survival <24 h; Servizi and Jensen 1977). Nevertheless, migration for several days at ≥19°C results in increased *en route* mortality in at least some sockeye salmon populations (Martins et al. 2011). Additionally, chronic exposure to 19°C in a laboratory study resulted in increased mortality and evidence of a stress response in the blood of sockeye and pink salmon (Chapter 3). Indeed, in the present study there was evidence of a thermal stress response in fish held at 19°C compared with fish held at 14°C in both the gene expression profiles and blood properties. Regardless of survival status, the genes HSP90AB1 and CIRBP, which are thermally responsive (Sonna et al. 2002), were transcriptionally modified because of the high temperature treatment in the present study. The directional changes in those genes were similar to the changes detected in thermally stressed arctic charr (*Salvelinus alpinus*; Quinn et al. 2011), and in wild sockeye salmon experiencing a 7–8°C increase in water temperature when transitioning from saltwater to freshwater (Evans et al. 2011). The upregulation of molecular chaperones in fish is a common response to elevated water temperatures. The collagen-specific endoplasmic reticulum resident chaperone SERPINH1, involved in collagen stabilization during

stress (Krone et al. 1997), was upregulated at 19°C. In addition to the heat shock proteins HSP90AB1 and SERPINH1, the molecular chaperone PDIA4, which is involved in protein folding and is thermally responsive in fish (Logan and Somero 2010), was also upregulated at 19°C. The gene CIRBP is involved in RNA stabilization and has been associated with osmotic stress in fish (Pan et al. 2004; Evans 2010); however, it is also involved in a cold shock response in vertebrates (Sonna et al. 2002). These results show that CIRBP is thermally responsive in sockeye salmon over a 5°C temperature range. However, given that fish in the 19°C treatment also showed an increase in plasma chloride, suggesting a disturbance in osmoregulatory homeostasis and consistent with previous work (Chapter 3), I cannot discern whether CIRBP was responding directly to temperature or to an osmotic perturbation. Additionally, there was an increase in plasma lactate and potassium in fish held at 19°C compared with those held at 14°C indicating that the 19°C treatment induced a stress response detectable in the blood properties.

Protein biosynthesis represents a significant portion of the cellular energy budget. In some eurythermal fish, protein biosynthesis may be upregulated to potentially compensate for higher rates of protein turnover at high temperatures (Logan and Somero 2010). However, the present study found that several genes involved in protein biosynthesis were downregulated at 19°C in sockeye salmon (CIRBP, hyperosmotic glycine rich protein, splicing factor, arginine/serine-rich 2, PRIC285, and CIP29). Additionally, genes involved in mRNA translation [elongation and initiation factors: EEF2, EIF3S6 (also EIF5 and EIF3H correlated with PC3) in survivors; EEF2L in moribunds] and various ribosomal proteins were downregulated in sockeye salmon held at 19°C (e.g., RPL7A and RPL3 correlated with PC3 in survivors; RPL31 and RPS25 in fish that became moribund). Many functional categories involved in protein biosynthesis were significantly enriched in both the survivors and moribund groups due to the temperature treatments. These results suggest that adult sockeye salmon may alter protein biosynthesis during periods of heat stress. Cellular energy expenditure may be significantly reduced by downregulating protein biosynthesis in energy stressed cells (Staples and Buck 2009); hence, a downregulation of nonessential protein biosynthesis may be a strategy to conserve finite energy stores during a temperature-induced increase in metabolic rate.

The upregulation of genes involved in immunity [e.g., CD74, H2-EB1, interferon regulatory factor 1 (IRF1), tumor necrosis factor alpha-induced protein 2 (TNFAIP2), all correlated with PC3; Ig kappa chainV-IV region B17 precursor (no gene symbol available) in

survivors; MGC84465 in moribund fish] in sockeye salmon held at 19°C may result from temperature stress, but could also be stimulated by the enhanced virulence of many salmon diseases at higher temperatures. Pacific salmon are exposed to a wide variety of pathogens and parasites when they transition back to freshwater during spawning migrations, many of which have temperature-dependant progressions (Rucker et al. 1954). ErmineJ ROC analysis of PC3 confirmed that functional categories relating to an immune response were significantly affected by the high water temperature treatment in survivors. Indeed, many of the genes most correlated with PC3 are involved in immunity, rather than a thermal stress response as determined by the supervised analysis approach. Fraser River sockeye salmon are often affected by diseases caused by the bacteria *Flexibactor columnaris*, the kidney parasite *Parvicapsula minibicornis,* and the fungus *Saprolegnia* sp., which all progress faster at higher temperatures (Servizi and Jensen 1977; Crossin et al. 2008).

4.5.2 Mortality patterns

The most significantly upregulated gene in sockeye salmon that became moribund compared with survivors was ODC1, which is involved in polyamine synthesis and has been linked to cellular apoptosis (Pignatti et al. 2004). Accumulation of polyamines in the cell may increase the permeability of mitochondrial membranes, which results in cyt c and other proteins being released into the cytoplasm and initiates the post-mitochondrial phase of apoptosis (Pignatti et al. 2004). Polyamines are also involved in the phosphorylation of MAPK, which leads to the upregulation of several transcription factors (Bachrach et al. 2001), like JUNB, that may be involved in apoptosis. Along with the upregulation of ODC1, I detected an upregulation of cyt c and JUNB, both of which are linked to cell apoptosis. The apparent role of ODC1 in cellular apoptosis in dying sockeye salmon is an interesting discovery and warrants further investigation.

The extracellular signal(s) that triggered the upregulation of ODC1 in gill tissue in moribund sockeye salmon is unknown. However, there is evidence that ODC1 is upregulated during periods of hypoosmotic stress (Watts et al. 1996; Lockwood and Somero 2011). Interestingly, sockeye salmon plasma ions decrease throughout their adult freshwater residency

(Shrimpton et al. 2005) and mortality is preceded by drastic decreases in plasma chloride, sodium, and osmolality that begin days before the fish dies (Chapter 2). I detected lower levels of plasma chloride and sodium in moribund fish at both temperatures, which could effectively create a hypoosmotic stress for cells and potentially lead to the increase in ODC1. Indeed, ODC1 was one of the genes most strongly correlated with plasma chloride levels. There is also evidence that cellular ODC1 activity may be stimulated by cortisol (Wu et al. 2000), a hormone that increases in the plasma of sockeye salmon at death (Hruska et al. 2010; Chapter 2). Therefore, the osmoregulatory failure and spike in plasma cortisol that occur in moribund sockeye salmon could be linked with the upregulation of ODC1 in gill tissue. To my knowledge, this is the first evidence of this potential relationship between apoptosis and ODC1 upregulation in dying Pacific salmon and in any other dying semelparous animal.

There was increased expression of transcription factors, in addition to JUNB, in fish that became moribund. Nuclear protein 1 (NUPR1), which is a stress-responsive transcription factor involved in a wide variety of cell functions (Chowdhury et al. 2009), including cell growth, was upregulated. An increase in NUPR1 may also be related to or correlated with elevated cortisol levels in a generalized fish stress response (Momoda et al. 2007). Because cortisol levels are known to be elevated in moribund Pacific salmon (Hruska et al. 2010; Chapter 2), a potential relationship between NUPR1 and cortisol is possible in moribund sockeye salmon. The transcription factor CCAAT/enhancer binding protein beta (CEBPB), which has a role in cell proliferation (Lekstrom–Himes and Xanthopoulos 1998), was also upregulated in moribund salmon. However, CEBPB is also involved in an inflammatory response (Lekstrom-Himes and Xanthopoulos 1998) and therefore its upregulation may also be related to processes involved in immune function.

Adult sockeye salmon become progressively immunosuppressed throughout their freshwater spawning migration, and disease has been suggested as the ultimate cause of death in adult Pacific salmon (Gilhousen 1990). Several genes involved in the immune and inflammatory response [e.g., chemokine C-C motif ligand 19 (CCL19), allograft inflammatory factor 1 (AIF 1), and complement factor D (CFD)] were downregulated in moribund sockeye salmon, consistent with immunosuppression. Indeed, ErmineJ ROC analysis indicated that the functional categories defense response and response to wounding were significantly enriched in PC1. Additionally, many of the genes strongly correlated with plasma chloride levels, used as a proxy for survival

status, were associated with an immune response (e.g., CD74, H2-EB1, ICLP2), indicating the complex relationship between plasma chloride, osmoregulatory ability, immunosuppression, and other molecular processes that occur in gill tissue as fish die. Many contigs of the antigen processing and presenting gene major H2-EB1, and the associated CD74, were also downregulated in moribund sockeye salmon. Additionally, the transcription activator IRF1, involved in regulating MHC class I and II related genes (Young et al. 2008), was downregulated in moribund fish. MHC II genes are involved in the humoral immune response generally associated with extracellular pathogens such as bacteria and parasites. By downregulating humoral immunity during senescence, bacterial infections like those caused by *F. columnaris*, common in Fraser River sockeye salmon and believed to cause significant mortality in some Pacific salmon populations (Servizi and Jensen 1977), could become more virulent.

4.6 Conclusions

The semelparous life history of Pacific salmon allows for only one opportunity to migrate to spawning grounds and successfully spawn. Spawning migrations during warm water periods may lead to increased levels of premature mortality for sockeye salmon, which results in a lifetime fitness of zero for those individuals. The present study is the first to demonstrate the effects of water temperature and mortality on the cellular-level physiology of wild-caught Pacific salmon held under controlled conditions. I show that the gill transcriptome of sockeye salmon held for seven days at 19°C showed an increase in heat shock and immune responses, and a decrease in expression of genes involved in protein biosynthesis compared with salmon held at 14°C. These patterns were common between the two populations. There were also indications of individual variability in adaptive responses to thermal stress that may have been associated with survival; the fish that did not respond similarly to other fish in the high temperature treatment, but survived seven days, were dead by day 9, potentially because of their lack of an appropriate cellular response to temperature stress. The potential role of a lack of an appropriate response to temperature stress in temperature-induced mortality is an interesting observation that warrants further investigation. My data also suggest that Pacific salmon may be increasingly affected by temperature-dependent diseases when migrating during periods of elevated (but not acutely lethal) water temperatures, which can lead to premature mortality. Despite the fact that neither

population nor sex were identified as strong factors in the gene expression profiles in the present study, recent work has shown the importance of considering population- (Eliason et al. 2011) and sex-specific (Clark et al. 2009; Sandblom et al. 2009; Chapter 3) differences in Fraser River sockeye salmon populations when interpreting physiological responses to high water temperature. Therefore, future studies should specifically examine the role of population- and sex-specific differences in thermal tolerance at the cellular level. This study also presents some of the first data showing the gene expression changes associated with premature mortality, which may or may not parallel final senescence, and suggests that ODC1-mediated processes are involved in cell death in dying Pacific salmon. Genetic and biochemical processes associated with mortality may be useful in developing assays for understanding causes of premature mortality and predicting the fate of adult Pacific salmon throughout the migration and upon arrival at spawning grounds.

Table 4.1. Primer sequences and expressed sequence tag (EST) numbers for the genes selected for quantitative real-time PCR (qRT-PCR) analysis.

Gene Group	Gene Name	Gene Symbol	EST Number	Primers (5'-3')
Temperature Responsive	Heat Shock Protein 90	HSP90AB1	CB493960	F-TGGGCTACATGGCTGCCAAG
				R-TCCAAGGTGAACCCAGAGGAC
	Serpin peptidase inhibitor	SERPINH1	CA063723	F-TCCACTTTCCACCCTGCAAAG
				R-AGTTTGGTTGGCAAATGGCATAG
	Cold-Inducible RNA Binding Protein	CIRBP	CB499204	F-AAGCTGTGATTGTGCTCTAAAGAC
			CA048095 CA064457	R-TCCCACTTAGCATTCCATCCTTG
Mortality Responsive	Cytochrome c	Cyc c	CB494539	F-CGAGCGTGCAGATCTTATAGC
				R-CTTCTCCGCTGAACAGTTGATG
	Transcription Factor Jun B	JUNB	N/A*	F-CTACACGCACAGCGATATTCG
				R-TCGTCGCTGCTCTGCATGT
Housekeeping Genes	Bone Morphogenetic Protein 4	BMP4	CA056395	F-TTGCCCATAGTCAGTGTTAGCG
				R-GTGCCATCTCCATGCTCTACC
	Si:dkey-78d16.1 protein [*Danio rerio*]	N/A	CA056739	F-AAAGGTCCCACGCTCCAAAC
				R-ACACACCCATCTGTCTCATCACC

* Primer sequences for JUNB were previously published in Momoda et al. (2007).

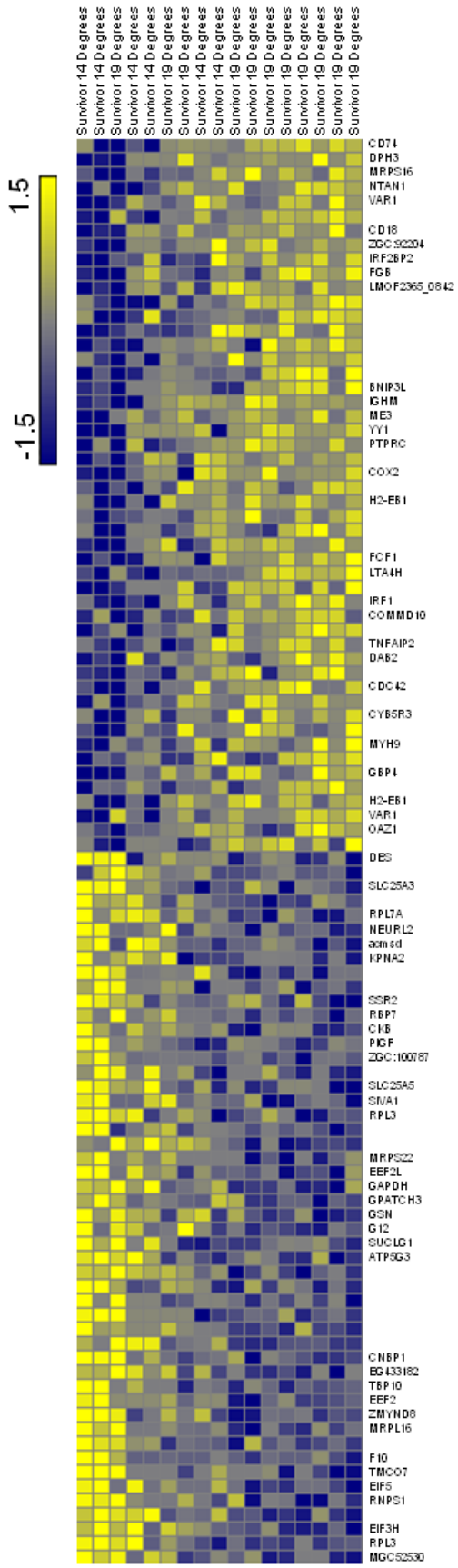
Table 4.2. Functional analysis performed using the receiver operator characteristic (ROC) scoring method in ErmineJ. The Gene Ontology (GO) categories Biological Process and Molecular Function are presented here. Only gene sets significant at false discovery rate corrected *P*-values < 0.1 are presented.

GO Category	GO ID	Name	GO ID	Name	# of Genes	Raw Score	Corrected P-value
Temperature effects on survivors							
Biological Process	GO:0065007	Biological Regulation	GO:0006446	regulation of translational initiation	14	0.81	0.0087
			GO:0006417	regulation of translation	14	0.79	0.011
			GO:0010608	posttranscriptional regulation of gene expression	18	0.74	0.015
			GO:0008361	regulation of cell size	14	0.72	0.065
	GO:0044237	Cellular Metabolic Process	GO:0006119	oxidative phosphorylation	8	0.86	0.011
			GO:0006091	generation of precursor metabolites and energy	19	0.74	0.016
	GO:0009058	Biosynthetic Process	GO:0006412	translation	44	0.65	0.020
			GO:0006633	fatty acid biosynthetic process	6	0.85	0.039
			GO:0006487	protein amino acid N-linked glycosylation	12	0.75	0.045
	GO:0002376	Immune System Process	GO:0006955	immune response	37	0.66	0.028
	GO:0009987	Cellular Process	GO:0006886	intracellular protein transport	45	0.63	0.045
			GO:0006605	protein targeting	20	0.69	0.049
			GO:0016197	endosome transport	7	0.81	0.054
			GO:0007005	mitochondrion organization	13	0.72	0.090
			GO:0006839	mitochondrial transport	7	0.79	0.089
			GO:0034613	cellular protein localization	40	0.62	0.098
	GO:0050896	Response to Stimulus	GO:0006952	defense response	38	0.64	0.063
			GO:0006954	inflammatory response	21	0.67	0.088
	GO:0043170	Macromolecule Metabolic Process	GO:0009100	glycoprotein metabolic process	19	0.68	0.081

GO Category	GO ID	Name	GO ID	Name	# of Genes	Raw Score	Corrected P-value
	GO:0055114	Oxidation-Reduction Process	GO:0045333	cellular respiration	5	0.84	0.090
Molecular Function	GO:0005488	Binding	GO:0003723	RNA binding	69	0.63	0.011
			GO:0008135	translation factor activity, nucleic acid binding	26	0.67	0.046
			GO:0008022	protein C-terminus binding	14	0.70	0.096
	GO:0003824	Catalytic Activity	GO:0016651	oxidoreductase activity, acting on NADH or NADPH	14	0.76	0.020
			GO:0016655	oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	8	0.78	0.084
			GO:0008137	NADH dehydrogenase (ubiquinone) activity	8	0.76	0.095
	GO:0005198	Structural Molecule Activity	GO:0003735	structural constituent of ribosome	27	0.68	0.025
			GO:0005198	structural molecule activity	53	0.61	0.080
Temperature effects on moribund fish							
Biological Process	GO:0065007	Biological Regulation	GO:0006355	regulation of transcription, DNA-dependent	98	0.61	0.030
			GO:0051252	regulation of RNA metabolic process	97	0.60	0.040
			GO:0051130	positive regulation of cellular component organization	7	0.83	0.074
			GO:0006446	regulation of translational initiation	14	0.73	0.072
			GO:0051246	regulation of protein metabolic process	36	0.64	0.091
			GO:0009892	negative regulation of metabolic process	58	0.61	0.092
	GO:0009058	Biosynthetic Process	GO:0006412	translation	46	0.64	0.043

GO Category	GO ID	Name	GO ID	Name	# of Genes	Raw Score	Corrected P-value
	GO:0051179	Localization	GO:0015031	protein transport	47	0.63	0.068
			GO:0008104	protein localization	54	0.61	0.088
	GO:0009057	Catabolic Process	GO:0009057	macromolecule catabolic process	50	0.62	0.080
	GO:0009987	Cellular Process	GO:0051649	establishment of localization in cell	98	0.58	0.087
Molecular Function	GO:0005198	Structural Molecule Activity	GO:0003735	structural constituent of ribosome	28	0.70	0.039
			GO:0005198	structural molecule activity	56	0.62	0.074
			GO:0005200	structural constituent of cytoskeleton	13	0.73	0.083
	GO:0051082	Binding	GO:0051082	unfolded protein binding	14	0.72	0.081
Moribund vs. Survivor Patterns							
Biological Process	GO:0009058	Biosynthetic Process	GO:0006412	translation	46	0.68	0.0027
	GO:0006950	Response to Stress	GO:0009611	response to wounding	40	0.65	0.042
			GO:0042221	response to chemical stimulus	50	0.62	0.094
			GO:0006952	defense response	41	0.63	0.091
	GO:0009987	Cellular Process	GO:0016192	vesicle-mediated transport	57	0.61	0.091
			GO:0006928	cellular component movement	33	0.65	0.086
			GO:0046907	intracellular transport	87	0.59	0.083
	GO:0044237	Cellular Metabolic Process	GO:0006690	icosanoid metabolic process	6	0.82	0.091
	GO:0048731	System Development	GO:0048513	organ development	100	0.58	0.092
			GO:0007399	nervous system development	75	0.59	0.097
	GO:0051179	Localization	GO:0051674	localization of cell	32	0.64	0.098
Molecular Function	GO:0005198	Structural Molecule Activity	GO:0003735	structural constituent of ribosome	28	0.73	0.0050
			GO:0005198	structural molecule activity	56	0.62	0.050

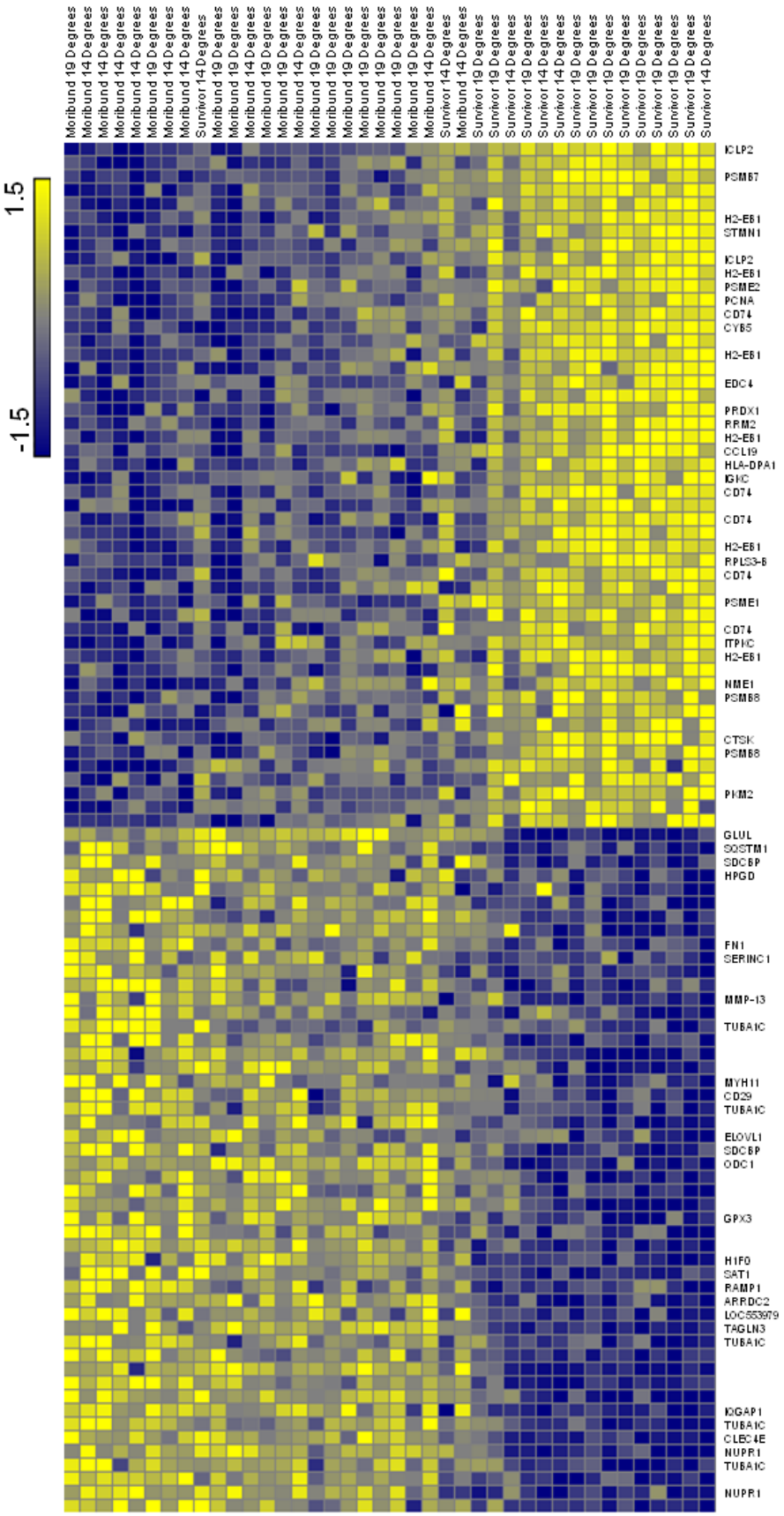
GO Category	GO ID	Name	GO ID	Name	# of Genes	Raw Score	Corrected P-value
	GO:0005488	Binding	GO:0003723	RNA binding	72	0.62	0.024
			GO:0003746	translation elongation factor activity	5	0.85	0.091
			GO:0005543	phospholipid binding	16	0.70	0.096
	GO:0016209	Antioxidant Activity	GO:0016209	antioxidant activity	7	0.84	0.046
	GO:0003824	Catalytic Activity	GO:0008238	exopeptidase activity	7	0.81	0.087
			GO:0070011	peptidase activity, acting on L-amino acid peptides	33	0.65	0.093
			GO:0008233	peptidase activity	35	0.64	0.090
	GO:0005215	Transporter Activity	GO:0008565	protein transporter activity	13	0.72	0.096



A)

Figure 4.1. Heat maps showing the top 50 positively and top 50 negatively loaded genes for the principal component (PC) most related with A) temperature in survivors (PC3) and B) for comparisons between survivors and fish that became moribund (PC1; next page). Relative expression levels are indicated by the color scale with yellow indicating upregulation and blue indicating downregulation. Ordering of fish reflects their relative PC scores along the PC axis. Gene symbols (if available) are presented along the right side of the heat map.

