Oxidative Stress in Pacific Salmon (*Oncorhynchus* spp.) during Spawning Migration

Samantha M. Wilson[1,2,3,*]
Jessica J. Taylor[1,2]
Trisha A. Mackie[2]
David A. Patterson[4]
Steven J. Cooke[1,3]
William G. Willmore[2]
[1]Fish Ecology and Conservation Physiology Laboratory, Ottawa-Carleton Institute for Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada; [2]Institute of Biochemistry, Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada; [3]Institute of Environmental Science, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario K1S 5B6, Canada; [4]Fisheries and Oceans Canada, Cooperative Resource Management Institute, School of Resource and Environmental Management, Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada

Accepted 11/9/2013; Electronically Published 2/19/2014

ABSTRACT

The energetic and physiological challenges of spawning migrations in semelparous Pacific salmon (*Oncorhynchus* spp.) have been well characterized. However, the accompanying costs associated with oxidative stress during this nonfeeding migration and the potential connection to senescence have not been explored. Oxidative stress is caused by an imbalance between free radical production and absorption, leading to irreparable cellular damage that accumulates over time and contributes to senescence. The objective of this study was to determine whether oxidative stress occurs during migration between river entrance and spawning for maturing pink salmon (*Oncorhynchus gorbuscha*), a semelparous species. Samples of plasma, liver, heart, brain, red muscle, and white muscle were collected from individual pink salmon at both the beginning and the end of the freshwater migration and then assayed for antioxidant capacity as well as for oxidative DNA damage. Antioxidant capacity and DNA damage changed between sites on a tissue-specific basis, demonstrating that oxidative stress may be experienced differentially between tissues. Consistent with our prediction, DNA damage was higher and antioxidant capacity lower in plasma (an integrative measure of body condition) and heart tissue at the spawning grounds compared with river entrance. The increased oxidative stress of these tissues is correlated with the senescence and deterioration associated with a semelparous reproductive strategy. However, similar changes were not seen in liver, red muscle, or white muscle. More surprisingly, the antioxidant capacity was higher and DNA damage was lower in the brains of spawning migrants at the spawning grounds than at river entrance. The latter results highlight the importance of tissue-specific variability in understanding the role that oxidative stress may play in spawning migration success.

* Corresponding author; e-mail: swilson471@gmail.com.

Physiological and Biochemical Zoology 87(2):346–352. 2014. 1522-2152/2014/8702-3030$15.00. DOI: 10.1086/674798

Introduction

Pacific salmon (*Oncorhynchus* spp.) experience a myriad of physical (e.g., increased water temperatures, high water flows) and biological (e.g., disease and predators) challenges throughout their migration to the spawning grounds (Hinch et al. 2006). These semelparous fish (i.e., die after spawning) face migration challenges that must be overcome utilizing finite resources, as salmon cease feeding in the ocean prior to freshwater migration (see Groot and Margolis 1991). Previous work has focused on the appropriate allocation of energetic resources (e.g., lipid and protein) for fueling upstream migration, maintenance of somatic tissue, and reproductive development to ensure the completion of migration and production of high-quality gametes (Kinnison et al. 2001, 2003; Crossin et al. 2003) before dwindling energetic resources, disease, and stress result in deterioration of physical condition and death (Hinch et al. 2006; Crossin et al. 2009). However, very little is known about the allocation of the finite supply of antioxidant reserves, the changes in oxidative stress during the nonfeeding migration, and the potential connection this may have to the organismal senescence associated with the semelparous reproductive strategy.

