Effects of maternal oxidative stress and gametic cortisol exposure on Fraser River sockeye salmon (*Oncorhynchus nerka)* offspring

By

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B.Sc., University of Ottawa, 2012

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Masters of Science
in
Biology

Carleton University
Ottawa, Ontario

Dedication

To Mom and Dad for constantly supporting me in every challenge I take on and to Cole for proving to us all that you can accomplish anything you put your mind to. To my Papa Pennell, who passed away during the completion of this thesis, for instilling in me his passion for fishing and love of being on the water.

Abstract

Though intergenerational effects have been reported in a wide range of taxa including insects, plants, birds, and fish, little is known about the potential effects of oxidative stress and gametic cortisol exposure on future generations of fish. The semelparous, migratory life history of Pacific salmon (*Oncorhynchus spp.*) requires a delicate balance of resource allocation and thus provides a unique opportunity to explore intergenerational and intraspecific effects of oxidative stress. In Chapter 2, I assessed oxidative health in sockeye salmon (*O. nerka*) from three distinct populations in the Fraser River (British Columbia) and demonstrated that neither maternal antioxidant concentration nor oxidative stress is conferred to offspring and that oxidative stress appears to be related to population at specific offspring life stages. In Chapter 3, I assessed the effect of maternal oxidative health on egg cortisol levels, and the effect of egg cortisol on offspring oxidative health. I also experimentally elevated egg cortisol to mimic maternal stress, and found that there may be a buffering mechanism in the mother and the egg to avoid negative effects of hypercortisolism on oxidative health. Given the population level differences in productivity and abundance, population-specific information on oxidative stress in sockeye salmon (both as adults and offspring) can help identify which sockeye salmon stocks may be at greater risk of the resonating effects of oxidative stress on offspring.

Acknowledgments

First I would like to thank my supervisors, S. Cooke and W. Willmore, for providing me with constant support, feedback and countless amazing opportunities throughout the completion of this thesis. I would like to thank the members of the DFO Environmental Watch Program, especially J. Hills for assistance with fish collection and D.A. McKay for assistance with offspring rearing and sampling. I would also like to thank C. Middleton of the Hinch lab for assistance with fish collection. I would like to thank the Willmore lab members, especially J. Mattice, and H. Bo, for help with laboratory assays. Finally, I would like to thank all my co-authors, especially N. Sopinka and S. Wilson for their constant mentorship and S. Wilson for assistance with statistical analysis. Support for my research was provided in the form of Natural Sciences and Engineering and Research Council (NSERC) Strategic and Discovery Grants to S.J.C., S.G.H., and W.G.W. Ocean Tracking Network Canada also provided logistical and financial support. S.J.C. is supported by the Canada Research Chairs program.

Co-Authorship

Chapter 2: Intergenerational and population-specific effects of oxidative stress in sockeye salmon *(Oncorhynchus nerka)*. Jessica J. Taylor, Samantha M. Wilson, Natalie M. Sopinka, Scott G. Hinch, David A. Patterson, Steven J. Cooke, William G. Willmore

While this study is my own, the research was undertaken as part of a collaborative effort and each co-author played a valuable role in its completion. The project was conceived by Taylor, Wilson, Sopinka, Cooke, and Willmore. Fieldwork was completed by Taylor, Sopinka, and Patterson, with logistical support from Hinch and Patterson. All data analysis was conducted by Taylor and Wilson. Data were interpreted by Taylor, Sopinka, Wilson, Patterson, Cooke and Willmore. All writing was conducted by Taylor. Sopinka, Wilson, Cooke and Willmore provided comments and feedback on the manuscript.

Chapter 3: Egg cortisol is not influenced by maternal oxidative stress and does not alter oxidative stress in developing sockeye salmon (*Oncorhynchus nerka*). Jessica J. Taylor, Natalie M. Sopinka, Samantha M. Wilson, Scott G. Hinch, David A. Patterson, Steven J. Cooke, William G. Willmore

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Glossary

24HPF: 24 hours post fertilization

AIC: Corrected Akaike information

ANOVA: Analysis of Variance

AUC: Area under the curve

CBP: Corticosteroid-binding protein

DFO: Department of Fisheries and Oceans

DTNB: 5,5'-dithiobis-(2-nitrobenzoic) acid

EDTA: Ethylenediaminetetraacetic acid

EIA: Enzyme immunoassay

EMG: Emergence

EYE: Eyed

GC: Glucocorticoid

GR: Glutathione reductase

GSH: Glutathione

GSI: Gonadosomatic index

GSSG: Glutathione disulfide

HA: Hatch

HPA: Hypothalmic-pituitary-adrenal

ID: Identity

NaCl: Sodium chloride

NADPH: Nicotinamide adenine dinucleotide 2'-phosphate

NP-40: Nonyl phenoxypolyethoxylethanol

NSERC: Natural Sciences and Engineering Research Council

ORAC: Oxygen radical absorbance capacity

PF: Pre-fertilization

PUFA: Polyunsaturated fatty acids

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SE: Standard error

SEM: Standard error of mean

TGSH: Total glutathione

Tris HCl: Tris hydrochloride

Chapter 1: General Introduction

Intergenerational Effects of Stress

Understanding environmentally-induced parental effects is fundamental to gaining an ecological and evolutionary perspective on the dynamics of populations across a wide range of taxa (Gagliano and McCormick, 2007). Fox and Mousseau (1998) speculated that adults may be able to "prime" their offspring to better handle future environments based on their own life history. The theory of adaptive intergenerational plasticity suggests that females can influence phenotypic differences in offspring traits to reflect the environmental conditions that would be met by the offspring, thereby increasing the chance of offspring survival and the female's reproductive fitness (Green, 2008). Intergenerational effects, defined here as effects initiated by an environmental stimulus in the parental generation and not exclusively attributed to parent's chromosomal contribution (Bernardo, 1996; Lacey, 1998), have been reported across a wide range of taxa including insects (Mousseau and Dingle, 1991), plants (Roach and Wulff, 1987; Donohue and Schmitt, 1998;), birds (Surai et al., 2001), and fish (Heath and Blouw, 1998). Several examples of the effect of stress on epigenetic modifications exist in human offspring including those that were exposed to prenatal intimate partner violence (Radtke et al., 2011), or cigarette smoke (Breton et al., 2009). However, the intergenerational effects of parental stress on offspring physiology, behavior and fitness have not been well characterized.

The vertebrate stress response (defined hereafter as "the biological response elicited when an individual perceives a threat to its homeostasis"; Moberg and Mench,

2000) involves a suite of physiological, hormonal and behavioural responses, one of which is the release of glucocorticoids (GCs), including cortisol (Romero, 2004), through the activation of the hypothalamic-pituitary-adrenal (HPA) axis (which is the HP-interrenal axis in fish; Sumpter, 1997). Across taxa, the release of these steroid hormones has been shown to negatively effect an individual's fitness by decreasing levels of circulating reproductive hormones, reduce number and size of gametes, and impair gonadal development (Marchlewska-Koj, 1997; Pankhurst and Van Der Kraak, 1997; Schreck et al., 2001; Wingfield and Sapolsky, 2003; Breuner, 2010). To provide a more comprehensive assessment of offspring health, methods have been developed to directly study how stress can alter functionally and ecologically relevant offspring traits (e.g. cortisol baths, Sloman, 2010; dietary cortisol administration, Lin et al., 2004). Recent studies have shown negative intergenerational effects of cortisol in a range of taxa including Japanese quail (*Coturnix coturnix japonica*), whose offspring experienced slower growth after maternal cortisol implantation (Hayward and Wingfield, 2004). Similarly, impairments were observed in rats (*Rattus norvegicus*), having lower birth weight after *in utero* exposure to glucocorticoids (Drake et al., 2005), and in tropical damselfish (*Pomacentrus amboinensis*) after abdominal injection of cortisol, whose offspring had decreased body length and smaller yolk sacs (McCormick, 1998). Importantly stress has also been shown to cause oxidative stress in adults (Liu et al., 1996; Welker and Congleton, 2004) and also in the offspring of mothers exposed to cortisol (Lin et al., 2004).

Oxidative Stress

When oxygen began to appear in the earth's atmosphere, organisms evolved aerobic metabolism. The byproducts of aerobic metabolism include reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can be toxic to organisms due to an unpaired electron in their molecular or atomic orbitals (Halliwell and Gutteridge, 1999). The evolution of a mechanism to resist oxygen's toxicity was then necessary and there is now a large body of evidence showing that not only have organisms adapted to these molecules, but have also developed mechanisms to use them to their advantage (Halliwell, 1999; Dröge, 2002; Valko et al., 2007). Beneficial effects of these free radicals at low/moderate levels include regulation of vascular tone, secondary messangers in signal transduction, and defense against infectious agents (Dröge, 2002; Valko et al., 2007). To balance the positive and negative effects of the free radicals exposure, organisms developed a series of defense mechanisms including preventative and repair mechanisms, physical defenses and antioxidant defenses (Cadenas, 1997; Valko et al., 2007). The antioxidant defense system is made up of specialized enzymes such as superoxide dismutases, glutathione peroxidase, and catalase, as well as non-enzymatic antioxidants including ascorbic acid (Vitamin C), tocopherol (Vitamin E), carotenoids, flavonoids, and glutathione (Halliwell 1999; Valko et al., 2007).

G-glutamylcysteinylglycine, or glutathione (GSH), is a tripeptide present in all cells at millimolar concentrations and is the major non-enzymatic redox buffer of the cell. Glutathione acts a protective molecule against oxidative stress, as it assists in amino acid transport across the plasma membrane and is able to regenerate key antioxidants, such as vitamins C and E (Masella et al., 2005). It is also a cofactor of several detoxifying

enzymes such as the glutathione peroxidases and glutathione S-transferases during the detoxification of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds (Masella et al., 2005). During this detoxification, GSH becomes oxidized at its active thiol and glutathione disulfide (GSSG) is formed and accumulates in cells (Pompella et al., 2003; Masella et al., 2005). Through the action of glutathione reductase, in a nicotinamide adenine dinucleotide 2'-phosphate (NADPH) dependent reaction, GSSG can be reduced back to GSH. For this reason, GSH is essential but also requires other molecules and enzymes to be a sufficient mechanism of defense against ROS (Masella et al., 2005). The ratio of reduced to oxidized glutathione (GSH/GSSG) has therefore become a common measure to evaluate oxidative stress *in vivo* (Jones et al., 2000).

In fish, factors including physiological stress, exposure to xenobiotics, and disease can make individuals more susceptible to the harmful effects of oxidative stress (Welker and Congleton, 2005). Also, the biochemical makeup of fish tissues predisposes individuals to the effects of unquenched ROS (Roald et al., 1981; Welker and Congleton, 2004). Fish tissues often contain high levels of polyunsaturated fatty acids (PUFAs) which, based on their high number of double bonds are easy targets for free radical oxidation (Pedrajas et al., 1995; Welker and Congleton, 2004). From an ecological-evolutionary perspective, little is known about the intergenerational effects of oxidative stress across taxa (Pamplona and Costantini, 2011), especially in the case of fish.

Pacific Salmon

Pacific salmon (*Oncorhynchus* spp.) migrate throughout their life span, but due to their anadromous nature, endure the most extensive migration as adults, swimming from

the ocean to spawn in their natal freshwater stream (Dingle, 1996; Hinch et al., 2006). In some cases, this migration can be over 1,000 km in distance and 1 km in elevation, which amounts to a physiologically challenging journey for returning salmon (Crossin et al., 2004; Hinch et al., 2006). Pacific salmon are also semelparous, spawning only once in their lifetime immediately before their death at the spawning grounds (Groot and Margolis, 1991; Dingle, 1996). Salmon must distribute their finite resources (such as time and energy) in order to maximize the fitness benefits of growth, maintenance and reproduction (Fleming and Gross, 1989). For example, females must face trade-offs having to allocate a fixed amount of energy to egg production, osmoregulatory transition from seawater to freshwater, migration to and female breeding competition on spawning grounds (Fleming and Gross, 1989).