B)


Moribund 19 Degrees
Moribund 14 Degrees
Moribund 14 Degrees
Moribund 14 Degrees
Moribund 14 Degrees
Moribund 19 Degrees
Moribund 19 Degrees
Moribund 14 Degrees
Survivor 14 Degrees
Moribund 19 Degrees
Moribund 19 Degrees
Moribund 14 Degrees
Moribund 19 Degrees
Moribund 19 Degrees
Moribund 14 Degrees
Moribund 19 Degrees
Moribund 19 Degrees
Moribund 19 Degrees
Moribund 19 Degrees
Moribund 14 Degrees
Moribund 14 Degrees
Survivor 14 Degrees
Moribund 14 Degrees
Survivor 19 Degrees
Survivor 19 Degrees
Survivor 14 Degrees
Survivor 19 Degrees
Survivor 14 Degrees
Survivor 14 Degrees
Survivor 19 Degrees
Survivor 19 Degrees
Survivor 19 Degrees
Survivor 19 Degrees
Survivor 19 Degrees
Survivor 19 Degrees
Survivor 19 Degrees
Survivor 14 Degrees
1.5
-1.5
ICLP2
PSMB7
H2-EB1
STMN1
ICLP2
H2-EB1
PSME2
PCNA
CD74
CYB5
H2-EB1
EDC4
PRDX1
RRM2
H2-EB1
CCL19
HLA-DPA1
IGHC
CD74
CD74
H2-EB1
RPLS3-8
CD74
PSME1
CD74
ITPKC
H2-EB1
NME1
PSMB8
CTSK
PSMB8
PKM2
GLUL
SQSTM1
SDCBP
HPGD
FN1
SERINC1
MMP-13
TUBA1C
MYH11
CD29
TUBA1C
ELOVL1
SDCBP
ODC1
GPX3
H1F0
SAT1
RAMP1
ARRDC2
LOC553979
TAGLN3
TUBA1C
IQGAP1
TUBA1C
CLEC4E
NUPR1
TUBA1C
NUPR1

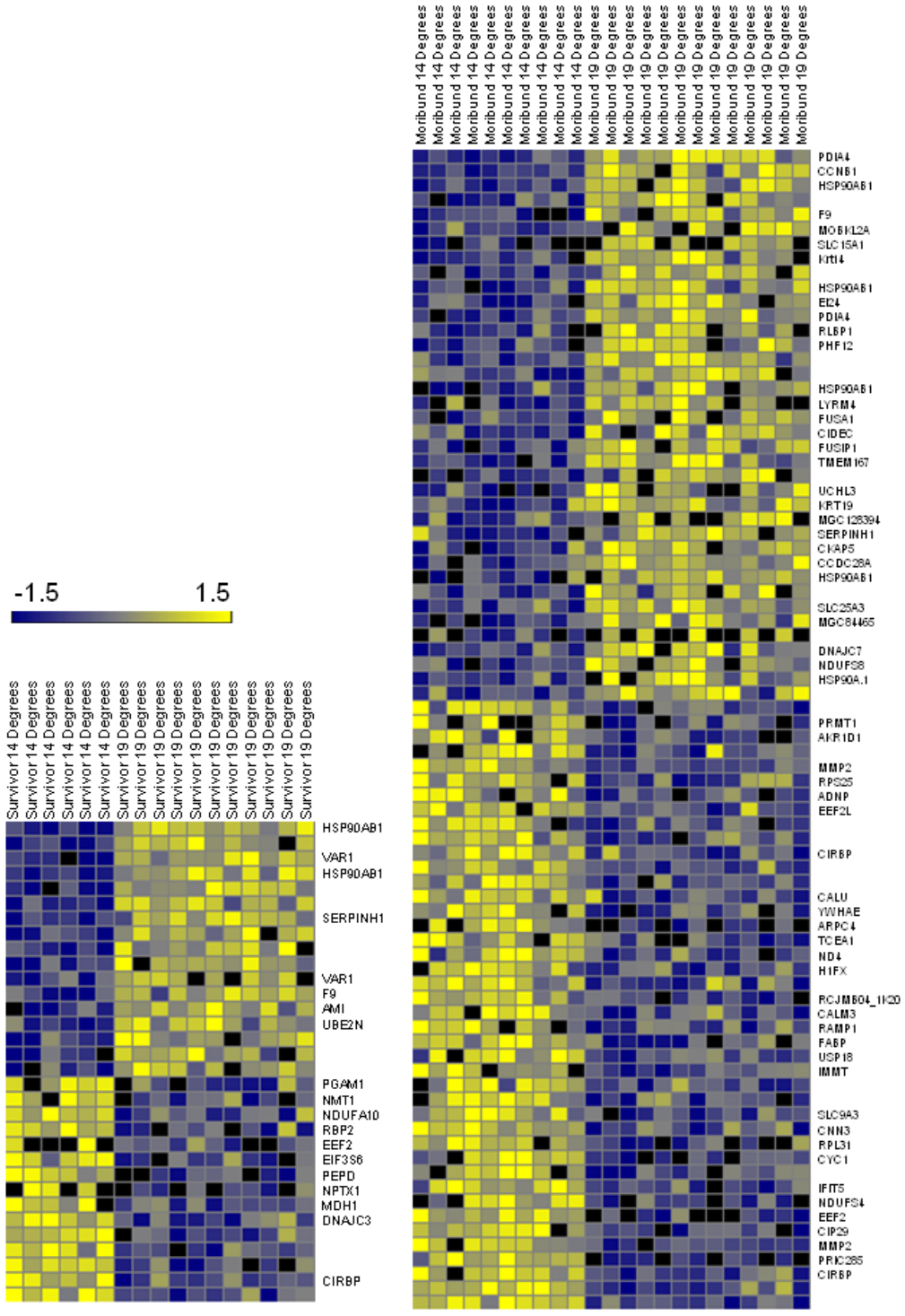


Figure 4.2. Heat maps showing the genes significantly different between temperature treatments for (left) survivors (32 genes total, 30 unique genes) and (right) moribund (80 genes total, 74 unique genes) sockeye salmon at $P < 0.001$ (Mann–Whitney U tests) when fish were grouped based on treatment. Relative expression levels are indicated by the color scale with yellow indicating upregulation and blue indicating downregulation. Missing values are shown in black. Gene symbols (if available) are presented along the right side of the heat map.

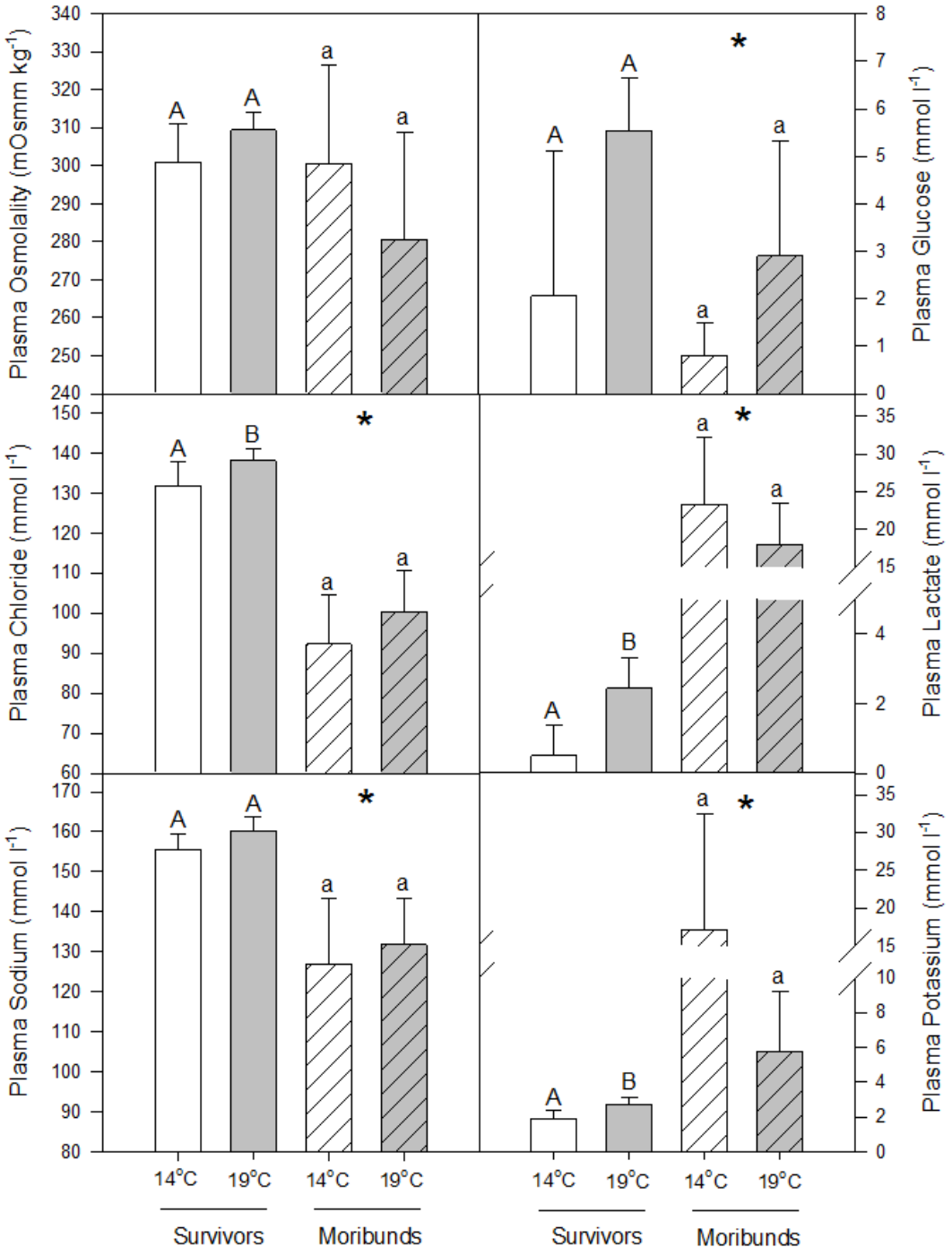


Figure 4.3. Blood plasma properties for survivors (open bars) and moribund (hatched bars) sockeye salmon held at 14°C and 19°C (white and gray bars, respectively). Capital letters indicate statistical differences between temperature treatments for survivors; lower case letters indicate statistical differences between temperature treatments for moribund fish. Statistical differences between moribund fish and survivors are indicated by (*).

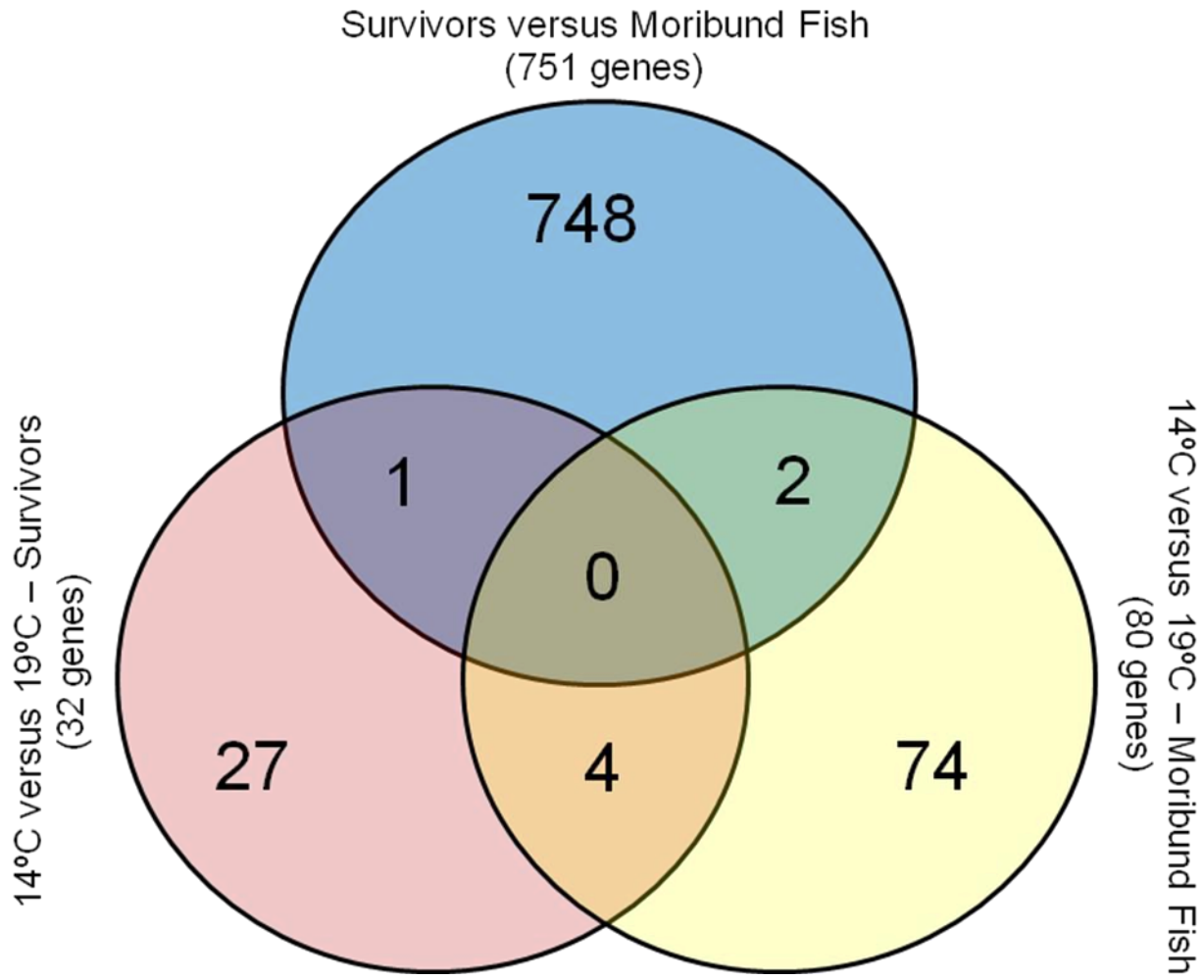


Figure 4.4. Venn diagram of genes that differed significantly in expression levels from the three main supervised comparisons in the present study (effects of the temperature treatments on survivors and moribund fish, and the effects of survival status on gene expression). Numbers represent the number of differentially expressed genes at $P < 0.001$ as determined by Mann–Whitney U tests.

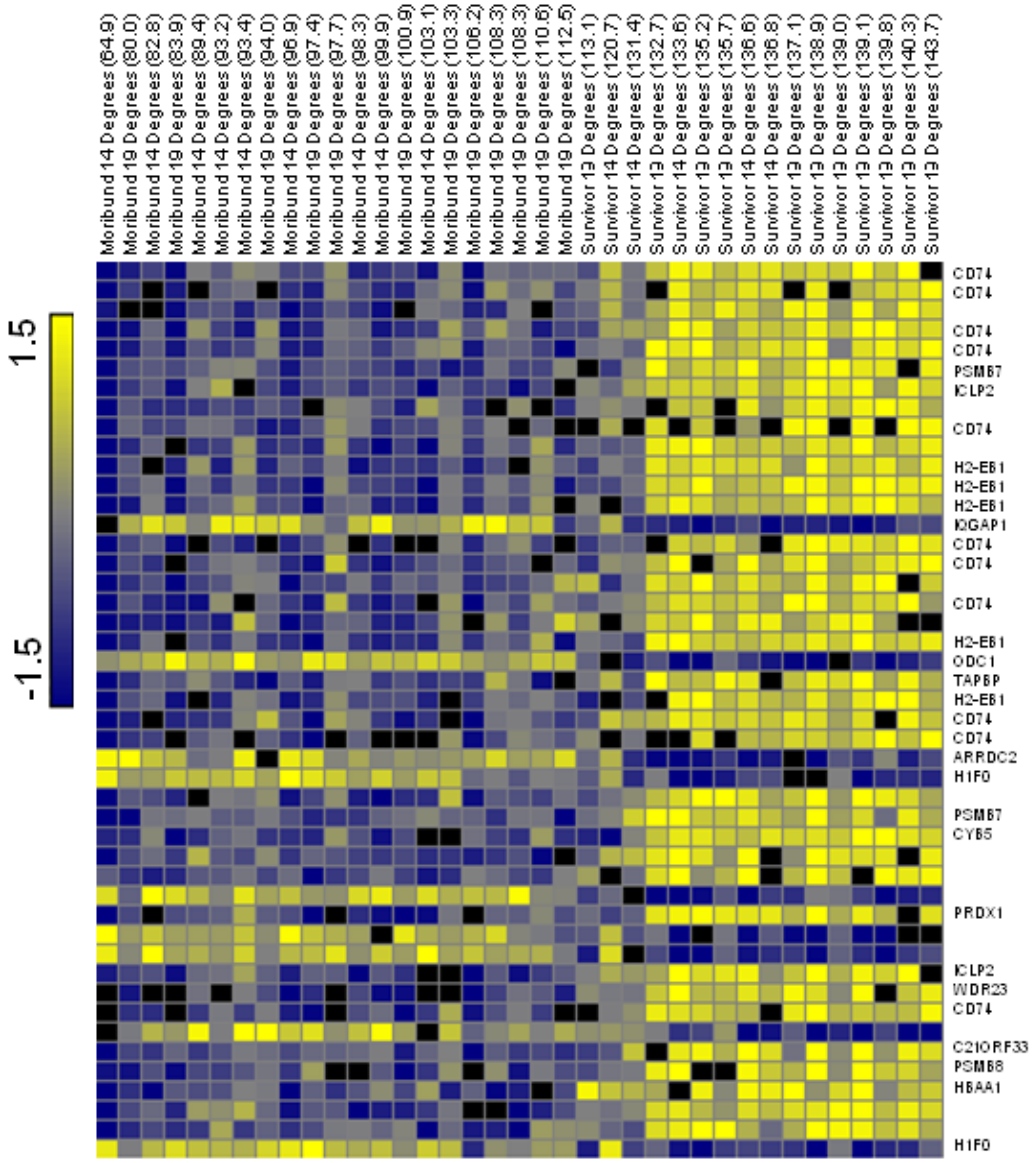
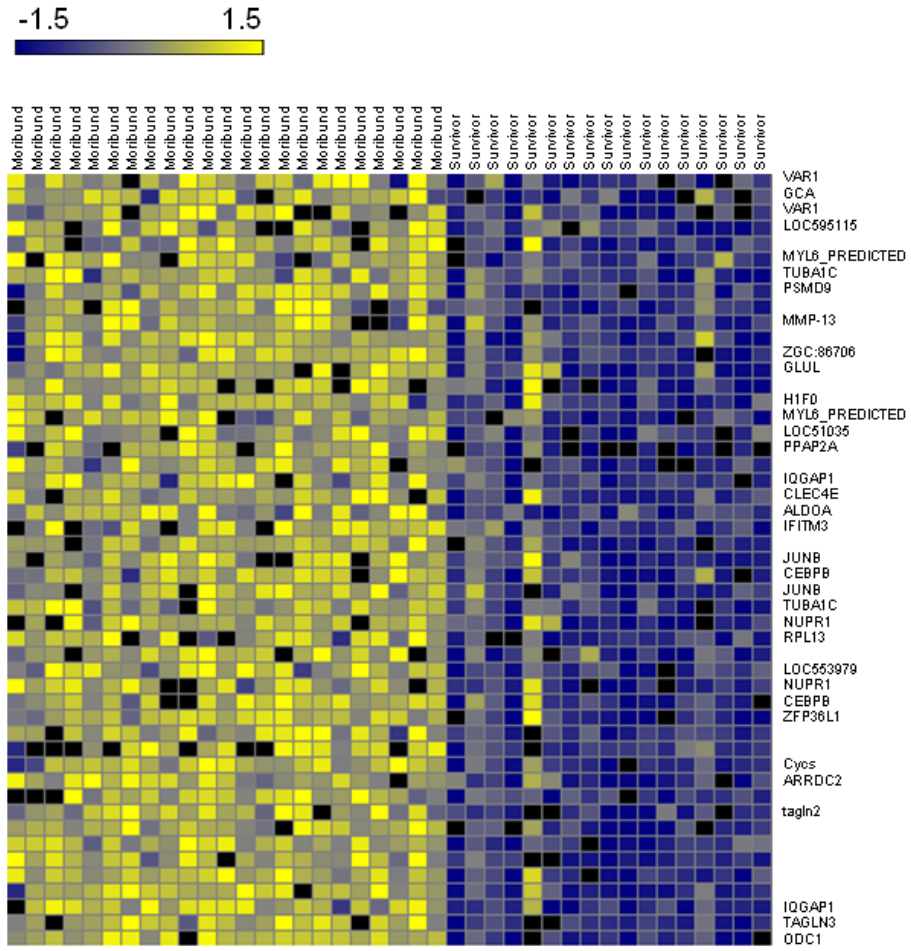