Oxidative stress is an imbalance between oxidative damage by free radicals, generated though aerobic metabolism, and repair by antioxidant resources (i.e., antioxidant enzymes, small molecular weight antioxidants) that results in irreparable tissue damage, which accumulates over time, contributing to the aging process (Das and White 2002; Valko et al. 2007). The evolutionary theory of aging has suggested that point mutations in DNA, resulting from free radical attack, accumulate throughout the life span of an organism and result in aging and eventually death (Beckman and Ames 1998; Finkel and Holbrook 2000;

Barja 2007; Buffenstein et al. 2008). It has been hypothesized that oxidative stress may be an added cost of reproduction and intense activity and therefore may constrain life history decisions (reviewed in Costantini 2008). To date, no studies have directly examined the influence of oxidative stress during migration in semelparous Pacific salmon. However, several studies have found that oxidative stress may occur throughout migration in other organisms. For example, Costantini et al. (2007) sampled both migratory garden warblers (*Sylvia borin*) and barn swallows (*Hirundo rustica*) at a stopover site and found that oxidative stress was condition specific, where individuals with better condition (higher fat and protein) had lower reactive oxygen metabolites and higher antioxidant capacity than those in lower condition. In another study, pigeons (*Columba livia*) that flew for more than 3 h had increased concentrations of reactive oxygen metabolites and decreased antioxidants, resulting in an overall increase in oxidative stress compared with pigeons that flew for shorter flights (Costantini et al. 2008). Studies of the reproductive migration of European eels (*Anguilla anguilla* L.) found that despite a 6,000-km-long fasting migration through the Atlantic Ocean, these semelparous eels experienced minimal oxidative stress (Amérand et al. 2010). Oxidative stress remains a largely unexplored physiological trade-off of long-distance migration.

Pacific salmon that return to the Fraser River, British Columbia, cease feeding prior to river entry and continue to migrate extraordinarily long distances (10–1,100 km) to their natal rivers to spawn (Hinch et al. 2006). They expend 75%–95% of energy stores throughout freshwater migration and rely on lipid stores to fuel egg production and river migration and protein to fuel secondary sexual characteristics and spawning (Idler and Clemens 1959). Cessation of feeding limits Pacific salmon to finite antioxidant stores to balance free radicals produced by metabolic pathways during aerobic exercise and may increase vulnerability to oxidative stress. Additionally, warm in-river water temperatures increase metabolic rate throughout the freshwater portion of the Pacific salmon migration (Brett 1995) and may further contribute to an increase in free radical production.

Fraser River pink salmon (*Oncorhynchus gorbuscha*) have the shortest lifecycle and are the most numerous and smallest of the Pacific salmon (Groot and Margolis 1991). Pink salmon were chosen to examine the role that oxidative stress may play as a proximate mechanism associated with senescence during spawning migration. More specifically, the objective of this study was to quantify changes in antioxidant capacity (the ability of a cell to withstand free radical damage) and DNA damage within different tissues from pink salmon captured just prior to river entry and at the spawning grounds to infer changes in oxidative stress. We predicted that antioxidant capacity would decrease, oxidative damage would be elevated, and therefore oxidative stress would increase between marine exit and spawning. To determine whether oxidative stress results from the freshwater spawning migration in pink salmon, samples from six tissue types, plasma, liver, heart, brain, red muscle, and white muscle, were collected from female pink salmon in the ocean just prior to river entry, as well as in spawning grounds. We compared the level of antioxidant capacity, DNA damage (8-hydroxy-2-deoxyguanosine [8-OHdG] concentration), and the oxidative stress ratio (ratio of DNA damage to antioxidant capacity × 1,000) in each tissue to determine whether oxidative stress had occurred and whether antioxidant defenses changed during migration. A decrease in antioxidant capacity is indicative of the potential for oxidative stress to occur, an increase in DNA damage indicates that oxidative damage is occurring, and the oxidative stress ratio compares the amount of oxidative stress between time points.