Although the evolutionary basis of migration is to gain a survival advantage by residing in a particular location at a given stage of an individual's lifecycle, the benefits are never without their costs (Dingle, 1996; Northcote, 1997). Indeed, Crossin et al. (2004) found an energetically driven reproductive trade-off in migrating female Fraser River sockeye salmon (*O. nerka*) as ovarian mass, egg mass, and egg number diminished with an increased migration difficulty. A recent study by Wilson et al. (2014) compared oxidative stress in Fraser River pink salmon (*O. gorbuscha*) at the river entrance and the spawning grounds. Results showed that oxidative stress was experienced on a tissue-specific basis with plasma and heart tissue showing signs of higher oxidative stress and brain tissue having lower oxidative stress when sampled at the spawning ground versus the river entrance. The following examples highlight key oxidative stress-related costs of migration imposed on adult sockeye salmon:

i. With the return to freshwater comes a fasting period for adult sockeye salmon, which do not feed for this final phase of life (Groot and Margolis, 1991; Quinn, 2005). With this lack of food intake, dietary-derived antioxidants are no longer available to the salmon to counter oxidative stress that is occurring (Welker and Congleton, 2005). Studies have shown that oxidative stress and oxidative lipid damage in fish are increased during food restriction and fasting despite their antioxidant defense systems being activated (Pascual et al., 2003; Morales et al., 2004) .

ii. The increase in muscular activity that accompanies migration (i.e., swimming) and reproduction (i.e., aggression) can also affect oxidative stress by increasing the rate of ROS production (Monaghan et al., 2009). Increased electron flow through the electron transport chain, and thus increased superoxide production from mitochondrial sources, results from an increase in physical activity, which can lead to measurable oxidative damage (Leeuwenburgh and Heinecke, 2001).

iii. Several studies have also shown that an increase in environmental temperature (as is experienced upon river entry) can induce oxidative stress in fish (Parihar and Dubey, 1995; Heise et al., 2006; Lushchak and Bagnyukova, 2006). An increase in temperature increases metabolic processes in ectotherms such as fish, causing an increase in oxygen consumption and the production of ROS (Lushchak, 2011).

iv. Harman (1956) suggested that damage by free radicals might be partly responsible for the process of aging. This along with the fact that telomeres, which are critical for genome stability, are vulnerable to attack from ROS resulting in accelerated reduction of telomere length (Richter and Zglinicki, 2007)

may possibly relate the en route mortality observed in sockeye salmon (Jeffries et al., 2012) to an acceleration in the natural senescence process.

Research Objectives

The general objective of this thesis was to study the intergenerational effects of stress, with a focus on oxidative stress, on sockeye salmon migrating in the Fraser River watershed (British Columbia, Canada). The objectives of Chapter 2 were to examine the effects of population origin on maternal adults and developing offspring, to determine effects on antioxidant capacity and oxidative stress, and to elucidate intergenerational relationships among populations of sockeye salmon with varying migration effort (e.g., distance). This was accomplished by measuring antioxidant capacity and oxidative stress in maternal tissues and developing offspring from 3 geographically distinct populations of Fraser River sockeye salmon. I expected that females completing more arduous migrations would have higher levels of oxidative stress and reduced antioxidant capacity, as longer flights have been shown to cause oxidative stress in birds (Costantini et al., 2008). A recent study on pink salmon revealed that salmon do experience oxidative stress during spawning migration, although on a tissue specific basis (Wilson et al., 2014). I also expected that levels of oxidative stress and antioxidant capacity in maternal tissues would be paralleled in offspring, as Surai et al. (2001) showed that in four species of birds, females influenced the future susceptibility of their offspring to oxidative stress based on the investment of antioxidants into the egg or milk. In Chapter 3 of my thesis, I studied the effect of maternal oxidative stress on egg cortisol deposition, as well as the relationship between oxidative stress in egg cortisol levels (both natural and experimental) and developing offspring. I artificially elevated egg cortisol using a

1000ng/mL cortisol bath to simulate an increase in maternal circulating cortisol due to stress. I expected that maternal oxidative stress in tissues would reflect the amount cortisol deposited into the eggs due to the effect of oxidative stress on key biological molecules (e.g., proteins, DNA, lipids; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010; Pamplona and Costantini, 2011) deposited into eggs during oogenesis. I also expected that levels of cortisol in PF eggs would correlate with levels of oxidative stress in offspring, and that this response would be amplified when cortisol levels were artificially elevated, as this was observed after glucocorticoid exposure in birds (Haussmann et al., 2012). Chapter 4 will integrate the findings of both Chapters 2 and 3 and will present future applications and directions.

Chapter 2: Intergenerational and population-specific effects of oxidative stress in sockeye salmon *(Oncorhynchus nerka)*

Abstract

Examples of intergenerational effects on offspring have been reported in a wide range of taxa including insects, plants, birds, and fish. However, little is known about the potential intergenerational effects of oxidative stress on offspring, and whether these effects vary intraspecifically. Oxidative stress occurs in living organisms when the products of aerobic metabolism (i.e., reactive oxygen species) remain unquenched by antioxidant defence systems and become detrimental to cells. In fishes, it is unknown how maternal oxidative stress and antioxidant capacity influence offspring quality, and whether these maternal traits and effects vary among wild populations. The semelparous, migratory life history of Pacific salmon (*Oncorhynchus spp.*) requires a delicate balance of resource allocation and thus provides a unique opportunity to explore intergenerational and intraspecific effects of oxidative stress. The objectives of this study were to examine the effects of population origin on maternal and developing offspring antioxidant capacity and oxidative stress, and elucidate intergenerational relationships among populations of sockeye salmon (*O. nerka*) with varying migration effort (e.g., distance). For 3 geographically distinct populations of Fraser River sockeye salmon (British Columbia, Canada), antioxidant capacity and oxidative stress were measured in female plasma, heart, brain, and liver, as well as in developing offspring (pre-fertilization, 24 hours post-fertilization, eyed, hatch, and emergence). Maternal and offspring oxidative stress and antioxidant capacity varied among populations but patterns were not consistent across tissue/developmental stage. Furthermore, neither maternal antioxidant

concentration nor oxidative stress had an effect on offspring oxidative health across any of the developmental stages or populations sampled. My results showed that offspring begin to develop their endogenous antioxidant system at varying rates across populations, in preparation for yolk sac absorption. However, by time of emergence antioxidant systems are similar among populations. Overall, although maternal oxidative stress did not translate directly across generations, offspring may be using maternal resources to ensure that their own oxidative stress is at a level that will allow for successful future migrations.

Introduction

Understanding environmentally-induced parental effects is fundamental to gaining an ecological and evolutionary perspective on the dynamics of populations across a wide range of taxa (Gagliano and McCormick, 2007). Fox and Mousseau (1998) speculated that adults may be able to "prime" their offspring to better handle future environments based on their own life history. The theory of adaptive intergenerational plasticity suggests that females can influence phenotypic differences in offspring traits to reflect the environmental conditions that would be met by the offspring, thereby increasing the chance of offspring survival and the female's reproductive fitness (Green, 2008). Intergenerational effects, defined here as effects initiated by an environmental stimulus in the parental generation and not exclusively attributed to parent's chromosomal contribution (Bernardo, 1996; Lacey, 1998), have been reported across a wide range of taxa including insects (Mousseau and Dingle, 1991), plants (Roach and Wulff, 1987; Donohue and Schmitt, 1998), birds (Surai et al., 2001), and fish (Heath and Blouw, 1998). Indeed, maternal environment and stressors such as pollutants (Heinz, 1976), disease (Buechler, 2002), temperature (Eriksen et al., 2008), and nutrition (Taborsky, 2006) have all been shown to have an effect on offspring health. However, little is known about the intergenerational effects of oxidative stress (Pamplona and Costantini, 2011).

Oxidative stress occurs in living organisms when the products of aerobic metabolism, reactive oxygen species (ROS), remain unquenched by the antioxidant defence systems and become detrimental to the cell (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010). Across taxa, factors such as environmental pollutants

(Bagnyukova et al., 2007), increased temperatures (Heise et al., 2006), exercise (Leeuwenburgh and Heinecke, 2001), and physical stressors such as immobilization (Liu et al., 1996) have been shown to cause oxidative stress. In order for the organism to balance the positive and negative effects of the free radical exposure, organisms have developed a series of defence systems including preventative physical and antioxidant defences and repair mechanisms (Cadenas, 1997; Valko et al., 2007). The antioxidant defence system is made up of specialized enzymes such as superoxide dismutases, glutathione peroxidase, and catalase, as well as non-enzymatic antioxidants including ascorbic acid (Vitamin C), tocopherol (Vitamin E), carotenoids, flavonoids, and glutathione (GSH) (Halliwell, 1999; Valko et al., 2007). When antioxidant defence systems are not capable of balancing ROS, key biological molecules such as DNA, proteins and lipids, can become damaged (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010; Pamplona and Costantini, 2011). These molecules are essential components of eggs deposited during oogenesis in oviparous species (e.g., fishes, Brooks et al., 1997). Consequently, the environment a mother experiences can influence not only maternal levels of oxidative stress and antioxidant capacity but also the quality of maternally—derived molecules in eggs and thus, offspring quality.

Fishes now encounter a multitude of environmental, anthropogenic and biological stressors that can increase oxidative stress and divert antioxidants away from developing eggs. Such compromises to reproductive effort could be especially detrimental for semelparous Pacific salmon (*Oncorhynchus spp.*) whose life history is a delicate balance of resource allocation (Fleming and Gross, 1989; Quinn, 2005; Hinch et al., 2006) and whose uptake of dietary-derived antioxidants ceases due to fasting during spawning

migration (Welker and Congleton, 2005). Pacific salmon are anadromous, migrating up to 1000 km in distance and 1 km in elevation from the Pacific Ocean to their natal freshwater river to spawn before dying (Crossin et al., 2004; Hinch et al., 2006). In addition to the natural obstacles and effort required for traversing such great distances, Pacific salmon also face a number of anthropogenic stressors including aquatic chemical pollution, (Bradford et al., 2010), capture by recreational, commercial and aboriginal fisheries (Donaldson et al., 2011), and unpredictable flows, potentially lethal river water temperatures, and temperature-mediated pathogen infections (Cooke et al., 2008; Jeffries et al., 2012). The oxidative stress-related costs of migration can include increased muscular activity during migration and reproduction (Leeuwenburgh and Heinecke, 2001; Monaghan et al., 2009), increased riverine temperatures (Parihar and Dubey, 1995; Heise et al., 2006; Lushchak and Bagnyukova, 2006), dwindling dietary-derived antioxidants due to cessation of feeding (Morales et al., 2004; Pascual et al., 2003), and telomere shortening due to ROS attack (Richter and Zglinicki, 2007). Research investigating oxidative stress processes in Pacific salmon is currently limited. When subjected to low-water stress after starvation, juvenile Chinook salmon (*O. tshawytscha*) experienced higher oxidative stress than control fish (Welker and Congleton, 2004). Wilson et al. (2014) found that oxidative stress was experienced in a tissue-specific manner during migration in pink salmon (*O. gorbuscha*), and that some tissues may be differentially protected to ensure successful spawning. However, the aforementioned studies do not address how oxidative stress varies intraspecifically or in an intergenerational manner.

Given the substantial decline over the past two decades in the productivity (adults produced per spawner) and abundance of adults returning for many of the major Fraser River (British Columbia, Canada) sockeye salmon (*O. nerka*) populations (Peterman et al., 2012), studying how oxidative stress may influence spawning adults and future generations has the potential to provide insight into mortality and cumulative effects of stressors. Importantly, population level differences in productivity and abundance are evident (Martins 2011; Peterman and Dorner, 2011). Identifying which populations (e.g., short distance/low effort versus long distance/high effort migrators) incur the greatest load of oxidative stress (both as adults and offspring) can help identify which sockeye salmon stocks may be at greater risk of the resonating effects of oxidative stress on offspring. Here, I compared oxidative stress, antioxidant capacity and glutathione levels in females (plasma, brain, liver, heart) from three geographically distinct populations [Harrison River (short distance/low elevation), Chilko River and Stellako River (long distance/high elevation)] of sockeye salmon in the Fraser River watershed. Oxidative metrics were also assessed in offspring at five stages of early development to provide insight into the effects of maternal oxidative stress on future generations of sockeye salmon. I anticipated that females completing more arduous migrations (i.e., Chilko River and Stellako River) would have higher levels of oxidative stress and reduced antioxidant capacity, and that these levels would be paralleled in offspring.

Methods

Ethical Note

Research conformed to protocols approved by Animal Care Committees at the University of British Columbia (#A11 0215), Simon Fraser University (#926R-94), and Carleton University (#B10-06) and met the Canadian Council for Animal Care guidelines.