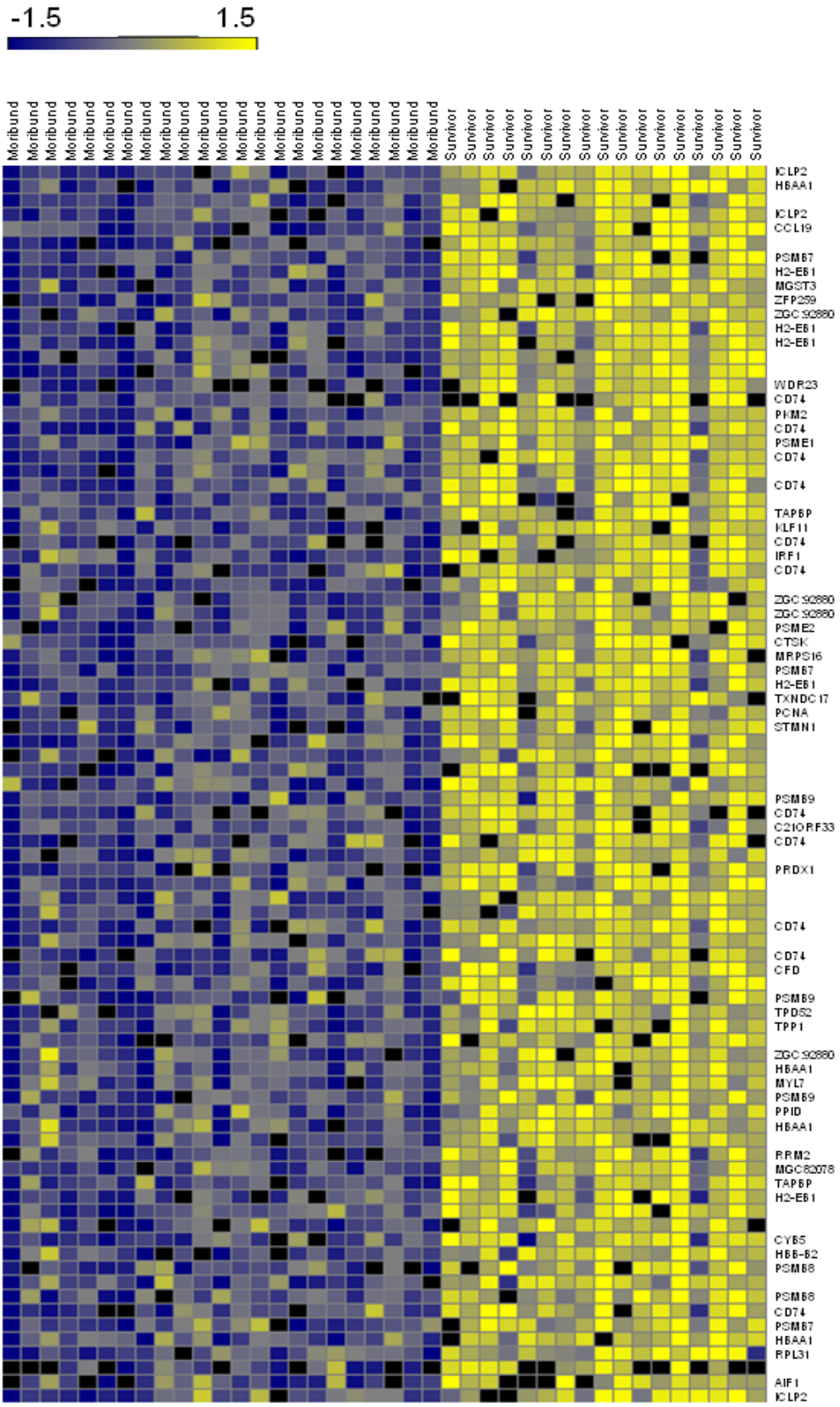
Figure 4.5. Heat map showing the genes most significantly correlated with plasma chloride concentration (mmol l^{-1}, in parentheses) at $P < 1.0 \times 10^{-7}$ in survivors and moribund sockeye salmon. Relative expression levels are indicated by the color scale with yellow indicating upregulation and blue indicating downregulation. Missing values are shown in black. Gene symbols (if available) are presented along the right side of the heat map.



A)

Figure 4.6. Heat maps showing the genes most significantly A) downregulated (49 genes total, 42 unique genes) and B) upregulated (following page; 87 genes total, 59 unique genes) in survivors at $P < 1.0 \times 10^{-6}$ (Mann–Whitney U tests) when sockeye salmon were grouped based on survival. Relative expression levels are indicated by the color scale with yellow indicating upregulation and blue indicating downregulation. Missing values are shown in black. Gene symbols (if available) are presented along the right side of the heat map.

B)


-1.5
1.5
Moribund
Survivor
ICLP2
HBAA1
ICLP2
CCL19
PSMB7
H2-EB1
MGST3
ZFP259
ZGC:92880
H2-EB1
H2-EB1
WDR23
CD74
PKM2
CD74
PSME1
CD74
CD74
TAPBP
KLF11
CD74
IRF1
CD74
ZGC:92880
ZGC:92880
PSME2
CTSK
MRPS16
PSMB7
H2-EB1
TXNDC17
PCNA
STMN1
PSMB9
CD74
C21ORF33
CD74
PRDX1
CD74
CD74
CFD
PSMB9
TPD52
TPP1
ZGC:92880
HBAA1
MYL7
PSMB9
PPID
HBAA1
RRM2
MGC82078
TAPBP
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HBB-B2
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PSMB8
CD74
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HBAA1
RPL31
AIF1
ICLP2

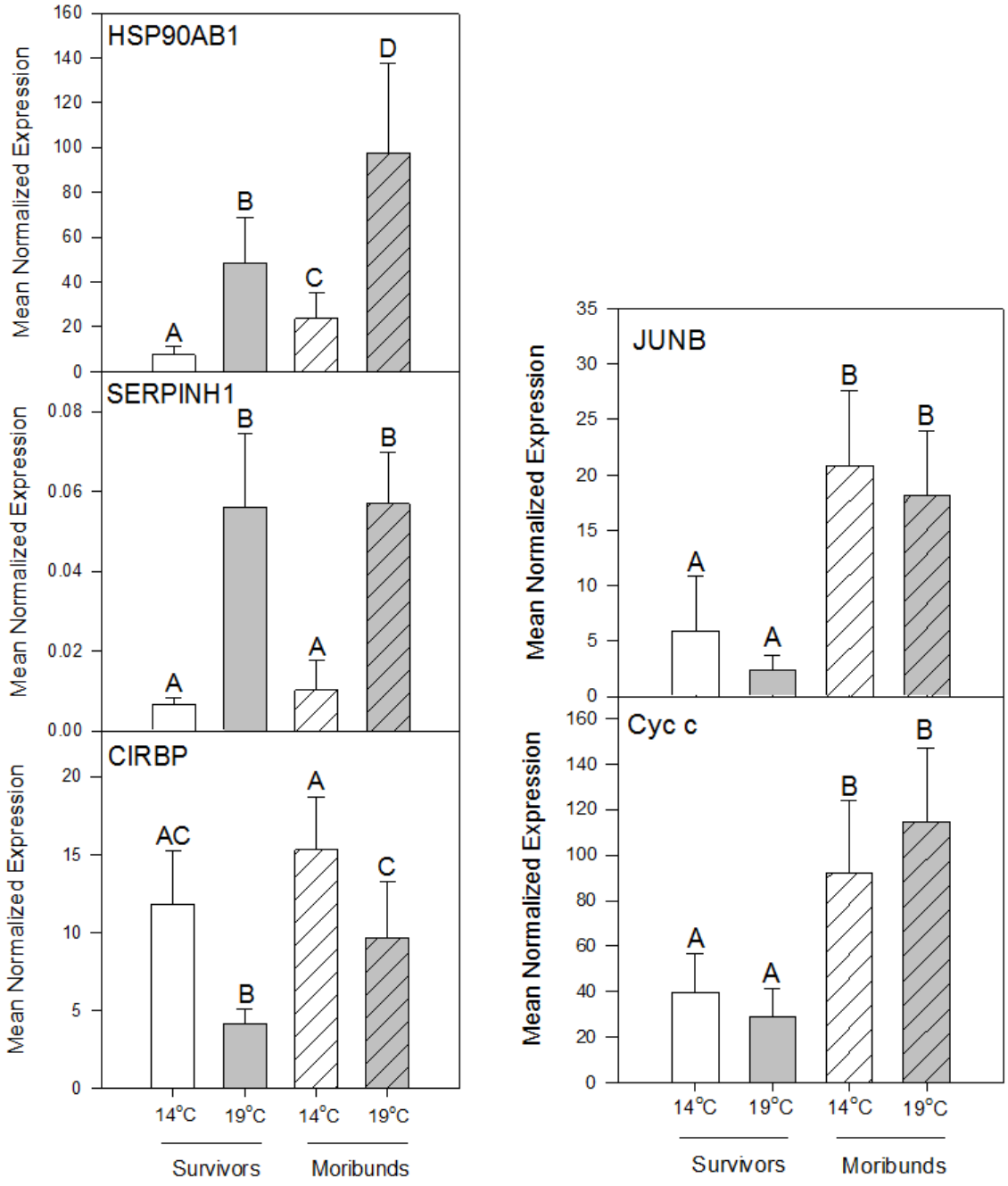


Figure 4.7. Differences in gene expression associated with the temperature treatments (14°C, white bars; 19°C, gray bars) or survival (Survivors, open bars; moribund fish, hatched bars) as determined by quantitative real-time PCR (qRT-PCR) to validate the microarray results. Different letters indicate statistically significant differences.

Chapter 5: Transcriptomic responses to high water temperature in Pacific salmon

5.1 Synopsis

Identifying a common cellular stress response (CSR) signature to high water temperature across species and populations is necessary for identifying the capacity of Pacific salmon to persist in current and future climate warming scenarios, especially for populations at the southern periphery of their species' distributions. In this study, multiple populations of wild adult pink (*O. gorbuscha*) and sockeye (*O. nerka*) salmon from the Fraser River, British Columbia, Canada, are experimentally treated to an ecologically relevant 'cool' or 'warm' water temperature to uncover common transcriptome responses to elevated water temperature. I detected the differential expression of 109 features (65 unique annotated genes) associated with protein folding, protein synthesis, oxidative stress and ion homeostasis at 19°C compared with fish held at a cooler temperature (13°C or 14°C) that was common between run-timings of sockeye salmon and pink salmon. These gene expression changes occurred at temperatures that resulted in high mortality, which suggests a possible relationship between a temperature-induced CSR and mortality in Pacific salmon. I also found that stage of senescence and maturation may affect some of the processes involved in a temperature-induced CSR as fish that were more mature had differential regulation of genes involved in maintenance of the cell cycle and apoptosis, cellular and oxidative stress response, and various metabolic processes compared with less mature fish. I found that water temperatures ≥19°C, which are capable of inducing a CSR, may increase risk of up-river spawning migration failure for Pacific salmon, especially if exposure to high water temperature occurs close to final maturation and peak spawning.

5.2 Introduction

Many fish populations are chronically or acutely exposed to water temperatures outside their species- or population-specific preferred temperature ranges and these occurrences are expected to become more frequent due to climate change. Ecologically and economically important Pacific salmon (*Oncorhynchus* spp.) species are of particular concern because they

experience some of the warmest water temperatures during a crucial life-history stage - their upstream spawning migration. Migration during warm water periods is associated with increased rates of *en route* and prespawn mortality (Gilhousen 1990; Heard 1991; Keefer et al. 2008; Taylor 2008; Keefer et al. 2010; Macdonald et al. 2010). Pacific salmon populations at the southern periphery of their species' distributions are particularly at risk of population declines or extirpation due to warming water temperatures (Beamish et al. 1997). Many southern populations already experience temperatures that may result in premature mortalities (e.g., Keefer et al. 2008; Martins et al. 2011) and warming river temperatures will likely contribute to declines in Pacific salmon populations in British Columbia and the Northwest U.S.A.

Water temperature has a profound influence on physiological processes in fish, which include temperature-dependent changes in metabolic rates (Brett 1971). Aerobic scope, the difference between routine and maximum oxygen consumption during aerobic activity (Fry 1947), has been recommended as a tool in determining the thermosensitivity of fish species (Portner 2002; Portner and Knust 2007; Farrell et al. 2008). The aerobic scope concept suggests that there is an optimal temperature for aerobic performance, and temperatures exceeding the optimal temperature will result in reduced aerobic performance and an eventual collapse in aerobic capabilities [termed critical temperature, (Portner 2002)]. As water temperature increases towards the critical temperature, cellular functions are maintained and protected by heat shock proteins and antioxidant defences (Portner 2002; Kassahn et al. 2009). This suggests that a CSR would be activated when a fish is experiencing a thermal environment close to their critical temperature. The length of time that a fish can maintain its CSR may depend on the thermosensitivity of the fish species or population. Eurythermal species, such as the annual killifish (*Austrofundulus limnaeus*) and longjaw mudsucker (*Gillichthys mirabilis*), have been shown to be able to maintain a CSR for periods of weeks (Podrabsky and Somero 2004; Logan and Somero 2010). However, it is currently unknown whether Pacific salmon can maintain a CSR for extended periods of time, which would be required for migration during periods of high water temperatures that would approach the population-specific critical temperature.

Although it is known that the aerobic scope varies among species and populations of Pacific salmon (Clark et al. 2011; Eliason et al. 2011), little is known about the CSR of Pacific salmon at elevated water temperatures and whether species- and population-specific differences exist. Additionally, it is unknown whether prolonged activation of a temperature-induced CSR is

detrimental to adult Pacific salmon. Identifying a common CSR signature to high water temperature across species and populations will aid in identifying the capacity of Pacific salmon to persist in current and future climate warming scenarios. However, because Pacific salmon are semelparous and die after spawning, they are senescing as they mature during upstream migrations. This can greatly complicate the interpretations of the physiological effects of elevated water temperature on Pacific salmon. It is currently unknown whether senescence, and consequently proximity to final maturation, influences the temperature-induced CSR of adult Pacific salmon. However, because genes associated with energy metabolism and protein biosynthesis have been shown to be affected by both temperature and maturation in wild migrating Pacific salmon (Miller et al. 2009), a logical expectation would be that advanced senescence could alter the 'normal' responses of these cellular processes in thermally stressed fish.

In this study, multiple populations of two species of wild adult Pacific salmon were experimentally treated to an ecologically relevant 'cool' (13°C or 14°C depending on the population studied) or 'warm' (19°C) water temperature to uncover common transcriptome responses to elevated water temperature for the first time. I used pink (*O. gorbuscha*) and sockeye (*O. nerka*) salmon from the Fraser River, British Columbia, Canada, as my model species as these well-studied species suffer increased *en route* mortality during migration in warm compared with cool water and will likely be adversely affected by future projected increases in water temperatures. The Fraser River is Canada's largest and most economically important salmon-producing watershed and is near the southern extent of the distributions of pink and sockeye salmon. Peak and average Fraser River summer water temperatures have been increasing over recent decades, with 13 of the past 20 summers being the warmest on record (Patterson et al. 2007). This has resulted in many Pacific salmon populations performing their spawning migration during increasingly warmer water temperatures, which is considered a major factor that results in *en route* mortality (Hinch and Martins 2011; Hinch et al. 2012, *in press*). I predicted that fish exposed to 19°C would induce a CSR and this temperature would result in higher incidences of premature mortality. I also predicted that a common CSR signature resulting from chronic exposure to a 19°C temperature treatment would include an up-regulation of heat shock proteins and genes involved in an oxidative stress response, consistent with previous work (chapter 4); however, this response would be common across both species and different populations of Pacific salmon. Additionally, I predicted that senescence and maturation would

influence the temperature-induced CSR by affecting energy metabolism and protein biosynthesis when compared with fish that are less mature.

5.3 Materials and methods

5.3.1 Fish collection and holding conditions

Wild migrating adult Pacific salmon were collected by beach seine from the mainstem of the Fraser River, British Columbia, September 5-7, 2007 (n = 130; summer-run sockeye salmon), and in the major Fraser River tributary Harrison River, British Columbia, September 15-18, 2008 (n = 128; late-run sockeye salmon), and September 22-24, 2009 (n = 156; Lower Fraser River pink salmon). Each year, fish were transported by truck in aerated tanks to the Fisheries and Oceans Canada Cultus Lake Laboratory, near Chilliwack, British Columbia, Canada (See Figure 2.1), where they were randomly distributed among four to ten 8,000 L aerated tanks with 10-12°C sand-filtered and UV-sterilized water at equal fish densities and sex ratios. Sexes were determined visually upon arrival at the Cultus Lake Laboratory and then confirmed during autopsies conducted as fish died or during terminal sampling conducted at the end of the holding period. Each tank contained a submersible pump that created a water flow of approximately 0.3 m s^{-1} into which the fish were able to orient and maintain position by continuous, gentle swimming. Because there are ~150 genetically distinct populations of sockeye salmon in the Fraser River, fish in 2007 and 2008 were PIT-tagged and an adipose fin clip was taken for DNA stock identification (see below).

Fish were given 2-6 d to recover from transport, after which they appeared vigorous and without external signs of disease. Water temperatures were subsequently raised at a rate of 2-3°C d^{-1} until the test temperatures were reached and maintained for 5-7 days. After 5-7 days at the test temperature, gill tissue was non-lethally sampled from surviving fish in both temperature groups to determine the effect of water temperature on gene expression. Only fish that were vigorous and maintaining position in the current were sampled for gill tissue. Gill samples were immediately flash frozen in liquid nitrogen and stored at -80°C until analysis. Chapters 2, 3, and

4 provide more detail on the holding environment and sampling protocols along with different physiological responses to the temperature treatments perform on subsets of the same fish as the present study. Fish that were observed to become moribund were immediately removed and enumerated throughout the holding studies. Survival patterns were continuously monitored post-sampling until the termination of the experiment in each particular year (see Figure 5.1). In 2008 and 2009, rapid mortality of females in the high water temperature groups required me to reduce the temperatures to ~7-9°C post-sampling in a subset of tanks to attempt to improve survival until maturation. It is important to note that this would not affect the gene expression profiles as the gill samples were taken while the females were still exposed to the warm water temperature.

DNA identification (Beacham et al. 2005) confirmed that the sockeye salmon sampled for gill tissue and used in the microarray study were summer-run populations in 2007 and were a late-run population in 2008. Because the summer-run fish used in the present study have similar thermal tolerances in terms of aerobic scope, and face similar migration challenges in terms of historical temperature, distance and elevation gain (Eliason et al. 2011), I pooled the summer-run stocks of Chilko River, Horsefly and Mitchell for the microarray analysis. Fraser River pink salmon are generally divided into Lower and Upper Fraser River populations, which can be distinguished based on migration timing and capture location (Groot and Margolis 1991; Crossin et al. 2003), but not by DNA identification (T.D. Beacham, personal communication). The pink salmon that spawn in the Harrison River (used in the present study) belong to the Lower Fraser River population complex and were grouped for the microarray analysis. Specific details of the fish populations studied and the temperature treatment experiments for each year are provided in Table 5.1.

5.3.2 Microarray analysis

Total RNA from gill samples was purified from individual fish gill tissue using Magmax™-96 for Microarrays Kits (Ambion Inc., Austin, TX) with a Biomek FXP (Beckman-Coulter, Mississauga, ON) automated liquid-handling instrument. Approximately 0.5 mg of gill tissue per fish was homogenized with stainless steel beads in TRI-reagent (Ambion Inc.) on a MM301 mixer mill (Retsch Inc., Newtown, PA). 1-bromo-3-chloropropane was added to the

homogenized sample and aliquots of 100 µl of the aqueous phase were pipetted into 96-well plates. Extractions were carried out using the 'Spin Procedure' according to the manufacturer's instructions. In the final step, RNA was eluted and RNA yield was determined by measuring the A260 using a DTX 880 Multimode Detector (Molecular Devices, Sunnyvale, CA). The RNA purity was assessed by measuring the A260/A280 ratio. Solutions of RNA were stored at -80°C until used for the microarray experiment.