Material and Methods

Collection

This study was conducted in accordance with guidelines of the Canadian Council of Animal Care, as administered by Carleton University (Animal Care CU-B-09-10). Pink salmon migrate from the open ocean and through the southern Strait of Georgia (SOG) to spawn in various tributaries in the Lower Fraser River. Pink salmon were collected at two sites (SOG on September 2, 2009, and Weaver Creek Spawning Channel on October 15, 2009) throughout the migration route. At the first site, nine female pink salmon were collected prior to freshwater transition and river entrance in the SOG (fig. 1). These individuals had the entire freshwater migration to complete (~100 km) but had already ceased feeding. Pink salmon were caught by purse seine and were immediately euthanized by cerebral percussion and sampled within 5 min of landing. At the second collection site, Weaver Creek (WVR), seven female pink salmon were captured at the spawning grounds while staging for spawning (fig. 1). These individuals were caught via a dip net, euthanized, and sampled as above. A blood sample was taken via caudal venipuncture using a vacutainer tube (4 mL, sodium–heparin anticoagulant, Becton-Dickinson, Franklin Lakes, NJ; 21 G, 1-1/2-inch-long long syringe, Becton-Dickinson), placed on ice-water slurry for <30 min, and centrifuged for 5 min at 3,200 rpm (Clay Adams Compact II Centrifuge, Becton-Dickinson) to separate erythrocytes and plasma. Approximately 3 g each of liver, red muscle, and heart was collected along with 1 g of white muscle and the entire brain. Tissues and plasma were flash frozen in liquid nitrogen and stored at −80°C until analysis. After tissue sampling was complete, morphometrics were collected including fork length and, when possible, gonadosomatic index (GSI).

Antioxidant Capacity

The samples were ground over liquid nitrogen and homogenized in 1 : 5 w/v in lysis buffer (20 mM Tris HCl, 137 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, 0.05 mM PMSF). The sample lysate was centrifuged at 13,000 rpm for 5 min at 4°C and stored at −80°C until the oxygen radical absorbance capacity (ORAC) assay was performed. White muscle, red muscle, and brain were diluted 1 : 200, whereas heart and liver were diluted 1 : 400 in lysis buffer prior to ORAC assay, as outlined

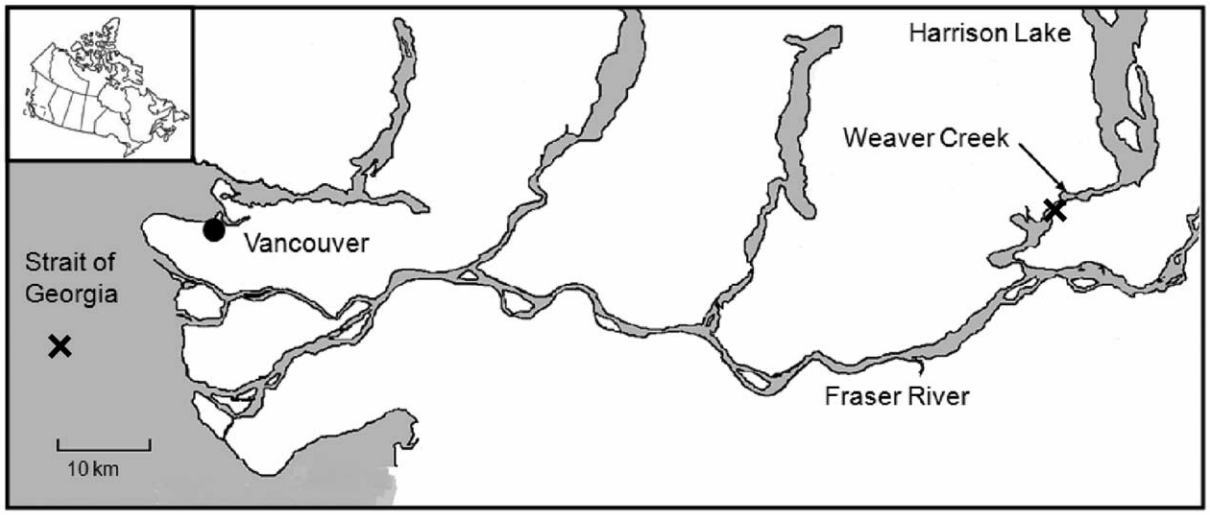

Figure 1. Map of the Lower Fraser River, Canada, showing the Weaver Creek Spawning Channel and the Strait of Georgia catch sites, indicated by crosses.

in Wilson et al. (2012). This assay measures the nonenzymatic portion of cellular antioxidant capacity (ascorbic acid, α-tocopherol, glutathione). Total protein of samples was determined using the Bradford (1976) assay (Bio-Rad, Hercules, CA), and final values were reported in Trolox equivalents per milligram total protein.