Sample Collection

This study was conducted using three geographically distinct sockeye salmon populations from the Fraser River watershed in British Columbia, Canada. Harrison River (Figure 2-1, Table 2-1), with a migratory effort of 25 (migratory effort = 0.001*Lower Fraser discharge (m^3/s)* migration distance (km), Eliason et al., 2011), was used in this study to represent a short distance. Chilko River and Stellako River (migratory effort 219 and 325, respectively, Figure 2-1) were used to study longer distance migrations of varying efforts. Sockeye salmon were collected at the three sites in the watershed while staging for spawning (Chilko River on September 24, 2012, Stellako River on September 26, 2012, and Harrison River on November 6 and 9, 2012). At the spawning grounds, sockeye salmon were collected by either angling (landed within 30 seconds, Chilko River) or by beach seine (Harrison River, Stellako River), immediately euthanized by cerebral percussion and sampled. Blood was sampled from each of the collected females using caudal venipuncture into heparinized vacutainers, inverted, and centrifuged at 3,200 rpm for 5 minutes (Clay Adams Compact II Centrifuge, Becton-Dickson; Sparks, MD) to separate erythrocytes and plasma. Plasma, along with samples

of liver (Chilko and Stellako River only), heart and brain from the same females were flash frozen in liquid nitrogen and stored at -80°C until analysis. All females and an equal number of males were stripped of their eggs and milt, which were stored on ice in clean, dry, containers until fertilization. Care was taken to avoid contamination of the milt and eggs with water or excreta. A sample of three pre-fertilized (PF) eggs from each female were also flash frozen in liquid nitrogen and stored at -80°C until analysis. When all sampling was complete, morphometrics including weight (to nearest 0.01 kg), fork length (to nearest 0.1 cm) and gonadosomatic index (GSI; gonad weight (to nearest 0.001 g) as a percentage of total body weight (Neat et al. 1998) were measured.

Collected eggs and milt were transported in coolers on ice to Simon Fraser University in Burnaby (British Columbia, Canada) for fertilization. In triplicate, 15 g of eggs from each female were transferred to clean, dry mason jars and 1 mL milt from each male was added to create full sibling crosses (i.e., within a population, each female was crossed with each male once). After milt application, 30 mL of water was added and swirled gently to activate milt and jars were topped off with an additional 400 mL of water. After incubation (one hour for Harrison River, and overnight for Chilko River and Stellako River), separated by population and family ID, fertilized eggs were transferred to flow through baskets and distributed in hatchery stacks with circulating de-chlorinated municipal water at ambient temperature (4.8 - 10.7°C). Wet and dry egg weight was determined for each female. Three replicates of ten eggs were weighed wet and after drying for 24 hours in an oven (65°C) to the nearest 0.0001 g. Baskets were checked daily and dead embryos removed.

At 24 hours post-fertilization (24HPF) a sample of eggs from each cross were flash frozen in liquid nitrogen and stored at -80°C until analysis. This same sampling procedure was also performed at eyed stage (EYE), hatch (HA), and emergence (EMG, full yolk sac absorption) for all crosses from each of the three populations.

Antioxidant Capacity

All samples were ground over liquid nitrogen and homogenized on ice in 1:5 lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 1 % NP-40, 10 % glycerol, 2 mM EDTA) using a handheld Tissue Master 125 (Omni International, Kennesaw, GA). Sample lysate was centrifuged at 13,000 rpm for 5 minutes at 4°C in a Hermle Labnet Z216MK (Mandel, Guelph, ON) and supernatant was stored at -80°C until the Oxygen Radical Absorbance Capacity (ORAC) assay as described in Lucas-Abellan et al. (2008) and Wilson et al. (2012) was performed. The ORAC analyses were completed using a Fluostar Optima microplate reader (BMG Labtech; Offenburg, Germany) and black 96-Well Costar microplates. Fluorescence was measured with an excitation wavelength of 485 nm and emission of 520 nm. Fluorescence data were analyzed using Optima software (9.15.31, Optima Technology Corporation, Rio Rico, Arizona).

Each reaction well contained 20 µL of either sample, blank (75 mM potassium phosphate (pH 7.4)), standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 0-400 µM), or a strong antioxidant positive control (Rutin; 200 µM), and 3.82 µM fluorescein in 75 mM potassium phosphate (pH 7.4). The plate was incubated at 37°C for 30 min before rapidly adding the free radical generator 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) to a final concentration of 79.83 mM. The

plate was placed immediately in the microplate reader and the fluorescence was read every 35 seconds for 60 minutes. The area under the fluorescence decay curve (AUC) was determined for the samples and Trolox standards to determine the Trolox equivalency, commonly used as a benchmark for antioxidant capacity. Total protein of samples was determined using the Bradford assay (Bradford 1976) and final values were reported in Trolox equivalents/mg total protein.

Glutathione

All samples were ground over liquid nitrogen and homogenized on ice in 1:5 5% sulfosalicylic acid solution (previously bubbled with N_2) using a handheld Tissue Master 125 (Omni International, Kennesaw, GA). Sample lysate was centrifuged at 13,000 rpm for 5 minutes at 4°C in a Hermle Labnet Z216MK (Mandel, Guelph, ON) and supernatant was used to assess total glutathione (TGSH) and oxidized glutathione (GSSG) [TGSH = GSH + 2GSSG]. Glutathione assays were performed using a SpectraMax 340PC microplate reader with SoftMax Pro 4.8 data analysis software (Molecular Devices, Sunnyvale, CA) and clear 96-Well Costar microplates. Glutathione assay was performed as previously described in Hermes-Lima and Storey (1996) by following the rate of reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) by GSH at 412nm compared to a standard curve of GSH.

For the measurement of TGSH, the reaction media contained 10uL of sample, 0.5 U/mL glutathione reductase (GR), 100mM potassium phosphate buffer (pH 7.0), 0.25 mM nicotinamide adenine dinucleotide 2'-phosphate (NADPH), and 0.60 mM DTNB and reduction was read for 20 minutes and compared to a GSH standard curve (0-20 μM).

To quantify only GSSG, 50 μL of the initial supernatant and the GSSG standards (0-10 μM) were treated with 44.7 mM 2-vinylpyridine and 227.27 mM KPi in a total volume of 110 μL and allowed to incubate at room temperature for 90 minutes to derivatize the GSH. Once complete, the GSSG was measured in the same manner as TGSH using the methods described above. GSH values were calculated using the equation described above. Final values were reported in GSH/g wet weight and GSH/GSSG.

Statistical Analysis

Statistical analyses were completed using RStudio (v. 0.98.501). Pearson's correlations were used to examine the antioxidant concentration and oxidative stress association between four maternal tissues and offspring at five developmental stages across three populations. To control for Type 1 errors, false discovery rate was calculated and significant p-values for antioxidant concentration and oxidative stress correlations were taken to be 0.011.

Mixed effects models were used to determine how maternal antioxidant concentration and oxidative stress were influenced by population, egg wet weight and GSI as predictor variables. An interaction term was included for egg weight and GSI. Additionally, fish ID and fish ID nested within population were tested as random variables. The best model for each maternal tissue was chosen with a stepwise procedure based on minimizing AICs. Tukey *post hoc* tests with planned contrasts were used to examine the differences between populations, within maternal tissues.

Two-way ANOVAs were run to test the effects of both population origin and offspring developmental stage on antioxidant concentration, oxidative stress and reduced

glutathione concentration. To further investigate the differences between stages and populations, Tukey *post hoc* tests were run. To control for Type 1 errors, false discovery rate was calculated and significant p-values for differences between populations and between offspring life stages are 0.011 and 0.010, respectively. Although data were taken from the same cohort through time, individuals were not sampled more than once so data were considered to be independent and thus I did not use repeated measures analyses.

Results

Antioxidant concentration, as measured by the ORAC assay, oxidative stress, as measured by the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG), and reduced glutathione for all maternal and offspring tissues across the three populations are presented in Table 2-2.

Does maternal oxidative stress and antioxidant capacity vary with migration effort?

For all maternal tissues, both antioxidant concentration and oxidative stress were not influenced by GSI or egg weight, however population was a significant predictor variable in the full factorial mixed effect model (ΔAIC=0). Tukey *post hoc* tests between populations showed significant differences in antioxidant concentration and oxidative stress for maternal brain and liver tissue only. Antioxidant concentration was significantly higher in brain tissue of Stellako River females compared to Chilko River ($z = 6.57$, $p < 0.01$) females, but no differences were detected between Chilko River and Harrison River ($z = 1.83$, $p = 0.793$) or Stellako River and Harrison River ($z = 3.41$, $p = 0.030$). There was also significantly higher liver antioxidant concentration in Stellako River females compared to Chilko River females ($z = 3.81$, $p < 0.01$). No differences in

liver antioxidant concentration were detected between Chilko River and Harrison River females ($z = 0.39$, $p = 1.000$) or Stellako River and Harrison River females ($z = 2.65$, $p = 0.244$). Oxidative stress was highest in brain tissue of Harrison River females, which was higher than levels in Stellako ($z = -4.49$, $p < 0.001$), but not Chilko River females ($z = 2.09$, $p < 0.001$). There were no differences in brain oxidative stress between Chilko River and Stellako River females ($z = -2.93$, $p = 0.110$). Liver oxidative stress was higher in Chilko River females compared to Stellako River females ($z = -5.33$, $p < 0.001$).

Does offspring oxidative stress and antioxidant capacity vary with developmental stage and maternal migration effort?

There was a significant interaction between population and offspring life stage for offspring antioxidant concentration ($F_{8,166} = 18.853$, $p < 0.001$), indicating inconsistent effects of population across developmental stages. For all life stages except EYE, Tukey *post hoc* tests revealed population differences in antioxidant concentration ($p < 0.011$; Figure 2-2A, Table 2-5). Antioxidant concentration of Harrison River offspring was higher than Chilko River offspring at HA and EMG, and higher than Stellako River offspring at HA (Table 2-5). Antioxidant concentration was lower in Chilko River offspring compared to Stellako River offspring at PF, 24HPF, and EMG (Figure 2-2A, Table 2-5). Within each population, differences in antioxidant concentration were observed among developmental stages ($p < 0.010$, Figure 2-2A, Table 2-6). Within a population, Harrison River HA had higher antioxidant concentration than PF, 24HPF, EYE, and EMG, Chilko River EMG had lower antioxidant concentration than EYE, and Stellako River HA had lower antioxidant concentration than PF, 24HPF, and EMG (Figure 2-2A, Table 2-6).

Similarly, a significant interaction effect was detected between population and development stage on concentration of reduced glutathione ($F_{8,165} = 9.349$, $p < 0.001$). Tukey *post hoc* tests revealed that at PF, 24HPF, EYE and EMG there were no significant differences in concentration of reduced glutathione among populations ($p < 0.011$; Figure 2-2B, Table 2-5). Only at HA were there population-level differences in offspring reduced glutathione; Chilko River and Harrison River offspring levels were higher than those measured in Stellako River offspring, but did not differ from each other (Figure 2-2B, Table 2-5). Tukey *post hoc* tests also revealed differences in concentrations of reduced glutathione among developmental stages, within a population ($p < 0.010$, Figure 2-2B, Table 2-6). For the Harrison and Chilko River populations, reduced glutathione was higher at HA than at PF, 24HPF and EYE, with concentrations highest at EMG compared to all other stages. Offspring from Stellako River had higher concentrations of reduced glutathione at HA than at PF and 24HPF, and the highest reduced glutathione concentrations at EMG compared to all other earlier life stages (Figure 2-2B, Table 2-6).

A significant interaction effect was also detected between population and offspring stage on oxidative stress ($F_{8,163} = 15.871$, $p < 0.001$). Tukey *post hoc* tests revealed that at PF there were no population-level differences in oxidative stress ($p < 0.011$; Figure 2-2C, Table 2-5). However, at other developmental stages differences were apparent. Harrison River offspring had higher oxidative stress than offspring from Chilko River at EYE and EMG, and offspring from Stellako River at 24HPF, EYE, HA, and EMG. Offspring from Chilko River had higher oxidative stress than offspring from Stellako River at 24HPF, HA, EMG (Figure 2-2C, Table 2-5). Differences in oxidative

stress among developmental stages were also observed ($p < 0.010$, Figure 2C, Table 2-6). Within the Harrison River population, PF oxidative stress was lower than all other stages and EMG oxidative stress was higher than all other stages (Figure 2-2C, Table 2-6). In the Chilko River population, oxidative stress in offspring at HA was higher than oxidative stress at PF, 24HPF, and EMG, and at EMG oxidative stress was higher than PF and EYE. Stellako River offspring at EYE had higher oxidative stress than levels measured at 24HPF and HA (Figure 2-2C, Table 2-6).

