Oxidative ecology of wild fish: investigating the effects of intrinsic and extrinsic factors on oxidative stress and its link to life-histories

By

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Dedication

To all of the wonderful people I have had the chance to meet during the completion of this thesis, from the folks at the Technical University of Denmark, to those I met as I travelled the world with Habitat for Humanity or to attend conferences. Thank you for instilling in me a passion that knows no bounds. To my mom and dad, for their constant support and encouragement, and for their patience in listening to me discuss this project despite their total lack of understanding. To Kim Aarestrup and Martin H. Larsen for their dedication and faith in my abilities, but most of all, for the moments of laughter that I will never forget. Thank you to each of you for saving my life at least once in the field – from electric fences to drowning in the stream. A very special thank you goes to all the fish that have made this project possible. Finally, thank you to my one and only, my very best of friends, you know who you are.

Abstract

Though oxidative stress has been reported to have important repercussions on the biology and ecology of animals, most research has thus far focused on mammals and birds, while relatively little is known about its role in fish. Fish display a wide range of life history strategies, for which resources must be allocated delicately, providing the perfect opportunity to investigate the effects of extrinsic (i.e., environmental) and intrinsic (i.e., life-histories) factors on oxidative stress, as well as the role of oxidative stress markers in determining life-history strategies. In Chapter 2, I reviewed the current literature on oxidative stress in fish from an ecological perspective, and found that oxidative ecology in fish is largely understudied. In Chapter 3, I assessed the short-term (two-weeks) and long-term (4 months) effects of cortisol on oxidative status in brown trout (*Salmo trutta*) from a Danish lowland stream, and demonstrated that cortisol caused an increase in glutathione in the short-term, and that oxidative stress levels as well as low molecular weight antioxidants decreased in the short-term in all treatments. No effects of treatment or time were detected in the long-term. Interestingly, I show that overwinter survival may be associated with low total glutathione and low oxidative stress levels. In Chapter 4, I investigated the oxidative status of migratory and resident individuals from the same Danish brown trout (*S. trutta*) population, and demonstrated that migratory individuals have a higher antioxidant capacity than their resident counterparts, which may represent an anticipatory response to the upcoming demanding journey that migration represents. Continuing studies on the oxidative ecology of fish may help to uncover the physiological mechanisms that influence behavior in relation to ecological phenomena.

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Co-Authorship

Chapter 2: A comparative and evolutionary approach to oxidative stress in fish: a complete review. Kim Birnie-Gauvin, David Costantini, William G. Willmore, Steven J. Cooke

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 Birnie-Gauvin, Costantini, Willmore and Cooke. All writing was conducted by Birnie-Gauvin. Costantini, Willmore and Cooke provided comments and feedback on the manuscript.

Chapter 3: Short-term and long-term effects of exogenous cortisol manipulation on oxidative stress in juvenile brown trout. Kim Birnie-Gauvin, Kathryn S. Peiman, Martin H. Larsen, Kim Aarestrup, William G. Willmore, Steven J. Cooke

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 Birnie-Gauvin, Peiman, Willmore and Cooke. Fieldwork was completed y Birnie-Gauvin, Peiman, and Larsen, with logistical support from Aarestrup. All data analysis was conducted by Birnie-Gauvin. Data were interpreted by Birnie-Gauvin, Peiman, Larsen, Aarestrup, Willmore and Cooke. All writing was conducted by Birnie-Gauvin. Peiman, Larsen, Aarestrup, Willmore and Cooke provided comments and feedback on the manuscript.

Chapter 4: Oxidative stress and partial migration in a salmonid fish. Kim Birnie-Gauvin, Kathryn S. Peiman, Martin H. Larsen, Henrik Baktoft, Kim Aarestrup, William G. Willmore, Steven J. Cooke

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 Birnie-Gauvin, Peiman, Willmore and Cooke. Fieldwork was completed y Birnie-Gauvin, Peiman, and Larsen, with logistical support from Baktoft and Aarestrup. All data analysis was conducted by Birnie-Gauvin and Baktoft. Data were interpreted by Birnie-Gauvin, Peiman, Larsen, Aarestrup, Willmore and Cooke. All writing was conducted by Birnie-Gauvin. Peiman, Larsen, Baktoft, Aarestrup, Willmore and Cooke provided comments and feedback on the manuscript.

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Glossary

ANOVA: Analysis of Variance

AUC: Area under the curve

DTNB: 5,5’-dithiobis-(2-nitobenzoic) acid

EDTA: Ethylenediaminetetraacetic acid

GC: Glucocorticoid

GR: Glutathione reductase

GSH: Glutathione or reduced glutathione

GSSG: Glutathione disulfide or oxidized glutathione

NaCl: Sodium chloride

NADPH: Nicotinamide adenine dinucleotide 2’-phosphate

NSERC: Natural Sciences and Engineering Research Council

ORAC: Oxygen radical absorbance capacity

RBCs: Red blood cells

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SEM: Standard error of mean

TBARS:

TGSH: Total glutathione

Tris HCl: Tri hydrochloride

Chapter 1: General Introduction

Oxidative Stress

The Earth's atmosphere was originally highly reduced (i.e., oxygen was limited) and dominated by microscopic organisms (Kasting and Siefert 2002). By the mid-to-early Archean, photosynthesizing cyanobacteria evolved and completely changed the composition of the atmosphere (Falkowski et al. 2004). As a result, oxygen accumulated quickly and changed both terrestrial and shallow aquatic habitats, providing strong selection pressures on anaerobic organisms to become aerobic (Lesser 2006). Aerobic metabolism is accompanied by various by-products including reactive oxygen species (ROS) and reactive nitrogen species (RNS), both of which can be highly toxic to organisms due to the presence of an unpaired electron (Halliwell and Gutteridge 2015). The evolution of a mechanism to cope with the toxicity of reactive species became necessary. Organisms have evolved methods to both use and resist oxygen's toxicity (Halliwell 1999; Dröge 2002; Valko et al. 2007).

Oxidative stress is defined as the production and accumulation of reactive oxygen species (ROS) to a point where an organism is no longer capable of quenching these reactive species. ROS include superoxide radicals, singlet oxygen, hydrogen peroxide and hydroxyl radicals (Asada and Takahashi 1987; Cadenas 1989; Fridovich 1998; Halliwell and Gutteridge 2015). Reactive oxygen species have a vast array of physiological effects including the acceleration of cellular aging, the decrease of fertility and the decrease of survival probability (Haenold et al. 2005; Monaghan et al. 2009). Physiologically, the damage caused by ROS can be rather extensive, resulting in damage of most macromolecules. More precisely, ROS can cause lipid peroxidation and impact membrane fluidity, modify, fragment, aggregate, degrade, remove and/or alter the electric charge of proteins, and cause deletions, mutations and lethal genetic effects in DNA (Asada and Takahashi 1987;

Cadenas 1989; Fridovich 1998; Halliwell and Gutteridge 2015). ROS also participate in normal cellular function at low-to-moderate levels (regulation of vascular tone, secondary messenger in signal transduction and defenses against infectious agents; Dröge 2002; Valko et al. 2007). In order to balance the positive and negative effects of exposure to reactive species, organisms developed preventative and repair mechanisms via the evolution of antioxidant defenses. This defense system comprises enzymatic antioxidants such as superoxide dismutase, catalase and peroxidase, as well as non-enzymatic antioxidants include ascorbic acid (vitamin C), glutathione, alpha-tocopherol (vitamin E), carotenoids and other small molecules (Cadenas 1997; Valko et al. 2007).

Oxidative damage arises from three main sources: (1) acute or prolonged physical activity (Costantini et al. 2008; Fletcher et al. 2013), (2) reproduction (Alonso-Álvarez et al. 2010; Christe et al. 2012; van de Crommenacker et al. 2012) and (3) exposure to anthropogenic pro-oxidative agents (Bonisoli-Alquati et al. 2010; Koivula and Eeva 2010). The physiological oxidative balance of organisms interacts with the constraints of environmentally-induced oxidative conditions, presenting animals with a tug-of-war situation where it is speculated that oxidative balance and demographic processes are interrelated (Beaulieu et al. 2013). In other words, ecological conditions can impact oxidative balance and oxidative status influences or is associated with ecological processes. In a study by Costantini et al. (2008), Adélie penguins (*Pygoscelis audeliae*) were found to have a decreased antioxidant capacity due to a depletion of Antarctic krill (*Euphasia superba*). The decrease in food led to increased foraging efforts, limiting the penguins' ability to invest in antioxidant production. In addition, the krill itself was a source of antioxidant (Tou et al. 2007), thus potentiating the decrease in antioxidant capacity, demonstrating further that ecological conditions can influence an organism's oxidative balance (Nicol et al. 2008; Tierney et al. 2009; Trivelpiece et al. 2011).

Oxidative balance has the potential to shape life-history trade-offs (Monaghan et al., 2009), but organisms are also likely to modify their oxidative balance with respect to their own life history traits (Beaulieu et al. 2013). Studies using the same experimental manipulations to increase breeding constraints have resulted in both increased and decreased antioxidant capacity in Adélie penguins and zebra finches (*Taeniopygia guttata*), respectively (Alonso-Álvarez et al. 2004; Wiersma et al. 2004; Beaulieu et al. 2011). This discrepancy can likely be explained by individual life-history traits such that self-maintenance is favored over current reproduction in Adélie penguins (Stearns 1989).

The generation of ROS is also prevalent in the aquatic world, where oxidative stress is an important component of the stress response (Lesser 2006). Organisms increasingly face changing environmental conditions such as thermal stress, pollution and UV radiation, all of which exacerbate the effects of oxidative stress (Lesser 2006). In fish, factors such as stress, disease and exposure to xenobiotics can render individuals more susceptible to oxidative stress (Welker and Congleton 2005). Fish tissues are often high in polyunsaturated fatty acids (PUFAs) and are thus easy targets for free radical oxidation due to the presence of many double carbon-carbon bonds (Pedrajas et al. 1995; Welker and Congleton 2004). Ecologically and physiologically speaking, relatively little is known about oxidative stress in the context of life-histories in fish.

Migration

Migratory fish often move from their birth location to environments in which growth is maximized, and often travel back to the vicinity of their original birth place to spawn. By doing so, fish are exploiting temporally and spatially variable food resources, adapting to and/or avoiding environmental changes, and maximizing reproductive success, but they also regulate population density and expand their distribution (Ueda 2012). The evolutionary basis for migration

is to gain survival advantage by residing in a particular location during a particular stage of an individual's lifecycle, but the benefits are accompanied by several costs (Dingle 1996; Northcote 1997). Migratory fish are limited by their energetic resources which must be dedicated to fitness traits such as growth, avoiding predation, and avoiding and/or fighting oxidative stress.

Brown Trout

The brown trout (*Salmo trutta*) is an iteroparous salmonid species with populations indigenous to many regions of Europe, North Africa and Western Asia (MacCrimmon et al. 1970). In addition to being an opportunistic carnivore, the brown trout is ecologically variable, and has thus adapted to a variety of habitat types (Klemetsen et al. 2003). It has been speculated that brown trout migrate to utilize the most appropriate habitat for specific life stages, moving within freshwater systems or between freshwater and marine habitats, in order to promote fitness (Jonsson and Jonsson 1993). Migration enables individuals to exploit better feeding grounds, thus accelerating growth rate, increase fecundity in females and attain larger sizes (Hendry et al. 2004). However, brown trout migration includes energetic costs pertaining to swimming, foraging efforts and avoiding mortality (Gross et al. 1988; Jonsson and Jonsson 1993).

Brown trout populations typically consist of two phenotypes: anadromous (herein referred to as sea trout or migratory individuals) and resident individuals, both originating from the same parents (Jonsson and Jonsson 1993), hence why they are referred to as semi-anadromous fish (also known as partial migration). The mechanisms that determine which individuals become resident and which ones become migratory are poorly understood (Acolas et al. 2012), but factors that play a role in this determination include genetics, phenotypic plasticity, metabolic rate, growth rate, body size, energy reserves and sex (Jonsson and Jonsson 1993; Thorpe 1987; Forseth et al. 1999; Wysujack et al. 2009). It appears that environmental conditions also participate in the balance

between migratory and residency via food availability, population density and competition (Pulido 2011).

In Europe, sea trout can enter freshwater streams throughout the year (Went 1962; Jonsson and Jonsson 2002, 2009). There is considerable variation in the time spent at sea between individual fish, as well as in the migration distance. For instance, intrapopulation differences in marine migration distance was observed in a Danish population of brown trout, where 47% of tagged sea trout postsmolts remained close to home in a coastal fjord, and 53% migrated to the open Kattegat Sea (del Villar-Guerra et al. 2014). The authors believe that the findings support the idea of partial migration, where individuals decide whether to stay in the stream or migrate to open sea.

Mechanisms determining whether an individual assumes residency or becomes migratory are not fully understood, and so it is both ecologically and physiologically relevant to investigate the role of oxidative stress on the biology of partial migration.

Current State of Research: Oxidative Stress in the Context of Life-Histories

Life-history theory is concerned with trade-offs between reproduction, growth and survival, and how an organism will organize its energetic investments to promote these fitness traits. It is widely recognized that physiology participates in mediating life-history trade-offs, but exactly how it does so remains unclear (Speakman et al. 2015). Oxidative stress and free radical production have been suggested to act as mediators of life-history trade-offs. There is currently a serious need for studies associating oxidative stress to functional outcomes like survival (Speakman et al. 2015).

Current methods for studying oxidative stress are rather invasive – sometimes lethal – making it difficult to study wild populations without compromising the objectives of the studies.

Laboratory settings provide opportunities to study oxidative stress in a more controlled environment but fail to reveal the underlying physiology of life-history trade-offs (Speakman et al. 2015). Thus far, the scientific community has only scratched the surface of the link between life-history trade-offs and physiology.

In the last decade, we have seen this area of research grow exponentially. Nonetheless, the results are often contradictory. It is likely that oxidative damage and the need for repair vary among different tissues and organisms. At present, it is unknown whether there is conservation in the patterns of damage and protection across different organisms, or whether different taxa have varying points of vulnerability (Speakman et al. 2015). Measuring oxidative stress markers at various time points within the same individuals provides a unique opportunity to assess the role of oxidative stress on life-history strategies (Nussey et al. 2009; van de Crommenacker et al. 2011).

The idea that there exists a link between reproduction, future survival and oxidative stress predicts that oxidative damage should be positively correlated with reproduction, thus limiting survival and future reproductive performance. This concept is however *different* from the idea that oxidative stress is a mediator of aging (i.e. free radical theory of aging), and with it come different predictions that may not encompass life histories (Speakman et al. 2015). Organisms are often viewed a single homogeneous entity, and predictions are made as such. However, we know far too well that organisms are complex, and that physiological systems have different functions, metabolic rates, levels of oxidants and antioxidants as well as repair mechanisms, all of which should be addressed specifically when formulating predictions. If a theory predicted that damage should be uniform across all tissues, then we would have sufficient data to refute it (Speakman et al. 2015). What is most challenging about the oxidative stress model for life-history effects is that the predictions are poorly defined, and therefore difficult to evaluate.

It is also important to consider reproductive form as a factor which influences oxidative stress. Viviparous organisms may not exhibit the same changes in oxidative damage as oviparous organisms, for example. Organisms also vary in their mechanisms for dealing with oxidative stress. In the naked mole rat, it appears as though certain proteins (i.e., trisephosphate isomerase and peroxiredin 1) serve as oxidative sinks and endure the bulk of oxidative damage, while remaining functional (De Waal et al. 2013; Rodriguez et al. 2014). Organisms differ in life-history strategies, which in turn engender different responses to oxidative stress. It is therefore ecologically and physiologically relevant and important to study a wide range of organisms, in a wide range of conditions.

Research revealed that knocking out important oxidative stress enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, in many organisms led to obvious oxidative damage, but does not impact lifespan (Perez et al. 2008, 2009; Jang et al. 2009; Zhang et al. 2009). Salmon et al. (2010) also found that when these animals were subjected to unfavorable environmental conditions, oxidative stress accelerated pathology and shortened lifespan, thus suggesting that the role of oxidative stress on aging is dependent on environmental conditions. After all, over 99.9% of all animals live and age under natural conditions, and not in a laboratory setting. Wild organisms therefore face all of nature's complexity and challenges, which begs for more oxidative stress studies to be performed in the field (Speakman et al. 2015).

At present, there are few studies associating oxidative stress with life-history traits in wild populations. There are even fewer studies investigating the link between oxidative stress and energetically demanding activities such as migration. These types of studies are rarely performed on wild populations of fish (see Wilson et al. 2014 for sockeye salmon, *Oncorhynchus nerka*, study), providing us with a unique opportunity to do so.

Research Objectives and Questions

The general objective of this thesis was to provide further understanding of oxidative stress in fish and more specifically, to link oxidative status to life-history strategies in a wild population of brown trout from the Gudsø stream (Jutland, Denmark). The objective of Chapter 2 was to provide a complete review of the current knowledge we have on oxidative stress in fish, through a comparative and evolutionary approach. I specifically investigated fish that vary in terms of the extrinsic and intrinsic factors they are exposed to, and made recommendations for future studies in that realm. The aims of Chapter 3 were to examine the short-term and long-term effects of intracoelomic cortisol injections on oxidative stress and to further understand how oxidative status may play a role in coping mechanisms. This was accomplished by measuring indicators of oxidative stress and antioxidant capacity in red blood cells of juvenile brown trout (*Salmo trutta*). The goal for Chapter 4 was to investigate the link between oxidative stress and partial migration in brown trout. More specifically, I studied whether differences in oxidative status existed between resident and migratory individuals of the same population. Chapter 5 will integrate the findings of Chapters 2 through 4, their implications, and present future research opportunities on the topic.