DNA Damage: 8-OHdG Determination

Ground samples were prepared as in Wilson et al. (2012). Tissue lysates were diluted to fall within a standard curve of 8-OHdG; plasma and liver (1 : 5 to 1 : 50), red muscle and heart (1 : 40), white muscle (1 : 35). Fifty microliters of the diluted sample was used for the determination of 8-OHdG with a commercial EIA kit (8-OHdG EIA Kit, Cayman Chemicals, Ann Arbor, MI). Values from each sample were calculated based on an absorbance at 420 nm of standard 8-OHdG concentrations (10.3, 23.1, 52.0, 117.1, 263.4, 592.6, 1,333, 3,000 pg/mL). Total DNA concentrations were determined using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) at an absorbance of 260 nm. All 8-OHdG concentrations were standardized against total DNA concentration (ng/mL).

Oxidative Stress

Oxidative stress ratios were calculated by dividing oxidative DNA damage by ORAC value and multiplying by 1,000 (see Costantini et al. 2008, which showed that long-distance migrants had higher oxidative stress ratio than short-distance migrants).

Statistical Analyses

Statistical analyses were completed using RStudio (v. 0.94.110). Oxidative stress ratio and antioxidant and DNA damage concentrations for each tissue were compared to potential covariants (catch location, fork length, hematocrit, and GSI) using multiple regressions. Data were tested for normality and homoscedasticity. Data failing homoscedasticity were log transformed. Of the covariants tested, only location was found to be a significant predictor. Therefore, Welch's *t*-tests comparing SOG samples to WVR samples were completed for each tissue individually ($P < 0.05$).

Results

Oxidative DNA damage changed between the initial and final migration sites in a tissue-dependent fashion (fig. 2*A*). DNA damage, as measured by 8-OHdG concentration, was significantly higher in white muscle ($t = -3.168$, df = 13, $P = 0.007$) and heart tissues ($t = -26.594$, df = 9, $P < 0.001$) for fish collected at WVR compared to fish collected in the SOG. Brain ($t = 2.289$, df = 12, $P = 0.041$) and red muscle ($t = 2.245$, df = 12, $P = 0.045$) 8-OHdG concentrations were significantly lower in fish collected at WVR compared to fish collected in the SOG. No significant changes in DNA damage concentrations were observed in plasma ($t = -0.475$, df = 12, $P = 0.643$) or liver ($t = 1.286$, df = 14, $P = 0.220$) tissues.

Changes in antioxidant capacity were also tissue dependent (fig. 2*B*). Brain antioxidant capacity was higher in fish collected at WVR compared to fish collected in the SOG ($t = -3.572$, df = 10, $P = 0.005$). Plasma antioxidant capacity was lower in fish collected at WVR ($t = -5.867$, df = 7, $P < 0.001$). Liver ($t = -0.354$, df = 8, $P = 0.733$), red muscle ($t =$