Does maternal oxidative stress and antioxidant capacity influence offspring oxidative health?

Despite evidence for population differences in maternal and offspring oxidative stress and antioxidant capacity, there were few significant correlations between maternal and offspring tissues. Antioxidant concentration in heart tissues sampled from Chilko River females positively correlated with offspring antioxidant capacity at emergence ($r = 0.78$, $p = 0.001$) (Table 2-3). Maternal liver tissue sampled from Stellako River females positively correlated offspring oxidative stress at 24HPF ($r = 0.70$, $p = 0.008$; Table 2-4).

Discussion

The semelparous life history of sockeye salmon is a delicate balance of resource allocation, the importance of which is highlighted during their final migration to natal freshwater rivers to spawn (Fleming and Gross, 1989; Quinn, 2005; Hinch et al., 2006). Contrary to my predictions, population origin had no clear effect on maternal oxidative stress nor antioxidant concentration, though some evidence emerged for tissue protection in Stellako River females that migrate the furthest of the populations studied. Offspring

oxidative stress and antioxidant capacity was affected in a population-dependent manner, however these differences were not related to maternal oxidative stress nor antioxidant concentration.

Linear mixed effects models of maternal tissues showed only population as a predictor for both antioxidant concentration and oxidative stress in brain and liver. Our results show that brain tissue from Stellako River females had higher antioxidant concentration and experienced lower oxidative stress than females from Chilko River and Harrison River, which are populations with shorter migration distances. Similar results have been shown in pink salmon sampled at both the Strait of Georgia (prior to fasting and freshwater entrance) and at Weaver Creek spawning grounds to compare oxidative stress pre- and post-spawning migration (Wilson et al., 2014). Wilson et al. (2014) found that brain tissue had both higher antioxidant concentration and lower DNA damage at the spawning ground than prior to their migration suggesting that brain may be preferentially protected over other tissues to ensure migratory success. My results support this theory of preferential tissue protection and suggest that populations with longer distance migrations have adapted to protect the brain from oxidative stress to ensure successful arrival at the spawning grounds despite the increased challenge. My results showed that liver might also be preferentially protected in populations making longer distance migrations, as antioxidant concentration was higher and oxidative stress lower in Stellako River compared to Chilko River. Wilson et al. 2014 also assayed liver tissue but found no difference between pre- and post- migration for oxidative stress, however the spawning migration studied in this case was only ~100 km and may not be long enough to observe the changes I observed in the longer distance migrations.

Once sockeye salmon fry complete yolk-sac absorption and emerge from the gravel redds, they must migrate to rearing areas (Quinn, 2005). The direction and difficulty of this migration varies between populations of sockeye salmon and therefore fry require population-specific migration strategies (Table 2-1; Sopinka et al., 2013). Specifically, Harrison River fry must migrate downstream to the Lower Fraser River to rear in estuaries, while Chilko River fry migrate upstream to Chilko Lake, and Stellako River fry migrate downstream to rear in Fraser Lake (Sopinka et al., 2013). While an upstream migration is taxing on the fry due to increased activity and risk of predation (Brannon, 1973), rearing in a riverine environment may also be very challenging making the migration to the rearing area for the Harrison River fry arguably the most difficult of the three populations studied (Birtwell et al., 1987). Significant differences between populations at most offspring developmental stages are observed. In general Harrison had the highest GSH/GSSG ratio indicative of the least amount of oxidative stress experienced, followed by Chilko River and finally Stellako River with the highest amount of oxidative stress. It is possible that in preparation for the upcoming migration, offspring have adapted to ensure their oxidative stress is at a level that will allow for the most successful migration and future life stages. This type of preparatory adaption has been seen in other migrant species including long distance migratory birds, the semipalmated sandpipers (*Calidris pusilla*), which prepare for their migration by priming their flight muscles by eating a diet rich in specific lipids as well as modifying dietary acids before storing them as fuel (Maillet and Weber, 2006). Although these types of adaptions prepare animals for their future migrations, it is a complex integration of multiple adjustments in morphology, biomechanics, behaviour, nutrition and metabolism

that ensure that individuals are set up for successful migrations (Weber, 2009). Although many factors will likely contribute to the health of the offspring as they prepare for their future migrations, my results suggest that oxidative stress may play a role in this preparation.

It has been suggested in previous studies that during the early stages of embryogenic and larval development in fish, oxidant stress is high due to metamorphosis and rapid growth rates (Rudneva, 1999; Ciarcia et al., 2000; Kalaimani et al., 2008). During this period, antioxidant enzymes have been typically observed to increase, while a decrease is seen in low molecular weight anti-oxidants such as vitamins C and E, and GSH (Cowey et al., 1985; Kalaimani et al., 2008). GSH levels measured across development in this study remained low in all three populations until EYE stage, with significant increases seen at HA and continuing to EMG. These results coincide with those seen by Cowey et al. (1985) in Atlantic salmon (*Salmo salar*), who suggested that these temporal increases indicate that an efficient system for removal of peroxidase is established soon after hatch. In Asian Seabass (*Lates calcarifer*) a decrease in GSH was observed between 3 days post hatch (dph) and 20dph (corresponding to yolk sac absorption and metamorphosis, respectively), however a drastic increase in GSH was observed at 25dph (Kalaimani et al., 2008). This increase was synchronous with increases in catalase and glutathione peroxidase activity, which are involved in the oxidation of GSH, suggesting that secondary enzymes are active at this stage of development (Kalaimani et al., 2008). My study indicates that glutathione concentration increases drastically at hatch, which may be attributed to the development of the larvae's endogenous antioxidant defence system. This is supported by the fact that antioxidant

concentrations remain relatively consistent across stages, suggesting that in preparation for yolk sac absorption and the loss of maternal stores, larvae must compensate by increasing their endogenous antioxidants.

In both antioxidant concentrations and reduced glutathione, differences between populations were most apparent at the HA stage throughout development. Specifically, Harrison had significantly higher antioxidant concentration than both Chilko and Stellako interior populations at hatch. It was shown by Beacham and Murray (1989) that interior sockeye salmon spawners were more efficient at converting yolk to body tissue and had proportionally longer and heavier alevin than coastal spawners relative to egg size. Based on this finding, it could be speculated that the increase in antioxidant concentration in Harrison offspring could be related to the proportionally smaller alevin size and not to an increase in maternal antioxidant deposition. The fact that population differences are not as pronounced in earlier developmental stages, despite different egg weights suggests that mothers may deposit similar amount of antioxidants into the eggs regardless of population. These antioxidants therefore become more dilute in larger alevin from interior spawners from Chilko and Stellako.

The amounts of reduced glutathione in offspring also revealed significant differences between populations at HA. As stated above, this study suggests that at HA, the antioxidant defence system of the alevin begins to develop as its maternal resources become depleted. A previous study showed that incubation requirements varied by population and that populations which experienced cooler water temperatures developed faster than those that experienced warmer water temperatures (Whitney, 2012). This

finding agrees with a study using rainbow trout (*O. mykiss*), in which populations that adapted to cooler water temperatures in early life had rapid development rates (Miller et al., 2012). Mean spawning temperatures for the three populations in the present study have been reported to be 8.50°C for Harrison, 9.95°C for Chilko, and 10.95°C for Stellako (Whitney et al., 2013). The increased reduced glutathione, reflective of an increase in endogenous glutathione stores, observed at HA in both cooler temperature populations Harrison and Chilko, suggests that their antioxidant defence systems experience the same increase in development rate as observed by Whitney (2012). Stellako, the population that experiences the warmest mean spawn temperature, had the lowest amount of endogenous glutathione at HA suggesting that their antioxidant defence system may develop at a slower rate. These population differences are all overcome by EMG suggesting that regardless of degree of development at HA, antioxidant defence system efficiency is no longer population-dependent by the time the fry emerge.

In this study, neither antioxidant concentration nor oxidative stress observed in maternal tissues greatly influenced oxidative stress in offspring at the developmental stages that were examined. This result was consistent across the three populations of varying migration difficulties, with the exception of a correlation observed between maternal heart tissue and emergent offspring antioxidant concentration in the Chilko River population, and between maternal liver tissue and 24HPF offspring oxidative stress in the Stellako River population. However, two results do not strongly infer the presence of intergenerational effects of oxidative stress. A lack of correlation between maternal and offspring oxidative health suggests that mothers may buffer offspring from oxidative stress. Such a strategy may be driven by the semelparous nature of Pacific salmon and the

importance of offspring survival on their own fitness. Although unrelated to oxidative stress, a similar strategy is seen in other semelparous species such as *Parastrachia japonensis*, a species of shield bug (Filippi et al., 2001). Females of this species provide progressive provisioning to their brood until the third larval stadium at which time the female dies (Filippi et al., 2001). This reproductive strategy comes with large costs and risks and the female ceases feeding while provisioning her brood to ensure success because the female's entire reproductive effort is expressed in the success of that one brood (Filippi et al., 2001). Although female sockeye salmon must allocate their resources strategically to ensure survival to spawn (Fleming and Gross, 1989), it appears that offspring oxidative health is not negatively affected by the maternal trade-offs that must occur.

Here I have shown that neither maternal antioxidant concentration nor oxidative stress is conferred to offspring and that oxidative stress appears to be related to population at specific life stages. I have provided further support to previous studies that have shown the brain to be protected from oxidative stress during sockeye salmon migration and also the importance of tissue-specific responses to oxidative stress (Wilson et al., 2014). This study also suggests that mothers, who do successfully spawn, ensure that investment into their offspring is not impeded by their own oxidative stress. To assess offspring oxidative stress specific to maternal stress as opposed to oxidative stress, experiments involving gametic cortisol exposure could be performed to simulate maternal deposition of cortisol into eggs (Auperin and Geslin, 2008). My results showed that offspring develop their endogenous antioxidant system as they prepare to absorb their yolk sac at varying rates across populations, however this population-dependent variation

is overcome by emergence. Overall, although maternal oxidative stress does not translate directly across generations, offspring are able to prepare themselves, in the context of oxidative stress, for future migrations based on the contributions of their mother despite stressors experienced during her migration. Based on the population-specific nature of the results in this paper, further investigation into whether these observations in offspring are based on adaptive transgenerational plasticity, genetics or a combination of both could provide insight into the long-term stability of this species in wake of increasing water temperatures (Jeffries et al., 2012), fisheries (Donaldson et al., 2011), and increase in aquatic pollution and bacterial and viral infections (Bradford et al., 2010).

Tables

Table 2-1. Population-specific migration characteristics for the three study populations of *Oncorhynchus nerka*, including adult migration distance, elevation, effort and temperature, a description of where the rearing areas are for fry, the direction and distance fry must migrate to reach these areas from spawning grounds following emergence (Eliason *et al*., 2011; Whitney *et al*., 2013; Sopinka *et* al. 2013).

	Adult				**Offspring**		
Population	**Migration Distance (km)**	**Elevation (m)**	**Mean Spawning Temperature (°C)**	**Migratory Effort (0.0001* discharge* distance)**	**Migration Direction**	**Rearing Area**	**Maximum Distance to Rearing Area (km)**
Harrison River	121	10	8.50	25	Downstream	Lower Fraser River & estuary	100
Chilko River	642	1174	9.95	219	Upstream	Chilko Lake	10
Stellako River	976	664	10.95	328	Downstream	Fraser Lake	10

Table 2-2. ORAC, GSH/GSSH, GSH levels in maternal tissues and offspring life stages in sockeye salmon. Data are means ± SEM, sample sizes n are in parentheses.