Chapter 2: A comparative and evolutionary approach to oxidative stress in fish: a complete review

Abstract

Oxidative stress results from an imbalance between the production of reactive oxygen species and antioxidant defenses, in favour of the former. In recent years, the association between oxidative processes, environmental change and life histories has received much attention. However, most studies have focused on avian and mammalian taxonomic groups, with less attention given to fish, despite their ecological and socio-economic relevance. Here we present a review of the extrinsic and intrinsic factors that influence oxidative processes in fish, using a comparative and evolutionary approach. We demonstrate that oxidative stress plays a key role in shaping fish's responses to environmental change as well as life history strategies. We focus on representative examples to compare and contrast how levels of oxidative stress respond to changes in temperature, salinity and oxygen availability. Furthermore, we describe how emerging threats (i.e., pollution) affect oxidative stress parameters in fish. Oxidative stress indicators are increasingly being used as biomarkers to understand the mechanisms of various human-induced stressors, but also to understand the physiological consequences of how animals are distributed in space and time and influenced by different life stages. Despite the expansion of the field of ecological oxidative stress, we are only beginning to understand the complex ways in which oxidative stress may interact with both extrinsic and intrinsic factors in fish. We conclude with a research agenda for oxidative research on fish and note that there is need for further research particularly in the area of life-history strategies and ecological impacts of oxidative status.

Keywords: antioxidants, oxidative ecology, evolution, fish, oxidative stress, reactive oxygen species

Introduction

Oxygen in the Earth's atmosphere became present approximately 2.4 billion years ago and was highly reduced (below 5%) until approximately 600 million years before present. Prior to this time, the Earth was dominated by anaerobic organisms (Kasting and Siefert 2002). Archean cyanobacteria evolved the ability to photosynthesize (Nisbet and Sleep 2001), after which the abundance of carbon dioxide and water as reductants, along with the availability of sunlight, greatly increased the production of oxygen, leading to the evolution of other photosynthetic organisms (Falkowski et al. 2004). Over time, atmospheric oxygen increased greatly, modifying both aquatic and terrestrial habitats. These changes brought along forceful selective pressures on the remaining anaerobic organisms, resulting in a world populated by aerobic species.

The presence of atmospheric oxygen comes with its own set of challenges, the most important one being the formation of oxygen-derived free radicals – an atom or molecule with an unpaired electron – or of non-radical reactive oxygen species (ROS) (e.g., hydrogen peroxide; hypochlorus acid) (Halliwell and Gutteridge 1999). The discovery of ROS in biological systems occurred approximately 60 years ago, and was immediately linked to diseases and aging (Harman 1956). The study of these ROS and oxidative stress (i.e., generation of biomolecular oxidative damage due to an imbalance between ROS and protective antioxidant mechanisms) has since become a "hot topic" in medicine, molecular biology, physiology and, more recently, ecology. While there is extensive literature on the underlying mechanisms of oxidative stress (Costantini 2008, 2014; Dowling and Simons 2009; Metcalfe and Alonso-Alvarez 2010; Blount et al. 2015), along with many reviews on specific aspects of oxidative stress in aquatic environments (marine

environments (Lesser 2006), or environmentally-induced oxidative stress in aquatic animals (Lushchak 2011)), we lack a unifying review that accounts for all aspects surrounding oxidative stress in fish. While other reviews have mainly focused on birds and mammals (e.g., Costantini 2008; Metcalfe and Alonso-Alvarez 2010), a similar review is lacking for fish.

Fish generate many ecosystems services (reviewed in Holmlund and Hammer 1999; Lynch et al. 2016) and are remarkably diverse (Helfman et al. 2009). They live in waters that vary from entirely fresh to hypersaline, from the shallows to the abyss, from supersaturated (with oxygen) to hypoxic, from polar seas to tropical oxbow lakes. They also vary dramatically in size, morphology and life history. Their important role in aquatic ecosystems (and for humans) coupled with this immense diversity makes them relevant models for further exploring oxidative stress in ecology. Moreover, because of the direct and indirect effects of human activities on fish (see Gray 1997; Dudgeon et al. 2006), there is need and desire to characterize the ways in which humans are potentially influencing their biology, including influences that affect oxidative stress in aquatic organisms. Here we present a comprehensive summary of oxidative stress in fish. This review will focus on the extrinsic and intrinsic factors associated with oxidative stress and antioxidants in fish and provide representative examples of the main findings associated with these factors, using a comparative and evolutionary approach. We then summarize the current gaps in knowledge in this field and provide suggestions for future research. Given that the assays for ROS are not taxon-specific, we avoid in-depth discussion of the technical aspects of *in vivo* and *in vitro* ROS assays and analytical methods because they are covered elsewhere (see e.g., "Free Radicals in Biology and Medicine" by Halliwell and Gutteridge 2015).

Reactive oxygen species, antioxidants and oxidative stress in fishes

Atmospheric oxygen has two unpaired electrons in its ground state, making oxygen paramagnetic, a characteristic that prevents it from easily interacting with organic molecules unless it is activated (Asada and Takahashi 1987; Cadenas 1989; Fridovich 1998; Halliwell and Gutteridge 1999). The reduction of oxygen leads to the production of reactive intermediates such as superoxide radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO\bullet$) (Asada and Takahashi 1987; Cadenas 1989; Fridovich 1998; Halliwell and Gutteridge 1999), referred to as ROS. All photosynthetic and aerobic organisms produce ROS, through their normal metabolism, from the electron transport chain at (1) complex I (NADH dehydrogenase) and (2) between ubiquinone and complex III (Brookes 2005). The superoxide radical is typically the first ROS formed, eventually leading to the formation of hydrogen peroxide and finally hydroxyl radicals which are, chemically, the most damaging of the ROS (Asada and Takahashi 1987; Cadenas 1989; Fridovich 1998; Halliwell and Gutteridge 1999), but it remains uncertain how superoxide affects organismal biology and fitness. While not a radical species, H_2O_2 can pass easily through membranes and is longer lived than most oxygen-derived radical species. In the presence of metals, H_2O_2 can be converted to the highly reactive hydroxyl radical which is capable of extracting electrons and protons from macromolecules (nucleic acids, proteins, lipids and carbohydrates) that it comes in contact with, thereby generating more unpaired electrons and radical species.

The production of ROS is positively associated with the concentration of oxygen within an organism (Jamieson et al. 1986). When the accumulation of ROS exceeds an organism's ability to quench these reactive species with the use of antioxidants, oxidative stress occurs, damaging cellular components (Asada and Takahashi 1987; Cadenas 1989; Fridovich 1998; Halliwell and

Gutteridge 1999). At low concentrations, however, ROS can serve as signaling molecules (Dröge, 2002; Valko et al. 2007). To protect themselves against the potentially highly damaging ROS, organisms have evolved a system to either prevent or repair the effects of oxidative stress. Prevention comes in the form of antioxidants, which can either be enzymatic (e.g., superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX)) or non-enzymatic molecules (e.g., ascorbic acid (vitamin C), glutathione, □-tocopherol (vitamin E), carotenoids and other small molecules (Cadenas 1997; Valko et al. 2007) in nature. Table 2-1 presents a summary of the commonly measured oxidative markers and antioxidants in fish biology.

Aquatic animals, like all other organisms, must have a balance between the production of ROS and antioxidant defenses (Winston 1991; Winston and Di Giulio 1991; Kelly et al. 1998; Valavanidis et al. 2006), and are of particular interest as they experience a multitude of stressors such as temperature fluctuations, osmotic stress (in the case of anadromous species), alterations in oxygen availability, pollution, and other anthropogenic impacts, which can directly affect free radical chemistry (Lushchak 2011).

Extrinsic factors affecting oxidative stress

While ROS are produced intrinsically through the mitochondrial electron transport chain or the activity of immune cells, environmental stressors can also result in the production of reactive species (reviewed by Lushchak 2011). Aquatic environments include freshwater and marine systems, each bringing their own sets of constraints and challenges. In this section, we review the extrinsic factors associated with oxidative stress, and provide examples of the ways in which these factors may influence fish's levels of ROS.

Temperature

It is predicted that global climate change will increase water temperature by 2°C by the end of the 21st century (IPCC 2013), with the potential to severely impact physiological mechanisms across a wide range of organisms. It is believed that organisms living in thermally stable environments (e.g., Arctic and Antarctic regions) have a reduced ability to cope with the effects of increasing temperature (Huey and Hertz 1984), but how this temperature increase will affect cellular mechanisms is still poorly understood, especially those related to redox chemistry (Almroth et al. 2015). According to known thermodynamics principles, an increase in temperature is associated with an increased metabolic rate (Q10 Effect), leading to an increase in oxygen consumption, an increased flux at the level of the electron transport chain, and a greater production in ROS (Halliwell and Gutteridge 2015). Consequently, one would expect a higher production of ROS when water temperatures are higher. This is especially true for stenothermal fish species of the Arctic and Antarctic regions, which have very narrow thermal windows (Levins 1968). Life at the Poles has led to the evolution of a wide range of adaptations in fish, such as the increased presence of polyunsaturated fatty acids (PUFAs; Tomanek 2010) and increased mitochondrial density (Bottino et al. 1967; Fangue et al. 2009), in order to maintain energy production and metabolic rates at lower temperatures (Cheng and Detrich 2007). Antarctic icefish have also lost their red blood cells and hemoglobin over evolutionary time as a result in living in oxygen-rich Antarctic waters. While these mechanisms are adaptive at cold temperatures, increasing water temperatures may render them maladaptive, especially in the context of oxidative stress (e.g., increased production of ROS in the mitochondria and increased likelihood of oxidation for PUFAs; Halliwell and Gutteridge 2015; Reznick and Packer 1994).

A study on the bald notothen (*Pagothenia borchgrevinki*), a species endemic to Antarctica (Clarke and Johnston 1996), exposed fish to an increase in temperature and examined the effects of such increase on antioxidant defenses, protein and lipid damage and transcriptional regulation of genes involved in redox chemistry (Almroth et al. 2015). The study showed that acute (12 hour) exposure to increasing temperature led to increased antioxidant defenses, but that these levels were similar to baseline levels when fish were exposed to chronic temperature elevation (3 weeks). These findings were also accompanied by greater levels of oxidative damage suggesting that, although stenothermal fish can modulate their antioxidant capacity, they can only do so in a transient manner, the latter which remains insufficient in quenching the increased ROS production accompanying the increase in metabolic rate with increased temperature (Almroth et al. 2015). It has been suggested that the accumulation of damage products from oxidative stress can lead to a decrease in fitness and proper physiological function (Sohal 2002); a situation which is likely to occur with the predicted ocean warming temperature across the globe. Hofmann et al. (2000) found that in one Polar fish species (emerald rockcod *Trematomus bernacchii*) the ability to induce heat shock proteins (which are for example important to help detoxification from oxidized proteins) of all size classes following either thermal or chemical stress was absent, possibly lost during evolution in these cold and thermally stable environments. *In vivo* metabolic labelling experiments that involved injection of ^{35}S-labelled methionine and cysteine into whole fish previously subjected to a heat stress of 10°C yielded no evidence for synthesis of any size class of heat shock protein (Hofmann et al. 2000). *In vivo* labelling experiments with isolated hepatocytes similarly showed significant amounts of protein synthesis, but no indication of enhanced expression of any class of heat shock proteins. Induction of chemical stress through exposure to the heavy metal cadmium also failed to induce synthesis of heat shock proteins. However, Western analysis

revealed that both the inducible and constitutively expressed forms of Hsp 70 chaperones are present in this species (Hofmann et al. 2000).

Mitochondria of Antarctic icefish also have higher densities of phospholipids per mg of mitochondrial proteins compared with related species from temperate regions (O'Brien and Mueller 2010). The high density of lipid-rich mitochondria in oxidative muscles of icefishes enhances oxygen delivery in the absence of haemoglobin and myoglobin (O'Brien 2011). However, these phospholipids are rich in PUFAs, which place these fish at increased risk of oxidative damage in increased water temperatures. Additionally, it was found that several tissues in icefishes have lower levels of antioxidants compared with related temperate species (Witas et al. 1984; Cassini et al. 1993; Abele et al. 2012). Heart mitochondria of icefishes were shown to have more tightly coupled electron transfer than those of red-blooded fishes at 2 or 10°C, which increased the production of reactive species in icefishes when the electron transport chain was disrupted (Mueller et al. 2011). The activity of superoxide dismutase and the non-enzymatic antioxidant capacity per mg of mitochondrial proteins did not differ between icefishes and red-blooded species, but the non-enzymatic antioxidant capacity normalized to mitochondrial phospholipid content was significantly lower in icefishes than in red-blooded fishes. It was also found that the membrane susceptibility to peroxidation was only detectable in icefishes at 1°C and not in red-blooded species (Mueller et al. 2011). These constitutive differences contribute to make icefishes more vulnerable to oxidative stress than red-blooded temperate fishes when exposed to a thermal challenge. However, as with heat shock proteins, icefishes also appear to have lost the ability of upregulating antioxidant enzymes in response to oxidative stress. Mueller et al. (2012) found that levels of oxidized proteins and lipids increased in the heart ventricle of some icefishes but not in red-blooded species in response to warming. Despite an increase in oxidative damage in

hearts of icefishes, there was no activation of the antioxidant response: neither transcript levels nor activity of antioxidants increased in any tissue of any species in response to exposure to the critical thermal maximum (thermal limit above which the ambient temperature becomes lethal).

Recent studies on Antarctic Notothenioidei fish showed, however, that their antioxidants might have the potential to buffer oxidative damage induced by increased temperatures. Enzor and Place (2015) have examined the potential synergistic effects that increased water temperature and partial pressure of CO_2 have on the level of protein damage in *Trematomus bernacchii*, *Pagothenia borchgrevinki*, and *Trematomus newnesi*, and combined these measurements with changes in antioxidant enzymes (SOD and CAT). Their findings indicated that the activity of both SOD and CAT displayed only small changes across treatments and tissues. Short-term acclimation to decreased seawater pH and increased temperature resulted in significant increases in oxidative damage. Surprisingly, despite no significant change in antioxidant capacity, cellular damage returned to near basal levels, and in *T. bernacchii*, significantly decreased, after long-term acclimation. Although, these data suggest that notothenioid fish may have an antioxidant capacity necessary to offset predicted future ocean conditions, it remains unclear if this capacity comes with physiological trade-offs that would impact on fitness traits. While the above is generally true, studies have also shown that the opposite can also occur. For example, Malek et al. (2004) found that a decrease in ambient water temperature appeared to induce oxidative stress in the skeletal muscle of adult zebrafish by upregulating genes related to oxygen and ROS metabolism as well as the response to oxidative stress. Similarly, compared to gilthead sea breams (*Sparus aurata*) acclimated to 20°C, fish maintained for 10 days at 8°C had higher levels of liver oxidative damage (thiobarbituric acid reactive substances; TBARS) and nitric oxide, as well as downregulated antioxidant genes (Ibarz et al. 2010).

There exists a wide range of studies surveying the effects of temperature on oxidative stress markers and antioxidants. These studies show that, although polar fish species might be more sensitive to increased water temperatures, fish species from other regions are not out of risk. Bagnyukova et al. (2007) investigated the effects of a rapid transfer from cold to warm temperatures on oxidative damage as well as antioxidant defenses in goldfish (*Carassius auratus*), and found that increases and/or decreases in oxidative markers were highly dependent on the tissues. Furthermore, it was concluded that short-term exposure to warm temperature disturbed multiple oxidative stress indicators, but only marginally impacted antioxidant capacity. Heise et al. (2006a) showed that at critical and severe temperature elevations (considered critical and severe for the species), the North Sea eelpout (*Zoarces viviparous* L.) had increased oxidative damage markers (TBARS) and reduced enzymatic activity of SOD. Another study on the North Sea eelpout demonstrated that cold exposure increased oxidative stress parameters only during the recovery phase at control temperature, while antioxidant capacity remained stable throughout (Heise et al. 2006b). Lushchak and Bagnyukova (2006) showed that goldfish exposed to high temperature (35°C) had elevated SOD levels in the brain, liver and kidney. This effect was reversed when fish were allowed to recover at 21°C. The same study also found that SOD activity was highly correlated to levels of lipid peroxidation products, which suggests that these products might play a role in the upregulation of antioxidants. A study on the coastal fish, the Rock goby (*Gobius paganellus*) further demonstrated that, while some tissues (muscle, liver) may suffer increased oxidative damage when fish are exposed to increasing water temperatures, other tissues (gills) may be unresponsive because their basal antioxidant defences are already very high (Vinagre et al. 2014). These results might indicate that basal antioxidant protection is kept high in those tissues that may have a stronger impact on health and, ultimately, fitness. Altogether, these findings also

suggest that the effects of temperature on oxidative stress mechanisms may be tissue- and species-dependent and that species may have evolved different mechanisms to deal with the oxidative challenges induced by thermal stress. Finally, we should not forget that conspecific populations may also differ in the way they regulate their redox state, depending on the selective pressures under which they evolved. A nice example is provided by Nikinmaa et al. (2013), where the transcription and redox enzyme activities at a steady state and in response to an acute temperature change in three populations of three-spined sticklebacks (*Gasterosteus aculeatus*) were analysed. They found that there was divergence in markers linked to antioxidant response, indicating that processes regulating the redox balance may be important targets of selection underlying adaptive divergence in this fish species.

Oxygen availability

The introduction of molecular oxygen into Earth's atmosphere billions of years ago has allowed animals to produce energy more efficiently (Falkowski et al. 2004). With this new ability also came a dependence on oxygen and sensitivity to changes in oxygen availability for most organisms, though some species are exceptions. In fact, ectothermic animals have developed ways to cope and survive extreme oxygen conditions (Welker et al. 2013). In the 1990s, scientists started to observe an increase in antioxidant levels under hypoxic conditions, a phenomenon later coined "preparation for oxidative stress" by Hermes-Lima et al. (1998). More specifically, anoxia- and hypoxia-tolerant fish appear to have an anticipatory response during low-oxygen conditions by increasing their antioxidant capacity under low oxygen conditions (hypoxia, anoxia) to enhance their ability to quench ROS production upon return to normal oxygen concentrations (Lushchak and Bagnyukova 2006).