Eleven µL of total RNA was used to make aRNA, which was purified, amplified and amino allyl-modified using Amino Allyl MessageAmp^{TM} II-96 kits (Ambion Inc.) following manufacturer's instructions. The aaRNA samples were purified, lyophilized and eluted in 12 µl of DNAse/RNAse-free water prior to the dye coupling reactions. Samples were labelled with Alexa dyes (Invitrogen, Carlsbad, CA) in the dark. After a1 h incubation at room temperature, the reactions were quenched using 4M hydroxylamine and the samples were purified, eluted in 35 µl of aRNA elution buffer and quantified using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Products, Wilmington, DE). Treatment samples were labelled with Alexa 555 and reference samples, comprised of a pool of RNA from all the fish used in the study, were labelled with Alexa 647. Labelled samples were stored at -80°C until used for hybridization.

The microarrays used in the present study were the cGRASP 4x44K Salmonid Oligo Arrays (Agilent Technologies, Santa Clara, CA; http://web.uvic.ca/grasp/microarray/array.html). Each individual in the microarray study was run on a single array against a reference control; to minimize technical artefacts, all labelling reactions were performed simultaneously and individuals were randomized between slides and hybridization days. Prior to hybridization, dye labelled treatment and reference samples (825 ng each) were combined along with 1.2 µl 25X fragmentation buffer, 6 µl blocking agent and nuclease-free water to bring the final volume to 30 µl. The fragmentation mix was incubated at 60°C for 30 minutes. The incubation was stopped by adding 30 µl of 2X GEx hybridization buffer HI-RPM. Samples were briefly centrifuged, placed on ice and 55 µl of the mix was loaded into hybridization chambers in a Tecan-HS4800 Pro Hybridization Station (Tecan Trading AG, Männedorf, Switzerland). The microarray slides were washed with Prehybridization Buffer (Agilent Technologies) at 65°C prior to sample loading. Samples were hybridized to the arrays for 17 hours at 65°C, followed by a wash with Gene Expression Wash Buffer 1 (Agilent Technologies) at room temperature, a wash with Gene Expression Wash Buffer 2 (Agilent Technologies) at 37°C and 2 minutes drying at 30°C. All

steps from washing, hybridization and slide drying were carried out automatically on the Tecan-HS4800 Pro Hybridization Station. Fluorescent images were scanned using a Tecan LS Reloaded Scanner (Tecan Trading AG, Männedorf, Switzerland) and the analysis software Array-Pro Analyzer (Media Cybernetics, Inc., Bethesda, MD). The images were quantified using the program Imagene (BioDiscovery, El Segundo, CA). Each slide was normalized in BASE using the print-tip LOESS method. A detailed description of how slide quality and outliers were assessed can be found in supplemental material from Miller *et al.* (2011).

5.3.3 Data analysis

I utilized both supervised and unsupervised analysis approaches on the microarray data. Unsupervised principal component analyses (PCA) were conducted to identify the major transcriptional trajectories in the data as detailed in Alter *et al.* (2000). Missing values were imputed for the PCA using the mean expression for the experiment for that gene. The PCA method provides a ranking of genes and individuals characterizing their contribution (in terms of variance) to each principal component (PC; i.e., the PC loading). Both positive and negative loadings were considered. The relationships between each PC and the temperature treatment groups, sex and species were assessed using Mann-Whitney U (MWU) tests. The relationship between each PC and sampling year was assessed using Kruskal-Wallis (KW) tests. Supervised analyses were used to compare between treatment groups using MWU tests for each year. Genes were considered significantly different between treatment groups at a very conservative $q < 0.01$ [the false discovery rate (FDR) analogue of the *P*-value]. It is important to note that multiple copies of the same gene may reflect either different regions of the same gene or duplicated copies of that gene due to pseudo-tetraploidy in the salmon genome (Quinn et al. 2011b); hence I included all copies of each gene that was significant for my analyses. All statistical analyses were performed using R (R Development Core Team 2008).

Because there were subtle differences in experimental design each year and there can be considerable variation in the condition of returning salmon between years and populations, direct comparisons between experiments conducted in the different years was not considered appropriate when performing the supervised analyses. Therefore, I conducted MWU tests for

each year rather than use a multifactor ANOVA, and used a Venn diagram approach (Kammenga et al. 2007) to infer common changes in the temperature responses for each run-time and species. This approach is useful for determining commonalities between multiple datasets and has been used previously in salmonid microarray studies to detect differences in responses to temperature treatments (Quinn et al. 2011a; 2011b).

Functional analysis of gene expression profiles was performed with ErmineJ using the receiver operator characteristic (ROC) scoring method (Lee et al. 2005). The ROC method is a non-threshold method performed on all gene rankings, which may be more robust than using raw gene scores. All three categories of the GO hierarchy (Biological Process, Molecular Function and Cellular Component) were considered, limited to groups with 5-100 genes. However, only the categories Biological Process and Molecular Function are presented. The 'best-scoring replicate' method was used in ErmineJ to handle repeated measurements of the same gene. Gene sets in the ErmineJ analyses were considered significant at Benjamini-Hochberg FDR < 0.05.

5.4 Results

5.4.1 Cumulative mortality

There was higher mortality at 19°C in each experimental year compared with fish held at a cooler temperature (Figure 5.1). There was also generally higher mortality in females than male sockeye salmon. Mortality rates generally decreased after the temperature treatments were dropped to cooler temperatures (~7-9°C), however in most cases, there was substantial mortality (i.e., ≥60%) after 10 days at 19°C.

5.4.2 PCA among years and populations

Based on the PCA performed on all fish among all years, PC1 explained 35.0% of the variance, with 6.0% and 4.9% of the variance explained by PC2 and PC3, respectively. The year that fish were sampled was overwhelmingly related with PC1 (KW, $P = 2.34E^{-100}$), with the pink

salmon sampled in 2009 being distinctly separated from the sockeye salmon sampled in 2007 and 2008 (Figure 5.2). Species was also significantly associated with PC1 (MWU, $P = 2.22E^{-17}$) further indicating that the strongest pattern in the PCA was differences between pink and sockeye salmon. Functional analysis of PC1 indicated that processes involved in oxidative stress, protein catabolism, lipid metabolism and calcium ion binding were significantly enriched (FDR < 0.05) in the PC1 gene list (Appendix A).

Functional analysis of PC2 suggests that it was associated with cell survival as processes involved in cellular apoptosis, caspase activity and cell death were significantly enriched (FDR < 0.05) in the PC2 gene list. Genes associated with these processes were upregulated in fish with more negative values on the PC2 axis. Furthermore, fish that were positioned on the negative end of the PC2 axis were generally among those that died shortly after sampling (~1-3 days; see figure 5.3 for dates when individual fish died), especially in the pink salmon.

While the temperature treatments were significantly related to PC1 (KW, P = 0.03) and PC2 (KW, P = 0.002), they were most strongly associated with PC3 (KW, P = 0.00016) with the fish held at 19°C generally being located on the positive end of the PC3 axis (Figure 5.2). Functional analysis of PC3 indicated that processes involved in the cell cycle and cell proliferation, cellular homeostasis (including cellular ion/calcium homeostasis), cell signalling pathways and immune response were significantly enriched (FDR < 0.05) in the PC3 gene list (Appendix A). Sex was not significantly associated with any of the PC axes (MWU, P > 0.05).

5.4.3 PCA on individual years

When PCAs were performed separately on data from each year, similarities among the different year's experiments emerged. Each year, the temperature treatments were significantly associated with PC1 (Table 5.2). However, the ranking of individuals along the PC1 axis appeared to be closely related with the length of time salmon survived post-sampling (Figure 5.3) and therefore PC1 (explaining 12.3-13.9% of the variance in the data) is likely associated with premature fish mortality. Indeed, functional groups involved in cell death, cell proliferation, apoptosis and caspase activity were enriched in the PC1 gene lists for each year (Appendix B), just as they were enriched in PC2 in the PCA analysis that included all three studies.

Additionally, biological processes involved in metabolism, protein catabolism, ion homeostasis, G-protein coupled receptor signalling, cellular stress response, oxidative stress, antigen binding and immune response were also enriched in the PC1 gene list for each year.

The temperature treatments were most significantly associated with PC4 in 2007 and 2009, and PC3 in 2008 (explaining 5.5-7.1% of the variation in the data; Figure 5.3). Functional groups involved in protein biosynthesis, metabolic processes, cell cycle, cell stress, immune response, apoptosis, cell proliferation, and cell structure were enriched in the PC4 gene lists in 2007 and 2009 (Appendix C). Relatively few functional gene groups were enriched in the PC3 gene list in 2008, potentially due to the fact that these fish were not as far along the senescence trajectory as the fish studied in 2007 and 2009, the overall signal was weaker or there simply were not as many functional groups that varied significantly. Only functional groups involved in protein biosynthesis were enriched in the PC3 gene list in 2008. Sex and population were only significantly associated with PC2 in 2007 and were not significantly associated with any other PC. Therefore, I believe the patterns detected in PC1 (mortality responses) in each year, and PC3 and PC4 (temperature responses) in each year were not sex- or population-specific.

5.4.4 Supervised analysis of temperature treatments

Warm water and cool water treatments were directly compared for each year using a supervised approach. At a threshold cut-off of $q < 0.01$, 200 microarray features in 2007, 808 features in 2008, and 5062 features in 2009 had significantly different levels of expression between the temperature treatments (Figure 5.4). For the gene lists that differed significantly with temperature, 109 features had common expression patterns between both species, and among populations within species. This represents 54.5%, 13.5% and 2.2% of the significant features in 2007, 2008 and 2009, respectively. Of those 109 features, there were 65 unique genes and 12 genes with unknown functions (Figure 5.5). Several of the 65 genes [HSP90A.1, HSP90AA1, HSPA8, SERPINH1 (also known as HSP47), EEF2 and CIRBP] are known to be thermally responsive or have been previously determined to be thermally responsive in Pacific salmon using microarray techniques. Other significant genes with multiple copies that were common among years were ATP1A1, COX6B1, FKBP10, SEPW1, TUBA1A and several

splicing factors (SFRS2, SFRS7 and SFRS9). The directional change in the expression of the 109 features between the temperature treatments was consistent between years. In addition to being thermally responsive, these 65 unique genes are generally involved in cell redox homeostasis, protein folding, calcium and ion homeostasis, transmembrane transport, protein biosynthesis and metabolism (Figure 5.5).

5.5 Discussion

Exposure to a 'warm' water treatment resulted in high mortality and evidence of a CSR in both species and the different populations of sockeye and pink salmon. The temperature treatments were most strongly associated with PC3 when PCA was performed using three independent years of data for the two species. Functional analysis of PC3 showed that genes involved in cell homeostasis, cell cycle regulation and immune response had greater expression with exposure to the high temperature treatment. Previous work has shown that sockeye salmon exposed to relatively high water temperatures have increased expression of genes involved in cell maintenance processes and immune function (Chapter 4). The present study suggests that this may be a general response to 19°C for sockeye and pink salmon. In all years, there was greater observed mortality in the 19°C treatment than in the 'cool' treatment, which suggests a potential relationship between a temperature-induced CSR and survival in Pacific salmon.

5.5.1 Common temperature responses

Identifying a characteristic response to temperature stress in Pacific salmon is crucial for determining the capacity for certain populations to persist in a warming climate. For the first time, I present the common transcriptomic responses of multiple run-timings and species of Pacific salmon exposed to high water temperatures for ecologically relevant periods of time in controlled laboratory conditions. There was evidence of a thermal stress response at 19°C across populations and two species of Pacific salmon compared with the fish held at the 'cool' temperature that was consistent between years. With the use of the largest salmonid microarray available (44K genes), along with sample sizes that are excellent for wild salmon and genomics

research, many genes were able to be classified as thermally responsive across Pacific salmon populations and species.

Many of the genes that were upregulated at 19°C were molecular chaperones and were consistent with an unfolded protein response associated with a heat shock response. Several heat shock proteins [HSPA8, HSP90AA1, HSP90AB1, SERPINH1] were differentially regulated in fish held at 19°C and these patterns were consistent with those previously described in Pacific salmon (Miller et al. 2009; Evans et al. 2011; Chapter 4). Certain heat shock proteins are up-regulated during acute exposure to high temperatures; however, during chronic high temperature, expression may return to a basal level or be downregulated (Meyer et al. 2011). This may be species-dependent as Hsc70 (≈HSPA8) and HSP90 remained elevated during chronic exposure/acclimation to extreme water temperatures for the eurythermal annual killifish (Podrabsky and Somero 2004). I found that after an ecologically relevant 5-7 day period of high water temperature exposure, the heat shock response was still activated, which suggests that either these fish could not acclimate to 19°C or that 5-7 days was not a sufficient amount of time for the heat shock proteins to return to what would be basal levels for Pacific salmon. However, given that adult Pacific salmon have a reduced ability to acclimate to high water temperatures in the adult stage of their life history (Clark et al. 2011), and the increased mortality in sockeye and pink salmon at 19°C, these gene expression results suggest that these fish are not acclimating to this temperature after 5-7 days of exposure.

There was differential expression with the temperature treatments of the molecular chaperones PDIA4, up-regulated at 19°C and consistent with patterns found in thermally treated moribund sockeye salmon (Chapter 4), and of FKBP10, down-regulated at 19°C. The latter has not previously been described as thermally responsive in Pacific salmon. The directional changes of PDIA4 and FKBP10 were consistent with those of the longjaw mudsucker acclimated for 4 weeks to a high temperature treatment (Logan and Somero 2010) and in Atlantic cod (*Gadus morhua*) exposed to a 3 hour heat shock treatment (Hori et al. 2010). Interestingly, PDIA4 expression may only be increased during periods of extreme temperature stress, and onset of expression depends on acclimation temperature (Logan and Somero 2011), which indicates an effect of acclimation similar to that of inducible heat shock proteins. The consistency between the temperature responses for these species may suggest that PDIA4 and FKBP10, both specific to the endoplasmic reticulum (ER) and involved in calcium ion binding processes, are indicative

of a thermal stress response across fish species. Additionally, SERPINH1, a collagen-specific ER resident molecular chaperone involved in collagen stabilization (Krone et al. 1997), was also significantly upregulated at 19°C. The differential regulation of ER-specific molecular chaperones suggests an activation of the unfolded protein response (reviewed in Rutkowski and Kaufman 2004).

Several genes commonly associated with protein biosynthesis were differentially regulated in response to the temperature treatments. These included the upregulation at 19°C of an initiation factor (EIF4A2) and the initiation factor transporter protein (EIF4ENIF1), 60S ribosomal proteins (RPL32, RPL36A), genes involved transcription regulation (CBX1, ZBTB46) and nucleo-cytoplasmic shuttling splicing factors (SFRS1, SFRS6, SFRS7; Twyffels et al. 2011). Interestingly, SFRS1 can indirectly influence phosphorylation of EIF4E (Karni et al. 2007), and EIF4E regulates a rate-limited protein involved in protein biosynthesis (Sukarieh et al. 2009), which could be related to the up-regulation of EIF4ENIF1 detected in the present study. Conversely, there was a downregulation of genes involved in polypeptide elongation (EEF2, C24G6.8), RNA stabilization and translation efficiency (CIRBP), and transcription regulation (CARM1, TCF12), along with other splicing factors (SFRS2, SFRS9) at 19°C. The downregulation of EEF2 and CIRBP has been demonstrated as thermally responsive in sockeye salmon (Chapter 4); however the present study suggests that this could be a temperature response that is common across two species of Pacific salmon. Because EEF2 is an essential factor in protein synthesis, its downregulation may result in an overall reduction in protein biosynthesis. Decreases in protein biosynthesis have been shown in rainbow trout exposed to a chronic elevated water temperatures and this response to water temperature may be more pronounced in fish with limited energy availability (Morgan et al. 1999), as would be the case with adult Pacific salmon. The complex variability in the regulation of genes involved in protein biosynthesis may suggest that there is an upregulation of genes required to respond to the temperature stress event, while still resulting in an overall downregulation of the synthesis of non-essential genes, a common response to periods of ER stress and cellular stress in general (Rutkowski and Kaufman 2004; Storey and Storey 2004). This may be reflected by the fact that genes associated with ER/Golgi-mediated transport mechanisms (AP3S1, SCFD1, TMED2), for transporting proteins out of the cell, were down-regulated at 19°C.

Oxidative stress is common in organisms during periods of extreme environmental challenges, which include high temperature stress (Kassahn et al. 2009). The hydrogen peroxide sensor gene, PARK7, which protects the cell against oxidative stress (Eltoweissy et al. 2011), along with SEPW1, were significantly upregulated at 19°C, which may indicate an oxidative stress response. While SEPW1 is often associated with selenium binding in the cell, it also functions as a glutathione-dependant antioxidant (Whanger 2009). The primary function of PDIA4 appears to be as a molecular chaperone; it also functions in an oxidative stress response as the ratio of reduced to oxidized PDIA4 contributes to initiating the unfolded protein response in the ER (Jeanson et al. 2012). Collectively, the upregulation of these genes suggests an activation of an oxidative stress response. Indeed, functional analysis of the individual years show that functional categories representing an oxidative stress response and oxidoreductase activity were significantly enriched in the 2007 and 2009 gene lists. Aging, senescence and disease also contribute to oxidative stress and susceptibility to oxidative stress (Ermak and Davies 2002; Martinez-Alvarez et al. 2005); these factors were likely stronger contributors in the 2007 and 2009 studies as these fish were closer to final maturation than the late-run fish sampled in 2008.

Oxidative stress can lead to fluctuations in cellular calcium levels that may cause physiological disturbances (protein phosphorylation and activation of signal transduction pathways) and apoptosis (Ermak and Davies 2002). Many genes associated with cellular calcium binding and homeostasis (PDIA4, FKBP10, CRELD1, TNNC1), and calcium-related processes, were differentially affected by the temperature treatments. There were several genes that were differentially regulated that are involved in protein phosphorylation (MAP3K14, NEK4) and a variety of signal transduction pathways (GPS2, NDRG2, PSME3, WASF2, ZMYND11, ZNF259) involved in stress-associated responses, cell proliferation, and apoptosis. Collectively, these results suggest that cellular calcium-mediated processes may be important in a temperature response in Pacific salmon. However, given the remarkably diverse role of calcium in cell function, it is difficult to make any direct associations between calcium signalling and the oxidative stress response in the present study; therefore, further investigation into the role of cellular calcium signalling in the Pacific salmon CSR is warranted.

The Na^+/K^+-ATPase protein ATP1A1 was downregulated in Pacific salmon held at 19°C. Sockeye salmon held at high temperatures for 24 days have previously been shown to have

decreased total Na^+/K^+-ATPase activity (Crossin et al. 2008). Because Na^+/K^+-ATPase can use a significant portion of the cellular energy budget, a downregulation of this process may help maintain metabolic balance in organisms (Staples and Buck 2009). Consistent with this theory, Richards (2010) suggested that this response to environmental stress was particularly strong in organisms with limited endogenous energy stores. This would be the case for migrating Pacific salmon which rely solely on endogenous energy stores to fuel freshwater migration (Groot and Margolis 1991). Moreover, the downregulation of several other transmembrane transporters (SLC45A2, TMEM16A, TMEM49, MFSD7) in fish held at 19°C may result from the need to maintain a proper electrochemical gradient across membranes (Staples and Buck 2009). However, the end result of this potentially energy conserving mechanism may be a dysfunction of ion homeostatic processes detectable in the blood. I have shown in previous analyses that plasma chloride and osmolality levels were increased in the high temperature treatment in sockeye salmon from these same holding experiments (Chapter 3; Chapter 4).

5.5.2 Species differences

There was evidence of differences in transcriptomic responses between pink and sockeye salmon in this study. Pink salmon had differentially regulated genes involved in oxidative stress and calcium-mediated processes, along with lipid metabolism and protein catabolism, which were upregulated when compared with sockeye salmon. There could be several causes of this pattern. Recent work suggests that pink salmon have a higher aerobic capacity, enabling them to be more resilient to warm temperatures (Clark et al. 2011). However, under controlled conditions they demonstrate an exaggerated thermal and handling stress response (based on blood plasma indices) compared with sockeye salmon (Chapter 3; M.R. Donaldson, Ph.D. Thesis, University of British Columbia). It should be noted though, that the fish used in the Clark et al. (2011) study were less reproductively mature than the fish used in the present study and were subjected to fluctuating acute temperature exposures rather than prolonged chronic temperatures. Consistent with higher blood plasma stress indices observed in fish from the same holding study (e.g., glucose and lactate; Chapter 3), there were substantially more genes differentially regulated in response to chronic warm temperature exposure (5062 genes, with 4662 genes unique to pink

salmon) in pink salmon compared with sockeye salmon. This could indicate that the temperature stressor, in combination with any potential handling and confinement stresses, could have resulted in a relatively more stressful treatment for pink salmon. Periods of extreme stress can cause increased production of reactive oxygen species and activate the oxidative stress response (Kassahn et al. 2009), which may induce calcium-mediated signalling pathways (Ermak and Davies 2002), as observed in pink salmon from my study. Additionally, chronic exposure to environmental stress may result in alterations in the expression of genes involved in fatty acid metabolism (McClelland 2004). Given that this pathway was only disrupted in pink salmon, I suggest that the pink salmon were more stressed overall throughout the holding period. Although proximity to final maturation and stage of senescence influences the responses of pink salmon to stress-related events, the summer-run sockeye salmon used in the present study were as close or closer to final maturation than the pink salmon, which suggests that the differences in gene expression between the two species was not simply due to differences in their life-history stages.

5.5.3 Effects of senescence and maturation

Survival post-sampling appeared to be associated with PC1 in each year, and with PC2 in the PCA across years and species. Functional analysis of PC1 for each year shows that genes involved in cell proliferation, caspase activity and apoptosis were enriched in PC1; these processes are ultimately associated with cell death (Rutkowski and Kaufman 2004). Genes involved in immune response and ion homeostasis were also associated with PC1. Immunosuppression leading to increased susceptibility to disease is known to occur in senescing and moribund salmon (Dickhoff 1989; Miller et al. 2009; Chapter 4), with disease likely being the ultimate cause of death (Gilhousen 1990). The freshwater component of spawning migrations is also characterized by a gradual loss of osmoregulatory ability (Shrimpton et al. 2005), followed by a sharp decrease in plasma ion levels days in advance of actual death (Chapter 2), and in this study, differential regulation of genes associated with ion transport. There is potential that these osmoregulatory shifts, which occur in advance of death, may be predictive of eminent mortality in Pacific Salmon.