Population		**ORAC** (TE/mg protein)	**GSH/GSSH**	**GSH** (nmol/g wet weight)
Harrison				
	Heart	1.05 ± 0.05 (7)	3.20 ± 0.58 (4)	517 ± 49 (7)
	Liver	0.32 ± 0.03 (7)	-	-
	Brain	0.84 ± 0.10 (7)	4.96 ± 1.78 (7)	381 ± 27 (7)
	Plasma	0.73 ± 0.06 (6)	2.08 ± 0.22 (7)	-
	PF	0.62 ± 0.07 (7)	1.50 ± 0.12 (7)	25 ± 2 (7)
	24HPF	0.77 ± 0.06 (7)	4.37 ± 0.69 (7)	18 ± 1 (7)
	EYE	0.52 ± 0.01 (7)	4.69 ± 0.75 (7)	60 ± 4 (7)
	HA	2.05 ± 0.30 (7)	4.53 ± 0.58 (7)	161 ± 11 (7)
	EMG	0.98 ± 0.07 (7)	7.27 ± 0.97 (7)	297 ± 25 (7)
Chilko				
	Heart	0.92 ± 0.09 (15)	2.25 ± 0.32 (12)	521 ± 32 (15)
	Liver	0.26 ± 0.04 (15)	5.52 ± 1.16 (14)	341 ± 58 (15)
	Brain	0.54 ± 0.09 (15)	2.95 ± 0.43 (14)	515 ± 34 (15)
	Plasma	0.98 ± 0.12 (15)	1.19 ± 0.08 (15)	-
	PF	0.61 ± 0.03 (15)	1.61 ± 0.05 (15)	14 ± 1 (15)
	24HPF	0.51 ± 0.04 (14)	3.09 ± 0.23 (14)	22 ± 1 (14)
	EYE	0.82 ± 0.08 (15)	1.49 ± 0.10 (15)	31 ± 3 (15)
	HA	0.60 ± 0.04 (13)	5.49 ± 0.55 (13)	222 ± 19 (13)
	EMG	0.25 ± 0.02 (14)	4.33 ± 0.52 (14)	308 ± 13 (14)
Stellako				
	Heart	0.68 ± 0.04(15)	2.71 ± 0.52(15)	921 ± 111 (15)
	Liver	0.69 ± 0.05(15)	1.41 ± 0.16(15)	1005 ± 133 (15)
	Brain	1.47 ± 0.20(15)	0.69 ± 0.09(15)	414 ± 33 (15)
	Plasma	0.76 ± 0.03(15)	1.67 ± 0.10(15)	-
	PF	1.10 ± 0.11(15)	0.79 ± 0.17(15)	13 ± 2 (15)
	24HPF	0.92 ± 0.05(15)	0.54 ± 0.05(13)	4 ± 1 (13)
	EYE	0.66 ± 0.06(15)	2.50 ± 0.23(15)	48 ± 4 (15)
	HA	0.68 ± 0.08(15)	0.64 ± 0.07(14)	94 ± 12 (14)
	EMG	0.91 ± 0.10(15)	1.81 ± 0.14(15)	288 ± 15 (15)

Table 2-3. Pair-wise Pearson Correlation matrix for maternal tissue and offspring ORAC values (μmol TE/mg protein). Listed are r values, *p* values are shown in parentheses. P-values < 0.011 (false discovery rate) are bolded to show significance.

	Plasma			**Liver**			**Heart**			**Brain**		
	Harrison	**Chilko**	**Stellako**	**Harrison**	**Chilko**	**Stellako**	**Harrison**	**Chilko**	**Stellako**	**Harrison**	**Chilko**	**Stellako**
PF	-0.37 (0.465)	0.30 (0.274)	-0.13 (0.634)	-0.46 (0.303)	0.14 (0.623)	0.52 (0.046)	0.42 (0.342)	-0.13 (0.643)	0.22 (0.437)	-0.42 (0.348)	-0.28 (0.313)	-0.45 (0.096)
24HPF	0.21 (0.694)	-0.41 (0.146)	0.03 (0.918)	-0.29 (0.527)	0.08 (0.776)	0.38 (0.162)	0.08 (0.862)	-0.13 (0.659)	-0.33 (0.226)	-0.39 (0.381)	-0.20 (0.501)	0.07 (0.794)
EYE	-0.17 (0.750)	-0.15 (0.598)	0.02 (0.936)	0.37 (0.410)	0.22 (0.440)	-0.17 (0.549)	-0.18 (0.698)	-0.14 (0.624)	-0.14 (0.616)	-0.05 (0.909)	0.24 (0.385)	0.20 (0.484)
HA	0.85 (0.032)	0.45 (0.127)	-0.27 (0.329)	-0.67 (0.100)	0.19 (0.526)	0.01 (0.990)	0.58 (0.170)	-0.05 (0.882)	0.33 (0.236)	-0.16 (0.726)	0.46 (0.113)	-0.37 (0.174)
EMG	-0.05 (0.928)	0.35 (0.226)	0.30 (0.278)	-0.39 (0.392)	0.51 (0.063)	0.12 (0.675)	0.06 (0.903)	0.78 (**0.001**)	-0.32 (0.242)	-0.63 (0.126)	0.19 (0.521)	-0.01 (0.963)

Table 2-4. Pair-wise Pearson Correlation matrix for maternal tissue and offspring GSH/GSSG ratios. Listed are r values, *p* values are shown in parentheses. P-values < 0.011 (false discovery rate) are bolded to show significance.

	Plasma			Liver			Heart			Brain		
	Harrison	**Chilko**	**Stellako**	**Harrison**	**Chilko**	**Stellako**	**Harrison**	**Chilko**	**Stellako**	**Harrison**	**Chilko**	**Stellako**
PF	-0.50 (0.310)	0.45 (0.094)	0.43 (0.105)		0.03 (0.906)	-0.15 (0.590)	0.88 (0.120)	-0.11 (0.742)	0.04 (0.888)	0.56 (0.192)	0.26 (0.373)	0.02 (0.946)
24HPF	0.15 (0.783)	0.40 (0.159)	-0.43 (0.143)		0.12 (0.697)	0.70 (**0.008**)	0.33 (0.672)	-0.43 (0.182)	-0.06 (0.847)	-0.70 (0.081)	-0.17 (0.579)	0.47 (0.107)
Eye	0.35 (0.491)	-0.13 (0.656)	-0.15 (0.605)		0.04 (0.885)	-0.21 (0.441)	-0.54 (0.460)	0.32 (0.313)	-0.38 (0.160)	-0.35 (0.448)	-0.13 (0.655)	0.26 (0.350)
Hatch	0.15 (0.773)	-0.56 (0.045)	0.43 (0.128)		0.36 (0.243)	0.15 (0.612)	-0.77 (0.226)	0.23 (0.464)	0.36 (0.212)	-0.59 (0.160)	-0.24 (0.449)	0.18 (0.540)
Emergence	-0.08 (0.886)	0.09 (0.750)	0.15 (0.606)		-0.31 (0.300)	0.24 (0.385)	-0.48 (0.525)	-0.09 (**0.777**)	-0.05 (0.870)	-0.30 (**0.511**)	-0.01 (0.977)	0.25 (0.365)

Table 2-5. Summary of Tukey *post hoc* tests for within stage differences for ORAC, GSH/GSSH and reduced GSH. P-values are shown and bolded to show significance ($p < 0.010$, false discovery rate).

		ORAC				GSH/GSSG				GSH			
		24HPF	**EYE**	**HA**	**EMG**	**24HPF**	**EYE**	**HA**	**EMG**	**24HPF**	**EYE**	**HA**	**EMG**
Harrison													
	PF	1.000	1.000	**<0.001**	0.778	**0.001**	**<0.001**	**<0.001**	**<0.001**	1.000	0.872	**<0.001**	**<0.001**
	24HPF	-	0.984	**<0.001**	0.998	-	1.000	1.000	**0.001**	-	0.627	**<0.001**	**<0.001**
	EYE	-	-	**<0.001**	0.376	-	-	1.000	**0.008**	-	-	**<0.001**	**<0.001**
	HA	-	-	-	**<0.001**	-	-	-	**0.003**	-	-	-	**<0.001**
	EMG	-	-	-	-	-	-	-	-	-	-	-	-
Chilko													
	PF	1.000	0.909	1.000	0.210	0.074	1.000	**<0.001**	**<0.001**	1.000	0.995	**<0.001**	**<0.001**
	24HPF	-	0.416	1.000	0.764	-	0.034	**<0.001**	0.298	-	1.000	**<0.001**	**<0.001**
	EYE	-	-	0.917	**0.001**	-	-	**<0.001**	**<0.001**	-	-	**<0.001**	**<0.001**
	HA	-	-	-	0.282	-	-	-	0.453	-	-	-	**<0.001**
	EMG	-	-	-	-	-	-	-	-	-	-	-	-
Stellako													
	PF	1.000	0.148	**0.003**	1.000	1.000	0.013	1.000	0.590	1.000	0.268	**<0.001**	**<0.001**
	24HPF	-	0.182	**0.004**	1.000	-	**0.003**	1.000	0.266	-	0.048	**<0.001**	**<0.001**
	EYE	-	-	0.996	0.108	-	-	**0.005**	0.963	-	-	0.038	**<0.001**
	HA	-	-	-	**0.002**	-	-	-	0.373	-	-	-	**<0.001**
	EMG	-	-	-	-	-	-	-	-	-	-	-	-

Table 2-6. Summary of Tukey *post hoc* tests for within population differences for ORAC, GSH/GSSH and reduced GSH. P-values are shown and bolded to show significance ($p < 0.011$, false discovery rate).

		ORAC		GSH/GSSG		GSH	
		Stellako	**Harrison**	**Stellako**	**Harrison**	**Stellako**	**Harrison**
PF							
	Chilko	**0.009**	1.000	0.870	1.000	1.000	1.000
	Stellako	-	0.138	-	0.994	-	1.000
24HPF							
	Chilko	**0.001**	0.923	**<0.001**	0.595	0.992	1.000
	Stellako	-	0.793	-	**<0.001**	-	1.000
Eye							
	Chilko	1.000	0.801	0.606	**<0.001**	0.986	0.879
	Stellako	-	0.993	-	**0.009**	-	1.000
Hatch							
	Chilko	1.000	**<0.001**	**<0.001**	0.933	**<0.001**	0.022
	Stellako	-	**<0.001**	-	**<0.001**	-	**0.005**
Emergence							
	Chilko	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.975	1.000
	Stellako	-	1.000	-	**<0.001**	-	1.000

Figures

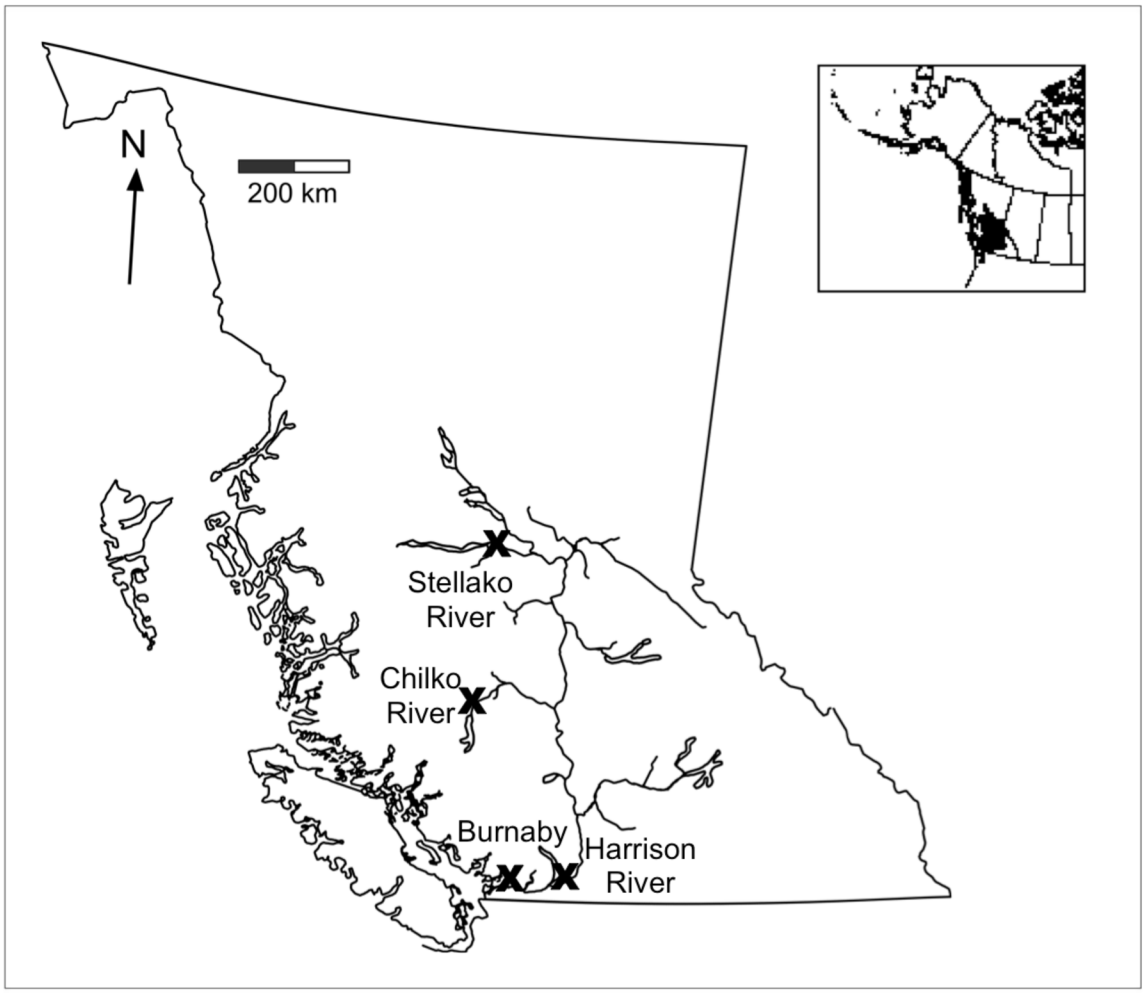

Figure 2-1. Figure 2-1: Map of the Fraser River, Canada, showing Burnaby (49°16′41 N, 122°55′12 W) and the Harrison River (49°17′5 N, 121°54′27 W), Chilko River (51°37′35 N, 124°8′35 W) and Stellako River (54°2'42 N, 124°55'28 W) catch sites, indicated by `X`s.