In the oceans, oxygen is not distributed evenly (Sewell and Fage 1948). Deep sea regions are often characterized as poorly oxygenated areas, and may provide refuges against oxidative stress for deep sea fish (Janssens et al. 2000). Furthermore, increased depth is associated with a decrease in metabolic rate (Childress 1995), resulting in lower ROS production in body tissues. Consequently, one could expect that deep sea fish face less oxidative stress, offering opportunities to study the mechanisms that evolved to regulate oxidative balance under low oxygen conditions. Janssens et al. (2000) found that reduced metabolic needs in deep sea fish were linked to lower antioxidant activities of SOD and GPX, but that CAT activity was neither affected by species depth nor metabolic rate. These findings suggest that SOD and GPX are primarily used against metabolically induced ROS, while CAT likely serves another purpose (Janssens et al. 2000). In fish that do not live in the deep sea, hypoxic conditions also appear to induce increased antioxidant activity, most notably that of SOD and catalase (in goldfish liver (Lushchak et al. 2001), and in common carp (*Cyprinus carpio*) liver (Lushchak et al. 2005b)).

In general, the production of ROS is correlated to the amount of oxygen within an organism (Jamieson et al. 1986). Logically, an increase in oxygen levels increases the chance of electrons to escape the complexes within the electron transport chain, and consequently form ROS. Fish have evolved ways to either avoid environments too rich in oxygen or to intrinsically reduce their efficiency for extracting environmental oxygen (Lushchak 2011). Goldfish (*C. auratus*) exposed to hyperoxic conditions experienced greater levels of oxidative stress (Lushchak et al. 2005a). Similar observations were made in Atlantic salmon (*Salmo salar*; Olsvik et al. 2005) and Senegal sole (*Solea senegalensis*; Salas-Leiton et al. 2009). Hypoxic conditions can also induce increased activities of CAT and GPX in goldfish liver and brain, respectively (Lushchak et al. 2001). Similar observations were found in the common carp (*Cyprinus carpio*) liver (Lushchak et al. 2005b).

Oehlers et al. (2007) also showed that glutathione S-transferase (GST) levels increased under low oxygen availability in medaka (*Oryzias latipes*). Hypoxia was found to increase oxidative stress in the rotan (*Perccottus glenii*) by increasing protein carbonyls in the brain, liver and skeletal muscle relative to control fish (Lushchak and Bagnyukova 2007). Additionally, lipid peroxidation products increased in all tissues, while thiols appeared to decrease with hypoxia. SOD increased significantly in the liver of hypoxic fish, while the activity of other enzymes appears to have decreased during hypoxia.

While the majority of studies demonstrate that hyperoxic conditions lead to increases in oxidative stress, some studies also show the opposite trend to be true. It is possible that oxidative stress, in the context of oxygen availability, is somewhat species-dependent. For example, fish inhabiting estuarine environments are exposed daily to episodes of anoxia and reoxygenation because they become exposed to air during low tides and their tissues are reoxygenated when the water level increases during high tides. Cycles of dissolved oxygen in estuaries can range from anoxia (complete lack of oxygen), hypoxia (oxygen concentrations lower than normoxia) to various levels of supersaturation (200–300 % higher than normoxia) over short time periods. Estuarine fish, as well as estuarine invertebrates, have evolved several metabolic adaptations to tolerate short-term anoxia, such as the use of fermentable fuels to produce energy and allow depression of metabolic rate (Storey and Storey 1990; Brooks and Storey 1997; Ross et al. 2001).

Salinity

In marine environments, the absorption of solar radiation as well as the presence of hydrothermal vents cause the greatest production of ROS (Mopper and Kieber 2000; Tapley et al. 1999). Marine fish species tend to experience additional environmental changes in comparison to freshwater fish due to the highly variable environment. Many fish species will undergo large

salinity changes due to their life history, for example as they migrate from freshwater rivers into marine waters (e.g., salmonids), or as they migrate on a global scale from waters with high to low salinity and vice versa (e.g., tuna, billfish). Salinity changes impose physiological and behavioural responses such as osmoregulatory demands. There is evidence that the stress associated by changes in salinity causes an increase in the production of ROS (Liu et al. 2007).

When olive flounder (*Paralichthys olivaceus*) were exposed to seawater for a 48h period, GPX and GST activity levels increased, demonstrating their role in quenching the increased production of ROS (Choi et al. 2008). Enzyme activities for CAT, GPX and SOD were measured in sturgeons (*Acipenser naccarii*) gradually acclimated to seawater. After 20 days spent in sea water, muscle water content, plasma osmolality and cellular constants had returned to normal, indicating that osmoregulatory processes were at work. However, cortisol levels, antioxidant activity and lipid peroxidation showed abnormal values, demonstrating that osmoregulation had caused significant physiological effects, consequently increasing oxidative stress (Martinez-Alvarez et al. 2002). Taken together, these findings suggest that changes in salinity induce oxidative stress in fish. Fish that undergo freshwater to marine transitions, and vise versa, may have evolved ways to cope with the increased ROS production imposed by this transition such as an anticipatory antioxidant build up in the months prior to migration (Birnie-Gauvin et al. *in prep*). Furthermore, we may expect that pelagic fish are less resistant to oxidative stress given that they do not undergo these periodic changes in salinity, however this kind of comparative research is lacking in the field.

Emerging threats: pollution and land use changes

Aquatic environments are increasingly receiving pollutants in the form of agricultural and industrial chemicals which, being taken up by fish, may initiate free radical processes (reviewed

in Lushchak 2011). Often these contaminants form radical species themselves, either through chemical or metabolic conversions. Mercury represents a serious contaminant, especially in tropical environments where gold mining results in the spreading of mercury (Malm 1998). Monteiro et al. (2010) found that mercury exposure leads to elevated levels of oxidative stress in matrinxã (*Brycon amazonicus*). Additionally, there findings suggest that oxidative processes may be the main pathway by which contaminants induce toxicity in fish. Catfish (*Channa punctatus* Bloch) exposed to paper mill effluent, both in the short-term and the long-term, showed a time-dependent increase in glutathione levels, GPX activity and GST activity in the liver (Ahmad et al. 2000). The same study also found that gills and kidney were less resistant to oxidative damage due to a lower antioxidant capacity. Oxidative stress was studied in the red blood cells of Nile tilapia (*Oreochromis niloticus*) in fish from a fish farm and from a polluted area of a reservoir (Bainy et al. 1996). Erythrocytes were found to be more susceptible to oxidative damage in fish from the polluted sites due to an increased oxygen uptake. The same fish showed an increase in SOD activity, and a decrease in both CAT activity and glutathione content; all of which are indicative of oxidative stress. Similar results were found in African catfish (*Clarias gariepinus*) obtained from the Ogun River of Nigeria, heavily polluted by heavy metals from local industries (Farombi et al. 2007), where SOD activity increased in liver, kidney and heart, but decreased in gills, and a decrease in CAT activity was consistently observed. Glutathione concentrations also increased in liver, kidney and heart, but decreased in gills. A similar study revealed that Indian freshwater fish (*Wallago attu*) from the highly polluted Panipat site had significantly higher levels of oxidative stress markers than fish from the less polluted Agra site (Pandey et al. 2003); a finding which was also observed in the European eel, in a tissue-specific manner (Ahmad et al. 2006), and the pearl cichlid (*Geophagus brasiliensis*, Wilhem Filho et al. 2001). Data obtained from such comparative

studies demonstrate that glutathione systems are a sensitive biochemical indicator of pollution exposure, and can be used to measure susceptibility to toxins (Almar et al. 1998).

Silver nanoparticles are becoming a growing concern in aquatic environments. To test their consequences on fish, Choi et al. (2010) exposed adult zebrafish (*Danio rerio*) to silver nanoparticles, and showed increased levels of malondialdehyde (a by-product of lipid peroxidation and measured by the TBARS assay) and total glutathione, as well as a decrease in both CAT and GPX activities. In addition, an induction of DNA damage was observed. These findings suggest that silver nanoparticle toxicity is associated with oxidative stress in zebrafish under laboratory conditions. Copper (Cu) is another element that tends to accumulate in fish tissues, generally at higher doses than in the environment (Chevreuil et al. 1995). Cu is an excellent Fenton reagent, with a greater ability than iron to convert H_2O_2 to the hydroxyl radical. In the eel, a single 24h exposure to Cu (2.5μM) caused a significant increase in GST concentration and a significant decrease in reduced glutathione content in gills (Ahmad et al. 2005). In kidney tissues, Cu exposure led to decreases in CAT activity, reduced glutathione and GPX activity, as well as an increase in GST activity. These results provide evidence that exposure to heavy metals in fish can unsettle the redox balance and alter antioxidant defences.

Pesticides have been shown to cause adverse effects in fish as they find their way into water systems (reviewed in Slaninova et al. 2009). Organochlorine pesticides were banned in many countries in the 1970s (still used in come tropical countries however) but their residues remain in the environment to this day. A prime example of such pesticide is dichlorodiphenyltrichloroethane (DDT) which causes an accumulation of lipid peroxides and protein carbonyls in fish (Grune 2000; Dalle-Donne et al. 2003). Fenthion, an organophosphate, causes large elevations of oxidized glutathione (GSSG; an indicator of oxidative stress) in the brain of Nile tilapia (*Oreochromis*

niloticus) at sublethal concentrations (Piner et al. 2007). A wide range of pesticides were found to have similar impacts in fish (reviewed in Slaninova et al. 2009). Aquatic environments are affected by other anthropogenic contaminants like xenobiotics, which can exert effects on the redox chemistry of fish (Rajkumar and Milton 2011). Ekambaram et al. (2014) investigated such effects in the brain tissue of flathead grey mullet (*Mugil cephalus*) and found that fish exposed to xenobiotics had significant increases in lipid peroxides and protein carbonyls, along with decreases in reduced glutathione and total antioxidant capacity. This suggested that water pollutants can increase oxidative damage and reduce antioxidant systems in fish. Another noxious pollutant of concern in aquatic ecosystems is fly ash leachate, which can have severe physiological effects on fish (Ghio et al. 2002). Spotted snakehead (*Channa Punctata*) exposed to fly ash leachate had higher lipid peroxidation and higher antioxidant enzyme activities than control fish. These effects were found to be most prevalent in gill tissues, suggesting that gills are the most vulnerable to pollutants (Ali et al. 2004).

Recent studies have shown that landscape modifications, like urbanization and agriculture, can have serious negative repercussions on adjacent aquatic environments (Allan 2004). More specifically, these landscape alterations can impact nutrient inputs, dissolved oxygen concentration and temperature regimes (Meador and Goldstein 2003) which, in turn, can impact community structure and biodiversity (Allan 2004). Physiological metrics have been used to evaluate the extent of anthropogenic impacts on individuals. For example, blood-based indicators (i.e., glucocorticoids, oxidative stress indicators) are highly sensitive to stressors associated with human activities and environmental change (Barton 2002; Cooke and Suski 2008).

To date, very few studies have considered changes in land use as drivers for free radical processes. However, Blevins et al. (2013) found that creek chub (*Semotilus atromaculatus*) from

agricultural and forested streams displayed no significant differences in glutathione concentration after high temperature exposure and acclimation to low temperature. A similar study by Blevins et al. (2014) found that creek chub from two stream types did not differ in plasma glutathione concentrations. Fish that swim in urban river systems are often exposed to high levels of contaminants, combined with the increased flow rates associated with channelized rivers (Winter et al. 2004); two factors which have the potential cause oxidative stress (Aniagu et al. 2006). While no studies that we know of have investigated this particular association, fish that were initially exercised to exhaustion had much lower performances when they were previously exposed to a polluted site (McKenzie et al. 2006).

Brinkmann et al. (2010) investigated the effects of sediment re-suspension on oxidative markers in rainbow trout by simulating a 5-day flood event; a phenomenon becoming more and more common in today's changing climate. While this approach demonstrated that sediment re-mobilization leads to the uptake of sediment-bound pollutants, there were no significant alterations in oxidative enzymatic activities. It was suggested that a 5-day event may not be sufficient to allow for the detection of changes at the protein and enzyme level. Alternatively, it is possible that antioxidants levels were sufficiently high to buffer against the oxidative stress levels in the rainbow trout utilized in this study.

The impact of contaminants on the oxidative balance might also depend on the interaction with other stressors. For example, under normoxia, exposure of silver catfish (*Rhamdia quelen*) to manganese increased lipid peroxidation in brain and kidney, increased glutathione in brain and decreased CAT activity in both brain and kidney (Dolci et al. 2013). Conversely, moderate hypoxia was able to prevent manganese-induced lipid peroxidation in brain and to reduce it in kidney;

glutathione was increased in brain, while activity of CAT was reduced in kidney and brain tissues (Dolci et al. 2013).

These findings suggest that in general, pollution of aquatic environments is reflected physiologically in fish by perturbing normal free radical processes, leading to increases in oxidative damage and disturbance of antioxidant defences.

Life histories and other intrinsic factors associated with oxidative stress

Life history theory is based on the assumption that increased allocation of resources into one function results in the diversion of resources from other functions (Stearns 1992). A classic example is reproductive trade-offs where increased breeding efforts result in lower future fecundity or survival (Stearns 1992). Other life history traits can be viewed using the same principle. An example of this is migration; investing more energy reserves into migration diverts such reserves from other functions such as growth, reproduction and survival. While there is extensive literature linking oxidative stress to life histories in birds (Costantini 2008) and mammals (Martin and Grotewiel 2006), there is a lack of literature regarding fish species. Recently, the role of ROS has received considerable attention as one of the key players in determining life-history strategies among various animal taxa.

Reproductive strategy

When resources are allocated to reproduction, an organism can no longer use these resources (e.g., energy, time, nutrients, etc.) for self-maintenance. The costs associated with reproduction have been widely studied and documented (Reznick 1992), though the underlying causes of these costs have only recently been investigated (Zera and Harshman 2001). Reproduction is a highly demanding activity which elevates metabolic rate for an extended period

of time, and likely induces an increase production in ROS (Alonso-Alvarez et al. 2004). However, fish (and other organisms) have evolved different breeding styles, each accompanied by their own benefits and constraints. Semelparity is defined by a single breeding event, after which an individual will die. In this case, fish may not invest resources into generating strong antioxidant defenses at all because all resources have been allocated to a single reproductive event. One might expect antioxidant capacity to be quite low during spawning of semelparous fish species. In pink salmon (*Oncorhynchus gorbuscha*), oxidative damage and antioxidant capacity changed on a tissue-specific basis during spawning migration (Wilson et al. 2014). More specifically, both DNA damage and antioxidant capacity were lower at the spawning grounds when compared to similar parameters from fish at the river entrance. These results demonstrate that oxidative stress, as well as antioxidant defense and repair, are tissue-specific during spawning migration, and support the prediction that semelparity is associated with little resource allocation toward antioxidant defenses. Alternatively, fish may be attempting to lower oxidative damage by consuming more antioxidants in order to produce eggs of higher quality.

As a life history strategy, fish may reduce investments in their own protection in favor of egg antioxidant protection which is essential for hatching success and survival chances (Fontagné et al. 2006). Taylor et al. (2015) measured oxidative stress indicators and antioxidants in the plasma, heart, brain and liver of adult female sockeye salmon (*Oncorhynchus nerka*) and developing offspring from three distinct populations from the Fraser river. The study revealed that oxidative stress and antioxidant status in the offspring were not affected by maternal oxidative stress. Rather, it appears that offspring produce their antioxidants (mainly glutathione) endogenously in the later stages of development, probably due to the fact that semelparous mothers invest the better part of their resources into spawning migration rather than antioxidant protection

during the migration (Taylor et al. 2015). Other studies have demonstrated that newly hatched fish and embryos rely heavily on maternal antioxidants, and subsequently generate endogenous antioxidants with time (Fontagné et al. 2006; Hung et al. 1981; Koshio et al. 1994).

In contrast, iteroparity is defined by multiple breeding events throughout life. Iteroparous species are expected to be less sensitive to the resource-based trade-off associated with reproduction (Stearns 1992). The first evidence of an association between reproduction and antioxidant defenses in iteroparous species was shown in zebra finches (Alonso-Alvarez et al. 2004). The study demonstrated that reproduction decreases antioxidant defences, which may suggest that oxidative stress is a cost of reproduction. One could assume a similar pattern in fish, but evidence for this is lacking. Despite its scarcity, studies linking oxidative stress to life histories in iteroparous fish do exist. In smallmouth bass (*Micropterus dolomieu*), a study revealed that paternal care was negatively correlated with oxidative stress resistance, but oxidative stress markers (lipid peroxides, protein carbonyl groups and 8-hydroxy-2-deoxyguanosine (8-OHdG)) did not increase as a result of parental care (Wilson et al. 2012). The cumulative effect of oxidative stress across the reproductive lifespan of an organism has been suggested to be the cause of the age-associated decline in performance observed in iteroparous species (Kirkwood and Austad 2000).

Individual strategies of investing in mate choice may also result in different oxidative stress states. For example, Pike et al. (2007) showed that male sticklebacks (*Gasterosteus aculeatus*) fed on a low-carotenoid diet allocated more carotenoids to their nuptial colouration in an attempt to maintain sexual attractiveness. Not only were they not chosen by females in mate choice trials, but also their capital investment in sexual colouration was paid in terms of higher oxidative damage and lower survival (Pike et al. 2007).

Diet and food deprivation

In recent years, the term dietary oxidative stress has been coined to describe an imbalance between pro-oxidants and antioxidants that results from an insufficient supply of nutrients (Sies et al. 2005). Additionally, it has been suggested that the extent to which oxidative damage is imposed on organisms is directly related to the ability to produce antioxidants and their effectiveness in oxidative stress defenses (Diguiseppi and Fridovich 1984). Many antioxidants contributing to a fish's antioxidant capacity originate from its diet, especially fat-soluble antioxidants as they cannot be synthesized *de novo* by animals (Goodwin 1984). Consequently, we may expect fish with different diets to cope with oxidative stress in different ways.

Selenium is an essential micronutrient used in many physiological functions, such as growth, development and antioxidant defences (Rayman 2000). It is a component of GPXs and thioredoxin reductases among many others, giving it a crucial role for cellular protection against oxidative stress (Rayman 2000). Fontagné-Dicharry et al. (2015) demonstrated that plant-based diets should be supplemented with selenium in rainbow trout fry (*Oncorhynchus mykiss*). Fish supplemented with selenium yeast had a significantly higher reduced:oxidized glutathione ratio compared to fish that were fed with sodium selenite or non-supplemental diets. Furthermore, Rider et al. (2009) found that supra-supplementation of selenium yeast did not affect oxidative stress prior to a exposure to a stressor in the form of daily handling and confinement. However, a trend toward higher GPX post-stress in selenium supplemented rainbow trout was observed. These findings suggest that physical stressors can increase the rate of selenium utilisation and, consequently, commercial fish diets should be supplemented with selenium (Rider et al. 2009). Similar findings were also observed in the crucian carp (*Carassius auratus gibelio,* Zhou et al. 2009).