Functional analysis of the PC most associated with the temperature response for each year suggested that there are additional effects of temperature stress on fish that are closer to final maturation and further along the senescence trajectory. Proximity to final maturation has been shown to influence the effect of temperature on survival in a subset of these pink and sockeye salmon (Chapter 3). At the time of the temperature holding experiments, the summer-run sockeye salmon in 2007 and pink salmon in 2009 were closer to final maturation than the late-run sockeye salmon from the 2008 experiment. Close proximity to final maturation resulted in differential regulation of genes involved in maintenance of the cell cycle and apoptosis, cellular and oxidative stress response, and various metabolic processes. These functional groups were not enriched in 2008, fish that were over a month away from the peak spawning period for that population for that year. The response of genes associated with apoptosis and oxidative stress may be related to processes involved in natural senescence (Miller et al. 2009; Chapter 4) or an interaction between senescence and the temperature stress response. Surprisingly few functional categories were enriched in the fish from 2008, and these were limited to functional categories involved in protein biosynthesis, processes that were also enriched in the 2007 and 2009 experiments. Collectively, these data suggest that proximity to final maturation may influence the transcriptomic response of Pacific salmon to temperature stress.

There was evidence of a lack of a response to the temperature treatments in some fish. Individuals from the warm water treatment that clustered with individuals from the cool water treatment in the PCA died shortly after sampling. This suggests that the warm-treated individuals with a gene expression profile similar to cool-treated individuals did not have an appropriate response to the high water temperature which could be is due to inter-individual differences in thermal tolerance, influences of disease or parasites, or because these fish were further along the senescence trajectory. However, variable responses to high water temperature that have been associated with elevated mortality have been previously observed in sockeye salmon gene expression profiles (Miller et al. 2009; Chapter 4) and suggest that a lack of a CSR by an individual is predictive of temperature-induced mortality in Pacific salmon.

5.6 Conclusion

The widely documented high rates of *en route* and prespawn mortality in Pacific salmon associated with high temperature and verified in laboratory-based thermal holding studies was coupled to a gene expression signature (CSR) that may be influenced by the proximity to final maturation. Despite knowledge that various populations of Pacific salmon have different aerobic capabilities at elevated water temperatures (Clark et al. 2011; Eliason et al. 2011), which have been used as a proxy for differences in thermal tolerance, a threshold temperature of 18°C (Macdonald et al. 2000) is often used for determining when spawning migrations are considered more difficult due to temperature stress in the Fraser River system. Indeed, migration during temperatures >18°C results in increased incidences of *en route* mortality for some populations of sockeye salmon (Hinch and Martins 2011; Martins et al. 2011). This threshold temperature has been reached or exceeded in six of seven years between 2005-2011 (eWatch 2011); my studies have shown that a common CSR is induced at 19°C for sockeye and pink salmon (herein and Chapter 4). It is currently unknown whether chronic activation of a CSR is harmful for Pacific salmon. However, given that chronic exposure to 18-19°C water temperature results in increased mortality in sockeye and pink salmon (Crossin et al. 2008; this thesis) and can result in an osmoregulatory disturbance in sockeye salmon (Chapter 3; Chapter 4), it appears that Pacific salmon that experience temperatures capable of inducing a CSR are at an elevated risk of dying prematurely. The ultimate cause of death is still unknown, as sub-lethal high water temperatures interact with disease and parasite progression, which are often considered factors in the actual death of senescing Pacific salmon (Gilhousen 1990). In conclusion, a water temperature of 19°C will induce a CSR in pink and sockeye salmon, which may increase risk of a failed spawning migration, especially if close to final maturation and peak spawning.

Table 5.1. Population and experimental design details for the sockeye salmon populations and pink salmon held at a warm and cool temperature in three different temperature holding experiments conducted from 2007-2009.

Species	Year	Run timing	Population	Treatment duration	Temperature (°C)	Sex	n	Date of Sampling
Sockeye Salmon	2007	Summer-run	Chilko/Horsefly/Mitchell[A]	7 days	19	M	10	Sept. 21, 2007
						F	3	
					14	M	3	
						F	5	
Sockeye Salmon	2008	Late-run	Harrison Rapids[B]	5 days	19	M	11	Oct. 1, 2008
						F	3	
					13	M	11	
						F	8	
Pink Salmon	2009	N/A	Lower Fraser[C]	5 days	19	M	11	Oct. 5, 2009
						F	11	
					13	M	11	
						F	11	

[A] 2007 peak spawning - Chilko: Sept. 28-Oct. 3, Horsefly: Sept. 5-15, Mitchell: Not available
[B] 2008 peak spawning - Nov. 11-13.
[C] 2009 peak spawning - Weaver Creek spawning channel: Oct. 13-16.

Table 5.2. *P*-values from Mann Whitney U tests to determine the relationship between temperature, sex and stock (if appropriate) and the first four principal components, along with the variance explained, from the principal component analysis conducted separately on each experimental year. Significant relationships at $P < 0.05$ are in bold.

Species	Year	Run timing	Population	Principal Component	Variance Explained (%)	Temperature	Sex	Stock
Sockeye Salmon	2007	Summer-run	Chilko/Horsefly/Mitchell	1	12.44	**2.46E-02**	7.56E-02	2.85E-01
				2	10.74	4.14E-01	**5.96E-03**	**3.13E-03**
				3	10.40	8.60E-01	8.60E-01	2.34E-01
				4	6.88	**1.97E-05**	6.45E-01	2.36E-01
Sockeye Salmon	2008	Late-run	Harrison Rapids	1	12.25	**2.88E-02**	1.54E-01	
				2	8.16	4.83E-01	8.36E-01	
				3	7.11	**2.94E-06**	6.65E-01	
				4	4.95	**2.61E-02**	8.95E-01	
Pink Salmon	2009	N/A	Lower Fraser	1	13.89	**3.82E-05**	6.67E-01	
				2	9.04	**1.31E-02**	1.44E-01	
				3	5.86	1.44E-01	4.22E-01	
				4	5.48	**2.02E-05**	6.01E-01	

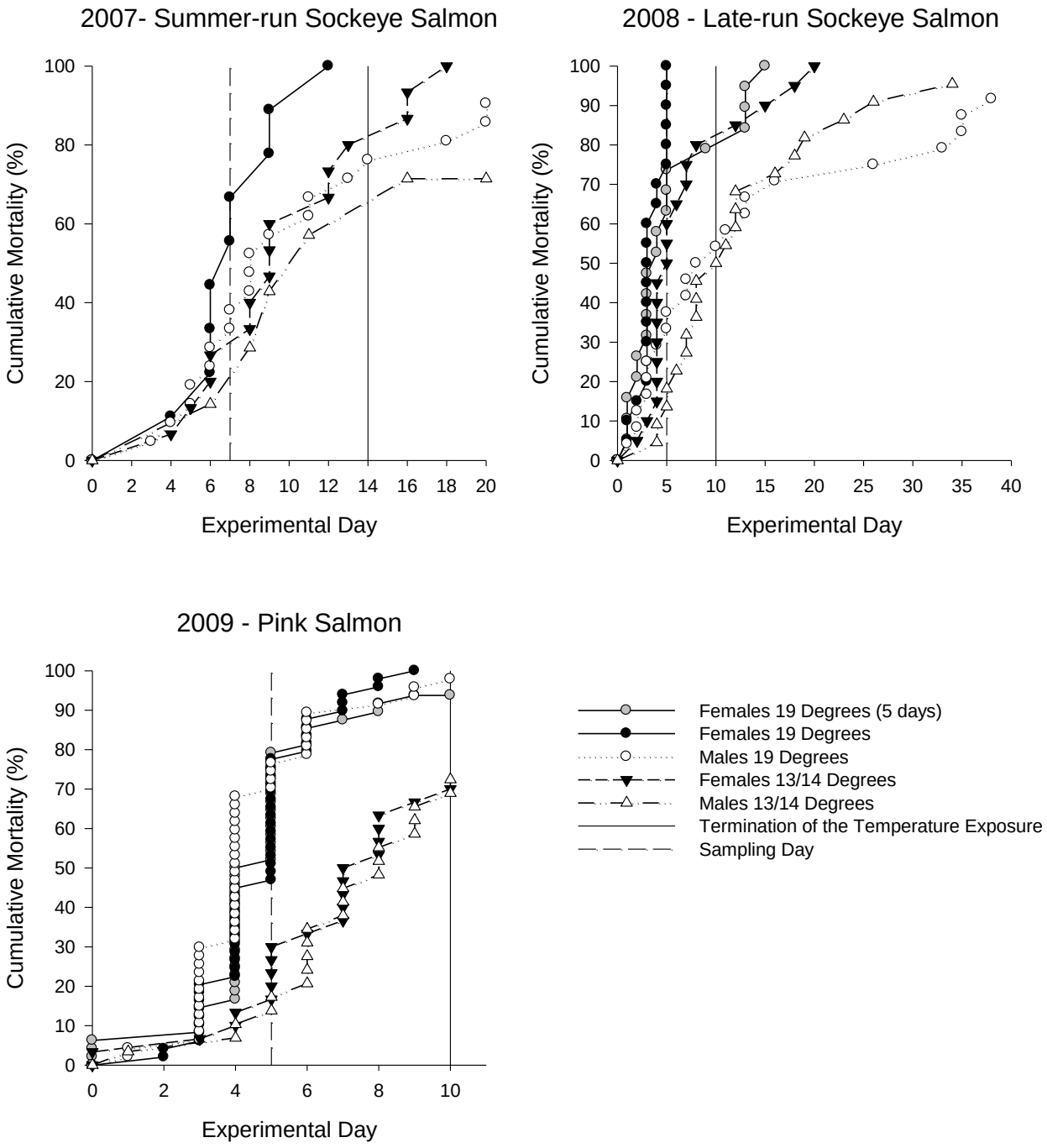


Figure 5.1. Cumulative mortality of male and female pink and sockeye salmon held at a warm and cool temperature for each experimental year. The dashed line indicates when the fish were sampled for gill tissue and the solid line indicates when the temperature treatments were terminated. In 2007 and 2008, water temperatures were reduced to ~7-9°C after the termination of the temperature treatments. Due to rapid mortality of females in 2008 and 2009, some of the temperatures in high temperature treatment tanks were reduced to ~7-9°C after 5 days to attempt to decrease mortality. Mortality patterns of females from these tanks are indicated.

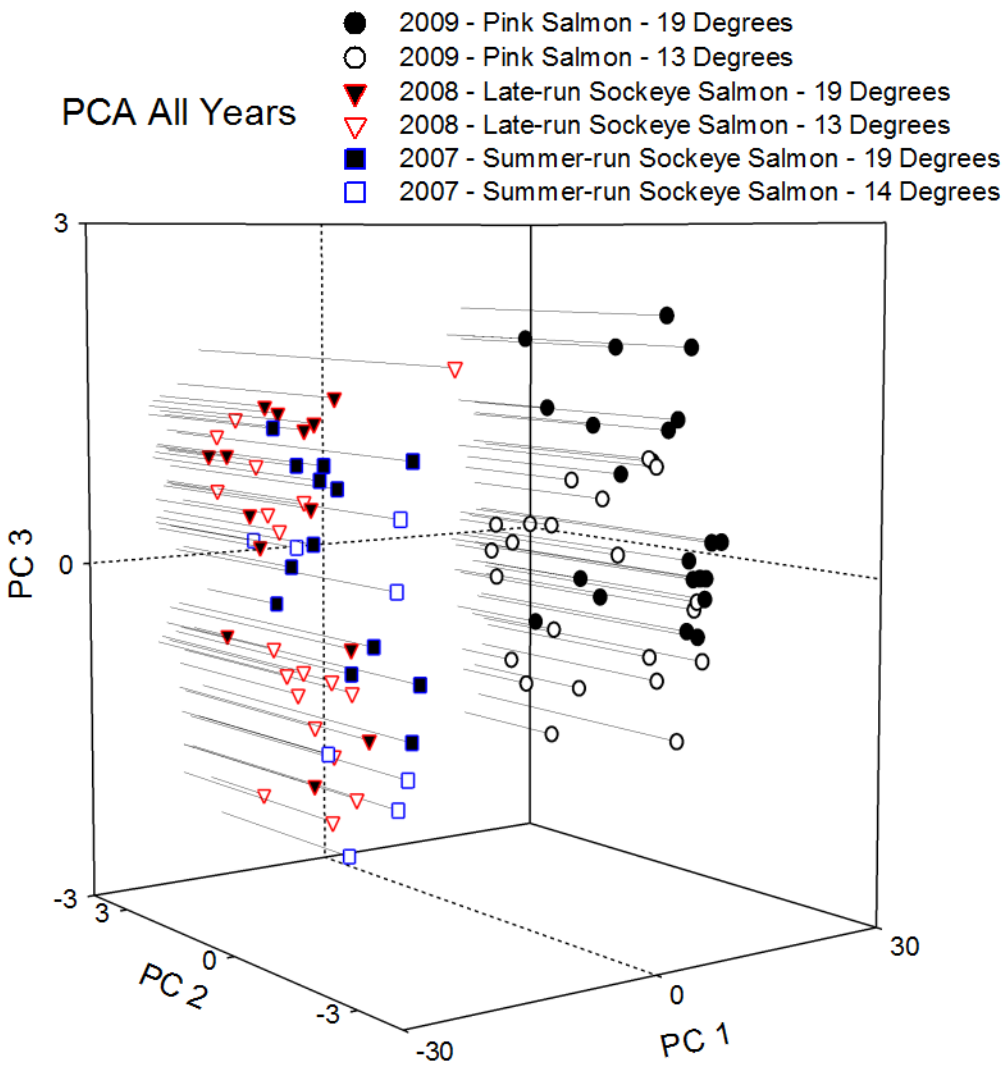


Figure 5.2. Position of each individual fish for the first three principal component (PC) axes for the principal component analysis (PCA) conducted on all fish from each experimental year.

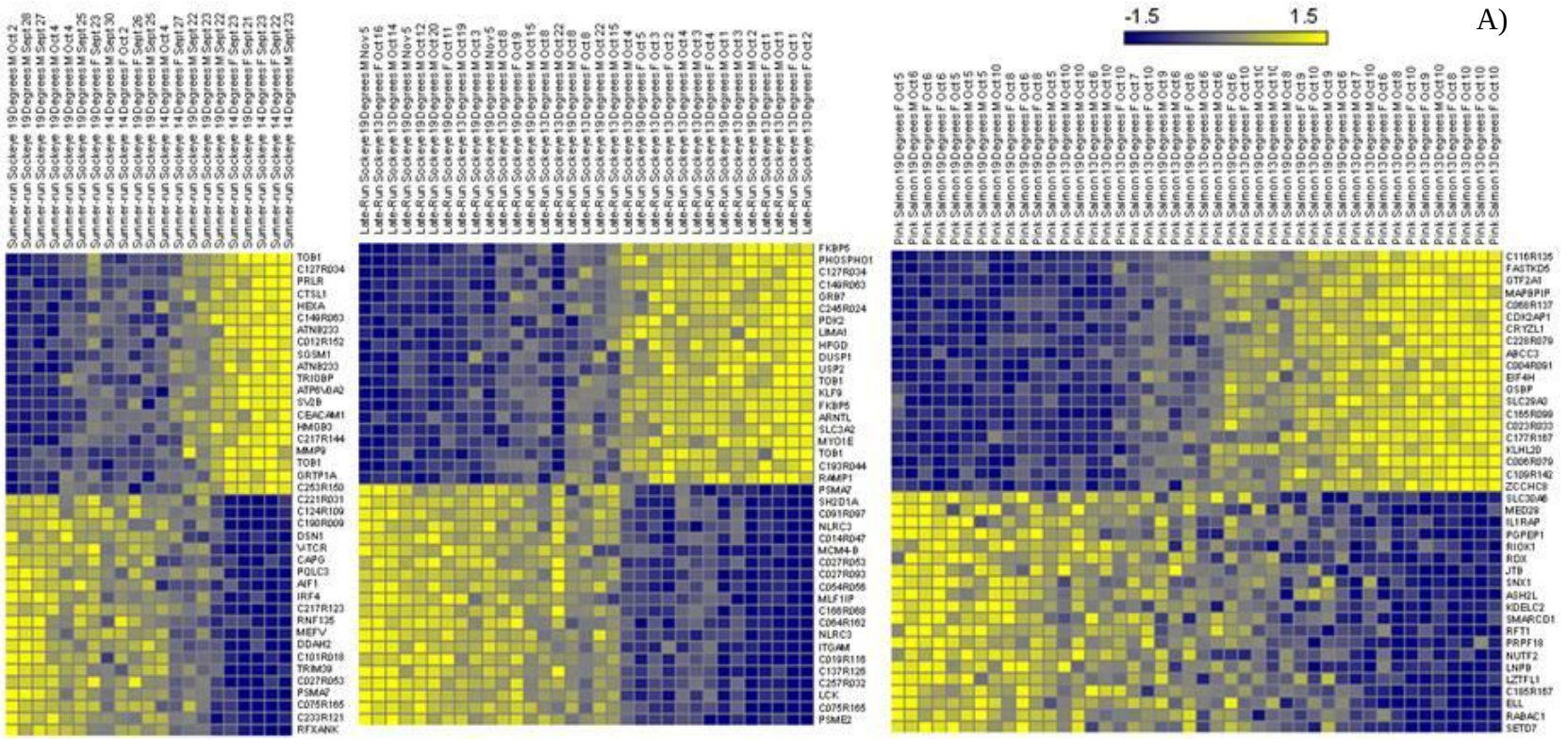
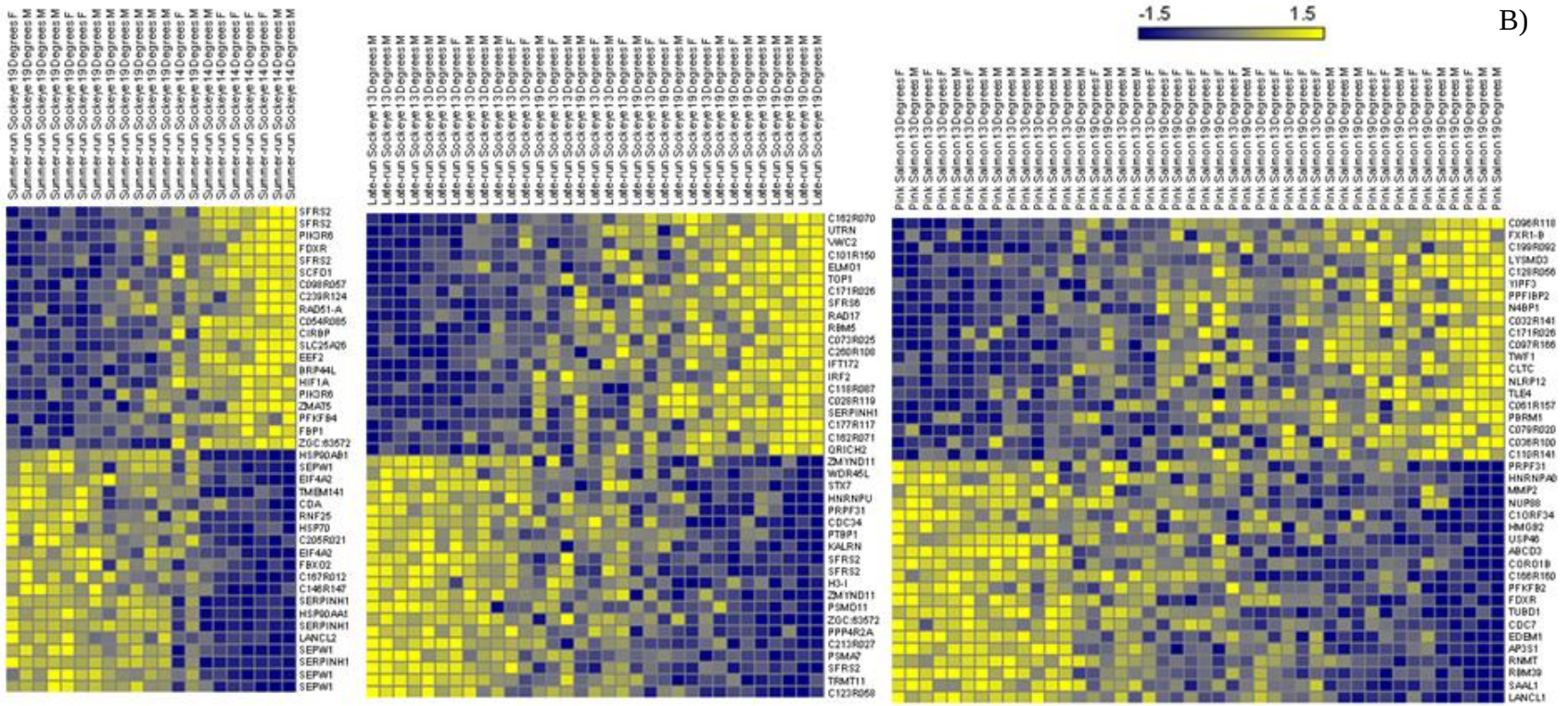


Figure 5.3. Heat maps showing the top 20 positively and top 20 negatively loaded genes for the principal component (PC) most related with A) survival (PC1 for each year; mortality date is indicated) and B) temperature (next page; PC4 for 2007 and 2009; PC3 for 2008; temperature is indicated). Fish were sampled for gill tissue on September 21, October 1 and October 5 in 2007, 2008 and 2009, respectively. Relative expression levels are indicated by the color scale, with yellow indicating upregulation and blue indicating downregulation. Ordering of fish reflects their relative PC scores along the PC axis. Gene symbols or annotation ID's (if gene symbols are not available) are presented along the right side of the heat map.