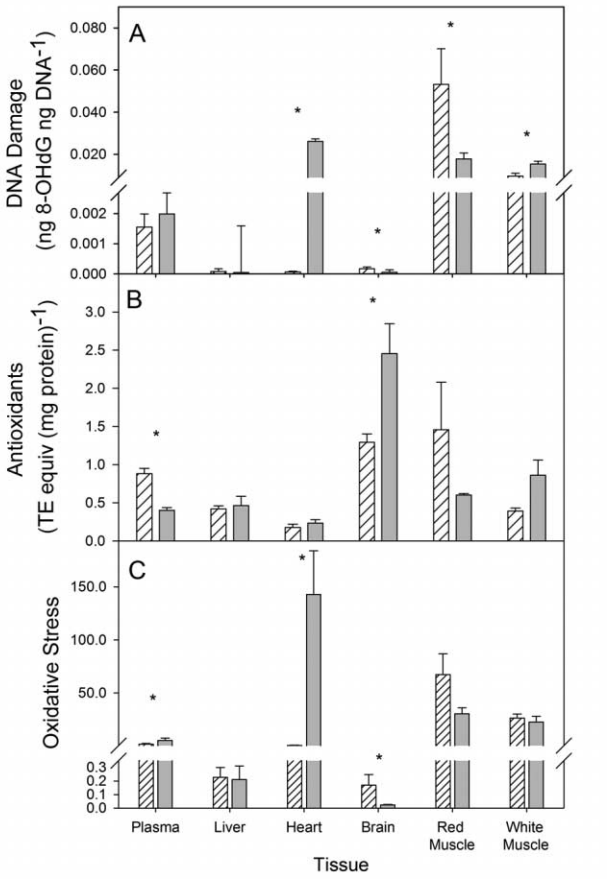

Figure 2. Levels of antioxidant capacity shown as Trolox equivalents (TE) per milligram protein (*A*); oxidative DNA damage represented as concentration of 8-OHdG per nanogram DNA (*B*); and oxidative stress ratio for liver, brain, heart, red muscle, white muscle, and plasma from female pink salmon captured in the Strait of Georgia (striped bars) or at Weaver Creek Spawning Channel (shaded bars). An asterisk denotes significant differences between sites as determined using Welch's *t*-test ($P < 0.05$).

-0.590, df = 9, $P = 0.570$), white muscle ($t = -2.332$, df = 6, $P = 0.056$), and heart ($t = -1.266$, df = 13, $P = 0.228$) antioxidant capacity did not change significantly between catch locations.

Oxidative stress ratio of female pink salmon significantly changed between WVR and the SOG in brain, heart, and plasma tissues (fig. 2*C*). Heart and plasma oxidative stress ratios were higher for fish collected at WVR compared to fish collected in the SOG ($t = -14.968$, df = 13, $P < 0.001$; $t = -2.276$, df = 13, $P = 0.041$, respectively). However, the oxidative stress ratio in brain was lower for fish collected at WVR compared to fish collected in the SOG ($t = 3.848$, df = 12, $P = 0.003$). Liver ($t = 0.564$, df = 11, $P = 0.584$), red muscle ($t = 1.813$, df = 9, $P = 0.102$), and white muscle ($t = 0.762$, df = 13, $P = 0.460$) oxidative stress ratios did not change significantly.

Discussion

Long-distance migrations, especially reproductive migrations, have been demonstrated to result in oxidative stress where, concurrent with increased energy expenditure, resources must be allocated to gonad maturation (Costantini et al. 2008; Amérand et al. 2010). We hypothesized that due to increased energetic demands and dwindling energetic reserves, semelparous pink salmon would experience systemic oxidative stress that would be higher in individuals sampled at the spawning grounds than in individuals sampled just prior to freshwater entrance. Contrary to our original hypothesis, oxidative stress was not consistent across tissue type; plasma and heart had increased oxidative stress at WVR; red muscle, liver, and white muscle had no significant change; and, interestingly, brain tissue had lower oxidative stress at WVR.

Plasma levels of antioxidants and 8-OHdG can indicate systemic oxidative stress conditions. Under conditions of oxidative stress, 8-OHdG is recognized and removed from cellular DNA by DNA repair mechanisms, transported from cells to the plasma, and then transported to the kidneys for excretion (Shigenaga et al. 1989). Plasma also functions as the transport system for transfer of antioxidants from storage organs to tissues experiencing oxidative stress. The higher oxidative stress ratio in plasma for individuals at WVR compared to individuals in the SOG was the result of a decrease in antioxidant capacity rather than an increase in DNA damage. This suggests that the total somatic tissue concentration of antioxidants in fish at WVR had decreased during the migration (as predicted), but large differences among tissues remain. Interestingly, a recent study by Hoogenboom et al. (2012) found that prior to spawning, female brown trout (*Salmo trutta*) with higher plasma testosterone levels had higher reactive oxygen metabolites and thus were at greater risk for oxidative stress. However, circulating testosterone levels slowly decrease throughout migration of female pink salmon (Dye et al. 1986), and corresponding decreases in DNA damage in plasma were not observed. Future studies could examine within a single site if individuals with higher circulating testosterone had higher DNA damage levels.