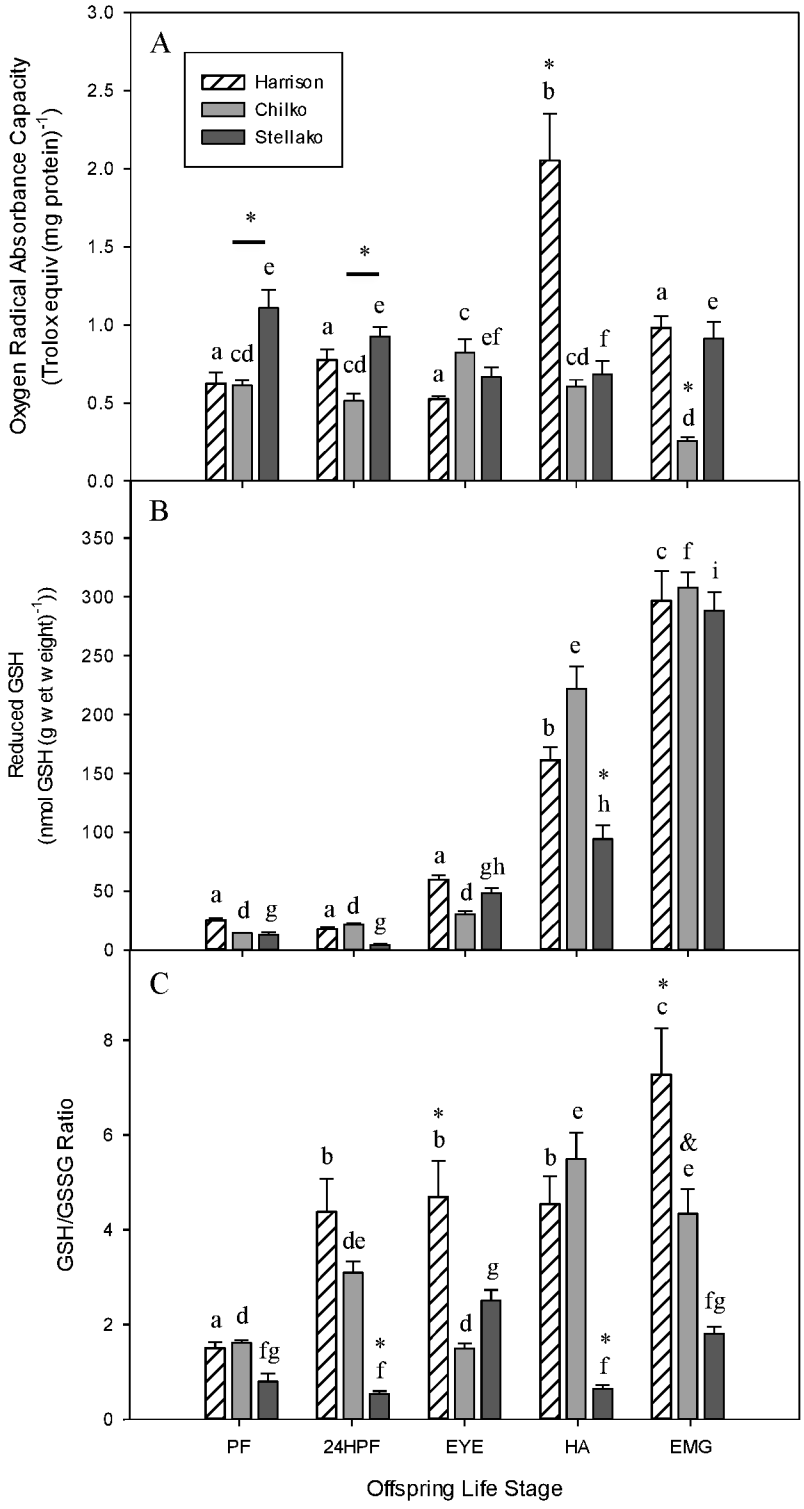
A
B
C
Harrison
Chilko
Stellako
Oxygen Radical Absorbance Capacity
(Trolox equiv (mg protein)-1)
Reduced GSH
(nmol GSH (g wet weight)-1))
GSH/GSSG Ratio
PF
24HPF
EYE
HA
EMG
Offspring Life Stage

Figure 2-2. Levels of antioxidant capacity shown as Trolox equivalents (TE)/mg protein (A), reduced glutathione (nmol/g wet weight) (B), and oxidative stress represented as a ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) (C) for pre-fertilized eggs (PF), eggs at 24 hours post-fertilization (24HPF), eyed embryos (EYE), hatchlings (HA) and emergent fry (EMG, full yolk sac absorption) reared from female sockeye salmon captured at Harrison River (hashed bars), Chilko River (light gray bars), or Stellako River (dark gray bars) spawning sites. Letters represent significant differences between offspring developmental stages ($p<0.010$, false discovery rate) and symbols represent significant differences between populations ($p<0.011$, false discovery rate).

Chapter 3: Egg cortisol is not influenced by maternal oxidative stress and does not alter oxidative stress in developing sockeye salmon (*Oncorhynchus nerka*)

Abstract

A key characteristic of oviparity, as a reproductive strategy, is the critical presence, at the time of oviposition, of all nutrients needed to sustain a viable embryo. Studies have shown the presence of various maternally-derived hormones in oocytes as early as the vitellogenic stage, and these hormones may play crucial roles during early embryo development. Though embryonic glucocorticoid exposure is positively correlated with oxidative stress in other species, the relationship between gametic hormone exposure and oxidative stress has yet to be characterized in fish. The objectives of this study were to examine the relationship between cortisol (the primary glucocorticoid in fishes and produced following activation of the hypothalamic-pituitary-interrenal [HPI] or stress axis), and oxidative stress to provide insight into the potential intergenerational effects of stressors in semelparous sockeye salmon (*Oncorhynchus nerka*) from the Fraser River, Canada. Antioxidant concentration and oxidative stress were measured in maternal tissues (plasma, brain, heart and liver) as well as at various offspring developmental stages (pre-fertilization (PF), 24 hours post-fertilization, eyed, and hatch) and were compared to both natural and experimental (i.e., cortisol egg bath at 1000ng/mL) levels of cortisol in PF eggs. This study revealed that the oxidative absorptive capacity of adult maternal sockeye salmon did not influence the amount of cortisol deposited into eggs during oogenesis. Also, artificially elevating cortisol levels in PF eggs did not further impair oxidative health of the offspring. My results suggest there may be mechanisms in both the mother and in her offspring, of this semelparous species,

to buffer the effects of hypercortisolism on oxidative stress. Although exact mechanisms underlying this observation remain unclear, this potentially represents an important form of maternal investment in offspring.

Introduction

Many reproductive strategies exist for animals. For oviparous organisms, a key characteristic is the critical presence, at the time of oviposition, of all nutrients and hormones needed to sustain a viable embryo (Brooks et al., 1997; Blackburn, 1999; Thompson and Speake, 2002). These necessary hormones and nutrients can include sex/thyroid and corticosteroids, amino acids, lipids, carbohydrates, vitamins, minerals, and metals, all of which must be protected from turnover and degradation until needed by the developing embryos (Brooks et al., 1997; Thompson and Speake, 2002). In many teleost fish, including salmonids, most of these gametic components are taken up by the oocyte during vitellogenesis, a process that involves the secretion of a yolk protein precursor, vitellogenin, by the maternal liver and its sequestration into the oocyte (Tyler et al., 1991; Brooks et al., 1997). This deposition of nutrients and hormones into eggs is critical for early larval development, particularly in fish, which are unable to endogenously produce certain enzymes, hormones, and growth factors until approximately the end of yolk sac absorption (Hwang et al., 1992; Mylonas et al., 1994; Tanaka et al., 1995), and these hormones could play a major role in directing development post-fertilization (Schreck et al., 2001). However, when in excess, maternally-derived gametic factors may prove to be disadvantageous to offspring development.

Studies on teleost fish development often focus on the presence of maternally-derived thyroid hormones (Greenblatt et al., 1989), sex steroids (Feist et al., 1990), and the primary glucocorticoid in fishes, cortisol (Hwang et al., 1992; McCormick, 1998), in either vitellogenic, unfertilized or fertilized eggs. Maternal environment can influence

gametic hormone content. In particular, stressors experienced by the mother can have varying effects on the egg hormone levels depending on severity, longevity and when in the reproductive process the stressor is experienced (Schreck et al., 2001). Stratholt et al. (1997) showed that female coho salmon (*Oncorhynchus kisutch)* that experienced an increase in plasma cortisol due to an applied stressor in the final two weeks of oogenesis, had an increased deposition of cortisol in their oocytes. This intergenerational effect was also observed in tropical damselfish (*Pomacentrus amboinensis*) where levels of cortisol in eggs strongly correlated with levels in maternal ovaries after abdominal injection of cortisol 3-5 days pre-spawn (McCormick, 1998). With the development of egg hormone baths (e.g., Sloman, 2010), and growing exposure of wild fishes to numerous aquatic stressors known to increase cortisol (e.g., fisheries capture, Donaldson et al., 2014), the effects of maternal stress (Sopinka et al., 2014) and egg cortisol (Burton et al., 2011), and interactions between these effects (Giesing et al., 2011) on offspring development has received much attention.

Several studies examining the effect of cortisol on offspring development in fishes have revealed changes in behavioural and physiological metrics including earlier hatch times (Japanese medaka (*Oryzias latipes*); Cloud, 1981), enhanced growth and increased yolk sac absorption (tilapia (*Oreochromis mossambicus*); Mathiyalagan et al., 1996), reduced length (damselfish (*Pomacentrus amboinensis*); McCormick, 1999; Burton, 2011), increased heart rate (damselfish; McCormick, 1999), and increased aggressiveness (brown trout (*Salmo trutta*); Sloman, 2010). It is currently unknown how egg cortisol influences offspring oxidative stress and whether egg hormone levels are mediated by maternal oxidative stress. Oxidative stress occurs in living organisms when

the products of aerobic metabolism, reactive oxygen species (ROS), remain unquenched by the antioxidant defense systems and become detrimental to the cell (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010). Little is known about the intergenerational effects of oxidative stress across taxa (Pamplona and Costantini, 2011), although it has been shown that embryonic glucocorticoid exposure in domestic chickens (*Gallus domesticus*) increased oxidative stress and accelerated telomere loss; both of which are linked to risk factors for disease and increased risk of mortality (Haussmann et al., 2012). However, the relationship between maternal oxidative stress and egg cortisol, and consequently offspring oxidative stress has yet to be established in oviparous species. Maternal oxidative stress and egg cortisol may have significant influences on offspring quality in semelparous Pacific salmon, whose lifetime fitness depends on a single spawning event.