B)
-1.5
1.5

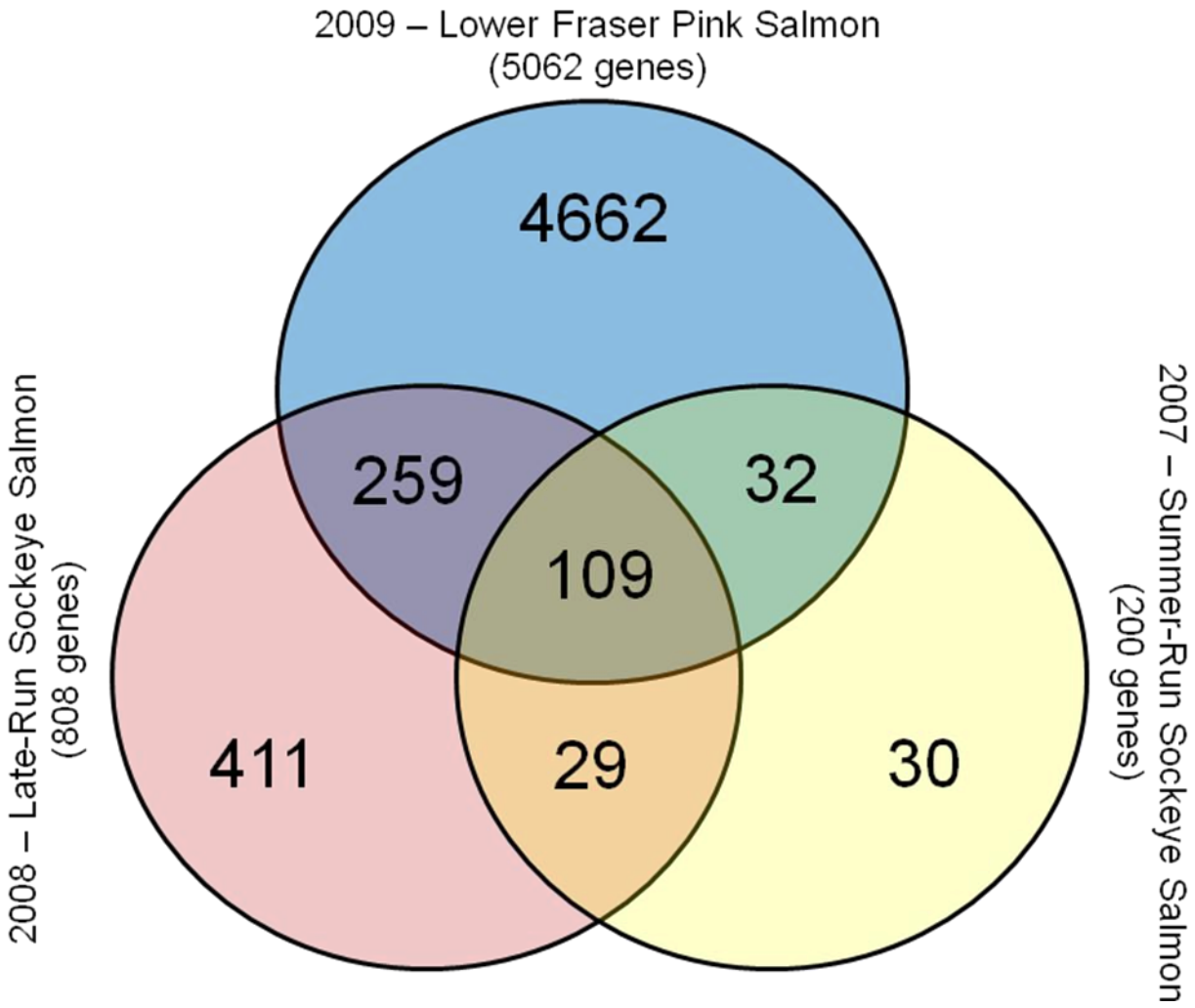


Figure 5.4. Venn diagram of genes that differed significantly in expression levels between warm and cool treatments from the supervised comparisons of Pacific salmon for each experimental year. Numbers represent the number of differentially expressed genes at $q < 0.01$ as determined by Mann-Whitney U tests.

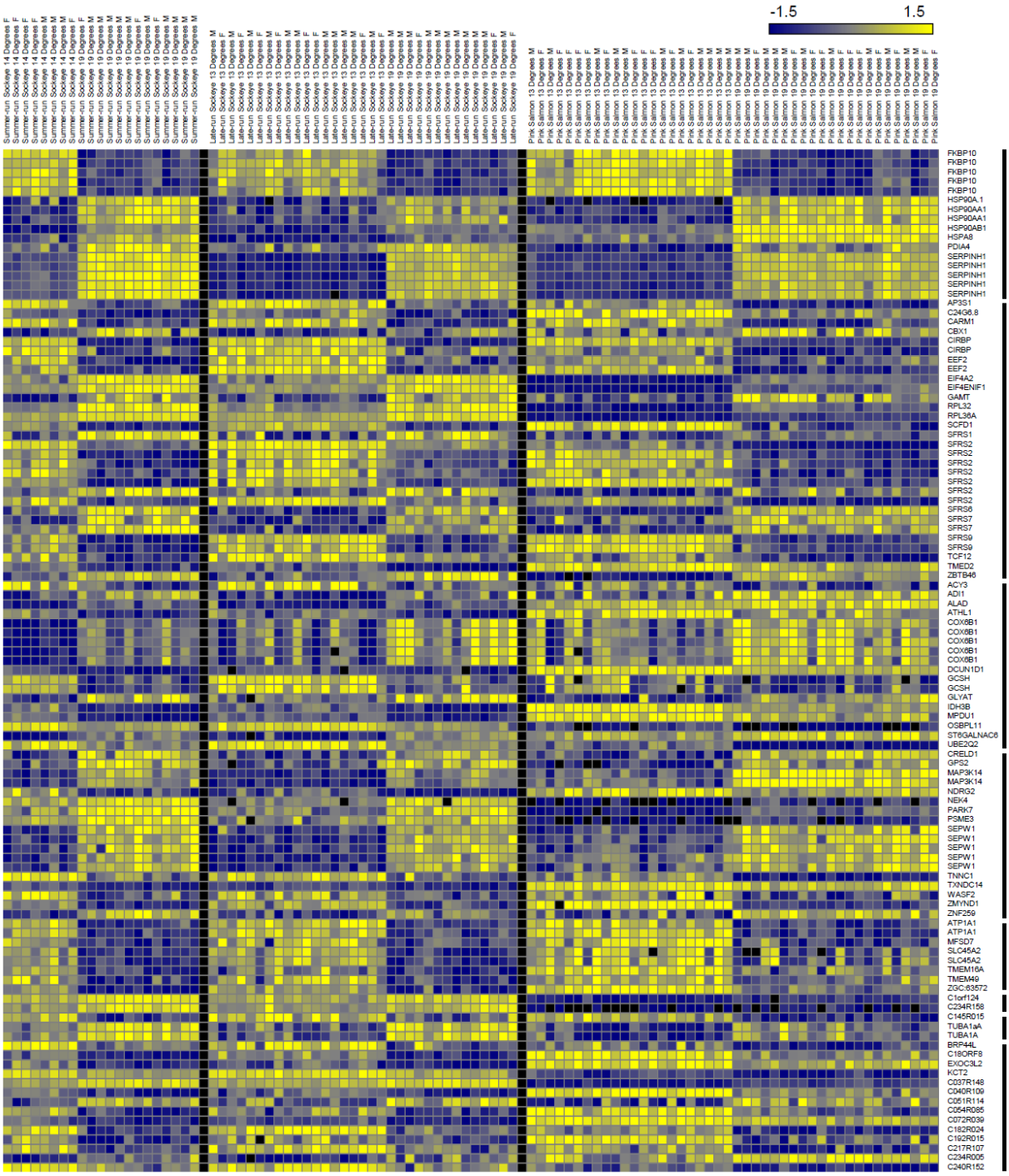


Figure 5.5. Heat map showing the 109 differentially regulated genes common between population and species as determined by Mann-Whitney U tests between warm and cool treatments. Genes are grouped based on function (1: Molecular chaperones; 2: Transcription/translation/protein transport; 3: Metabolic processes; 4: Oxidative stress/ion homeostasis/signal transduction/apoptosis; 5: Transmembrane transport; 6: DNA repair; 7: Cell structure; 8: Unknown function/no gene symbol available). Relative expression levels are indicated by the color scale with yellow indicating upregulation and blue indicating downregulation.

Chapter 6: Conclusions - synthesis and implications

6.1 Overview

This thesis sought to understand the effects of high water temperatures on adult Pacific salmon at the level of the cell, tissue and individual. The main hypotheses were that Pacific salmon exposed to a high temperature would suffer higher mortality (which would show sex-specific patterns); have indices of stress that were detectable in their blood plasma; and would elicit a cellular stress response characterized by the differential expression of genes associated with a heat shock and immune response as well as alterations in expression of genes involved in protein biosynthesis and metabolism compared with fish exposed to a cooler temperature. These hypotheses were tested through a series of laboratory-based temperature-exposure experiments conducted on wild-caught adult sockeye and pink salmon. Chapters 3, 4, and 5 found elevated mortality at 19°C compared with fish held at a cooler temperature. Chapter 3 compared the effects of water temperature on sockeye and pink salmon from the same river system. Consistent with my original hypothesis, female sockeye salmon suffered greater mortality than males; however, this pattern was not detected in pink salmon. Contrary to my *a priori* hypothesis and some of the existing literature, pink salmon did not have better survival at 19°C relative to sockeye salmon. A possible explanation for the differences in survival for pink salmon from those predicted is discussed in the subsequent section. In the blood plasma, sockeye salmon consistently showed higher chloride levels when held at 19°C (chapters 3 and 4) compared with those held at a cooler temperature, which may suggest an osmoregulatory disturbance for sockeye salmon exposed to high water temperature. Both sockeye and pink salmon had reduced levels of the plasma sex steroids testosterone and 17β-estradiol at 19°C compared with fish held at 13°C. Collectively, these findings demonstrate that indices of stress due to high water temperature can be detected in the blood plasma of Pacific salmon, which supports my original hypothesis. At the cellular level, both sockeye and pink salmon showed evidence of a CSR in their gill transcriptome that was characterized by the upregulation of genes associated with a heat shock response. Many of these results are novel for Pacific salmon and provide insight into some of the physiological effects of warm river water temperatures on adult Pacific salmon.

The other main objective of my thesis was to characterize some of the physiological changes in Pacific salmon associated with senescence and mortality. It was apparent early on in my research that to fully understand the physiological effects of temperature on Pacific salmon, I would need to separate out the potentially confounding effects of senescence. Therefore, by holding adult Pacific salmon during the period of final maturation, I tested the hypotheses that there would be changes in blood properties that are characteristic of senescence and mortality in sockeye salmon and that there would also be differential expression of genes involved in cellular processes associated with cell death (i.e., apoptosis) that was detectable in the transcriptome of dying sockeye salmon. Chapter 2 demonstrated that there are dramatic declines in plasma osmolality, chloride and sodium levels that occur days in advance of a male sockeye salmon's death, and that plasma chloride levels are strong predictors of longevity in maturing sockeye salmon. Chapter 4 presents the first data that characterize the effect of mortality on the sockeye salmon transcriptome. Sockeye salmon that became moribund were characterized by an upregulation of several transcription factors associated with apoptosis and the downregulation of genes involved in immune function and antioxidant activity, which is consistent with immunosuppression. Both chapters 2 and 4 examined the changes in blood properties that occur in sockeye salmon that become moribund, which were consistent with recent literature. Below I elaborate on these findings, highlight future research directions and suggest management implications of my thesis results.

6.2 Effects of high temperature on Pacific salmon and recommendations for future research

It has been widely documented that spawning migrations during warm water periods result in high rates of *en route* and prespawn mortality in Pacific salmon (Gilhousen 1990; Macdonald et al. 2000; Keefer et al. 2008; Taylor 2008; Keefer et al. 2010; Macdonald et al. 2010); results that have been verified in laboratory-based temperature exposure studies (Servizi and Jenson 1977; Jensen et al. 2004; Crossin et al. 2008; Gale et al. 2011; this thesis). Surprisingly, prior to this thesis, few data existed that demonstrated physiological effects (especially at the cellular level) of high water temperatures on Pacific salmon. With this thesis, I

attempted to provide some of the first data on the physiological effects of water temperature on different species of Pacific salmon at multiple levels of biological organization.

This was the first transcriptome-wide characterization of the CSR to temperature stress conducted on Pacific salmon. Therefore, I was able to determine many promising potential biomarkers of high temperature stress in Pacific salmon. Some of these are 'classic' heat shock response genes (e.g., HSP90AB1, SERPINH1), or are associated with other environmental stressors along with a temperature response (e.g., CIRBP), and some that have never been previously demonstrated to be associated with a thermal CSR in salmonids (e.g., ATP1A1, FKBP10, PDIA4, SEPW1) to my knowledge. It is currently unclear what role semelparity has in the differential expression of some of these genes associated with a temperature-induced CSR in Pacific salmon. Interestingly, some of the gene expression patterns detected by the microarrays were consistent with previous studies on fishes with diverse evolutionary histories, such as the eurythermal species, the longjaw mudsucker, common killifish and annual killifish (Podrabsky and Somero 2004; Healy et al. 2010; Logan and Somero 2010), and the stenothermal Arctic charr (Quinn et al. 2011). Collectively, these studies suggest that parts of the CSR associated with temperature stress are evolutionarily conserved. For example, upregulation of certain heat shock proteins in response to unfolded/misfolded proteins is common across taxa (Feder and Hofmann 1999) and demonstrates the widespread occurrence of protein denaturation during periods of temperature stress (Somero and Hofmann 1997). Hence, similarities between these diverse fish species in regards to the temperature-induced CSR could be expected. Therefore, some of these findings are likely applicable to many fishes and consequently, the implications of this thesis may go beyond Pacific salmon biology.

It is important to note that the mRNA transcript levels measured in microarray studies do not always correspond with protein levels or indicate protein turnover rates (Cossins et al. 2006; Prunet et al. 2008). Future work should attempt to determine the relationship between cellular mRNA transcript and protein levels during temperature stress in Pacific salmon to gain an understanding of the physiological significance of the changes in gene expression in response to the temperature stressor. Additionally, I report in chapters 4 and 5 that there is a downregulation of several genes involved in protein biosynthesis (e.g., EEF2, SFRS2) despite an upregulation of genes associated with a heat shock response and suggest that a decrease in protein biosynthesis may be an energy saving mechanism at the cellular level. Chronic exposure to high water

temperature has been linked to altered protein biosynthesis and turnover rates in salmonids (Reid et al. 1998; Morgan et al. 1999). Future studies should attempt to determine the effects of water temperature on cellular protein biosynthesis and turnover rates to attempt to fully understand how water temperature affects the cellular energy budget in adult Pacific salmon. As discussed in the introduction, biochemical and enzymatic processes have adapted to perform optimally at species-specific temperatures (Somero and Hofmann 1997). By using a microarray approach, I am unable to assess enzyme activity and therefore cannot determine the impact of the temperature treatments on vital cellular processes associated with rate-limiting enzyme function. Previous work has shown decreased total Na^+/K^+-ATPase activity in sockeye salmon gill tissue held at a warm temperature compared with fish held at a cooler temperature (Crossin et al. 2008). This corresponds nicely with a downregulation of Na^+/K^+-ATPase (ATP1A1) at higher temperatures demonstrated in this thesis and suggests that Na^+/K^+-ATPase activity may be controlled by alterations in gene expression levels. Future work should be conducted to directly show this relationship and to provide information on other enzyme activities that may be affected by water temperature, such as the enzymes involved in sex steroid biosynthesis.

The common temperature-induced CSR reported in this thesis occurred at a temperature that resulted in high mortality in both of the different species and all run-timings of the adult Pacific salmon populations tested, despite the expectation of population- and species-specific thermal tolerances. This segues into a possible limitation to this project. By holding fish at a static temperature (19°C) that induced a CSR in each year of experimentation, I was unable to determine the onset temperature of the CSR. The onset temperature for a temperature-induced CSR, along with the constituent levels of certain thermally-responsive proteins, has been suggested to be important in determining a species' ability to tolerate thermal stressors (Tomanek 2010). However, it has been suggested that it is the whole-organism thermal tolerance that is most important in setting species-specific thermal tolerances (Schulte 2004). This may be consistent with what has been observed in Pacific salmon in this thesis as previous work has shown that there are population and species level differences in whole animal thermal tolerances in Pacific salmon (Brett 1952; Lee et al. 2003; Eliason et al. 2011). Additionally, because many factors can influence the CSR and overall gene expression, the results of laboratory holding studies may not accurately reflect the complex response that would be detected in migrating fish. However, laboratory-based holding studies are a good first step in determining the effects of

water temperature on wild Pacific salmon. What can be concluded is that 19°C is sufficient to induce a CSR; however the exact onset temperatures of the CSR for the populations studied in this thesis are still unknown. I was also unable to determine how long Pacific salmon are capable of maintaining an activated CSR, which may have implications for assessing how long a fish can survive exposure to temperatures capable of inducing a CSR. Future work should be conducted to identify whether there are population- and species-specific differences in the onset temperature and the duration of the CSR. Additionally, future work should examine the role of fluctuating temperatures on the Pacific salmon CSR as fish often experience variable temperatures in nature. These differences may be important in determining how certain Pacific salmon populations will be required to respond to future warming water temperatures.

The elevation of plasma osmolality and chloride levels at 19°C, a temperature that has been commonly encountered by some Pacific salmon populations in recent years, suggests that exposure to high temperatures may cause an osmoregulatory disturbance in sockeye salmon. This may be related to the downregulation of ATP1A1 that was part of the common temperature-induced CSR reported in this thesis. Interestingly, the pink salmon examined did not show signs of an osmoregulatory disturbance in their blood plasma despite a downregulation of ATP1A1. Species-specific osmoregulatory abilities have been previously demonstrated in salmonids (Bystriansky et al. 2006); however, the species considered in that study were not as closely related as sockeye and pink salmon. This observation may suggest an inherent species difference in the thermal stress response at the level of the blood or that pink salmon have a way of physiologically compensating for the downregulation of ATP1A1 potentially through ion retention mechanisms associated with the kidney or due to regulation of other ion transport mechanisms in the gill, however this purely speculative. The ecological significance of the relatively small change in absolute values of plasma osmolality and chloride could be argued, however, given the consistency of the finding over multiple years combined with a corresponding change in the expression of genes associated with ion transport, it appears to be characteristic of a thermal stress response in sockeye salmon and warrants further investigation.

Pacific salmon invest into gonad development throughout the upriver spawning migration (Crossin et al. 2003; Crossin et al. 2004). High water temperatures may interfere with some of the hormone cascades involved in reproductive development. High water temperatures can delay or inhibit final maturation in salmonids by affecting steroid biosynthesis and inhibiting the pre-

ovulatory shift to maturational hormone production (Pankhurst and King 2010). For example, water temperatures during final maturation can inhibit the production of sex steroids in Atlantic salmon (King et al. 2003). For Fraser River sockeye salmon, it has been suggested that the threshold for biosynthesis of sex steroids is between 15 and 19°C (Macdonald et al. 2000). My results demonstrated reduced plasma sex steroid levels in both sockeye and pink salmon held at 19°C, which may be a result of reduced sex steroid biosynthesis. However, the mechanistic cause of this finding warrants further investigation.

Pink salmon were less likely to become ripe when held at 19°C, which suggests a link between reduced plasma sex steroids and inhibited or delayed final maturation. This pattern was not detected in sockeye salmon, likely due to differences in the reproductive schedules of the fish used in the study. If exposure to high temperatures were to happen during natural migrations, it may lead to higher incidences of prespawn mortalities detected on spawning grounds. The relationship between migration during high water temperatures and premature mortality on spawning grounds has been suggested previously (Gilhousen 1990; Hinch and Martins 2011); however these studies were correlative in nature and were not able to determine a physiological link between prespawn mortality and water temperature. A thermally-induced delay in final maturation would extend the freshwater residency period prior to spawning, which increases the likelihood of the fish succumbing to disease and parasite infection (Hinch and Martins 2011).

Proximity to final maturation, which can be assumed to mean that the fish is further along the senescence trajectory, may also influence the response to elevated water temperatures in sockeye and pink salmon. The sockeye salmon used in the 2007 study and the pink salmon used in the 2009 study were closer to their natural peak spawning (i.e., final maturation and eventual death) than the sockeye salmon used in the 2008 study. This may have factored into some of the patterns detected in their survival and gene expression. For example, some of the differences in relative expression of certain stress-responsive genes between the temperature treatments were greater in the fish used in the 2007 and 2009 studies compared with the 2008 study (e.g., FKBP10, HSP90 alpha and beta). Additionally, functional analysis indicated that fewer functional groups were significantly enriched in the 2008 dataset compared with 2007 and 2009. These patterns may reflect a relatively greater temperature-induced stress response in the fish closer to maturity and death. In terms of survival, there are several examples that suggest that pink salmon are more thermally tolerant than sockeye salmon; however ten days at 19°C resulted

in nearly 100% mortality whereas male sockeye salmon ~1 month away from their natural peak spawning period suffered just 54% observed mortality. This higher than expected mortality for pink salmon may have been related to the increased expression of genes associated with oxidative stress, calcium-mediated processes, lipid metabolism and protein catabolism when compared with both summer-run and late-run sockeye salmon. The mortality and gene expression patterns could have been influenced by the proximity to final maturation and processes associated with natural senescence.

My thesis results clearly demonstrate increased mortality in Pacific salmon held at 19°C compared with fish held in a cool temperature treatment (13°C or 14°C). Additionally, there was sex-specific mortality that was evident in sockeye salmon, but not in pink salmon, which may indicate that this is a species-specific phenomenon. However, because I was only able to sample one species each year due to limited tank space, any conclusions regarding a species effect must be viewed cautiously. The differences that I detected may be influenced by the populations considered and any potential inter-annual variation that may have existed, rather than true species level differences. To adequately test for species level differences, multiple populations from both species would need to be compared during the same holding experiment. Logistical constraints would make this very difficult to accomplish; however, future research needs to move in this direction to be able to fully determine if species and population level differences in Pacific salmon thermal tolerances exist.