Radi et al. (1985) found that SOD activity was generally higher in herbivorous fish (grass carp (*Ctenopharyngodon idella*) and silver carp (*Hypophthalmichthys molitrix*)) than in omnivorous fish (barbel (*Barbus barbus*), crucian carp (*C. carassius*) and common carp (*C. carpio*)), suggesting that plant-based diets may have micronutrients which are absent or in lower levels in other food sources. In the Senegalese sole (*S. senegalensis*), the activity of CAT and SOD were found to be significantly higher in the liver of fish fed high lipid diets, while low-lipid diets appeared to minimize susceptibility to oxidative stress (Rueda-Jasso et al. 2004).

Food deprivation can also have a wide range of physiological impacts (e.g., accelerated ageing, toxicity to chemicals), most of which can be attributed to the production of ROS (Robinson et al. 1997). For example, in the gilt-head bream (*Sparus aurata*), it was found that both complete and partial food deprivation for a 3-week period significantly increased malondialdehyde (a measure of lipid peroxidation by the TBARS assay) and GSSG levels (Pascual et al. 2003). The same study also showed that these levels returned to normal levels when fish were returned to control conditions (2% w/w food/fish). Similar results were obtained in a study on common dentex (*Dentex dentex*) where fish deprived of food for 5 weeks showed significant increases in lipid peroxidation, SOD, CAT and GPX and a significant decrease in GR activity (Morales et al. 2004). Taken together, these findings suggest that omnivorous and herbivorous fish may cope with oxidative stress differently by using different components of their diet to generate antioxidants. These findings also show that changes in food availability or food quality during an organism's lifetime may impact its ability to produce antioxidants, defend against oxidative stress and have impacts on its fitness.

Physical activity

It was postulated in 1988 by Alessio and Goldfarb that exercise may cause oxidative stress due to an increase in oxygen metabolism at the level of the mitochondria. This association has been experimentally shown in mammalian studies (Bejma and Ji 1999), but has only been studied in fish twice that we know of. After exhaustive exercise, adult chub (*Leuciscus cephalus*) had reduced antioxidant capacity (SOD and GSH concentration) compared to non-exercised control fish (Aniagu et al. 2006). Exercised fish also showed significantly higher DNA damage, but demonstrated no significant changes in SOD activity or GSH concentration relative to control fish. The authors reported that the degree of oxidative stress in exercised fish may not have been sufficient to elicit a defensive response or that the antioxidant defense mechanisms may have been evoked at a different timescale than the one used for the exhaustive swim and the tissue sampling. It is also possible that the antioxidant defenses were sufficient to handle the small amounts of oxidative stress which occurs during exercise, but not exhaustive swim.

In another study, Amérand et al. (2010) investigated the effects of exposure to 10.1 MPa (equivalent to 1,000 m in depth) hydrostatic pressure on silver European eel oxidative stress parameters. The pressure exposure, which is associated with eel spawning migration, resulted in an inverse correlation between ROS production and metabolic rate, suggesting that oxygen consumption can be elevated without the harmful oxidative damage, usually sustained during increased metabolic rate associated with continuous swimming (Amérand et al. 2010).

While the link between oxidative stress and physical activity may appear to be obvious, evidence suggests that the association may be more complex than first thought in fish. The few studies on the topic have observed variable results in response to different degrees of exercise, limiting the generality of their conclusions. Physical effort in fish is often associated with

important aspects of fitness and survival such as reproduction or predator avoidance. Given our current lack of understanding of the links between physical activity and oxidative stress parameters, further studies are needed.

Aging and senescence

The idea that aging and oxidative stress are linked has been around for decades (Harman 1956). As a result, a shift in the delicate balance between the production of ROS and antioxidant defences leads to a deterioration of physiological functions, and is in fact thought to be the major cause of senescence (Murphy et al. 2011). However, very few studies investigated the effects of age on antioxidant protection in fish. The free-radical theory of ageing emerged more than 50 years ago, postulating that the production of ROS increases with age, and that antioxidant defenses decline (Harman 1956). While this link has been established in humans, very little information is available for fish and the correlation between antioxidant defenses and age remains unclear (Martinez-Alvarez et al. 2005).

Wdzieczak et al. (1982) found that the younger fish of various species (roach [*Rutilus rutilus* L.], carp [*Cyprinus carpio* L.], burbot [*Lota lota* L.], the bream [*Abramis brama* L.], the perch [*Perca fluviatilis*], silver carp [*Hypophthalmichthys molitrix* Vol.], sea bass [*Dicentrarchus labrax*], eel [*Anguilla Anguilla*], and scorpion fish [*Scorpaena porcus*]) had higher antioxidant capacity than older fish. Otto and Moon (1996) compared the enzymatic activity of antioxidants in rainbow trout (*O. mykiss*) and black bullhead (*Ameiurus melas*) from two age classes; 1+ and 3+. They found that the activities of SOD and GR declined with age in liver and extrahepatic tissues, while age-associated changes were not observed for GPX and CAT. In the freshwater murrel (*Channa punctatus*), brain and liver GPX activity decreased throughout maturation but not during senescence where enzyme activities appeared to increase in the liver and remain stable in

the brain (Nayak et al. 1999). Similarly, Sanz et al. (2001) found that the activities of GPX and CAT increased with age in the plasma and erythrocytes of sturgeon (*A. naccarii*). The broad spectrum of observations described here suggests that long-lived species, such as the sturgeon, have evolved ways to increase their antioxidant capacity with age, while other more short-lived species appear to have a relatively constant decline in antioxidant defenses throughout life. These differences could play a significant role in expanding the lifespan of iconic long-lived fish species. The aforementioned studies are cross-sectional. We highlight the importance of doing longitudinal studies here, in order to better understand how life-history trajectories are linked to oxidative status regulation.

Summary and research needs

Fish are amongst some of the most diverse (Helfman et al. 2009) yet imperiled groups of organisms on Earth (Richter et al. 1997). Consequently, it is particularly relevant to study fish to understand how they will cope with future environmental change, and to understand the underlying physiological mechanisms associated with life history strategies. Oxidative stress, as well as antioxidant defenses, can be modulated in complex ways, and may play a key role in the coping mechanisms that fish will use to adapt to environmental variability. Based on our review, it became apparent that life histories of fish are likely to be significantly influenced by oxidative stress and antioxidant capacity through interactions with growth, reproduction, and body maintenance, and hence future fitness and survival (Costantini 2008; Metcalfe and Alonso-Alvarez 2010, Figure 2-1).

The last decade has seen explosive development in the field of ecological oxidative stress (see Speakman et al. 2015). However, birds (Wiersma et al. 2004; Costantini 2008) and mammals (Bergeron et al. 2011; Castillo et al. 2005) continue to be the best-studied taxonomic groups. Fish

typically have more variable reproductive strategies and environments in which they can live, in comparison to other species (e.g., warm vs cold; well oxygenated vs poorly oxygenated). Given these circumstances, fish may have evolved ways to cope with oxidative stress that differ from mechanisms observed in other taxa. Furthermore, fish may offset the costs of oxidative processes due to their indeterminate growth (see Charnov and Berrigan 1991) compared to species with determinate growth patterns. In addition, most studies investigating life histories have been correlative, with contradicting results, and few have been carried out in the wild (Costantini 2014; Speakman et al. 2015). The need for more field studies is pressing, especially within ectotherms (including fish), which would provide a more complete understanding of the implications of oxidative stress in the wild. There is also a need for a wider range of diversity of model species which may yield unexpected insights in the field, and help partition contrasting results in the current literature. There is a need for additional research with non-lethal endpoints, especially in the context of life-histories; following changes in oxidative status of a fish over time could be highly informative. In the last two decades, only 117 research articles have been published specifically on the oxidative ecology of fish (Figure 2-2), suggesting that this area of research is largely unexplored.

Furthermore, no studies that we know of have used a comparative approach to investigate differences in oxidative coping mechanisms among fish with different life cycles, predatory tactics, feeding strategies, and that live in different habitats. Evidence also suggests that oxidative parameters can be used to monitor population health, though the majority of studies investigating that link have focused on birds (e.g., Beaulieu et al. 2013). Given that antioxidant defenses are typically associated with fertility and survival while oxidative stress negatively affects reproduction and growth, the use of fish as models to study oxidative parameters to examine

population health would be valuable. Experimental approaches are also highly valuable in providing fundamental information on oxidative stress mechanisms. Further research is needed to validate the development of techniques to experimentally manipulate oxidative status and antioxidant capacity (e.g., buthionine sulfoximine and N-acetylcysteine manipulation) in fish (e.g., reviewed in Kock and Hill 2016). For the aforementioned reasons, research on oxidative stress in fish is essential to fully understand redox chemistry in an ecological context.

Tables

Table 2-1. Oxidative stress measures commonly used in fish biology.

OS Biomarker	Method of detection	Applications
Protein carbonyls	Protein carbonyl formation (Levine et al. 1990; Stadman and Berlett 1997)	Levels of protein damage, fragmentation; insight into overall oxidative stress levels
DNA damage	8-hydroxy-2'-deoxyguanosine assay (Kasai 1997)	Levels of DNA damage; insight into overall oxidative stress levels
Lipid peroxidation	Thiobarbituric acid reactive substances test (Draper et al. 1993)	Levels of lipid damage; insight into overall oxidative stress levels
Catalase (CAT)	CAT enzymatic activity assay (Sinha et al. 1972)	Insight into antioxidant defenses; higher activities may be associated with higher levels of H_2O_2
Superoxide dismutase (SOD)	SOD activity assay (Beauchamp and Fridovich 1971; Oyanagui 1984)	Insight into antioxidant defenses; higher activities may be associated with higher levels of $O_2^{\bullet -}$
Glutathione peroxidase (GPX)	GPX activity assay (Paglia and Valentine 1967; Flohé and Günzler 1984)	Insight into antioxidant defenses; higher activities may be associated with higher levels of ROS
Glutathione reductase (GR)	GR activity assay (Carlberg and Mannervik 1975; Wheeler et al. 1990)	Insight into antioxidant defenses; GR reduces GSSG back to GSH; higher activities of GR may be associated with higher levels of GSSG
Glutathione (GSH); total glutathione (TGSH); glutathione disulfide (GSSG)	Glutathione assay (Akerboom and Sies 1981; Smith et al. 1988)	Provides insight into oxidative damage (GSH to GSSG ratio or vice versa) and antioxidant defenses (GSH)
Vitamin C (ascorbic acid)	Ascorbic acid assay (Roe and Kuether 1943; Deutsch and Weeks 1965)	Insight into antioxidant defenses (provides an electron to quench ROS)
Vitamin E (α-tocopherol)	Vitamin E assay (Prieto et al. 1999)	Insight into antioxidant defenses (peroxyl radical scavenger)
Low molecular weight antioxidants	Oxygen radical absorbance capacity (ORAC) assay (Cao et al. 1993)	Insight into total low molecular weight antioxidant defenses.

Figures

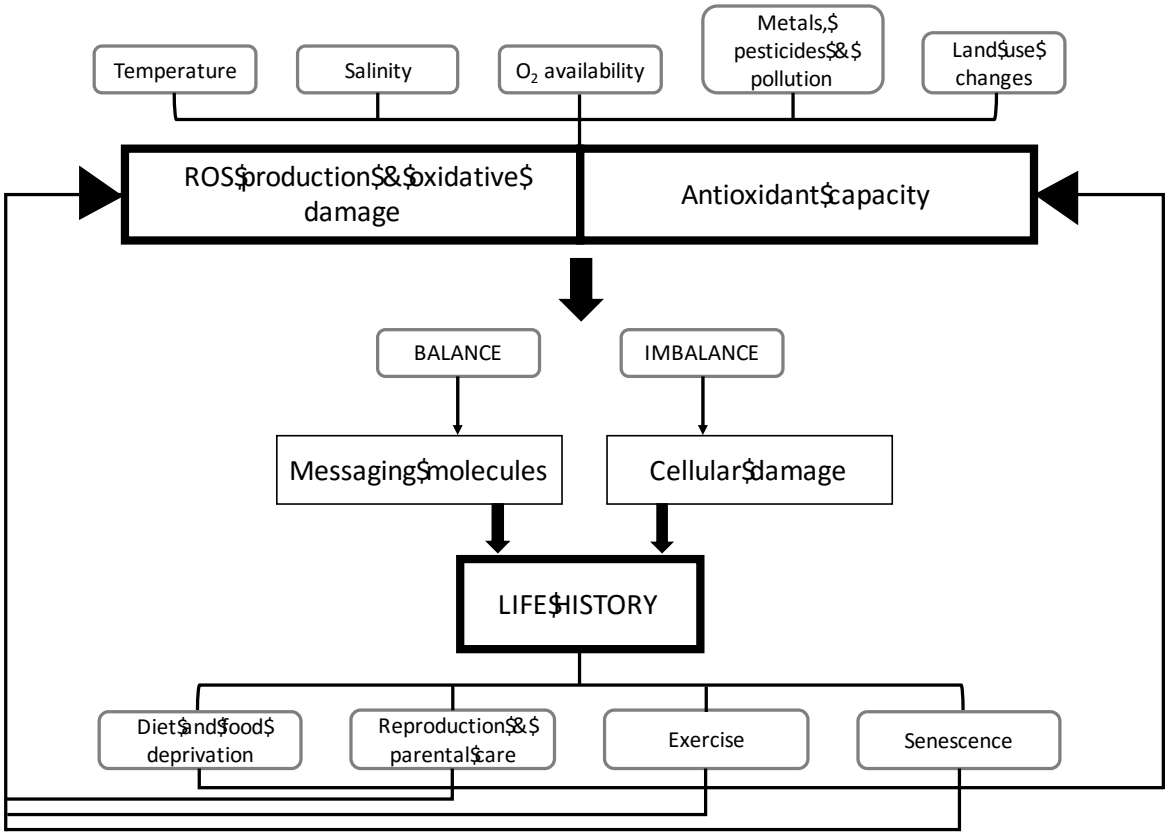

Figure 2-1. Interaction of intrinsic and extrinsic factors on oxidative stress in fish. The interplay between environmental factors and biological (life-history) factors in the context of oxidative biology. The level of oxidative stress experienced by a fish likely plays an important role in shaping its life history strategies, which in turn affects the type of environment the fish lives in.

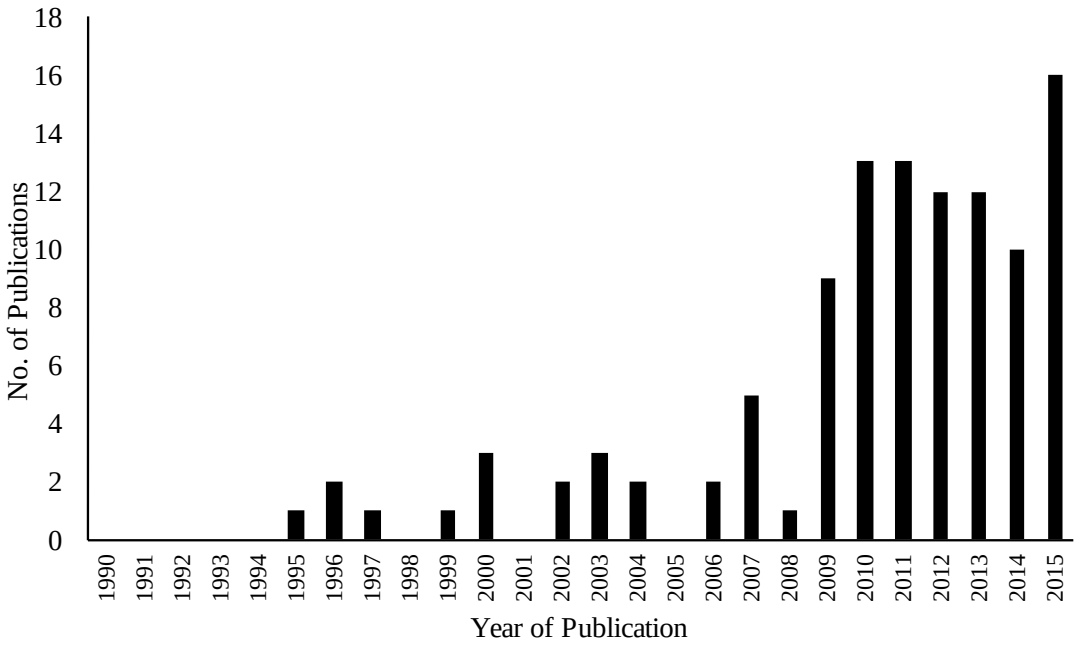

Figure 2-2. Publications in oxidative ecology of fish. Number of publications per year obtained from a Web of Science search within the years of 1900 to 2015, using the following Boolean search: ((fish*)AND(oxidative stress))OR((fish*)AND(antioxidant*)). The search was further refined for the fields of Ecology and Evolutionary Biology. The results were then processed to identify relevant studies using keywords within the article titles and abstracts.

Chapter 3: Short-term and long-term effects of exogenous cortisol manipulation on oxidative stress in juvenile brown trout

Abstract

In the wild, animals are exposed to a growing number of stressors with increasing frequency and intensity as a result of human activities and human-induced environmental change. To fully understand how wild organisms are affected by stressors, it is crucial to understand the physiology that underlies an organism's response to a stressor. Prolonged levels of elevated glucocorticoids are associated with a state of chronic stress and decreased fitness. Exogenous glucocorticoid manipulation reduces an individual's ability to forage, avoid predators and grow, thereby limiting the resources available for the defence against oxidative stress. Using the brown trout (*Salmo trutta*), we evaluated the short-term (2 weeks) and long-term (4 months over winter) effects of exogenous cortisol manipulations (as well as relevant shams and controls) on the oxidative status of wild juveniles. Cortisol caused an increase in glutathione over a two-week period but a reduction in glutathione overwinter. Cortisol treatment did not affect oxidative stress nor low-molecular weight antioxidants. Cortisol caused a significant decrease in growth rates in the short- and long-term. Overwinter survival in the stream was associated with low levels of oxidative stress and glutathione. Thus, transient exogenous cortisol administration does impact oxidative status in wild juvenile brown trout, and survival may be linked to low oxidative stress levels.