Over the past two decades, sockeye salmon (*O. nerka*) in the Fraser River (British Columbia, Canada) have experienced a substantial decline in abundance of returning adults and productivity (adults produced per spawner) (Peterman et al., 2012). Declines have been associated with various environmental and anthropogenic stressors that reliably elicit cortisol production. For this reason, Fraser River sockeye salmon were chosen to study the relationship between egg cortisol and oxidative stress to provide insight into the potential intergenerational effects of stressors. The objectives of this study were to examine how 1) maternal oxidative stress (measured as a ratio of reduced to oxidized glutathione) and antioxidant concentration in plasma, heart, brain, and liver influence cortisol deposition in eggs and 2) experimentally elevated egg cortisol influences offspring oxidative metrics at 24 hours post fertilization (24HPF), eyed (EYE),

and hatch (HA) stages. I hypothesized that 1) mothers with higher oxidative stress and reduced antioxidant capacity would deposit higher levels of cortisol into their eggs, and 2) eggs exposed to experimentally elevated levels of cortisol would develop into offspring with higher oxidative stress and lower antioxidant capacity.

Methods

Ethical Note

Research conformed to protocols approved by Animal Care Committees at the University of British Columbia (#A11 0215) and Carleton University (#B10-06) and met the Canadian Council for Animal Care guidelines.

Collection

This study was conducted using sockeye salmon collected at the Harrison River spawning grounds on the Fraser River watershed in British Columbia, Canada. Sockeye salmon were sampled on November 6 and 9, 2012 by beach seine net and immediately euthanized by cerebral percussion. Blood was sampled immediately from each of the collected females using caudal venipuncture into heparinized vacutainers, inverted, and centrifuged at 3,200 rpm for 5 minutes (Clay Adams Compact II Centrifuge, Becton-Dickson; Sparks, MD) to separate erythrocytes and plasma. Plasma, along with samples of liver, heart and brain from the same females were flash frozen in liquid nitrogen and stored at -80°C until analysis. All females and an equal number of males were stripped of their eggs and milt, which were stored on ice in clean, dry, containers until fertilization. Care was taken to avoid contamination of the milt and eggs with water or excreta. Samples of three pre-fertilized (PF) eggs from each female were also flash frozen in

liquid nitrogen and stored at -80°C until further analysis. When all sampling was complete, morphometrics including weight (to nearest 0.01 kg), fork length (to nearest 0.1 cm) and gonadosomatic index (GSI; gonad weight (to nearest 0.001 g) as a percentage of total body weight (Neat et al. 1998), were measured.

Collected eggs and milt were transported (~2 hours) in coolers on ice to University of British Columbia in Vancouver, Canada for fertilization. Fifteen grams of eggs were weighed and transferred to clean, dry mason jars and mixed with 1 mL milt from males to create full sibling crosses. After milt application, 30 mL of water was added and swirled gently to activate milt. Jars were topped off with an additional 400 mL of water with either 1000 ng/mL (Auperin & Geslin, 2012) cortisol that was initially dissolved in 95% ethanol (0.002% final ethanol concentration) or 0 ng/mL with the same concentration of ethanol as the cortisol exposed eggs (0.002%). Baseline and stressor-induced plasma cortisol levels can approach and exceed 1000 ng/mL in mature Pacific salmon (Cook et al., 2011; Hruska et al., 2010; McConnachie et al., 2012). After a two-hour incubation, fertilized eggs were rinsed thoroughly with fresh water, transferred to flow-through baskets and distributed in hatchery stacks with circulating de-chlorinated municipal water kept at 8-10°C. Wet and dry egg weight was determined for each female. Three replicates of ten eggs were weighed wet and after drying for 24 hours in an oven (65°C) to the nearest 0.0001 g. Baskets were checked daily and dead embryos removed. Offspring were sampled at 24 HPF, EYE, and HA for each cross and flash frozen in liquid nitrogen and stored at -80°C until analysis.

Egg cortisol concentrations were quantified with an enzyme immunoassay according to the manufacturer's instructions (EIA, Neogen Corporation, http://www.neogen.com; see Sopinka et al. 2014 for further details). Briefly, three PF eggs were homogenized in assay buffer, vortexed with diethyl ether, centrifuged and then flash frozen at -80°C. The liquid phase was poured off, evaporated under nitrogen and reconstituted in 1200 μL of assay buffer. Reconstituted samples were warmed for 10 min at 65°C, and a 250 μL subsample was removed and stored at -80°C for use on the EIA plate. In duplicate, samples were run on two plates with intra- and inter-assay coefficients of variation 4.3 % and 5.9 %, respectively.

Antioxidant Capacity

See Chapter 2.

Glutathione

See Chapter 2.

Statistical Analysis

Statistical analyses were completed using RStudio (v. 0.98.501). Pearson's correlation was used to examine the relationships between PF egg cortisol levels and maternal (plasma, liver, heart, brain) and offspring (24HPF, EYE, HA) antioxidant concentration and oxidative stress. To control for Type 1 errors, false discovery rate was calculated and significant p-values for antioxidant concentration and oxidative stress correlations are 0.019.

Two-way ANOVAs were run to test the effects of egg cortisol treatment (0 versus 1000 ng/mL) and offspring developmental stage (24HPF, EYE, HA) on offspring antioxidant concentration, reduced glutathione concentration, and oxidative stress. Significant main effects were investigated using Tukey *post hoc* tests. To control for Type 1 errors, false discovery rate was calculated and significant p-values for differences between egg cortisol treatments and among developmental stages are 0.027 and 0.020, respectively. Although data were taken from the same cohort through time, the same individuals were not sampled more than once so data were considered to be independent and thus I did not use repeated measures analyses.

Results

Naturally-occurring egg cortisol concentrations varied considerably among sampled females (mean ± SE, 17.35 ± 12.47 ng/g) and mirrored values previously reported in newly fertilized chum salmon (*O. keta*; deJesus and Hirano, 1992). Egg cortisol levels did not correlate with oxidative stress or antioxidant concentration in any maternal or offspring tissues (Table 3-1).

Following 2 h cortisol exposure, egg cortisol levels were significantly elevated (33.71 ± 3.80 ng/g) compared to unexposed eggs (10.83 ± 11.76 ng/g, $t = -4.899$, $p < 0.001$, Welch's Two Sample t-test). Neither egg cortisol treatment nor developmental stage influenced antioxidant concentration ($p > 0.020$, Figure 3-1A). Offspring developmental stage ($F_{2,26} = 105.391$, $p < 0.001$), but not egg cortisol treatment ($F_{1,26} = 2.807$, $p = 0.106$), affected concentration of reduced glutathione (Figure 3-1B). Tukey *post hoc* tests revealed that HA had reduced glutathione differed significantly from levels

at 24HPF ($p < 0.001$) and EYE ($p < 0.001$), but there were no differences in reduced glutathione between 24HPF and EYE ($p = 0.801$, Figure 3-1B). Similarly, offspring oxidative stress varied across developmental stage (two-way ANOVA, $F_{2,26} = 10.717$, $p < 0001$, Figure 1C), but not between egg cortisol treatments ($F_{1,26} = 0.105$, $p = 0.748$. Tukey *post hoc* tests showed that there were significant differences in offspring oxidative stress between 24HPF and EYE ($p < 0.001$), but not between 24HPF and HA ($p = 0.068$) or EYE and HA ($p = 0.116$, Figure 3-1C).

Discussion

Environmental factors that elicit increases in glucocorticoids (GCs; e.g., low water levels and food availability) can also elicit increases in oxidative stress (Welker and Congleton, 2004). However, very little information exists on the link between oxidative stress and GCs. Lin et al. (2004) showed that broiler chickens *(Gallus gallus domesticus)* with long-term administration of cortisol showed signs of oxidative stress during the first three days of treatment but the development of further oxidative injury was prevented, possibly by the enhancement of non-enzymatic antioxidant capacity during stress. In oviparous species, increases in maternal GCs can subsequently increase gametic GCs, which can have a range of repercussions. In fish, elevated egg GCs can compromise competitive ability (Burton et al., 2011; Eriksen et al., 2011), increase metabolic processes (Sloman, 2010), dampen growth (McCormick, 1999), and reduce survival (Gagliano and McCormick, 2009). Here, I explored how maternal oxidative stress relates to egg cortisol, and in turn how egg cortisol alters offspring oxidative metrics. Contrary to predictions, maternal oxidative health had no clear effect on cortisol deposition in eggs. Also, egg cortisol at experimental levels affected neither oxidative

stress nor antioxidant concentration in offspring further than at natural levels observed in unmanipulated eggs. Potential buffering of both maternal oxidative stress on egg cortisol, and egg cortisol levels on offspring oxidative health would be advantageous in semelparous species such as sockeye salmon that only have a single opportunity to reproduce.

Maternal antioxidant concentration and oxidative stress were not related to levels of cortisol in PF eggs for any of the tissues sampled. Due to the potential negative effects of elevated egg cortisol on offspring development (see above), it has been suggested that in fishes, females may control the amount of cortisol deposited into eggs as a means of protection from hypercortisolism (Schreck et al., 2001). This notion is supported by a study using rainbow trout (*O. mykiss*), which showed that at ovulation, both stressed and unstressed mothers had 30 times less cortisol in their eggs than in their circulation (Contreras Sánches, 1995). Previous studies have suggested that the presence of corticosteroid-binding protein (CBP) that binds cortisol in maternal circulation may be a mechanism used to avoid excess cortisol from entering the eggs (Caldwell et al., 1991; Contreras Sánches, 1995; Schreck et al., 2001). Schreck et al. (2001) also suggested that cortisol-metabolizing enzymes in the follicle might convert cortisol leaving the circulation into the inactive cortisone. Li et al. (2012) detected conversion of cortisol to cortisone in rainbow trout developing oocytes and fertilized embryos. The results of this study do not necessarily confirm the presence of a buffering strategy that protects eggs from maternal oxidative stress as it is possible that other egg components are targeted (e.g., genetic material, sex/thyroid hormones, lipids). Though Chapter 2 did not find correlations between maternal oxidative stress and egg/developing offspring, Wilson et

al. (2014) found tissue-specific buffering of oxidative stress in relation to migratory experience and sexual maturation, suggesting high buffering capacity against oxidative stress in adult salmon. Furthermore, effects of maternal oxidative stress on egg GCs may differ among reproductive strategies (i.e., semelparous versus iteroparous) and life histories (i.e., migratory versus non-migratory).

Sockeye salmon encounter many biological and environmental stressors during their reproductive migration from the Pacific Ocean to freshwater spawning grounds that can induce production of cortisol. Circulating levels of maternal cortisol correlate with egg cortisol (Mingist, 2007) and chronic exposure to a stressor can increase both maternal and egg cortisol (*O. kisutch*; Stratholt et al., 1997) in hatchery salmon. In this study, oxidative stress in offspring was not affected when egg cortisol was manipulated to mimic a migration whereby females repeatedly encounter stressors (e.g., predator, fisheries capture) that could result in increased deposition of cortisol eggs. In contrast to iteroparous salmonids, plasma cortisol is known to increase dramatically in wild female Pacific salmon during their spawning migration (Schmidt and Idler, 1962), suggesting that increases in egg cortisol beyond already potentially elevated egg hormone levels will not lead to expected declines in oocyte quality (Stratholt et al., 1997). Indeed, wild-caught sockeye salmon exposed to a stressor during final oogenesis did not have increased egg cortisol levels (Sopinka et al., 2014 but see Stratholt et al., 1997). Finally, sockeye salmon are semelparous and have only a single breeding episode to secure fitness before dying. Taken together, it is perhaps not surprising that naturally-migrating sockeye salmon have evolved biological processes that buffer eggs from detrimental effects of increased egg cortisol. It is noted that gametic cortisol exposure experiments

have revealed a range of outcomes for offspring within a single performance trait, including neutral (no differences, Stratholt et al., 1997), adaptive (increased, Sampath-Kumar et al., 1993) and detrimental (reduced, Gagliano and McCormick, 2007) effects on survival, re-emphasizing the diversity of responses across species and life histories.