The ultimate causes of death during the temperature exposure periods are still unknown. Because sub-lethal high water temperatures can enhance disease and parasite progression, these factors were likely contributors to mortality in the Pacific salmon used in my studies. It is important to note that I did not directly assess disease and parasite progression in the fish in my holding studies using histopathological or molecular techniques. Therefore, conclusions about diseases and parasites as a cause of death are speculative. However, I did detect many genes associated with immunity that showed different levels of expression in moribund fish compared with surviving fish and I visually identified symptoms of some diseases and parasites during autopsy. Also, the fish used in my 2007 study were from populations that have been shown to be affected by the unknown virus that has been linked with premature mortality in recent years (Miller et al. 2011). Furthermore, the knowledge of many diseases and parasites that have temperature-dependent progressions and are common in Fraser River Pacific salmon (e.g.,

Flexibactor columnaris, *Parvicapsula minibicornis* and *Saprolegnia* spp.), led me to conclude that these factors may have been involved in the mortality observed in the holding experiments.

Cumulative stress associated with handling and confinement likely contributed to the mortality detected in this thesis either directly through scale loss and other wounding that increases the likelihood of disease and parasite infection, or indirectly by contributing to stress-induced immunosuppression. The interaction between handling, disease and salmon survival warrants further investigation as it may be particularly pertinent to catch and release fishing. These additional stressors may be exacerbated by high water temperature. Therefore, the mortality reported throughout this thesis is likely higher than what would naturally occur during spawning migrations at 19°C. However, by conducting these experiments in a controlled laboratory environment, this thesis provides valuable insight into relative mortality patterns in Pacific salmon held at various temperatures using sample sizes that are excellent for adult Pacific salmon laboratory-based studies.

6.3 Management implications - effects of high water temperatures on Pacific salmon

The Pacific Salmon Commission (PSC) and the Department of Fisheries and Oceans Canada (DFO) are the agencies responsible for harvest management of Pacific salmon in Canada. Fraser River sockeye salmon are 'intensively' managed in that adjustments to harvest rates are made 'in-season' and often are changed on a daily basis as managers decide if their 'escapement targets' are being met – that is, are adequate numbers of fish arriving on spawning grounds (Hinch et al. 2012). Managers have developed 'escapement adjustment models' which are based on long-term correlations between 'escapement discrepancies' (i.e., *en route* mortality) and environmental conditions (e.g., river temperature and discharge) and river entry timing (Cummings et al. 2011). These models estimate levels of *en route* mortality based on forecasted water temperature, discharge or entry timing (Macdonald et al., 2010), but are not based on physiological knowledge. One of the problems that managers have encountered in applying these models is getting buy-in from stake-holders (e.g., commercial, recreational, and First Nations fishers; and non-governmental groups) because these are in essence 'black-box' models and not based on a direct understanding of mortality processes (Hinch et al. 2012). My research has demonstrated some of the physiological mechanisms responsible for high temperature mortality

in migrating salmon and confirms that harvest restrictions in high temperature circumstances are grounded in an understanding of physiological processes.

I found that proximity to final maturation is important to consider when determining how exposure to high water temperatures will affect Pacific salmon survival, especially for pink salmon. Both reproductive development and senescence progress throughout the migration and therefore the location and timing of exposure to high water temperatures may be important. For example, adult Early Stuart sockeye salmon in the Fraser River may experience some of the warmest water temperatures approximately 1000 km into their freshwater spawning migration (Macdonald et al. 2000); consequently they experience high temperatures in a more reproductively mature and senesced state. Indeed, adult Early Stuart sockeye salmon are one of the run-timing aggregates that suffer high rates of *en route* mortality during periods of warm water temperatures (Macdonald et al. 2012; Hinch and Martins 2011). The management adjustment models (reviewed above) used by the DFO and PSC only focus on temperatures that migrants encounter during their migration through the Fraser River mainstem and do not explicitly consider thermal experience during subsequent passage through tributaries *en route* to spawning grounds. My thesis suggests that exposure to elevated water temperatures at a late stage of a relatively long freshwater migration may increase premature mortality. Therefore, incorporating information on upper river water temperatures will be important in determining the full effects of temperature on migrating Pacific salmon.

Despite knowledge that various populations of Pacific salmon have different aerobic capabilities at elevated water temperatures (Lee et al. 2003; Clark et al. 2011; Eliason et al. 2011), a threshold temperature of 18°C has been suggested as a point where spawning migrations are considered more difficult due to temperature stress in the Fraser River system (Macdonald et al. 2000; Hinch and Martins 2011). Field-based telemetry studies have confirmed that spawning migration while temperatures are above 18°C indeed result in an increase in *en route* mortality for some populations of sockeye salmon (Martins et al. 2011). This threshold temperature has been reached or exceeded in six of the past seven years in the Fraser River (eWatch 2011). I found that a common CSR was induced at 19°C for different run-timings of sockeye and pink salmon, which indicates that these fish are physiologically responding to the effects of the warm water temperature. Whether this temperature would elicit a whole-organism level response, such as reduced aerobic scope, for these particular fish is currently unknown. However, previous

studies show that this temperature is within the range of temperatures that do not affect aerobic scope for at least some of these populations (Clark et al. 2011; Eliason et al. 2011). It is currently unknown whether chronic elevation of a CSR is harmful for Pacific salmon. However, given that chronic exposure to 19°C water temperature results in increased mortality in sockeye and pink salmon, along with an osmoregulatory disturbance in sockeye salmon, it appears that Pacific salmon experiencing temperatures capable of inducing a CSR are at an elevated risk of dying prematurely, especially if exposure to ≥19°C occurs close to final maturation and peak spawning. Therefore, this thesis provides evidence of a physiological response to temperature-stress that may explain some of the *en route* mortality that has been observed in recent years. This information may be useful as further biological evidence of the effects of water temperature that fisheries managers can use when justifying in-season harvest regulation adjustments during warm water temperature episodes.

6.4 Effects of senescence on Pacific salmon and recommendations for future research

Despite the fact that all Pacific salmon die after spawning, the physiological effects of senescence and ultimately, mortality, have remained relatively unknown. Prior to this thesis, no study had determined the effects of mortality on gene expression in Pacific salmon. I was able to examine changes in the transcriptome as sockeye salmon became moribund and identify differential expression of several transcription factors and genes associated with apoptosis. These results can be used to determine specifically which cell signalling pathways are involved in Pacific salmon apoptosis. Many genes and cell signalling pathways associated with apoptosis have been characterized in fishes (Krumschnabel and Podrabsky 2009), which include gene expression changes that occur sequentially in apoptotic cells. Therefore, it may be possible to identify what stage of apoptosis an individual Pacific salmon is at as they senesce or whether individuals are close to dying during migration by examining differential gene expression in fish. Future studies should address the temporal changes in the expression of the genes identified in this thesis to further characterize the sequential gene expression changes associated with rapid senescence and mortality that occur on spawning grounds. Sampling for this would be similar to the approach I used in chapter 2 examining blood properties, however at a finer temporal scale.

This thesis characterized the gene expression changes in sockeye salmon as they became moribund, however these changes may have been specific to the conditions in the holding environment (e.g., bacterial infections that are common to fish in confinement). Future studies should attempt to build on these results by further characterizing gene expression changes associated with mortality and by developing predictive biomarkers specific to various diseases and parasites common in dying Pacific salmon.

No previous study had examined the temporal changes in blood properties associated with final maturation and senescence in individual sockeye salmon for an extended period of time (i.e., 44 days; chapter 2). I found that plasma chloride, sodium and osmolality began to decrease 2-10 days in advance of the fish dying and before other blood properties started to change. A particularly intriguing pattern that emerged from this thesis is the potential association between the decrease in plasma ions and expression of ODC1 in gill tissue, the gene most significantly up-regulated in moribund sockeye salmon. Because ODC1 is up-regulated in response to hypo-osmotic stress in ectotherms (Lockwood and Somero 2011) and is associated with apoptosis (Pignatti et al. 2004), future research should attempt to confirm a direct link between ODC1 expression, apoptosis and the low plasma ion levels that are characteristic of dying sockeye salmon. Collectively, this thesis greatly increases our knowledge of physiological changes associated with senescence and mortality and enhances our ability to interpret blood physiology and gene expression patterns in wild-caught migrating Pacific salmon.

6.5 Management implications - physiological effects of senescence and mortality on Pacific salmon

Premature mortality is highly variable between years and populations in Pacific salmon (Gilhousen 1990; Hinch and Martins 2011), therefore molecular tools, such as disease-specific biomarkers, would aid management agencies in determining causes of premature mortality and potentially for predicting the levels of premature mortality that may occur during certain years. This approach has been demonstrated previously as a potential predictive tool for fishery managers as wild migrating fish that had gene expression profiles characteristic of a response to an unknown viral pathogen were 13.5 fold more likely to die *en route* to spawning grounds than fish that did not have the same genomic signature (Miller et al. 2011). Disease-specific

biomarkers could potentially be useful for predicting the percentages of runs that will survive to successfully spawn. For example, knowledge of the health of Pacific salmon during their coastal approach combined with river water temperatures forecasting could allow fishery managers to have a predictive estimate of *en route* mortality prior to the Pacific salmon arriving at the river. As with the 'adjustment models' used by managers to alter harvest rates in high temperature years to compensate for high river mortality, advanced indications of premature mortality rates based on pathogen information would further enable fishery managers to adjust harvest rates in-season to ensure spawning escapement targets are reached. This information could also be useful for hatcheries and recovery programs (e.g., the Cultus Lake Sockeye Salmon recovery program – Hinch et al. 2012) that maintain broodstock for extended periods of time during final maturation. By enabling hatchery managers to predict the levels of premature mortality of broodstock during maturation, they could more accurately estimate the minimum level of broodstock they need to ensure successful artificial fertilizations with sufficient genetic variation. The predictive capability of these molecular markers, of course, needs to be established experimentally before they can truly be implemented as management tools.

A novel outcome of this thesis was the discovery that plasma chloride levels are predictive of mortality in sockeye salmon. Because plasma chloride could predict mortality up to 10 days prior to death, this information is relevant to Pacific salmon at or near spawning grounds. Using a tool for rapid assessment of plasma ion levels (e.g., hand held ion meters), fisheries managers could conceivably predict the longevity of Pacific salmon on spawning grounds and potentially predict the levels of prespawn mortality that might occur during a given year. Information such as this would be useful for managing artificial spawning channels where managers strive to meet pre-determined spawning targets by allowing a certain number of fish into the spawning channel when appropriate. Spawning channel operators often try to allow a full representation of the run (i.e., early, peak and late arrivals) to spawn, if they are able to predict how many fish will die prematurely early on in the run, it would be possible to increase stocking densities later to ensure sufficient numbers of fish are able to successfully spawn in a given year.

6.6 Conclusion

This thesis provides valuable and novel information regarding the effects of temperature and senescence/mortality on blood properties and gene expression of wild-caught experimentally-treated sockeye and pink salmon. By using state-of-the-art microarray technology, I was able to characterize the transcriptome-wide response to mortality and temperature in ecologically relevant scenarios. Rarely is a single researcher involved in as many components of an ecological genomics study as I was for the studies presented in this thesis. I believe this provided me with an intimate connection between the study system, study organisms and the molecular approaches utilized. It potentially allows for a more holistic understanding of the ecological question being studied. By combining molecular approaches with existing already validated biopsy techniques, I was able to evaluate these physiological processes at multiple levels of biological organization more comprehensively than has ever been examined before. This thesis also provides further steps in the development of physiological research tools (see Cooke et al. 2008, Cooke et al. 2012), which can aid in the conservation and management of ecologically and economically important Pacific salmon species.

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Appendix A. Functional analysis performed using the receiver operator characteristic (ROC) scoring method in ErmineJ for the first three principal components (PC) for the principal component analysis performed on all experimental years. The GO categories Biological Process and Molecular Function are presented here. Only gene sets significant at a false discovery rate corrected *P*-value < 0.05 are presented for each PC.

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
1	Biological Process	Catabolic process	GO:0009056	protein catabolic process	GO:0030163	72	36	0.660	0.033		
1		Cellular metabolic process	GO:0044237	sphingolipid metabolic process	GO:0006665	20	10	0.814	0.029		
1				cellular lipid metabolic process	GO:0044255	171	71	0.620	0.032		
1		Nitrogen compound metabolic process	GO:0006807	polyamine metabolic process	GO:0006595	10	5	0.898	0.028		
1		Primary metabolic process	GO:0044238	lipid metabolic process	GO:0006629	195	83	0.630	0.014		
1				carbohydrate metabolic process	GO:0005975	179	70	0.615	0.033	0.040	
1		Response to stress	GO:0006950	response to oxidative stress	GO:0006979	48	21	0.774	0.004		
1	Molecular Function	Enzyme regulator activity	GO:0030234	enzyme inhibitor activity	GO:0004857	72	22	0.722	0.027		0.029
1		Ion binding	GO:0043167	cation binding	GO:0043169	187	81	0.628	0.015		
1				calcium ion binding	GO:0005509	85	35	0.667	0.031		
1		Lipid binding	GO:0008289	lipid binding	GO:0008289	92	30	0.687	0.027		
2	Biological Process	Biosynthetic process	GO:0009058	protein amino acid glycosylation	GO:0006486	56	23	0.701		0.018	
2				glycoprotein biosynthetic process	GO:0009101	59	21	0.697		0.029	
2		Cell death	GO:0008219	apoptosis	GO:0006915	119	53	0.638		0.017	
2		Cell death	GO:0008219	cell death	GO:0008219	139	55	0.634		0.017	

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
2				programmed cell death	GO:0012501	127	54	0.634		0.017	
2				activation of caspase activity	GO:0006919	25	11	0.759		0.028	
2		Cellular component organization or biogenesis at cellular level	GO:0071841	ribonucleoprotein complex assembly	GO:0022618	56	15	0.715		0.035	
2		Cellular localization	GO:0051641	cellular protein localization	GO:0034613	142	66	0.620		0.018	
2				intracellular protein transport	GO:0006886	140	67	0.614		0.026	
2				nucleocytoplasmic transport	GO:0006913	80	33	0.648		0.033	
2		Macromolecule localization	GO:0033036	protein localization	GO:0008104	195	89	0.632		0.005	
2				protein transport	GO:0015031	154	71	0.620		0.017	
2		Macromolecule metabolic process	GO:0043170	biopolymer glycosylation	GO:0043413	55	20	0.700		0.026	
2				glycoprotein metabolic process	GO:0009100	73	26	0.659		0.041	
2		Microtubule-based process	GO:0007017	microtubule-based process	GO:0007017	95	34	0.646		0.032	
2				microtubule cytoskeleton organization	GO:0000226	68	22	0.669		0.046	
2		Nitrogen compound metabolic process	GO:0006807	RNA processing	GO:0006396	187	73	0.626		0.015	
2		Nitrogen compound metabolic process	GO:0006807	RNA splicing	GO:0008380	104	42	0.666		0.022	
2				mRNA processing	GO:0006397	90	34	0.644		0.035	
2		Regulation of biological process	GO:0050789	positive regulation of programmed cell death	GO:0043068	135	67	0.609		0.028	0.041
2				positive regulation of developmental process	GO:0051094	188	91	0.591		0.031	

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
2		Regulation of biological process	GO:0050789	regulation of neuron apoptosis	GO:0043523	9	6	0.824		0.038	
2		Regulation of biological quality	GO:0065008	negative regulation of cell size	GO:0045792	16	7	0.821		0.028	
2		Vesicle-mediated transport	GO:0016192	vesicle-mediated transport	GO:0016192	189	81	0.607		0.018	
2	Molecular Function	Enzyme regulator activity	GO:0030234	GTPase regulator activity	GO:0030695	125	53	0.624		0.027	
2		Hydrolase activity	GO:0016787	protein serine/threonine phosphatase activity	GO:0004722	19	8	0.828		0.018	
2				inositol or phosphatidylinositol phosphatase activity	GO:0004437	27	10	0.772		0.029	
2				hydrolase activity, acting on acid anhydrides	GO:0016817	235	87	0.596	0.045	0.029	0.050
2		Ligase activity	GO:0016874	small conjugating protein ligase activity	GO:0019787	131	50	0.650		0.013	0.031
2				acid-amino acid ligase activity	GO:0016881	134	53	0.646		0.014	
2				ubiquitin-protein ligase activity	GO:0004842	109	47	0.650		0.016	0.017
2				ligase activity	GO:0016874	181	74	0.617		0.017	0.038
2				ligase activity, forming carbon-nitrogen bonds	GO:0016879	143	58	0.630		0.018	
2		Nucleic acid binding	GO:0003676	RNA binding	GO:0003723	232	84	0.607	0.035	0.019	
2		Nucleotide binding	GO:0000166	guanyl ribonucleotide binding	GO:0032561	52	20	0.726		0.015	
2				GTP binding	GO:0005525	61	24	0.709		0.016	
2				guanyl nucleotide binding	GO:0019001	54	21	0.729		0.017	
2		Protein binding	GO:0005515	actin binding	GO:0003779	91	35	0.673		0.014	
2		Structural molecule activity	GO:0005198	structural constituent of ribosome	GO:0003735	74	28	0.667		0.028	

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
2				structural molecule activity	GO:0005198	195	72	0.595		0.046	
2		Transcription activator activity	GO:0016563	transcription activator activity	GO:0016563	149	69	0.604		0.031	
2		Transcription factor binding transcription factor activity	GO:0000989	transcription coactivator activity	GO:0003713	95	43	0.647		0.018	
2		Transporter activity	GO:0005215	substrate-specific transporter activity	GO:0022892	125	44	0.645		0.019	
2				transporter activity	GO:0005215	172	67	0.635		0.020	
3	Biological Process	Actin filament-based process	GO:0030029	actin cytoskeleton organization	GO:0030036	92	38	0.671		0.035	0.006
3				actin filament-based process	GO:0030029	104	42	0.640			0.015
3				actin filament organization	GO:0007015	33	17	0.695		0.037	0.029
3		Anatomical structure development	GO:0048856	tissue development	GO:0009888	84	37	0.671			0.006
3				ectoderm development	GO:0007398	41	15	0.734			0.014
3		Anatomical structure morphogenesis	GO:0009653	anatomical structure formation involved in morphogenesis	GO:0048646	71	28	0.680			0.011
3		Biological adhesion	GO:0022610	biological adhesion	GO:0022610	195	85	0.585			0.035
3		Catabolic process	GO:0009056	macromolecule catabolic process	GO:0009057	156	65	0.609	0.029		0.019
3				cellular macromolecule catabolic process	GO:0044265	145	60	0.610			0.023
3		Cell activation	GO:0001775	cell activation	GO:0001775	70	28	0.695			0.006
3		Cell adhesion	GO:0007155	cell adhesion	GO:0007155	206	93	0.588			0.023
3		Cell cycle	GO:0007049	cell cycle	GO:0007049	210	93	0.650			0.000
3				cell cycle process	GO:0022402	184	84	0.644			0.001
3				mitotic cell cycle	GO:0000278	92	41	0.681			0.002

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
3				cell cycle phase	GO:0022403	141	64	0.639			0.003
3				mitosis	GO:0007067	47	23	0.691			0.014
3				M phase	GO:0000279	103	46	0.630			0.019
3				interphase of mitotic cell cycle	GO:0051329	42	20	0.674			0.034
3				interphase	GO:0051325	45	22	0.658			0.043
3		Cell junction organization	GO:0034330	cell junction assembly	GO:0034329	20	8	0.780			0.030
3		Cell proliferation	GO:0008283	cell proliferation	GO:0008283	152	71	0.636		0.033	0.003
3		Cellular component biogenesis	GO:0044085	cellular component biogenesis	GO:0044085	271	97	0.649		0.031	0.0003
3		Cellular component biogenesis	GO:0044085	protein complex biogenesis	GO:0070271	132	53	0.620			0.020
3		Cellular component organization	GO:0016043	cellular component assembly	GO:0022607	230	82	0.646		0.038	0.001
3				macromolecular complex subunit organization	GO:0043933	213	77	0.643			0.001
3				macromolecular complex assembly	GO:0065003	208	74	0.632			0.003
3		Cellular component organization	GO:0016043	protein complex assembly	GO:0006461	128	55	0.613			0.024
3		Cellular component organization or biogenesis at cellular level	GO:0071841	cytoskeleton organization	GO:0007010	187	71	0.644		0.009	0.001
3				ribonucleoprotein complex biogenesis	GO:0022613	94	28	0.677		0.028	0.013
3		Cellular component organization or biogenesis at cellular level	GO:0071841	ribosome biogenesis	GO:0042254	42	13	0.740			0.018
3				cellular macromolecular complex subunit organization	GO:0034621	116	41	0.627			0.029

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
3				chromatin assembly or disassembly	GO:0006333	20	5	0.819			0.044
3				nuclear division	GO:0000280	40	19	0.665			0.049
3		Cellular developmental process	GO:0048869	keratinocyte differentiation	GO:0030216	26	7	0.917			0.001
3		Cellular homeostasis	GO:0019725	cellular ion homeostasis	GO:0006873	63	25	0.685			0.013
3				cellular homeostasis	GO:0019725	71	30	0.661			0.018
3				cellular chemical homeostasis	GO:0055082	64	26	0.665			0.023
3				cellular calcium ion homeostasis	GO:0006874	15	7	0.793			0.031
3		Cellular metabolic process	GO:0044237	sulfur metabolic process	GO:0006790	43	19	0.672			0.040
3		Cellular response to stimulus	GO:0051716	G-protein coupled receptor protein signaling pathway	GO:0007186	65	41	0.648			0.012
3				phosphoinositide-mediated signaling	GO:0048015	25	8	0.798			0.019
3				integrin-mediated signaling pathway	GO:0007229	10	6	0.833			0.022
3				cellular response to stimulus	GO:0051716	171	87	0.588			0.030
3				cellular response to stress	GO:0033554	165	84	0.586			0.035
3		Coagulation	GO:0050817	coagulation	GO:0050817	16	10	0.746			0.033
3		Establishment of localization	GO:0051234	amino acid transport	GO:0006865	21	5	0.837			0.034
3		Growth	GO:0040007	growth	GO:0040007	48	23	0.677			0.023
3				developmental growth	GO:0048589	13	6	0.789			0.048
3		Immune response	GO:0006955	immune response	GO:0006955	117	49	0.687		0.037	0.001
3		Immune system development	GO:0002520	immune system development	GO:0002520	91	42	0.637			0.018
3				myeloid cell differentiation	GO:0030099	41	18	0.705			0.019