Introduction

Wild animals are constantly exposed to intrinsic and extrinsic stressors that challenge their homeostatic balance, which arise from anthropogenic (e.g. climate change, habitat disturbances) and natural (e.g. predation, social interactions, disease and nutritional limitations) sources (Johnstone et al., 2012; Boonstra, 2013b). To fully understand how organisms in the wild are affected by stressors, it is crucial to understand the physiology that underlies an organism's response (Baker et al., 2013; Dantzer et al., 2014). Although there has been much work on this topic, most has been done in laboratory settings (reviewed in Barton and Iwama, 1991; Barton 2002; Sopinka et al. in press), and much less is known about how wild animals in their natural environment respond to different stressors (Boonstra, 2013a). The neuroendocrine system is responsible for translating environmental signals into physiological and behavioural responses (Denver et al., 2009). It is therefore reasonable to assume that all mechanisms by which an individual responds to environmental stressors are mediated by hormones (Sapolsky et al., 2000). Understanding these basic underpinning concepts is crucial to discern the links between stressors and their impacts on behaviour, survival and life history trade-offs (Denver et al., 2009; Ball and Balthazart, 2008).

Stressful events lead to the activation of the hypothalamic-pituitary-interrenal (HPI) axis in fish. Circulating levels of glucocorticosteroid (GC) hormones such as cortisol increase rapidly, followed by the mobilization of fatty acids and liver glycogen to provide energy resources to cope with the stressor (Barton, 2002; Mileva et al., 2009). The purpose of this complex cascade of events is to re-establish homeostasis and it initiates both physiological and behavioural responses (Barton, 2002). However, prolonged elevation of cortisol can have detrimental effects as resources are diverted from other activities to cause individuals to focus on survival, having

important consequences on behaviour and ultimately, life-history trade-offs (Wingfield et al., 1998).

Stressful conditions generate important ecological pressures, modulating adaptive responses in natural populations (Romero, 2004). In recent years, much attention has been given to the role of redox chemistry in the context of life-history theory (Metcalfe and Alonso-Alvarez, 2010; Speakman et al., 2015). However, there is a growing interest in the study of oxidative stress in an ecological context (i.e., oxidative ecology; Beaulieu et al., 2013). Reactive oxygen species (ROS) production and an animal's capacity to fight oxidative stress varies depending on developmental stage, environmental conditions and life-history strategy (reviewed in Metcalfe and Alonso-Alvarez, 2010). Redox homeostasis is preserved by a balance between pro-oxidants and antioxidants (Sies, 1991). Once disturbed in favor of pro-oxidants for prolonged periods, chronic oxidative stress may arise (Finkel and Holbrook, 2000). As part of an organism's physiological mechanisms to maintain homeostasis, GCs can be used to signal the presence of a physiological stress condition (Dantzer et al., 2014). However, few studies have investigated the link between oxidative stress and GCs. To date, research has focused on mammalian and avian studies over short-term periods (days to weeks; Alonso-Alvarez et al., 2004; Costantini, 2008). Oxidative damage occurs as a result of an imbalance between pro-oxidants and antioxidants (Sies, 1991). ROS produced as a result of aerobic metabolism remain unquenched by antioxidant defences and negatively impact the cell (Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010). The physiological effects of ROS include severe damage to most macromolecules (i.e., DNA, RNA, proteins and lipids; Asada and Takahashi, 1987), decreased fertility (Halliwell and Gutteridge, 1999), and accelerated cellular aging, all of which are accompanied by a decrease in survival probability (Haenold et al., 2005; Monaghan et al., 2009). Several studies have

demonstrated that an organism's level of oxidative stress can be impacted by the ecological conditions which the organism encounters, therefore making the study of oxidative stress, in an ecological context, highly relevant (Beaulieu et al., 2013; Costantini et al., 2008; Trivelpiece et al., 2011).

Glucocorticoid circulation may change depending on an individual's current life history trajectory, as animals energetically invest more in aspects of their life histories that contribute most to fitness (Ricklefs and Wikelski, 2002). Elevated levels of GCs can reduce an individual's ability to forage, avoid predators and grow (Wingfield et al., 1998), therefore limiting the energetic resources available to the defence and repair against oxidative damage. Studies that manipulate circulating levels of GCs via exogenous manipulations are becoming increasingly common, not only to understand fundamental aspects of organismal function, but to also understand the ecology of stress in wild animals (Sopinka et al., 2015; Crossin et al., 2016). Elevated levels of GCs have been suggested to increase oxidative stress via an elevation in metabolic rate which causes an increased flux of electrons at the level of the electron transport chain (Wingfield et al., 1998; Roussel et al., 2004). GC administration also causes increased lipid peroxidation and decreased total antioxidant capacity (Behl et al., 1997; Orzechowski et al., 2002). Due to the increased catabolic activity, uncoupling and proton leak that may result from GC manipulation (Wingfield et al., 1998; Roussel et al., 2004), ROS production and oxidative stress levels should increase when GCs are manipulated (reviewed in Costantini et al., 2011). Additionally, these effects were dependent on the duration of treatment as long-term GC manipulation generally showed larger effects on oxidative stress (Costantini et al., 2011). To date, however, only a few studies have investigated the link between GC manipulation and

oxidative status, and they focused on avian and mammalian taxa (reviewed in Costantini, 2011). Fewer studies have focused on oxidative stress in wild fish (Taylor et al., 2015).

Brown trout (*Salmo trutta*) are a semi-anadromous salmonid species native to many regions of Europe (MacCrimmon et al., 1970). Their populations consist of two subpopulations: anadromous (i.e., migratory; sea trout) and resident (i.e., non-migratory) individuals, both originating from the same parents (Jonsson and Jonsson, 1993). Brown trout can be implanted with small passive integrated transponders (PIT tags) to uniquely identify individuals that are recaptured to enable repeat sampling of individuals (e.g., for growth or oxidative status) or to estimate mortality (see Gibbons and Andrews, 2004). During early life stages (e.g., juveniles) when all fish are in stream environments, fish can be easily captured with electrofishing as part of mark-recapture protocols. Here we used an experimental approach, comparing oxidative status, growth and survival among a control group, a sham group and a group that received an intracoelomic injection of cortisol. The approach we used consisted of implanting a cortisol-bearing vehicle to transiently elevate cortisol levels, an approach commonly used in fish (Gamperl et al., 1994; Sopinka et al., 2015). Though a single (transient) exogenous manipulation of cortisol is a common approach for studying "stress", it fails to fully emulate a stress response *per se* in that it does not include the process of the organism perceiving a stressor and the associated neuroendocrine cascade (Sopinka et al., 2015). Nonetheless, this approach does have merit for testing the effects of experimental elevation of GCs on organismal biology (Sopinka et al., 2015; Crossin et al., 2016). Given that increased GC levels have been shown to increase metabolic rate and may reduce the availability of resources to fight oxidative stress, we predicted that growth rate will be lower in fish manipulated with cortisol relative to the control and sham groups. We also predicted that oxidative stress levels and antioxidant capacity will be higher in

fish injected with cortisol relative to the control and sham groups in the short-term (herein defined as two weeks). We further predicted that these effects will not be maintained in the long-term (herein defined as four months) given that the manipulation we used is aimed to provoke a transient increase in circulating plasma cortisol levels. This study is among the first of its kind to explore the link between cortisol and oxidative stress in a wild population of fish.

Materials and Methods

Study location

The Gudsø stream is located in east-central Jutland, Denmark. The stream runs through agricultural land over approximately 16 km, and several tributaries flow into the main stem, before reaching the sea at Kolding Fjord (Figure 3-1). The stream has natural populations of semi-anadromous brown trout (*Salmo trutta*), eel (*Anguilla anguilla*) and lamprey (*Lampetra planeri*).

Fish sampling and tagging

Fish were captured in the main stem of the Gudsø stream, starting 2000m from the mouth of the stream and continuing upstream for approximately 2500m (indicated by insert in Figure 3-1) from October 20 to October 25, 2015. All trout greater than 120 mm in length were captured using single-pass electrofishing gear (Stampes Elektro A/S, Ringkøbing, Denmark) and placed in a 60L container of fresh stream water (~50 fish per container for less than 1 hour). The water was changed continually until processing (water was changed approx. every 15-20 minutes). A total of 793 juvenile brown trout were captured. Fish were placed in a solution of 0.03g l^{-1} of

benzocaine until their opercular rate had slowed significantly and fish were unresponsive to external stimuli (usually less than 3 minutes). Total length (±1 mm) and wet mass (±0.1 g) were measured for individual fish. Fish were then tagged with a 23mm PIT tag (Texas Instruments, RI-TRP-RRHP, 134 kHz, 0.6g mass in air, Plano, TX, USA) inserted into their body cavity. Larsen et al. (2013) demonstrated that the retention of these tags in Atlantic salmon (*Salmo salar*) was of 97% with no effects on mortality rate and growth. A condition factor (K) was calculated using equation (1).

$$K = \left(\frac{mass}{length^3}\right)(100) \qquad (1)$$

Blood sampling and cortisol treatment

Blood samples of 0.1ml were obtained from the caudal vasculature of individual fish using a 1.5 inch 25-gauge heparinized needle. Within 10 minutes of sampling, blood was centrifuged at 6,000 rpm for 2 minutes in the field (samples were kept on ice meanwhile), after which plasma was separated from red blood cells (RBCs). RBCs were flash-frozen in liquid nitrogen and later placed at -80°C. Fish were then randomly assigned to control (n = 426), sham (n = 282) or cortisol (n = 276) treatment groups. Cortisol fish received an intracoelomic injection of hydrocortisone 21-hemisuccinate (Sigma-Aldrich, St. Louis, MO, USA) suspended in vegetable shortening (100% vegetable shortening, Crisco, OH, USA) using a dosage of 0.01ml vehicle (concentration of 0.01g cortisol per ml) per 1 g of fish (equivalent to 100mg kg^{-1}). This dosage has been validated to elevate circulating baseline plasma cortisol levels in juvenile brown trout for at least 9 days post-treatment: at day 3, levels are approximately 900ng ml^{-1}; at day 6, they decrease to approximately 400 ng ml^{-1}; and at day 9, levels are approximately 200ng ml^{-1} all of which were higher than controls and shams (Birnie-Gauvin et al., *in review*). Though cortisol

values at day 3 were beyond the physiological levels seen in fish, by day 6, average values were within the range of stress-induced responses (Gamperl et al 1994). The validation study used the same population and the same products (cortisol and vehicle) as we used here, and similar methods for elevating cortisol have been used in several other studies of the same trout population (Midwood et al., 2014, 2015, 2016). We therefore did not measure individual cortisol levels in the current study as the objective was to investigate average treatment effects. Sham fish received the same injection of vegetable shortening, with no cortisol. Fish from all treatments were allowed to recover in a 60L container of fresh stream water, where cortisol fish were separate from control and sham fish. These standardized techniques were approved by the Danish Animal Experiments Inspectorate (License Number: 2012-DY-2934-00007).

Resampling of fish

To evaluate the short-term effects of cortisol, Gudsø stream was resampled from November 5 to November 7, 2015 using the same techniques described above. All captured fish were scanned for PIT tags. A total of 80 controls, 95 shams and 99 cortisol-treated fish were recaptured and resampled, after which sampling efforts were stopped. Tagged trout were placed in a 60L container of fresh stream water until processing. Total length and mass were measured; the mass of the PIT tag (0.6g) was subtracted from the overall wet mass. A blood sample was obtained from recaptured trout (as per above description). The same methodology was applied for recaptures from February 29 to March 2, 2016 to evaluate the long-term effects of cortisol, where 34 control fish (9.50%), 18 sham fish (7.69%), and 4 cortisol fish (1.70%) were recaptured. The resampling started 750m downstream and ended 1,600m upstream of our initial sampling locations. A growth rate was determined both in terms of mass and length for the short-

term and long-term effects of treatments. A condition factor (K) was also determined using these measurements.

Choice of oxidative stress assays

We opted to measure glutathione (GSH) given that it is the most abundant antioxidant in aerobic cells (millimolar concentrations in tissue), and that it is critical to protect vital organs against oxidative damage (Owen and Butterfield 2010). Metabolically, generating glutathione is costly, and hence the molecule is not typically broken down. For this reason, it is useful when measuring effects over a longer timescale. We also chose to measure oxidative stress levels via the ratio of oxidized to reduced glutathione (GSSG:GSH) given its current use to indicate cellular health (Owen and Butterfield 2010). Additionally, we opted for the ORAC (Oxygen Radical Absorbance Capacity) as a second method to measure overall antioxidant capacity of low molecular weight antioxidants because it is one of the few methods that takes the quenching reaction of ROS to completion. In essence, it combines both the time and percentage of ROS quenching by antioxidants, and converts it into a single quantity (Cao and Prior 1999).

Glutathione antioxidant (GSH) and oxidative stress levels (GSSG:GSH)

All RBC samples were ground and homogenized on ice in non-denaturing lysis buffer (20mM Tris-HCl, 137mM NaCl, 1% NP-40, 10% glycerol, 2mM EDTA and 100mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol), and centrifuged at 13,500 rpm for 10 minutes at 4°C in a Hermle Labnet Z216MK (Mandel, Guelph, ON). Supernatants were further homogenized in 1:5 5% sulfosalicylic acid solution (bubbled with N_2 gas). Sample lysates were centrifuged at 13,500 rpm for 10 minutes at 4°C. Supernatants were used to assess total

glutathione (TGSH), oxidized glutathione (GSSG) and reduced glutathione (GSH). The latter is measured indirectly using the following equation: TGSH = GSH + 2GSSG. Glutathione assays were performed using an Epoch microplate reader with Gen5 data analysis software (2.00.18, BioTek Instruments Inc., Winooski, VT, USA) and clear 96-Well Costar microplates. Glutathione assays were performed 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.

To measure TGSH, the reaction media contained 20µl of sample, 5 IU/ml glutathione reductase, 0.5M potassium phosphate buffer (pH 7.0), 0.3 mM nicotinamide adenine dinucleotide 2'-phosphate (NADPH), and 60 mM DTNB. The reduction was read for 30 minutes and compared to a GSH standard curve (0-4 mM). To quantify only GSSG, 50 µl of the initial supernatant and the GSSG standards (0-0.5263 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. All samples were run in duplicates (mean values were calculated and used for analysis), with an inter-assay variation of 3.74%.

Low molecular weight antioxidants (ORAC)

Samples of RBCs were homogenized on ice in 1:5 lysis buffer (20mM Tris-HCl, 137mM NaCl, 1% NP-40, 10% glycerol, 2mM EDTA) using a handheld Tissue Master 125 (Omni International, Kennesaw, GA). Lysates were centrifuged at 13,000 rpm for 5 minutes at 4°C in a Hermle Labnet Z216MK and supernatant were stored at -80°C until the Oxygen Radical Absorbance Capacity assay was performed (as described in Wilson et al., 2012). ORAC analyses

were performed using a Cytation 5 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) and black 96-Well Costar microplates. Fluorescence was measured with an excitation wavelength of 485 nm and emission of 520 nm. Gen5 data analysis software (2.07.17, BioTek Instruments Inc., Winooski, VT, USA) was used to analyze the fluorescence data.

Each reaction well contained 20 µl of either sample, blank (75 mM potassium phosphate (pH 7.4)), or standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 0-400 µM), and 3.82 µM fluorescein in 75 mM potassium phosphate (pH 7.4). The plate was incubated at 37°C for 20 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 80 seconds for 90 minutes. The area under the fluorescence decay curve (AUC) was determined for the samples and Trolox standards to determine the Trolox equivalency. Total protein of samples was determined using the BioRad assay and final antioxidant capacity values are reported in Trolox equivalents (TE) per µg total protein. All samples were run in duplicates (mean values were calculated and used for analysis), with an inter-assay variation of 2.34%.

Evaluation of predation

Two cormorant (*Phalacrocoracidae* sp.) colonies are located near the Gudsø stream. During the same time frame as long-term fish resampling was conducted (March 14 and 15), each colony was scanned to detect excreted PIT tags by two people, each sweeping the entire area of the colonies once. Scanned PIT tags allowed us to determine which fish had died from cormorant predation.

Statistical analysis

To assess short-term changes, twenty recaptured individuals were randomly chosen within each treatment group to assay TE/protein, GSH and GSSG:GSH. All long-term overwinter recaptures were used for these assays. We then ran these assays on the initial samples from these same individuals, thus forming two different initial groups. As long-term recaptures may not be representative of the whole population in the fall (as these individuals survived and were still in the stream, meaning they were either late migrators or had chosen the residency strategy), we used one-way ANOVAs to compare treatments at each time point (short- and long-term) separately.

To determine whether long-term survivors differed from the individuals randomly chosen for the short-term group, we used a one-way ANOVA to evaluate differences between the initial fish used in these two analyses. We note that 7 individuals were used in both 'initial' analyses (2 control, 4 sham and 1 cortisol) as they were long-term recaptures but also randomly selected for short-term analysis; otherwise the two groups represent unique individuals.

GSSG:GSH contained true zero values and was highly skewed, so we used nonparametric Wilcoxon tests, which precludes testing for interactions, and so for this metric we analyzed treatment separately at each time point (short-term initial sample, long-term initial sample, short-term sample, and long-term sample) and used the Steel-Dwass method for analyzing which treatments differed.

Changes in GSH, protein, and TE/protein due to treatment, time point (initial vs short-term, or initial vs long-term) and their interaction were analyzed using two-way ANOVAs with individual ID as a random effect, with a Tukey *post-hoc* test to determine which groups differed.

We also calculated specific (daily) growth rate (mass) and specific size (length) using the equation $(\log Y_2 - \log Y_1)/(t_2 - t_1)$, where Y_1 is the length or mass at the time of tagging (t_1) and Y_2 is the length or mass at the time of recapture (t_2). Specific growth rate and specific size were analyzed using one-way ANOVAs to test for treatments effects for short-term and long-term groups separately. A Kolmogorov-Smirnov test was performed to determine if the data were normally distributed. Mass, length, GSH, protein and TE/protein were log transformed to achieve normality.