Here I have shown that neither maternal oxidative stress nor antioxidant concentration affects the amount of cortisol deposited in to the egg during oogenesis in the semelparous sockeye salmon. Previous studies have shown the importance of timing and severity of cortisol responses during reproduction in female fish (Schreck et al., 2001). Therefore, as individual and interacting stressors (see Johnson et. al., 2012) such as increasing water temperatures (Jeffries et al., 2012), fisheries interactions (Donaldson et al., 2011), aquatic pollution and bacterial and viral infections (Bradford et al., 2010) create additional challenges to migrating Pacific salmon, the effect of timing and severity of these stressors on intergenerational oxidative stress may be an interesting avenue for future research. Beaulieu et al. (2013) also highlighted the importance of life-history traits, life stages and environmental conditions in future research in order to use oxidative markers as indicators of population health in conservation ecology. This research has provided further support to studies that have suggested that a buffering mechanism may exist to protect offspring from hypercortisolism (Caldwell et al., 1991; Contreras Sánches, 1995; Schreck et al., 2001). My results also suggest that spawning Pacific salmon may already transfer a high level of cortisol into the eggs, beyond which no further oxidative damage is incurred. The concepts of a threshold level of cortisol and buffering within the egg, however, are speculative and further research would be required to fully address these hypotheses. Although there appears to be no direct relationship

between cortisol levels and oxidative metrics measured here, research on those aspects individually may provide insight into the long-term stability of this ecologically and economically relevant species.

Tables

Table 3-1. Pearson Correlations for maternal and offspring tissue ORAC values, GSH concentration and GSH/GSSG with egg cortisol pre-fertilization. Listed are r values, p values are shown in parentheses. P-values <0.019 (false discovery rate) are considered significant.

		Egg Cortisol		
		ORAC	**GSH**	**GSH/GSSH**
Maternal				
	Plasma	-0.09 (0.856)	-	0.30 (0.569)
	Brain	0.17 (0.752)	-0.53 (0.223)	-0.53 (0.222)
	Liver	0.36 (0.430)		-
	Heart	-0.21 (0.645)	-0.40 (0.380)	-0.12 (0.792)
	PF	-0.47 (0.282)	-0.78 (0.039)	-0.67 (0.097)
Offspring				
	24HPF	0.38 (0.406)	-0.37 (0.411)	0.58 (0.172)
	Eyed	0.18 (0.777)	0.01 (0.988)	-0.49 (0.402)
	Hatch	0.37 (0.631)	0.77 (0.231)	0.98 (0.022)

Figures

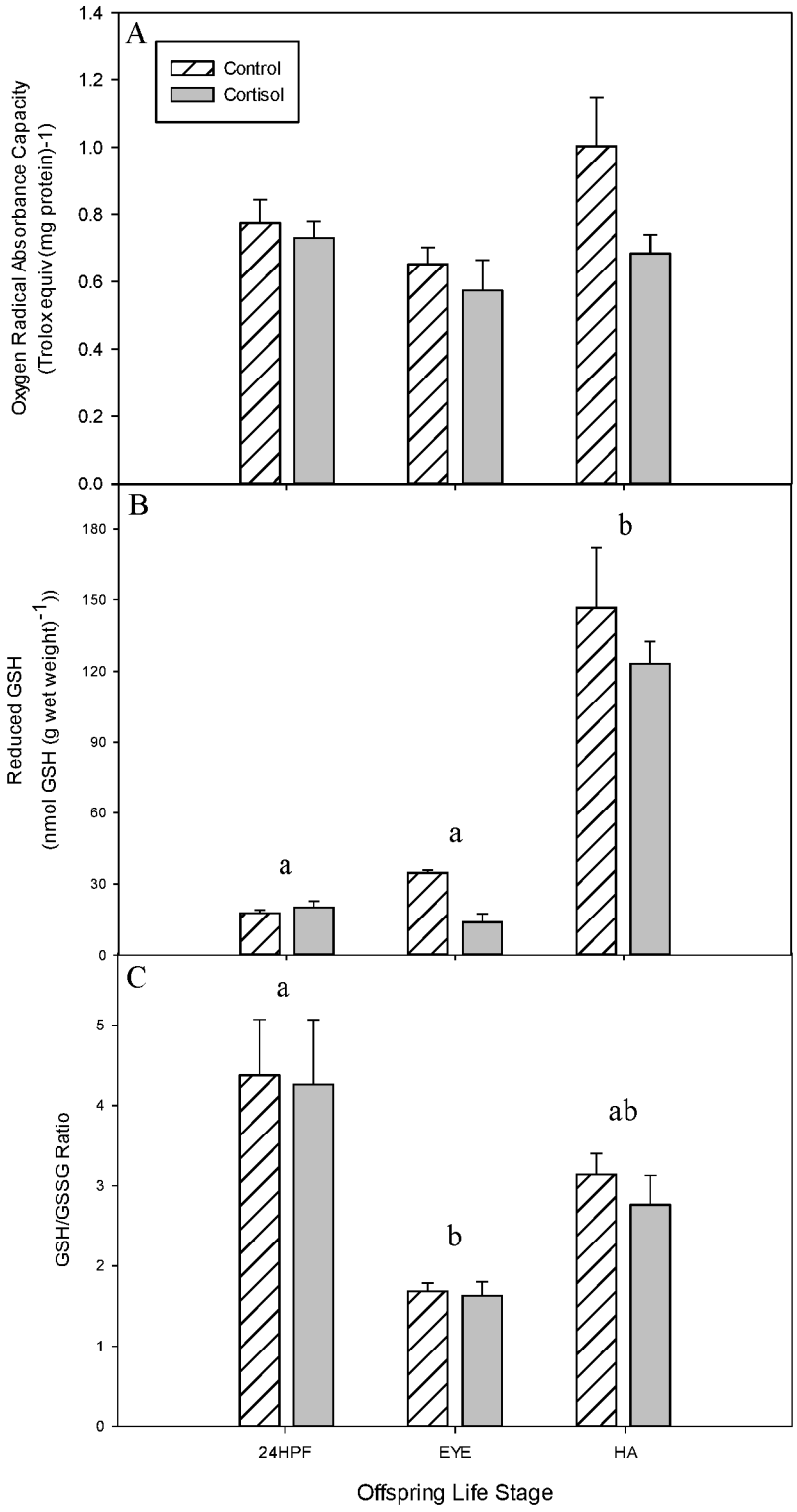
A
Control
Cortisol
Oxygen Radical Absorbance Capacity
(Trolox equiv (mg protein)-1)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B
Reduced GSH
(nmol GSH (g wet weight)-1))
180
150
120
90
60
30
0
a
a
b
C
GSH/GSSG Ratio
5
4
3
2
1
0
a
b
ab
24HPF
EYE
HA
Offspring Life Stage

Figure 3-1. Levels of antioxidant capacity shown as Trolox equivalents (TE)/mg protein (A), reduced glutathione (nmol/g wet weight) (B), and oxidative stress represented as a ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) (C) for eggs at 24 hours post-fertilization (24HPF), eyed embryos (EYE), and hatchlings (HA) reared from female sockeye salmon captured at Harrison River. Offspring were reared from eggs fertilized with control water (0 ng/mL cortisol, hashed bars) or cortisol-dose water (1000 ng/mL, grey bars). Letters represent significant differences between offspring stages ($p < 0.020$, false discovery rate).

Chapter 4: General Discussion

This thesis assesses the intergenerational effects of stress and oxidative stress in adult migrating sockeye salmon (*Oncorhynchus nerka*) and their offspring. In Chapter 2, population effects were studied by measuring metrics of oxidative stress in tissues of adult female sockeye salmon from three geographically distinct populations, at the end of their spawning migration. Eggs were also collected from the females, fertilized with milt to create full sibling crosses, and reared in hatcheries. Oxidative stress and antioxidant concentrations were measured throughout early development to assess maternal effects. In Chapter 3, the influence of maternal oxidative stress on egg cortisol deposition was evaluated by comparing oxidative stress in maternal tissues to the cortisol concentration measured in unmanipulated, pre-fertilized eggs. Egg cortisol levels were also artificially elevated to mimic a migration whereby females repeatedly encounter stressors, and oxidative stress was measured at early stages of offspring development.

Findings and Implications

Understanding the health of migrating sockeye salmon and the resonating impacts on their subsequent generations that maintain this ecologically and economically significant species has the potential to provide insight into the decline of many of the major Fraser River sockeye salmon populations (Peterman et al., 2012). Current research focuses on how adult sockeye salmon physiology (e.g. blood biochemistry, genomic expression) and survival is influenced by stressors (e.g., fisheries capture (Donaldson et al., 2011) and thermal extremes (Eliason et al. 2011; Jeffries et al. 2012)); information necessary for evidence-based fisheries management (Hinch et al. 2006; Green 2008). In

Chapter 2, I studied the intergenerational and population-specific effects of oxidative stress and found that neither maternal antioxidant concentration nor oxidative stress is conferred to offspring and that oxidative stress appears to be related to population at specific developmental stages. I found that offspring oxidative stress at various stages of development reflected the degree of difficulty of future migrations on a population-specific basis. Despite maternal stressors during migration, my results suggest that an oxidative stress-related preparatory adaptation in offspring may exist to ensure future success. I also found that offspring develop their endogenous antioxidant system as they prepare to absorb their yolk sac at varying rates across populations, however this population-dependent variation is overcome by emergence. Given the population level differences in productivity and abundance, population-specific information on oxidative stress in sockeye salmon (both as adults and offspring) can help identify which sockeye salmon stocks may be at greater risk of the resonating effects of oxidative stress on offspring.

In Chapter 3, I further studied the effect of maternal oxidative stress on offspring by measuring cortisol levels in pre-fertilized eggs. This revealed that oxidative stress in maternal tissues did not affect the amount of cortisol deposited into oocyte by the mother. The natural cortisol concentration in eggs was also compared to offspring oxidative stress over the course of development, and once again, no relationship was observed. Based on this finding, cortisol levels were experimentally elevated to mimic an increase in circulating maternal cortisol caused by a stressor. This treatment did not cause further oxidative stress in the offspring than observed in the untreated eggs, suggesting that spawning Pacific salmon may already transfer a high level of cortisol into the eggs,

beyond which no further oxidative damage is incurred. The levels of antioxidant capacity and reduced glutathione over the course of offspring development for both untreated and treated eggs mirrored those seen in Chapter 2, further supporting the notion that the increase in reduced glutathione at hatch is attributed to the development of larvae's endogenous antioxidant system. In contrast to trends observed in Harrison River offspring examined in Chapter 2, increases in oxidative stress at eyed in both control and cortisol treated offspring were observed. The cause of this apparent increase is unknown and does not appear to be related to rearing conditions (similar accumulated thermal units (ATUs) at sampling) or sampling techniques (rapid placement into liquid nitrogen, long-term storage in -80^{o}C). It is possible that this anomaly is an artefact of small sample sizes, and not treatment as both groups experienced the same increase. This study has provided further support to studies that have suggested that a buffering mechanism may exist to protect offspring from hypercortisolism (Caldwell et al., 1991; Contreras Sánches, 1995; Schreck et al., 2001).

Future Research Opportunities

This thesis contributed important information on the intergenerational effects of oxidative stress in sockeye salmon. Based on the population-specific nature of the results in Chapter 2, further investigation into whether the preparatory observations in offspring are based on adaptive transgenerational plasticity, genetics or a combination of both could provide insight into the long-term stability of this species in wake of increasing water temperatures (Jeffries et al., 2012), fisheries (Donaldson et al., 2011), and increase in aquatic pollution and bacterial and viral infections (Bradford et al., 2010). Furthermore, given that skin colour is responsible for sexual signalling in sockeye

salmon, and reflects antioxidant (i.e. carotenoid deposition) availability (Craig & Foote, 2001), the relationship between skin pigmentation and offspring oxidative health would be an interesting avenue for future research. Based on the lack of cortisol treatment effect on offspring oxidative health in Chapter 3, and the previously reported importance of timing and severity of cortisol responses during reproduction in female fish (Schreck et al., 2001), future research could study stressors at varying times and intensities during female oogenesis. Additionally, effects of maternal oxidative stress on egg glucocorticoids may differ among reproductive strategies (i.e., semelparous versus iteroparous) and life histories (i.e., migratory versus non-migratory). Chapter 3 also introduced the notion that there may be a threshold level of cortisol and a buffering mechanism in the egg. The extent to which that may exist as a protective mechanism for offspring is speculative, and future research is necessary.

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