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
3				hemopoietic or lymphoid organ development	GO:0048534	90	41	0.631			0.024
3				hemopoiesis	GO:0030097	86	41	0.630			0.026
3		Leukocyte activation	GO:0045321	leukocyte activation	GO:0045321	67	26	0.678			0.015
3				lymphocyte activation	GO:0046649	57	22	0.671			0.030
3		Macromolecule metabolic process	GO:0043170	proteolysis	GO:0006508	158	79	0.582			0.049
3		Microtubule-based process	GO:0007017	spindle organization	GO:0007051	29	9	0.747			0.041
3		Multicellular organismal development	GO:0007275	epidermis development	GO:0008544	38	13	0.759			0.011
3				blastocyst development	GO:0001824	20	8	0.763			0.039
3		Nitrogen compound metabolic process	GO:0006807	DNA metabolic process	GO:0006259	269	88	0.605			0.010
3				DNA replication	GO:0006260	99	35	0.651			0.017
3				rRNA metabolic process	GO:0016072	28	10	0.738			0.038
3				DNA repair	GO:0006281	99	48	0.610			0.040
3				transcription, DNA-dependent	GO:0006351	81	37	0.619			0.050
3		Regulation of biological process	GO:0050789	regulation of cell proliferation	GO:0042127	187	79	0.646		0.019	0.001
3		Regulation of biological process	GO:0050789	regulation of cell cycle	GO:0051726	138	63	0.655			0.001
3				negative regulation of cell proliferation	GO:0008285	109	49	0.661		0.016	0.003
3				regulation of DNA metabolic process	GO:0051052	37	19	0.736			0.006
3				regulation of DNA replication initiation	GO:0030174	21	12	0.791			0.006
3				regulation of mitotic cell cycle	GO:0007346	55	26	0.692			0.008

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
3				regulation of cell cycle process	GO:0010564	39	14	0.760			0.008
3				regulation of DNA replication	GO:0006275	28	14	0.750			0.011
3				positive regulation of signal transduction	GO:0009967	68	35	0.656			0.014
3				cell cycle checkpoint	GO:0000075	50	30	0.667			0.014
3				regulation of mitosis	GO:0007088	20	10	0.780			0.015
3				induction of apoptosis	GO:0006917	114	55	0.623			0.015
3				positive regulation of cell communication	GO:0010647	69	36	0.641			0.023
3				regulation of protein kinase cascade	GO:0010627	69	32	0.649			0.024
3				positive regulation of metabolic process	GO:0009893	203	90	0.587			0.028
3				regulation of protein metabolic process	GO:0051246	124	47	0.620			0.028
3				regulation of hydrolase activity	GO:0051336	80	30	0.649			0.029
3				regulation of caspase activity	GO:0043281	38	14	0.713			0.030
3				regulation of peptidase activity	GO:0052547	39	15	0.703			0.033
3				positive regulation of cellular metabolic process	GO:0031325	200	89	0.584			0.034
3				positive regulation of cytokine biosynthetic process	GO:0042108	13	5	0.830			0.038
3				positive regulation of programmed cell death	GO:0043068	135	67	0.593		0.028	0.041
3				positive regulation of hydrolase activity	GO:0051345	56	21	0.663			0.041
3				positive regulation of protein kinase cascade	GO:0010740	50	23	0.655			0.043

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
3				positive regulation of biosynthetic process	GO:0009891	154	71	0.588			0.044
3				positive regulation of cell proliferation	GO:0008284	64	31	0.631			0.048
3		Regulation of biological quality	GO:0065008	homeostatic process	GO:0042592	141	60	0.644			0.003
3				cation homeostasis	GO:0055080	31	13	0.766			0.008
3				chemical homeostasis	GO:0048878	68	29	0.663			0.018
3		Regulation of body fluid levels	GO:0050878	regulation of body fluid levels	GO:0050878	28	14	0.759			0.008
3				hemostasis	GO:0007599	26	13	0.750			0.014
3		Response to chemical stimulus	GO:0042221	response to chemical stimulus	GO:0042221	176	80	0.586			0.039
3		Response to external stimulus	GO:0009605	response to external stimulus	GO:0009605	155	67	0.635			0.004
3		Response to stress	GO:0006950	response to wounding	GO:0009611	98	41	0.662			0.006
3				wound healing	GO:0042060	25	14	0.724			0.023
3				inflammatory response	GO:0006954	68	29	0.642			0.038
3	Molecular Function	Antigen binding	GO:0003823	antigen binding	GO:0003823	24	8	0.863			0.004
3		Chromatin binding	GO:0003682	chromatin binding	GO:0003682	61	28	0.685			0.009
3		Cofactor binding	GO:0048037	cofactor binding	GO:0048037	35	14	0.702			0.039
3		Enzyme regulator activity	GO:0030234	enzyme regulator activity	GO:0030234	243	99	0.626	0.029	0.029	0.001
3		Enzyme regulator activity	GO:0030234	enzyme activator activity	GO:0008047	104	44	0.654			0.007
3				enzyme inhibitor activity	GO:0004857	72	22	0.672	0.027		0.029
3				GTPase activator activity	GO:0005096	65	26	0.654			0.034
3		Hydrolase activity	GO:0016787	phosphoprotein phosphatase activity	GO:0004721	49	23	0.690			0.014
3				phosphatase activity	GO:0016791	96	44	0.629		0.030	0.022
3				phosphoric ester hydrolase activity	GO:0042578	109	53	0.608			0.034

PC	GO Category	Name	GO ID	Name	GO ID	Probes	# of Genes	Score	PC1	PC2	PC3
3				GTPase activity	GO:0003924	51	22	0.660			0.041
3		Ion binding	GO:0043167	zinc ion binding	GO:0008270	86	45	0.611			0.043
3		Ligase activity	GO:0016874	ubiquitin-protein ligase activity	GO:0004842	109	47	0.631		0.016	0.017
3				small conjugating protein ligase activity	GO:0019787	131	50	0.614		0.013	0.031
3				ligase activity	GO:0016874	181	74	0.590		0.017	0.038
3		Peptide binding	GO:0042277	peptide binding	GO:0042277	29	13	0.701			0.047
3				cytoskeletal protein binding	GO:0008092	177	72	0.631		0.027	0.004
3				protein domain specific binding	GO:0019904	80	37	0.673			0.006
3				protein binding, bridging	GO:0030674	56	26	0.682			0.013
3				histone deacetylase binding	GO:0042826	10	7	0.796			0.029
3				growth factor activity	GO:0008083	34	10	0.746			0.033
3				SH3 domain binding	GO:0017124	16	9	0.755			0.035
3				protein dimerization activity	GO:0046983	131	65	0.596			0.038
3				receptor binding	GO:0005102	189	90	0.582			0.038
3				cytokine binding	GO:0019955	33	13	0.705			0.042
3				SH3/SH2 adaptor activity	GO:0005070	28	19	0.671			0.042
3		Signal transducer activity	GO:0004871	transmembrane receptor activity	GO:0004888	126	69	0.633			0.004
3				G-protein coupled receptor activity	GO:0004930	62	33	0.673			0.008
3				receptor activity	GO:0004872	180	99	0.581			0.030

Appendix B. Functional analysis performed using the receiver operator characteristic (ROC) scoring method in ErmineJ for the principal component (PC) most associated with survival (PC1 for each year) for the principal component analysis performed on each individual experimental year. The GO categories Biological Process and Molecular Function are presented here. Only gene sets significant at a false discovery rate corrected *P*-value < 0.05 are presented for each experimental year.

GO Category	Name	GO ID	Name	GO ID	2007	2008	2009
Biological Process	Biosynthetic process	GO:0009058	prostaglandin biosynthetic process	GO:0001516		0.044	
	Catabolic process	GO:0009056	carbohydrate catabolic process	GO:0016052			0.049
			catabolic process	GO:0009056	0.044		
			cellular catabolic process	GO:0044248	0.045		
	Cell death	GO:0008219	activation of caspase activity	GO:0006919		0.029	
			apoptosis	GO:0006915		0.045	0.010
			cell death	GO:0008219			0.019
			programmed cell death	GO:0012501			0.018
	Cell proliferation	GO:0008283	cell proliferation	GO:0008283	0.012	0.002	
	Cellular homeostasis	GO:0019725	cellular homeostasis	GO:0019725		0.034	
			cellular ion homeostasis	GO:0006873	0.049	0.035	
	Cellular metabolic process	GO:0044237	carboxylic acid metabolic process	GO:0019752		0.037	
			cellular carbohydrate metabolic process	GO:0044262		0.045	
			cellular lipid metabolic process	GO:0044255	0.050	0.024	
			glucose metabolic process	GO:0006006	0.024		
			monocarboxylic acid metabolic process	GO:0032787	0.045		
			organic acid metabolic process	GO:0006082		0.037	
			prostaglandin metabolic process	GO:0006693	0.043		
	Cellular response to stimulus	GO:0051716	cell surface receptor linked signal transduction	GO:0007166	0.015		
			enzyme linked receptor protein signaling pathway	GO:0007167	0.048		
			G-protein coupled receptor protein signaling pathway	GO:0007186		0.034	

GO Category	Name	GO ID	Name	GO ID	2007	2008	2009
			second-messenger-mediated signaling	GO:0019932		0.021	
	Immune response	GO:0006955	immune response	GO:0006955	0.036	0.005	
	Immune system process	GO:0002376	immune system process	GO:0002376	0.046		
	Macromolecule metabolic process	GO:0043170	proteolysis	GO:0006508			0.048
	Primary metabolic process	GO:0044238	carbohydrate metabolic process	GO:0005975			0.022
			lipid metabolic process	GO:0006629	0.042	0.021	
	Regulation of biological process	GO:0050789	regulation of catalytic activity	GO:0050790		0.011	
			induction of apoptosis	GO:0006917	0.039	0.045	
			negative regulation of apoptosis	GO:0043066			0.011
			negative regulation of cell proliferation	GO:0008285			0.025
			negative regulation of programmed cell death	GO:0043069			0.019
			positive regulation of caspase activity	GO:0043280		0.030	
			positive regulation of cell proliferation	GO:0008284		0.037	
			regulation of caspase activity	GO:0043281	0.049	0.008	
			regulation of cell cycle	GO:0051726	0.046	0.001	
			regulation of cell proliferation	GO:0042127		0.024	0.009
	Regulation of biological quality	GO:0065008	cation homeostasis	GO:0055080		0.026	
			chemical homeostasis	GO:0048878	0.041	0.035	
			homeostatic process	GO:0042592		0.039	
	Response to chemical stimulus	GO:0042221	response to chemical stimulus	GO:0042221		0.010	0.040
	Response to endogenous stimulus	GO:0009719	response to endogenous stimulus	GO:0009719		0.037	
	Response to external stimulus	GO:0009605	response to external stimulus	GO:0009605	0.046	0.004	
	Response to stress	GO:0006950	response to oxidative stress	GO:0006979	0.049		
	Defence response	GO:0006952	inflammatory response	GO:0006954		0.035	

GO Category	Name	GO ID	Name	GO ID	2007	2008	2009
			response to wounding	GO:0009611		0.017	
Molecular Function	Antigen binding	GO:0003823	antigen binding	GO:0003823	0.031	0.001	
	Carbohydrate binding	GO:0030246	carbohydrate binding	GO:0030246		0.029	
	Ion binding	GO:0043167	cation binding	GO:0043169		0.006	
			transition metal ion binding	GO:0046914		0.019	
			zinc ion binding	GO:0008270		0.002	
	Ligase activity	GO:0016874	ubiquitin-protein ligase activity	GO:0004842			0.037
	Lipid binding	GO:0008289	lipid binding	GO:0008289		0.045	
	Oxidoreductase activity	GO:0016491	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	GO:0016709		0.027	
	Protein binding	GO:0005515	receptor binding	GO:0005102		0.046	
	Signal transducer activity	GO:0004871	G-protein coupled receptor activity	GO:0004930		0.035	
	Transmembrane transporter activity	GO:0022857	active transmembrane transporter activity	GO:0022804		0.046	0.009
	Transporter activity	GO:0005215	primary active transmembrane transporter activity	GO:0015399			0.021

Appendix C. Functional analysis performed using the receiver operator characteristic (ROC) scoring method in ErmineJ for the principal component (PC) most associated with temperature (PC4 for 2007 and 2009, PC3 for 2008) for the principal component analysis performed on each individual experimental year. The GO categories Biological Process and Molecular Function are presented here. Only gene sets significant at a false discovery rate corrected *P*-value < 0.05 are presented for each experimental year.

GO Category	Name	GO ID	Name	ID	2007	2008	2009
Biological Process	Biosynthetic process	GO:0009058	translation	GO:0006412	0.012		
	Catabolic process	GO:0009056	catabolic process	GO:0009056	0.009		
			cellular catabolic process	GO:0044248	0.013		
	Cell cycle	GO:0007049	cell cycle	GO:0007049	0.001		
			cell cycle phase	GO:0022403	0.001		
			cell cycle process	GO:0022402	0.001		
			G1/S transition of mitotic cell cycle	GO:0000082	0.022		0.027
			interphase	GO:0051325	0.008		
			interphase of mitotic cell cycle	GO:0051329	0.004		
			M phase	GO:0000279	0.029		
			M phase of mitotic cell cycle	GO:0000087	0.008		
			mitosis	GO:0007067	0.005		
			mitotic cell cycle	GO:0000278	0.000		
	Cell proliferation	GO:0008283	cell proliferation	GO:0008283	0.043		
	Cellular component organization or biogenesis at cellular level	GO:0071841	chromosome organization	GO:0051276	0.015		
			nuclear division	GO:0000280	0.006		
			cellular macromolecular complex assembly	GO:0034622	0.027		
			organelle fission	GO:0048285	0.004		
			mitochondrion organization	GO:0007005	0.022		
			establishment or maintenance of chromatin architecture	GO:0006325	0.043		0.025
			cellular macromolecular complex subunit organization	GO:0034621	0.043		

GO Category	Name	GO ID	Name	ID	2007	2008	2009
			ribonucleoprotein complex assembly	GO:0022618	0.048		
			establishment or maintenance of chromatin architecture	GO:0006325	0.043		0.025
			ribonucleoprotein complex biogenesis	GO:0022613			0.026
			nucleosome organization	GO:0034728			0.046
			chromatin modification	GO:0016568			0.047
	Cellular metabolic process	GO:0044237	organic acid metabolic process	GO:0006082	0.041		
			sulfur metabolic process	GO:0006790	0.022		
			cofactor metabolic process	GO:0051186	0.042		
	Cellular response to stimulus	GO:0051716	cellular response to DNA damage stimulus	GO:0034984	0.010		
			cellular response to stress	GO:0033554			0.047
			response to DNA damage stimulus	GO:0006974	0.015		
	Detection of stimulus	GO:0051606	detection of stimulus	GO:0051606	0.015		
			histone methylation	GO:0016571			0.043
			histone modification	GO:0016570			0.029
			protein amino acid autophosphorylation	GO:0046777			0.045
	Microtubule-based process	GO:0007017	microtubule cytoskeleton organization	GO:0000226	0.043		
			microtubule-based process	GO:0007017	0.036		
	Multicellular organismal development	GO:0007275	embryonic development	GO:0009790	0.013		
			appendage development	GO:0048736	0.041		
			in utero embryonic development	GO:0001701	0.046		
	Multi-organism process	GO:0051704	multi-organism process	GO:0051704	0.041		
	Nitrogen compound metabolic process	GO:0006807	mRNA metabolic process	GO:0016071	0.022		0.041
			mRNA processing	GO:0006397	0.049		0.025
			RNA processing	GO:0006396	0.021	0.045	0.047
			aspartate family amino acid metabolic process	GO:0009066	0.031		
			cellular amine metabolic process	GO:0009308	0.043		
			cellular nitrogen compound metabolic process	GO:0034641	0.031		

GO Category	Name	GO ID	Name	ID	2007	2008	2009
			methionine metabolic process	GO:0006555	0.009		
			nitrogen compound metabolic process	GO:0006807	0.041		
			sulfur amino acid metabolic process	GO:0000096	0.025		
			DNA metabolic process	GO:0006259	0.000		0.045
			DNA repair	GO:0006281	0.005		
			DNA replication	GO:0006260	0.001		
			DNA-dependent DNA replication	GO:0006261	0.007		
	Regulation of biological process	GO:0050789	negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	GO:0045934	0.022		
			positive regulation of biosynthetic process	GO:0009891	0.015		0.034
			positive regulation of gene expression	GO:0010628			0.048
			positive regulation of macromolecule biosynthetic process	GO:0010557	0.024		0.048
			positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	GO:0045935			0.044
			positive regulation of transcription	GO:0045941	0.047		0.028
			regulation of transcription from RNA polymerase II promoter	GO:0006357	0.010		
			negative regulation of cellular metabolic process	GO:0031324	0.016		
			negative regulation of macromolecule metabolic process	GO:0010605	0.022		
			negative regulation of metabolic process	GO:0009892	0.011		
			positive regulation of cellular metabolic process	GO:0031325	0.004		0.028
			positive regulation of macromolecule metabolic process	GO:0010604	0.005		0.037
			positive regulation of metabolic process	GO:0009893	0.004		0.028
			negative regulation of DNA metabolic process	GO:0051053	0.008		
			negative regulation of DNA replication	GO:0008156	0.013		
			regulation of cell cycle	GO:0051726	0.009		

GO Category	Name	GO ID	Name	ID	2007	2008	2009
			regulation of cell cycle process	GO:0010564	0.011		
			regulation of DNA metabolic process	GO:0051052	0.007		
			regulation of DNA replication	GO:0006275	0.027		
			regulation of mitosis	GO:0007088	0.034		
			regulation of mitotic cell cycle	GO:0007346	0.008		
			anti-apoptosis	GO:0006916	0.012		0.036
			negative regulation of apoptosis	GO:0043066	0.018		
			negative regulation of cell proliferation	GO:0008285	0.041		
			negative regulation of programmed cell death	GO:0043069	0.033		
			regulation of cell proliferation	GO:0042127	0.031		
	Reproductive process	GO:0022414	viral reproductive process	GO:0022415	0.034		
	Response to biotic stimulus	GO:0009607	response to biotic stimulus	GO:0009607	0.047		
			response to other organism	GO:0051707	0.027		
			response to virus	GO:0009615	0.009		
	Response to stress	GO:0006950	response to oxidative stress	GO:0006979			0.036
	Viral reproduction	GO:0016032	viral reproduction	GO:0016032	0.011		
Molecular Function	Antioxidant activity	GO:0016209	antioxidant activity	GO:0016209			0.032
	DNA binding	GO:0003684	damaged DNA binding	GO:0003684	0.047		
	Hydrolase activity	GO:0016787	ATPase activity	GO:0016887			0.047
			ATPase activity, coupled	GO:0042623	0.044		
			hydrolase activity, acting on acid anhydrides	GO:0016817	0.007		0.030
			exonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 5'-phosphomonoesters	GO:0016796	0.008		
			nucleoside-triphosphatase activity	GO:0017111	0.009		0.047
			exonuclease activity	GO:0004527	0.009		
			hydrolase activity, acting on acid anhydrides	GO:0016817	0.007		0.030
			nucleoside-triphosphatase activity	GO:0017111	0.009		0.047

GO Category	Name	GO ID	Name	ID	2007	2008	2009
	Isomerase activity	GO:0016853	intramolecular oxidoreductase activity	GO:0016860	0.047		
	Ligase activity	GO:0016874	ligase activity	GO:0016874	0.022		0.035
			ligase activity, forming carbon-nitrogen bonds	GO:0016879	0.050		
			ligase activity	GO:0016874	0.022		0.035
	Nucleic acid binding	GO:0003676	RNA binding	GO:0003723	0.013	0.046	0.023
			translation factor activity, nucleic acid binding	GO:0008135			0.047
			translation initiation factor activity	GO:0003743		0.018	
	Nucleotide binding	GO:0000166	GTP binding	GO:0005525	0.009		
			guanyl ribonucleotide binding	GO:0032561	0.028		
			guanyl nucleotide binding	GO:0019001	0.034		
			ATP binding	GO:0005524			0.045
	Oxidoreductase activity	GO:0016491	oxidoreductase activity, acting on peroxide as acceptor	GO:0016684			0.037
	Protein binding	GO:0005515	transcription factor binding	GO:0008134	0.027		
			histone binding	GO:0042393	0.034		
			calmodulin binding	GO:0005516	0.046		
			steroid hormone receptor binding	GO:0035258			0.045
	Structural molecule activity	GO:0005198	structural constituent of cytoskeleton	GO:0005200	0.045		
			structural molecule activity	GO:0005198	0.033		
	transcription activator activity	GO:0016563	transcription activator activity	GO:0016563	0.048		0.035
	Transcription factor binding transcription factor activity	GO:0000989	transcription cofactor activity	GO:0003712	0.034		
	Transferase activity	GO:0016740	S-adenosylmethionine-dependent methyltransferase activity	GO:0008757	0.011		
			methyltransferase activity	GO:0008168	0.021		0.037
			transferase activity, transferring one-carbon groups	GO:0016741	0.027		0.028
			histone methyltransferase activity	GO:0042054			0.022
			protein methyltransferase activity	GO:0008276			0.025

GO Category	Name	GO ID	Name	ID	2007	2008	2009
			transferase activity, transferring one-carbon groups	GO:0016741	0.027		0.028
			transferase activity, transferring acyl groups	GO:0016746			0.034
			methyltransferase activity	GO:0008168	0.021		0.037
			transferase activity, transferring acyl groups other than amino-acyl groups	GO:0016747			0.046