A Pearson's chi-square analysis was performed to evaluate whether mortality as a result of cormorant predation differed among treatment groups, and to evaluate whether the percentage of overwinter recaptures differed among treatment groups. Statistical analyses were conducted using JMP v12.1.0.

Results

In the short-term, cortisol fish increased their glutathione antioxidants (GSH) while sham and control fish did not change (treatment X time interaction, $F_{2,113}=3.51$, $p=0.033$, Figure 3-3A). Oxidative stress levels (GSSG:GSH) decreased in the short-term in all treatments ($\chi^2=7.59$, $p=0.022$, Figure 3-3C) and there were no initial differences among treatments ($p=0.94$). Protein concentration decreased overall in the short term ($F_{1,109}=4.82$, $p=0.030$) but did not differ among treatments ($F_{2,109}=0.5$, $p=0.61$). Low molecular weight antioxidants (TE/protein) also decreased in the short term ($F_{1,109}=45.5$, $p<0.0001$, Figure 3-3E) but did not differ among treatments ($F_{2,109}=2.87$, $p=0.06$).

In the long-term (overwinter), GSH decreased in cortisol-treated fish while control and sham fish did not change (treatment X time interaction, $F_{2,100}=3.75$, $p=0.027$, Figure 3-3B).

Protein concentration decreased in the long term ($F_{1,101}$=14.48, p=0.0002) but did not differ among treatments ($F_{2,101}$=2.97, p=0.056). Neither oxidative stress levels (GSSG:GSH; p>0.37, Figure 3-3D) nor low molecular weight antioxidants (TE/protein; p>0.26, Figure 3-3F) were affected by treatment or time.

The two initial groups use for the short-term and the long-term studies differed from each other: both glutathione (GSH) (F1,30=17.66, p=0.0002, Figure 3-3A and B) and oxidative stress levels (GSSG:GSH) (χ^2=66.35, p<0.0001, Figure 3-3C and D) were higher in the random group selected for short-term analysis than the overwinter long-term group. Protein (p=0.99), TE/protein (p=0.99), mass (p=0.36), length (p=0.26) and condition factor (p=0.067) did not differ between these initial groups).

Specific growth rate was lower in cortisol-treated fish than control or sham in the short-term ($F_{2,56}$=5.70, p=0.0056, Figure 3-2A) and long-term ($F_{2,50}$=7.53, p=0.0014, Figure 3-2B). Specific size was not affected by short-term treatment (p=0.30) but was also lower in cortisol fish than control or sham in the long-term ($F_{2,51}$=6.58, p=0.0029).

The proportion of mortality as a result of cormorant predation did not differ among treatments (Pearson's χ^2 = 0.10, df = 2, *P* = 0.995). In total, 12 control fish were predated, 6 sham fish, and 6 cortisol-treated fish. However, the overwinter recapture rates were lower for cortisol-treated fish (1.70%) than control (9.50%) and sham (7.69%) (Pearson's χ^2 = 12.629, df = 2, *P* = 0.002).

Discussion

It has been hypothesized that prolonged secretion of GCs result in increased oxidative stress levels (Agostinho et al., 2010), and thus oxidative stress may provide a potential mechanism for

the costs associated with chronic stress (Costantini et al., 2011). The ratio of oxidized (GSSG) to reduced (GSH) glutathione is commonly used as a measure of oxidative stress, where a larger ratio represents a redox imbalance in favour of pro-oxidants. We found that cortisol manipulation did not increase oxidative stress levels in the short-term, but it did increase glutathione (GSH), an important antioxidant in fish, suggesting that the increase in GSH potentially counteracted ROS production. Hence, cortisol may protect against, rather than generate oxidative stress, and may upregulate antioxidant defenses via genomic pathways as well as other mechanisms that limit ROS production (Costantini et al., 2011). However, we found that in the long-term, cortisol caused a decrease in GSH antioxidants. This indicates that the increased GSH in the short-term could not be maintained, and that cortisol may have caused the diversion of resources away from GSH production, likely to counteract other cortisol-induced effects (e.g., increase susceptibility to disease; Wingfield et al., 1998).

Short-term administration of GCs has been studied in various species of birds and mammals. In broiler chickens (*Gallus gallus domesticus*), a 14-day corticosterone diet manipulation led to elevated lipid peroxidation and plasma antioxidant activity (Lin et al., 2004). A similar study in captive kestrels (*Falco sparverius*) showed that corticosteroid administration through diet increased reactive oxygen metabolites, but did not impact total antioxidant capacity or oxidative stress levels (Costantini et al., 2008). In rats (*Ratus norvegicus*), cortisol treatment did not affect the rate of ROS production in liver, but did increase DNA oxidative damage (Caro et al., 2007). We found that cortisol induced an increase in GSH in the short-term, a decrease in GSH in the long-term, but did not affect oxidative stress levels or low molecular weight antioxidants. These findings suggest that the effects of GCs on oxidative stress processes vary between species, and remain poorly understood. Furthermore, it appears that many of the GC-

caused oxidative stress changes are tissue-specific (e.g., McIntosh et al., 1998; Costantini, 2011). Our sampling approach did not involve lethal sampling to examine heart, liver and brain tissue, therefore limiting us to the use of blood samples. Our findings suggest that the mechanisms by which cortisol affects oxidative status may differ in fish.

Interestingly, a decrease in oxidative stress levels and GSH was observed in the short-term, in all treatments. Two possible explanations arise: (1) seasonal variation caused all individuals to decrease oxidative stress levels and GSH, possibly through the diversion of resources away from GSH to other forms of antioxidants (e.g., enzymatic antioxidants) through unknown mechanisms; or (2) the potential stress of handling may have caused fish to activate mechanisms that lowered oxidative stress levels and GSH, possibly to increase other forms of antioxidants which we did not measure (e.g., enzymatic antioxidants). The latter has important implications for future studies that aim to measure oxidative stress parameters shortly after fish have been handled.

Only fish that survived and stayed in the stream over winter could be used to evaluate the long-term changes of oxidative status. To our surprise, these overwinter fish initially had different oxidative statuses than randomly-chosen fish: long-term fish initially had lower GSH antioxidants and oxidative stress levels (GSSG:GSH) levels than the general population (Figure 3A,B). This suggests that individuals that survive overwinter and/or migrate later are already physiologically different from other fish in the fall. Bize et al. (2008) showed that in the Alpine Swift (*Apus melba*), oxidative stress may participate in shaping survival and fecundity. More specifically, they found that males with higher resistance to oxidative stress tended to survive to the next season more often, and that females with higher resistance tended to have larger clutches. It was also found that the eggs of females with lower resistance to oxidative stress were

less likely to hatch (Bize et al. 2008). Taken together, these findings suggest that lower oxidative stress levels may promote survival in wild organisms.

Although the evaluation of survival was not the focus of this study and the ultimate fate of each individual cannot be known for certain, past research has shown that overwinter mortality is highly variable in brown trout (Elliot, 1993). Additionally, exogenous cortisol manipulation causes increased overwinter mortality in brown trout of the same stream (Midwood et al., 2015). In general, high GC levels are associated with decreased fitness (Romero and Wikelski, 2001). However, the level of known predation at two cormorant colonies did not differ among treatment groups, suggesting that cortisol manipulation did not make fish more susceptible to predation by cormorants. Nonetheless, cortisol-treated fish showed significantly lower recapture rates which may be entrained by other causes of death, such as decreased foraging ability (Wingfield et al., 1998) and increased susceptibility to disease (Davis et al., 2008).

Glucocorticoid manipulation may affect body mass through its role in the hormonal control of appetite and food intake (Friedman and Halaas, 1998). Both baseline and acute GCs covary with body mass (e.g., Schoech et al., 1997). Growth depression is a common observation following such GC manipulation in birds, reptiles, fish and mammals (Davies et al., 2013; Cote et al., 2006; O'Connor et al., 2011; Brooks and Mateo, 2013). We found that cortisol manipulation caused a decrease in growth rate (mass) over two weeks and a decrease in growth rate (mass) and lower growth rate (length) over the long-term. The reduced growth rate observed in cortisol treated fish may be a result of a reduction in food intake (Morales et al., 2004) and decreased foraging ability (Wingfield et al., 1998). Caloric restriction has been found to increase the expression of heat shock proteins, which have the ability to quench ROS (Sørensen 2010).

This would provide an explanation for the decrease in mass in cortisol-treated fish, and may explain why we did not observe an increase in oxidative stress levels in those same fish. Alternatively, it is also possible that cortisol-treated fish became less active after receiving the treatment, though no studies that we know of have made such observations, and thus may have decreased metabolic rates. If this is the case, both ROS production and food consumption would have decreased, resulting in lower oxidative stress levels, and lower growth rates in cortisol-treated fish. In either case, it appears the link between GCs and oxidative stress is still poorly understood in fish, and may be more complex than first thought.

Conclusions

This study demonstrates that exogenous cortisol manipulation does not change oxidative stress levels but does affect antioxidant capacity, though these patterns differ with time, providing evidence that cortisol has different short and long term effects in fish. We also provide the first evidence that overwinter survival may be associated with low oxidative stress levels and low antioxidant capacity in the fall. This may have important implications for the survival of hatchery-reared salmonids that are released in the wild before winter. Ensuring low oxidative stress levels in those fish may provide them with better chances of survival overwinter. Alternatively, those fish could be released after winter. This study also emphasizes the need to measure indicators of both oxidative stress levels and antioxidant capacity when studying oxidative stress, as their interactions remain largely unpredictable. This emphasizes the need for more manipulative studies of oxidative stress in wild organisms in their natural environment.

Figures

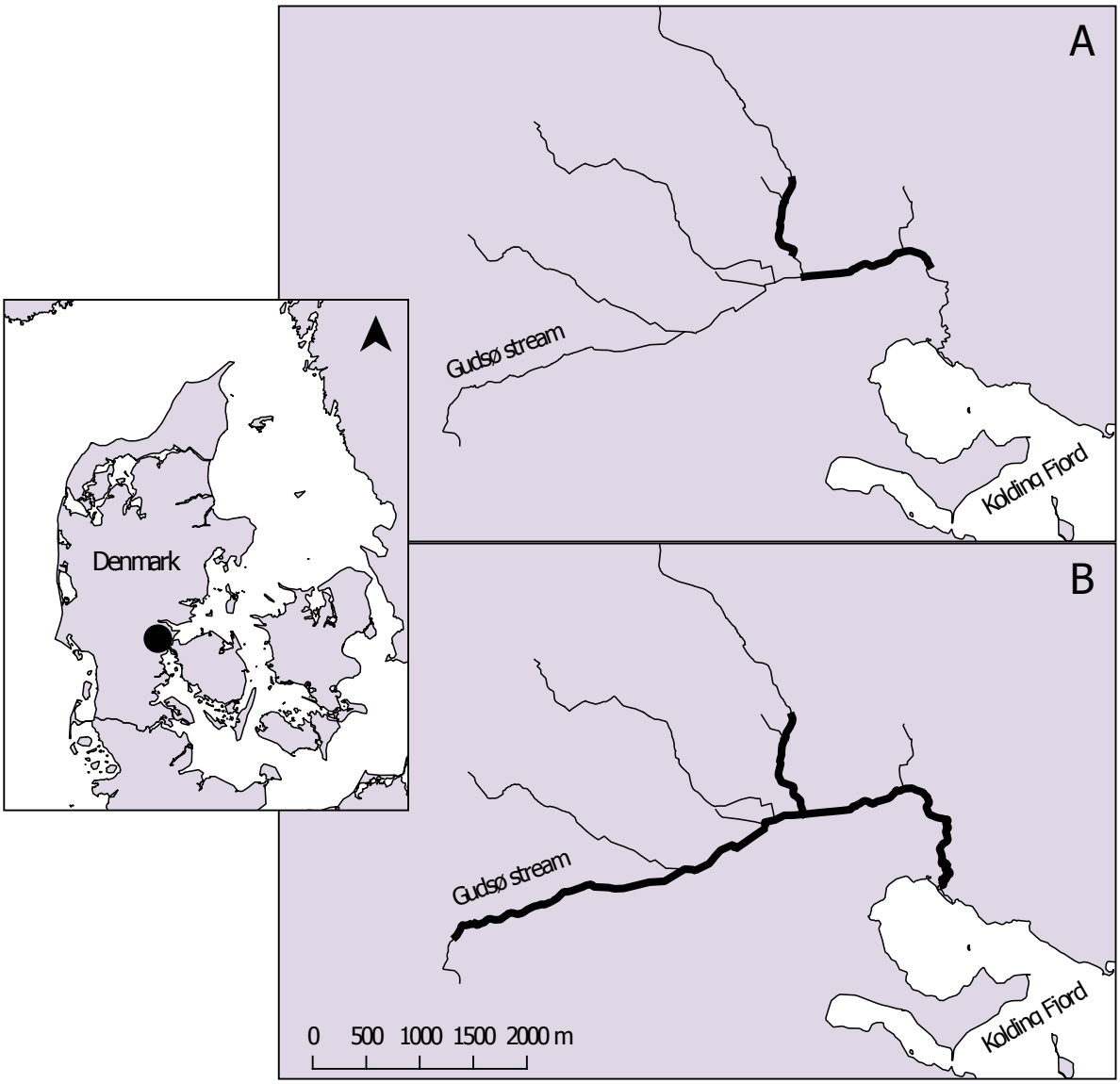

Figure 3-1. Map of the Gudsø stream, Denmark. Black circle shows location of the stream in Denmark. Sampling locations are highlighted in the thick black trace, (A) for initial and two-week capture locations in October and November 2015, and (B) for overwinter capture locations in February/March 2016.

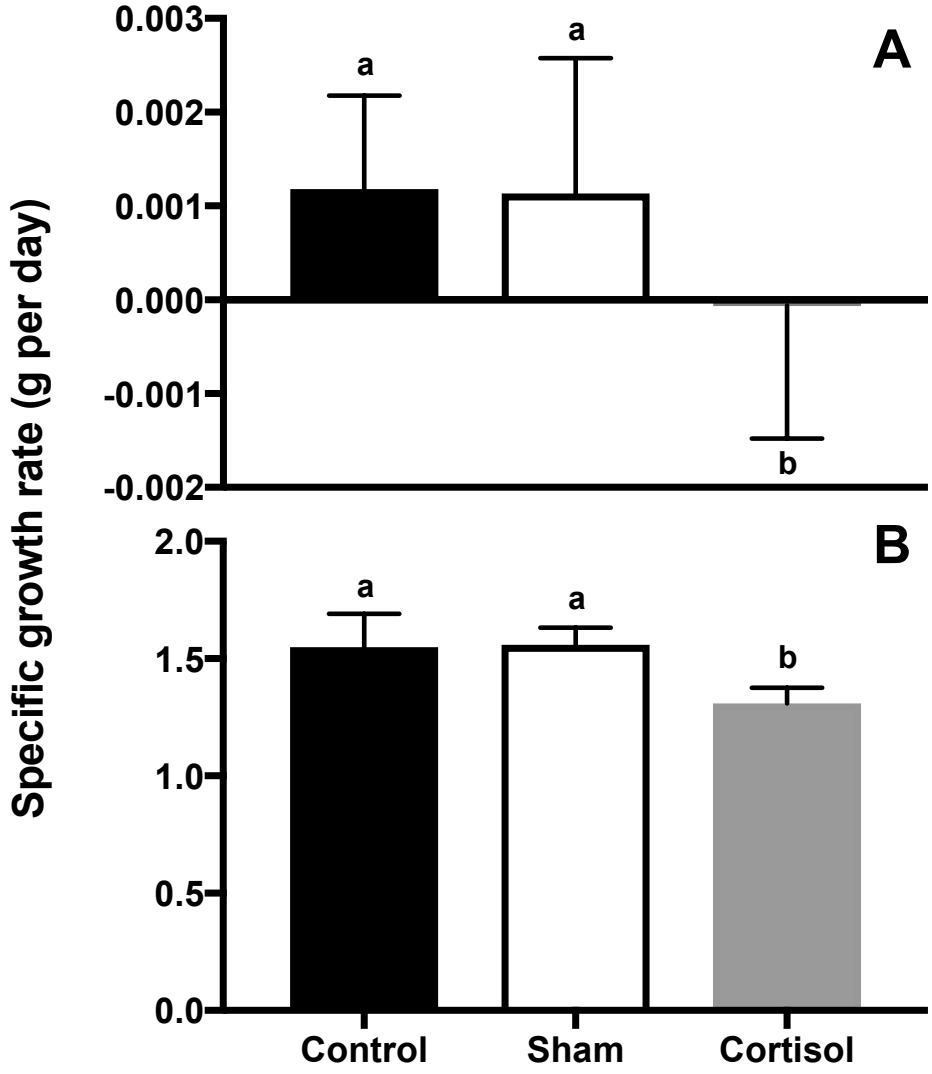

Figure 3-2. Growth rate. Specific growth rate for (A) two-week changes in mass (g per day), (B) overwinter changes in mass (g per day). Two-week study used n = 20 (control), n = 20 (sham), and n = 20 (cortisol); overwinter study used n = 34 (control), n = 18 (sham), and n = 4 (cortisol). Mean values are presented with standard deviations. Dissimilar letters (a, b) denote significant differences between groups (Tukey *post-hoc* test $P < 0.05$).