The liver is thought to be a main storage organ for acquired antioxidants (α-tocopherols); however, the liver tissue demonstrated no predicted increase in oxidative stress, DNA damage, or decrease in antioxidant capacity. Since we sampled maturing female fish, liver antioxidant resources are likely being acquired from other tissues and reallocated to vitellogenin for deposition in eggs. Antioxidants may be mobilized from other tissues, such as the skin (which was not measured) or the red muscle (nonsignificant decrease), or from other major storage organs of the antioxidant carotenoids astaxanthin and canthaxanthin (Moaka 2011). Overall, this makes antioxidant dynamics in liver difficult to interpret in isolation of other tissues.

Tissues with higher concentrations of mitochondria, such as those responsible for aerobic and anaerobic metabolism, are more susceptible to oxidative stress through mitochondrial leakage of free electrons (Leary et al. 2003). Typically, caloric restriction, such as with the cessation of feeding that occurs in

Pacific salmon migrations, is accompanied by mitochondrial uncoupling, whereby mitochondria become more efficient and reduce reactive oxygen species (ROS) production. However, in senescing heart tissue an accumulation of damaged and dysfunctional mitochondria lead to increased production of ROS (see review in Chen et al. 2012). Indeed, a 200-fold increase in oxidative stress was observed in heart tissue between SOG and WVR. This was driven by a 400-fold increase in 8-OHdG concentrations between SOG and WVR without sufficient changes in antioxidant capacity to offset the DNA damage. The increase in DNA damage may be partially attributed to an increase in heart rate driven by increased temperature. Heart rate can increase by as much as 30% with the ~7°C increase between the SOG (~9°C) and WVR (~16°C). Additionally, heart rate on the spawning grounds is higher in females than in males (Sandblom et al. 2009). An increase in heart rate can result in an increase in free radical production (see review in Chen et al. 2012) and an increase in DNA damage. Cardiac function is imperative for survival and reproductive success in Pacific salmon, and cardiac failure has been postulated as the driving factor in temperature-driven mortality in salmon (Farrell 2009; Clark et al. 2011; Eliason et al. 2011). Oxidative stress in heart tissue and senescence, under various temperature- and activity-related conditions, remains to be investigated to elucidate whether oxidative stress contributes to cardiac deterioration in salmon.

The white muscle of individuals captured at WVR had higher 8-OHdG concentrations compared to individuals captured in the SOG. Similar to the heart, this may be attributable to an increase in mitochondrial leakage associated with increased activity and cellular senescence (Leary et al. 2003; Chen et al. 2012). In contrast to the heart, the oxidative stress ratio and antioxidant capacity for white and red muscle did not significantly differ between SOG and WVR. Moreover, the opposite trend was seen in the red muscle for DNA damage, which was lower in WVR individuals. Since both routine and active metabolic rates are higher in saline waters compared to freshwater (Wagner et al. 2006), it is possible that higher levels of free radical damage occurred in red muscle in the marine SOG environments compared to the freshwater WVR environments. However, this is impossible to determine conclusively, given our experimental results, as temperature is a confounding variable (increased water temperatures between the SOG [~9°C] and WVR [~16°C]). Additionally, individuals caught at WVR were no longer engaged in active migration, and, thus, aerobic demands are less for individuals on the spawning grounds, potentially contributing to a decrease in free radical production.