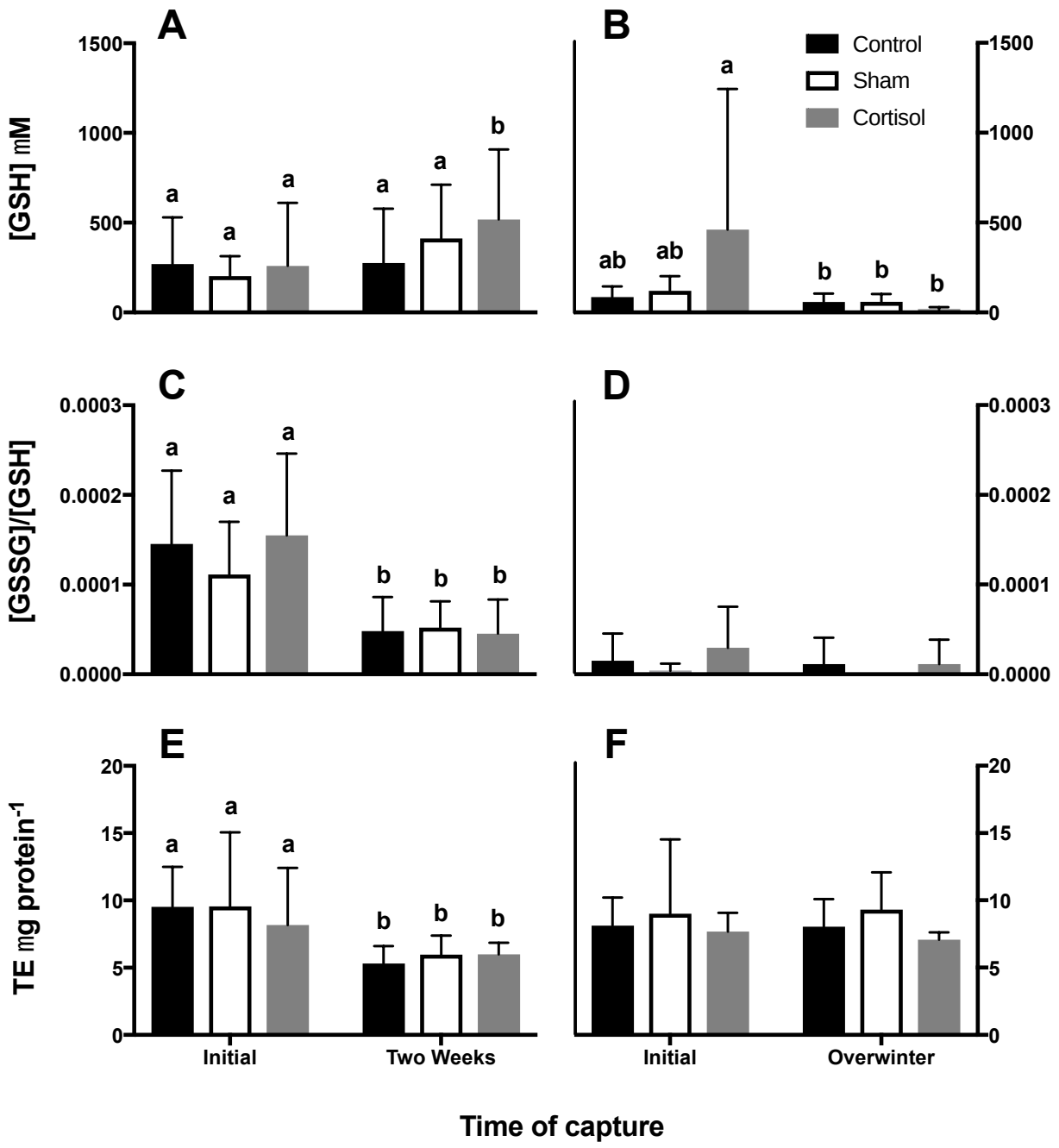

Figure 3-3. Oxidative and antioxidant parameters. Glutathione concentration (GSH, mM) at (A) initial and two-week recapture, and (B) initial and overwinter recapture. Oxidized to reduced glutathione ratio (GSSG:GSH; oxidative stress) at (C) initial and two-week recapture, and (D) initial and overwinter recapture (note that the ratios for cortisol-treated fish were 0 for all overwinter recaptures). Low molecular weight antioxidants (ORAC) in Trolox equivalents per μg

protein, at (E) initial and two-week recapture, and (F) initial and overwinter recapture. Two-week study used n = 20 (control), n = 20 (sham), and n = 20 (cortisol); overwinter study used n = 34 (control), n = 18 (sham), and n = 4 (cortisol). Mean values are presented with standard deviations. Dissimilar letters (a, b) denote significant differences between groups (Tukey *post-hoc* test for GSH and ORAC, Steel-Dwass *post-hoc* test for GSSG:GSH, $P < 0.05$). No letters are provided for D and F as no groups differed from each other.

Chapter 4: Oxidative stress and partial migration in a salmonid fish

Abstract

During migration, animals are typically limited by their endogenous energetic resources which must be allocated to the physiological costs associated with locomotion (i.e., increased metabolic rate), as well as predator avoidance, and avoiding and/or compensating for oxidative stress. To date, there have been few attempts to understand the role of oxidative status in migration biology, particularly in fish. Semi-anadromous brown trout (*Salmo trutta*) exhibit partial migration, where some individuals smoltify and migrate to sea, and others become stream residents, providing us with an excellent model to investigate the link between oxidative stress and migration. Using the brown trout, we tested the hypothesis that oxidative status is an early predictor of smolt migration and residency. Blood samples were obtained from juveniles from a coastal stream in Denmark in the fall prior to peak seaward migration which occurs in the spring, and assayed for antioxidant capacity (oxygen radical absorbance capacity) and oxidative stress levels (ratio of oxidized to reduced glutathione). We found that individuals that migrated had higher antioxidant capacity than residents and that future migration date was negatively correlated with both antioxidant capacity and body length in the fall. This study provides the first evidence that oxidative status is associated with migration strategy and timing, months in advance of the actual migration, and provides insight into the role of oxidative status in animal migration.

Introduction

Migrations represent some of the most fascinating and energy demanding phenomena in the animal world and are often typified by prolonged elevation in metabolic rate associated with high levels of locomotor activity (Leffler 1993; Jonsson et al. 1997). The idea that elevated metabolism leads to increased production of reactive oxygen species (ROS) via an increased flux of electrons at the level of the electron transport chain is widespread in the literature (Wingfield et al. 1998), though not well supported empirically (Salin et al. 2015 and references therein). An increase in ROS can result in an imbalance between ROS and antioxidants (molecules that delay or inhibit oxidation; Halliwell and Gutteridge 2007) and can lead to oxidative stress and the damage of macromolecules (Asada and Takahashi 1987). When organisms allocate their limited resources to enhance their antioxidant capacity and resist oxidative stress, energy is diverted from other activities such as growth, locomotion, immunity and avoiding predators (Denver et al. 2009; Ball and Balthazart 2008).

The relationship between oxidative stress and life history traits is still poorly understood (Speakman et al. 2015). The majority of studies have focused on immediate and short-term effects of ROS but there is increasing realization that oxidative stress will impact life histories over longer timescales (Monaghan et al. 2009). For example, Costantini et al. (2008) provided evidence that homing pigeons which undergo longer flights experience greater oxidative stress than individuals which undergo shorter flights as well as control individuals (no flight). The few studies published to date that investigated the link between oxidative stress parameters and life-history strategies are often limited to birds (Costantini 2014 and references therein), therefore restricting the generality of those conclusions to other taxa. As a result of elevated metabolism and high energetic demands during migration, we would expect migratory species to be better

equipped to deal with oxidative stress than non-migratory species (Costantini 2008). The same pattern may exist between migratory and resident individuals within a species (i.e., when a species exhibits partial migration, Chapman et al. 2011). No studies that we know of have investigated the potential role of oxidative status as a determinant of partial migration strategy.

Semi-anadromous brown trout, *Salmo trutta*, undergo partial migration, where migratory and resident individuals coexist within the same population (Jonsson and Jonsson 1993). The decision to smoltify and migrate to sea or to assume residency in their native stream is a complex interaction between environment and physiology that is particularly sensitive to stressful conditions (Thorpe et al. 1992; Metcalfe 1998; Cucherousset et al. 2005; Dingle and Drake 2007; Boel et al. 2014). The decision to migrate or not is thought to be made at the end of the summer before spring migration (Metcalfe 1998). We used blood samples collected in the fall to determine whether oxidative stress was associated with migration strategy and migration timing in the spring. Consequently, our goal was to evaluate the link between oxidative stress and partial migration in wild juvenile brown trout in relation to within-year variation in timing of migration and migration strategy (migratory versus resident), and not related to whether migratory individuals undertake migration in one year versus the next year (between-year variation). Larger individuals are more constrained by low food availability in stream environments than smaller individuals and are thus more likely to migrate to marine environments, where food availability is greater (Økland et al. 1993; Thorpe et al. 1998). Additionally, larger individuals often have faster growth rates and higher metabolic rates (Økland et al. 1993; Thorpe et al. 1998), and thus may have higher levels of oxidative stress than smaller individuals. Consequently, we predicted that: (1) larger individuals will migrate, (2) individuals with higher levels of oxidative stress and lower levels of antioxidants will migrate,

(3) within migratory individuals, larger individuals will migrate sooner, and (4) individuals with higher levels of oxidative stress and lower levels of antioxidants will migrate sooner.

Materials and Methods

Study location

The Gudsø Stream (mean width ~ 2 m) is located in east-central Jutland, Denmark (Figure 4-1). The stream runs over approximately 16 km and is surrounded by agricultural land. Several tributaries flow into the main stem, before reaching the northwest Baltic Sea at Kolding Fjord. The stream supports natural populations of semi-anadromous brown trout (*Salmo trutta*), eel (*Anguilla anguilla*) and lamprey (*Lampetra planeri*). Two passive integrated transponder (PIT) reading stations (PIT stations 1 and 2) located approximately 1 km from the outflow of the stream into fjord record the passage of PIT tagged fish (Figure 4-1). The distance between the two PIT reading stations is 150 m. Each reading station consists of two loop-shaped antennas spaced 5 m apart, each covering the entire cross-section of the stream. This allowed us to determine the swimming direction of migrating trout. We evaluated the tag detection efficiency of PIT station 1 using the formula described in Zydlewski et al. (2006), and found it to be 88%.

Fish sampling and tagging

Fish were captured in the main stem of the Gudsø Stream, approximately 2 km upstream of the entrance to the fjord (Figure 4-1) from October 20 to October 25, 2015. Additional fish were captured from a tributary on November 2 and November 3, 2015. All trout greater than 120 mm in length were captured using single-pass electrofishing gear (Stampes Elektro A/S, Ringkøbing,

Denmark) and placed in a 60L container of fresh stream water. The water was changed continuously to provide freshly oxygenated water. Fish were placed in a 0.03g L^{-1} benzocaine solution until their opercular rate had slowed and fish were unresponsive to external stimuli (usually less than 4 minutes). Total length (±1 mm) and wet mass (±0.1 g) were measured for individual fish. A relative condition factor (K) was calculated using equation 1 (Bolger and Connolly 1989). Fish were then tagged with a 23mm PIT tag (Texas Instruments, RI-TRP-RRHP, 134 kHz, 0.6g mass in air, Plano, TX, USA) inserted into their body cavity. Larsen et al. (2013) and Acolas et al. (2007) demonstrated that these tags in Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*), respectively, had no effects on mortality or growth.

$$K = \left(\frac{mass}{length^3}\right)(100) \qquad (1)$$

Blood sampling

Blood samples of 0.1mL were obtained from the caudal vasculature of individual fish using a heparinized 1.5 inch 25-gauge needle. Within 10 minutes of sampling, blood was centrifuged at 6,000 rpm for 2 minutes, after which plasma was separated from red blood cells (RBCs). RBCs were flash-frozen in liquid nitrogen and then stored at -80°C. Fish were allowed to recover in a 60L container of fresh stream water and were released near their site of capture. These standardized techniques were approved by the Danish Animal Experiments Inspectorate (License Number: 2013-15-2934-00808).

Antioxidant capacity

Samples of RBCs were homogenized on ice in 1:5 lysis buffer (20mM Tris-HCl, 137mM NaCl, 1% NP-40, 10% glycerol, 2mM EDTA) using a handheld Tissue Master 125 (Omni

International, Kennesaw, GA). Lysates were centrifuged at 13,000 rpm for 5 minutes at 4°C in a Hermle Labnet Z216MK (Mandel, Guelph, ON) and supernatants were stored at -80°C until the Oxygen Radical Absorbance Capacity (ORAC) assay was performed (as described in Wilson et al. 2012). ORAC assays were performed using a Cytation 5 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) and black 96-Well Costar microplates. Fluorescence was measured with an excitation wavelength of 485 nm and emission of 520 nm. Gen5 data analysis software (2.07.17 BioTek Instruments Inc., Winooski, VT, USA) was used to analyze the fluorescence data.

Each reaction well contained 20 µL of either sample, blank (75 mM potassium phosphate (pH 7.4)), or standard (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox); 0-400 µM), and 3.82 µM fluorescein in 75 mM potassium phosphate (pH 7.4). The plate was incubated at 37°C for 20 min before rapidly adding the free radical generator 2,2"-azobis (2-amidinopropane) dihydrochloride to a final concentration of 79.83 mM. The plate was placed immediately in the microplate reader and the fluorescence was read every 80 seconds for 90 minutes. The area under the fluorescence decay curve 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 BioRad assay and final values are reported in Trolox equivalents/µg total protein.

Oxidative stress levels

All samples were ground and homogenized on ice in non-denaturing lysis buffer (20mM Tris-HCl, 137mM NaCl, 1% NP-40, 10% glycerol, 2mM EDTA and 100mM PMSF in isopropanol), and centrifuged at 13,500 rpm for 10 minutes at 4°C. Supernatants were further homogenized in

1:5 5% sulfosalicylic acid solution (bubbled with N_2 gas). Sample lysates were centrifuged at 13,500 rpm for 10 minutes at 4°C. Supernatants were used to assess total glutathione (TGSH), reduced glutathione (GSH) and oxidized glutathione (GSSG) [TGSH = GSH + 2GSSG]. Glutathione assays were performed using an Epoch microplate reader with Gen5 data analysis software (Biotek Instruments, Winooski, VT, USA) and clear 96-Well Costar microplates. Glutathione assays were performed 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 20µL of sample, 5 IU/mL glutathione reductase, 0.5M potassium phosphate buffer (pH 7.0), 0.3 mM nicotinamide adenine dinucleotide 2'-phosphate (NADPH), and 60 mM DTNB and reduction was read for 30 minutes and compared to a GSH standard curve (0-8 µM). To quantify only GSSG, 50 µL of the initial supernatant and the GSSG standards (0-0.5263 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 are reported in mM concentrations.

Evaluation of predation and migration

Two cormorant (*Phalacrocoracidae* sp.) colonies are located approximately 2 and 5 km away from the Gudsø stream. Each colony was scanned on March 14-15, 2016 by two people, each sweeping the entire area of the colonies once to detect excreted PIT tags. Scanned PIT tags allowed us to determine which fish had died from cormorant predation.

The main stem and one tributary of the Gudsø stream were resampled entirely between February 29 and March 2, 2016 to evaluate residency. Recaptured fish were assumed to be residents unless the fish was later detected at the PIT reading stations. PIT data was downloaded on October 27th, 2016, past the peak migration period for smolts which occurred in March-April 2016. All fish detected at the PIT antennas were considered to be migrants. Fish that were neither detected at the PIT antennas, recaptured in the spring or found in the cormorant colonies were defined as unknown.

Statistical analysis

Statistical analyses were conducted using R version 3.2.3 (R Core Team 2015; nlme and AICcmodavg packages by Pinheiro et al. 2016 and Mazerolle 2016, respectively). To explore potential relationships between individual life history strategy (migratory vs. resident) and oxidative stress levels (OSL) or antioxidant capacity (AOX) obtained for each fish, we fit the following generalized linear model, with length and condition (K) as covariates:

$$\text{strategy}_i \sim \text{Bin}(\pi_i, 1)$$

$$E(\text{strategy}_i) = \pi_i$$

$$\text{var}(\text{strategy}_i) = \pi_i * (1 - \pi_i)$$

$$\text{logit}(\pi_i) = \eta_i$$

$$\eta_i = \beta_1 + \beta_2 * \text{length}_i + \beta_3 * K_i + \beta_4 * OSL_i + \beta_5 * \log(AOX_i)$$

The model states that the life history strategy of fish *i* follows a binomial distribution with probability parameter π_i and n = 1, i.e. a Bernoulli distribution. π_i is specified through a logit-link by the predictor function η_i to be a linear function of the included covariates (length_i, K_i, OSL_i and AOX_i).

For the migratory fish, we modelled day of migration (DOM, unit: day of year, where Day 300 = October 28, 2015) as a function of individual oxidative stress metrics and day of tagging (DOT, unit: day of year) by the following model fitted using generalized least squares:

$$DOM_i \sim N(\mu_i, \sigma^2 \times length_i^{2 \times \delta})$$

$$E(DOM_i) = \mu_i$$

$$var(DOM_i) = \sigma^2 \times length_i^{2 \times \delta}$$

$$\mu_i = \beta_1 + \beta_2 * length_i + \beta_3 * K_i + \beta_4 * OSL_i + \beta_5 * \log(AOX_i) + \beta_6 * DOT_i$$

The model assumes DOM of fish i follows a Gaussian distribution with mean μ_i specified as an identity linked predictor function of the included covariates ($length_i$, K_i, OSL_i, AOX_i and DOT_i). A covariate variance structure incorporating $length_i$ was used to accommodate variance heterogeneity.

For both models, OSL was transformed to a categorical variable representing whether or not individual OSL values were zero (i.e., true zeros) or not. Additionally, AOX was log-transformed as preliminary analysis indicated the models would otherwise violate underlying assumptions.

We tested whether length was correlated with OSL or AOX using a linear regression independently within migratory and resident individuals.

Results

A total of 414 juvenile brown trout were initially captured, tagged and sampled in the fall. Of those fish, 24 were recaptured in the spring, of which only 13 (3.1%) were not detected at the PIT antennas, and are therefore assumed to be residents. We found that 147 (35.5%) of all tagged individuals were known migrants, 11 (2.7%) were predated by cormorants and 241 (58.2%) had

an unknown fate (presumably mortalities). Of the 147 migratory individuals from the study, a subsample of 48 was randomly chosen to perform oxidative stress assays (with some individuals randomly chosen within specific time intervals to ensure coverage across the migratory season), while all 13 residents were assessed. Two of the migratory individuals were considered as outliers and therefore removed from subsequent analyses (n = 46).