Contrary to the original hypothesis that tissue oxidative stress levels would be higher at the spawning grounds, brain tissue oxidative stress levels were lower at WVR. This ratio was the result of both significantly higher antioxidant capacity at WVR compared to SOG and significantly lower 8-OHdG concentrations at WVR. The significantly higher antioxidant capacity of this tissue likely prevented free radical damage, including DNA damage. Thus, brain tissue may be preferentially protected, at the expense of other tissues such as muscle, to ensure migratory success in individuals. The high antioxidant capacity of brains of individuals found spawning at WVR may be a result of condition-dependent arrival. Condition-dependent arrival has been observed in migratory barn swallow (*Hirundo rustica* L.), whereby individuals with the highest blood carotenoid concentration arrived at the breeding area first (Ninni et al. 2004). The sampled individuals were collected at peak spawn and may be in a different condition from those that arrive at the spawning grounds early or late. An alternative explanation may be that individuals of lower condition (i.e., lower brain antioxidant capacity) spawned at areas closer to the mouth of the Fraser River rather than traveling the ~100 km to WVR (Groot and Margolis 1991). Regardless, the lack of free radical damage compared to other tissues indicates that the brain may be differentially protected from oxidative stress.

It is unclear whether the protection of vital organs from the oxidative stress associated with long-distance migrations is similar among salmon species that exhibit physical deterioration and senescence during migration. Arguably, sockeye salmon have higher rates of en route mortality in the Fraser River (D. Patterson, personal communication). Therefore, oxidative stress may be experienced differently between species of Pacific salmon. Sockeye salmon that did not successfully spawn had decreased expression of oxidative stress–related genes and increased expression of DNA damage–related genes in gill tissue (Miller et al. 2011). Decreased antioxidant-related gene expression was observed in the gills of moribund sockeye salmon versus surviving sockeye salmon (Jeffries et al. 2012). We suggest further research into oxidative stress in sockeye salmon, as well as how migrant pathogen load, immune response, and migrant spawning success are influenced by oxidative stress. In this study, we assumed a low rate of en route mortality of pink salmon, but migration success and spawning success of sampled individuals cannot be known due to the lethal nature of the sampling. Survival to the spawning grounds does not necessarily imply successful reproduction and hence fitness. Therefore, we suggest examining success of parents and offspring with varying levels of antioxidant capacity as a future avenue of research.

Oxidative stress is quickly becoming recognized as a physiological consequence of both natural (i.e., migration; Costantini et al. 2008; Amérand et al. 2010; parental care; Wilson et al. 2012) and anthropogenic (i.e., bycatch; Romero et al. 2007) activities. In semelparous pink salmon, the final reproductive migration is an enormously challenging phase with numerous selective pressures including osmoregulatory challenges, increased temperatures, and increased pathogen burden (Hinch et al. 2006). Here we have demonstrated that oxidative stress is experienced in a tissue-specific manner, and we provide further support for previous studies that have shown that antioxidant depletion does not always indicate oxidative stress (Costantini and Verhulst 2009). The oxidative stress ratio was developed as a way to simultaneously compare antioxidant status and oxidative damage levels. Interestingly, this ratio shows a clear decrease in oxidative stress in brain, with a corresponding increase in heart and plasma between sites. It appears that the brain is differentially protected from oxidative

stress during migration in this fish species. Further, oxidative stress in the heart and plasma (indicative of systemic oxidative stress) may lead to the tissue deterioration and rapid senescence observed on the spawning grounds (Hruska et al. 2010). Future work that tries to connect oxidative stress to differential survival in Pacific salmon needs to consider the tissue-specific responses to both DNA damage and antioxidant capacity that occur during freshwater spawning migration in salmon.

Acknowledgments

We would like to thank the members of the Department of Fisheries and Oceans Canada Environmental Watch Program for collecting tissue samples, especially L. Donaldson. We would also like to thank the Willmore lab members, especially G. MacDonald, S. McBride, A. McBride, and C. Ha, for help with laboratory assays. Finally, we thank the Natural Sciences and Engineering Research Council (NSERC) of Canada for an NSERC Undergraduate Student Research Award (USRA) and Canada Graduate Scholarships–Master's award to S.M.W. and an NSERC USRA to T.A.M. Additional support was provided in the form of NSERC Strategic and Discovery Grants to S.J.C. and W.G.W. S.J.C. is supported by the Canada Research Chairs program.

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