In the fall, migratory individuals had a higher antioxidant capacity than resident individuals (GLM, Z = -2.05, $P = 0.042$; Figure 4-2). No significant relationships were detected between life-history strategy and oxidative stress levels (Z = -0.688, $P = 0.49$), length (Z = -1.63, $P = 0.10$) or condition (Z = 0.25, $P = 0.80$). Within migratory individuals, day of migration was negatively correlated with antioxidant capacity (t = -2.20, $P = 0.0393$, Figure 4-3A) and body length (t = -3.81, $P = 0.0005$, Figure 4-3B), but was not associated with oxidative stress levels (t = -1.35, $P = 0.18$) or condition (t = 0.64, $P = 0.52$).

Within migratory individuals, body length was positively correlated with oxidative stress levels (t = 2.36, $P = 0.023$), but not antioxidant capacity (t = -1.89, $P = 0.065$). Within resident individuals, body length was not correlated with oxidative stress levels (t = -0.35, $P = 0.73$) or antioxidant capacity (t = -0.68, $P = 0.51$).

Discussion

Migration is an energetically demanding activity, which involves physiological costs such as a consistently elevated metabolic rate, thus depleting finite resources more rapidly and potentially increasing the production of reactive oxygen species (ROS) compared to non-migratory individuals (Leffler 1993; Jonsson et al. 1997; Costantini et al. 2007). Migratory individuals must therefore have the ability to cope with the increased ROS production that

migration induces, which implies having sufficient repair mechanisms and antioxidants (Costantini 2008). Here we tested whether this overall greater ability to deal with high levels of ROS production is apparent long before migration actually occurs.

Migrant and resident brown trout show differences in morphological (e.g., color and body form) traits, which could play a role in forging an individual's oxidative status. For example, resident individuals display yellow bellies and red spots on their sides, both of which can result from the presence of carotenoids, an important source of antioxidants (Youngson et al. 1997). Smolts in contrast, undergo massive physiological and morphological changes to prepare for migration, such as silvering in colour and increased sodium potassium ATPase activity in the gills (Hoar 1988; Aarestrup et al. 2000; Nielsen et al. 2004), but there is no indication that they divert antioxidant resources to do so. We found that antioxidant capacity, measured as the oxygen radical absorbance capacity (i.e., low molecular weight antioxidants), was higher in migratory individuals than in resident individuals. These antioxidants were elevated days to months in advance of migration, and likely required large amounts of ATP (adenosine triphosphate) which must be replenished through diet (reviewed in Costantini 2014). It is possible that residents deflect resources from building antioxidant capacity to invest in coloration (i.e., carotenoids in this case). In contrast, migratory individuals may invest their resources into building antioxidant capacity to deal with the demands of migration. This hypothesis is further supported by our finding that migrants with higher antioxidant capacity migrate earlier, perhaps as a sign that fish are ready to migrate (i.e., physiologically prepared to deal with oxidative stress during migration). This also predicts that later migrating individuals would increase their levels of antioxidants as they approached their migration date, an intriguing area for future study.

We observed that larger individuals migrated sooner, which is a well-supported pattern in the literature (Metcalfe et al. 1990; Bohlin et al. 1996). Because fish of larger size may have higher growth rates and higher metabolic rates than their smaller counterparts (Økland et al. 1993; Thorpe et al. 1998), we would predict that larger individuals have higher levels of oxidative stress. Our data suggests this is the case only within migrants, where oxidative stress levels were positively correlated with length, possibly emphasizing that larger individuals are more constrained by low food availability in freshwater stream environments.

It is possible that our findings reflect other physiological differences such as differences in growth rate or standard metabolic rate (SMR) among individuals, which may make an individual more likely to adopt one strategy over the other. Specifically, individuals with higher growth rates and metabolic rates may have higher antioxidant capacities to compensate for higher metabolic demands, and may be more likely to migrate as these individuals are more constrained by food availability. For example, Sloat and Reeves (2014) showed that rainbow trout (*Oncorhynchus mykiss*) with high SMR were more likely to smoltify and migrate than those with lower SMR. As such, the higher antioxidant capacities we observed in migrant individuals in the present study may reflect a compensatory mechanism rather than individual readiness to migrate. Future studies should consider measures of SMR and oxidative stress indices in the context of partial migration to answer this question, though this may represent a challenge in field studies.

We cannot exclude the possibility that sex and age played a role in the patterns in oxidative parameters observed in this study. While it has been reported that females tend to migrate more often than males (reviewed in Jonsson and Jonsson 1993), most studies of partial migration do not sex juvenile fish (e.g., Morinville and Rasmussen 2003) as this process requires

lethal sampling or expensive laboratory assays. However, even if the observed resident/migrant differences were due to sex, this itself is an intriguing possibility as no other studies that we know of have documented differences in oxidative parameters due to sex in immature wild fish. Similarly, age was not determined for these fish and may have impacted oxidative status. Though the link between oxidative stress markers and age has been established in humans (Harman 1956), we know very little about fish in that aspect (Martinez-Alvarez et al. 2005), which would provide an interesting avenue for future studies. Nonetheless, only fish between 12 and 20 cm were used, which are typically thought to be 16 to 18 months of age (personal observation, K. Aarestrup; Jonsson 1985) and so it is unlikely that the relationships between oxidative parameters and migratory strategy can be attributed to age. Furthermore, because some individuals may migrate after 3 years of stream residency (Økland et al. 1993), we cannot say for certain that individuals identified as residents in this study will always remain residents; they may in fact migrate during the following year. However, the factors affecting migration between years may be different than those affecting migration within any given year and thus, our study focused on the potential physiological aspects that underpin partial migration within one year.

Conclusion

To better understand the physiological factors that may promote the evolution of partial migration in fish, we examined oxidative stress markers in both migrant and resident individuals of brown trout. During migration, these fish must distribute their limited resources toward swimming efforts, immunity, and predator avoidance among other physiological demands, in addition to coping with the elevated production of ROS. We show that for migrant fish, these resources are also invested toward building antioxidant capacity days to months before migration

occurs. Our study suggests that antioxidant capacity is associated with migratory status and migratory timing in brown trout: migrants have higher antioxidant capacity than residents, and within migrants those with higher antioxidant capacity migrate sooner. This has important ecological implications: (1) increased antioxidant capacity may be a component of smoltification for migratory fish, and (2) fish exposed to stressful conditions may be less able to invest resource in antioxidants, which may delay fish in their migration, and impact population dynamics (Theriault et al. 2008). Our findings support the hypothesis that migrants have mechanisms to cope with the added ROS production induced by a sustained increase in metabolic rate during migration.

Figures

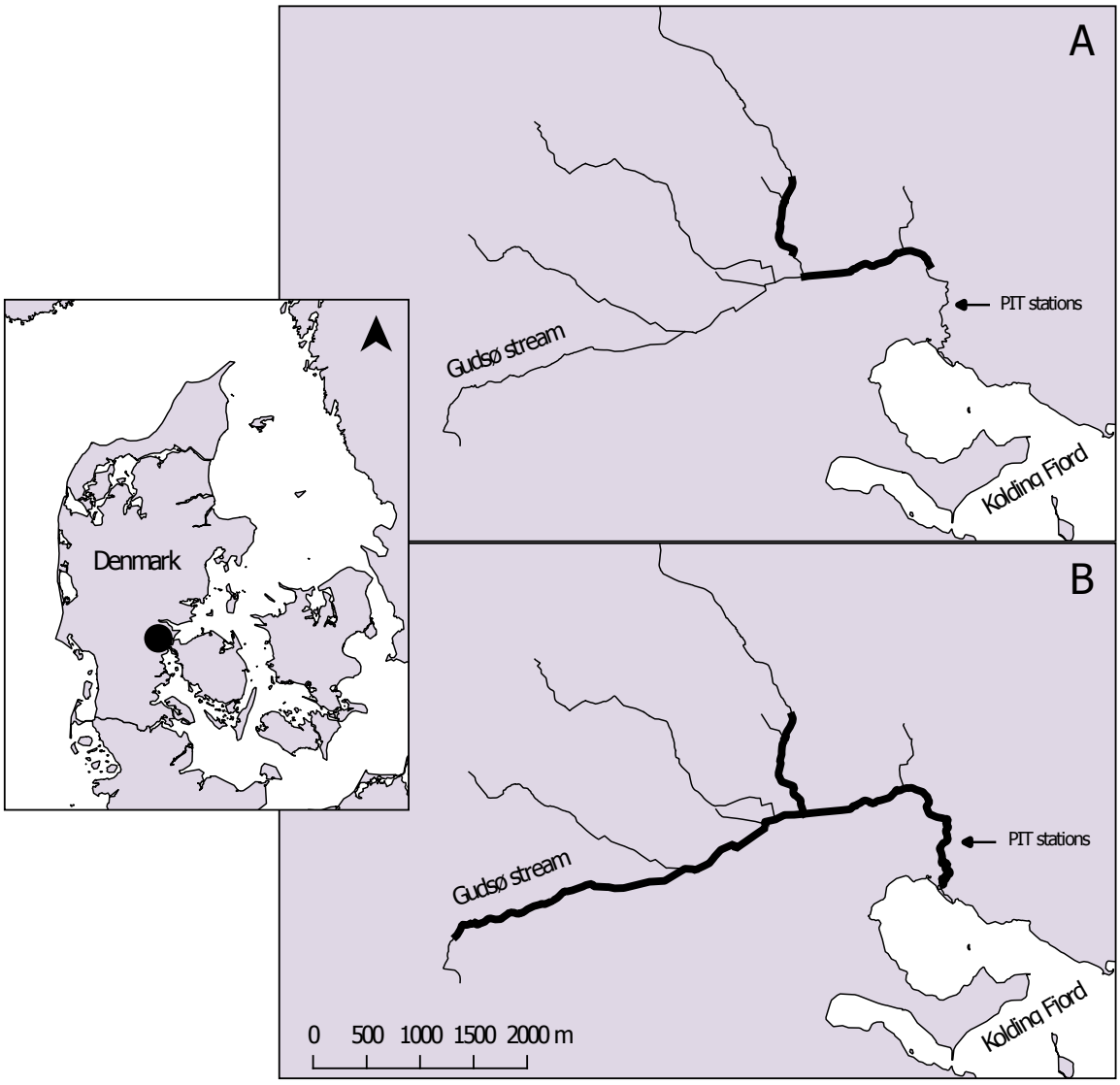

Figure 4-1. Map of the Gudsø stream, Jutland, Denmark. Inserted circle shows location of the stream in Denmark. Sampling locations are highlighted in the thick black trace, A) for the fall sampling, and B) for the spring sampling (adapted from Chapter 3).

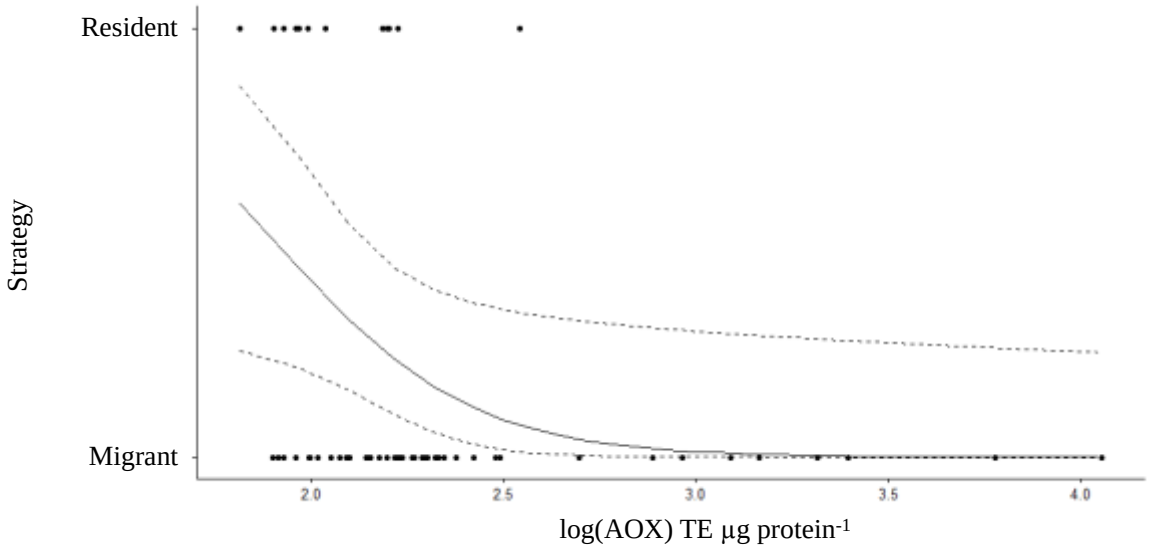

Figure 4-2. Antioxidant capacity. Antioxidant capacity (ORAC; Oxygen Radical Absorbance Capacity) in Trolox equivalents per μg protein for migrants (n = 46) and resident individuals (n = 13). There is a significant association between life-history strategy and antioxidant capacity (GLM, $P < 0.05$).

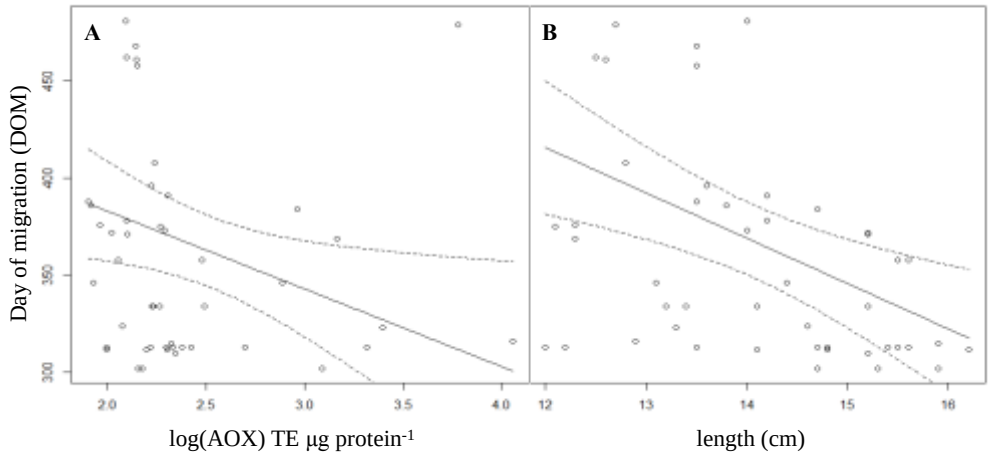

Figure 4-3. Migration timing. (A) migration day (Day 300 = October 28, 2015) as a function of antioxidant capacity (ORAC; Oxygen Radical Absorbance Capacity) in Trolox equivalents per µg protein (n = 46, GLM, $P = 0.0393$). (B) migration day as a function of body length (n = 46, GLM, $P = 0.0005$). Lines represent model predictions, dotted lines indicate 95% confidence intervals.

Chapter 5: General Discussion

This thesis assesses the link between oxidative stress, environmental factors and life-history strategies. In Chapter 2, a complete review of oxidative stress in fish was presented, in which it was demonstrated that different temperature, oxygen availability, salinity, exposure to pollutants, reproductive strategies, level of physical activity as well as age had a significant role in determining an individual's oxidative status. In Chapter 3, exogenous cortisol manipulations were studied to examine the short-term and long-term effects of cortisol on oxidative stress and antioxidant levels. In Chapter 4, the link between oxidative status and partial migration was investigated by comparing oxidative stress levels and antioxidant capacity of fish that migrated to sea with those that assumed residency within the stream.

Findings and Implications

In recent years, ecologists have made use of a variety of physiological markers as measurements of population health in animals (Wikelski and Cooke 2006). Though most studies have focused on the use of endocrine and immunological markers for these assessments (Stevenson et al. 2005), oxidative stress is highly relevant in the context of ecology due to its involvement in biological systems and overall impacts on individual fitness (Beaulieu et al. 2013). In Chapter 2, I reviewed the current knowledge on oxidative stress in fish in the context of extrinsic and intrinsic factors and found that fish's oxidative status can be impacted by virtually all environmental factors, and that oxidative stress parameters appear to play an important role in determining life-history strategies. Furthermore, reviewing the current literature on the topic suggested that relatively little is known about oxidative stress in the context of ecology.

In Chapter 3, I studied the effects of exogenous cortisol manipulation on oxidative stress markers both in the short and long-term, defined as 2 weeks and 4 months, respectively. I additionally investigated how this manipulation correlated to overwinter survival and predation. This study revealed that in the short-term, total glutathione increased, and oxidative stress levels decreased, while no changes were observed overwinter in any of the three oxidative stress markers (total glutathione, oxidized to reduced glutathione ratio and antioxidant capacity). This study further revealed that overwinter survival may be associated with lower total glutathione and lower oxidative stress levels, emphasizing the importance of oxidative stress markers in an ecological context. I additionally found that cortisol manipulation did not result in higher predation, but fewer cortisol-treated fish were recaptured overwinter, suggesting that while mortality may increase with higher cortisol levels, it may not be a direct consequence of higher predation risk, but likely lower feeding rates. Understanding the underlying physiological changes at play and the resonating impacts of exposing organisms to stressors is a crucial component of understanding behavior related to life history strategies.

The brown trout is an ecologically and economically significant species, especially in Denmark, where sea trout provide some of the most important sportfish for local anglers as well as some of the most ecologically important species in terms of ecosystem sustainability (personal communication, K. Aarestrup, N. Jepsen, A. Koed). Current research has focused on how migration is influenced by stressors (e.g., cortisol manipulation, Midwood et al. 2015) and environmental factors (e.g., water temperature, Aarestrup et al. 2002), with very little attention paid to physiology. In Chapter 4, I compared oxidative stress levels and antioxidant capacity in migratory and resident individuals of the same brown trout population and found that migrants had higher antioxidant levels, but found no associated differences in oxidative stress levels. My results

suggest that migrants may invest their limited resources to build resilient antioxidant defenses in preparation for migration and its associated metabolic demands. Oxidative ecology may help to understand what drives specific migration patterns in fish. Furthermore, oxidative ecology may be highly relevant to fisheries management where antioxidants may provide a valuable tool to increase migration success in hatchery-reared fish.

Empirical studies, such as those presented in Chapter 3 and 4, lack in the field of oxidative ecology (Speakman et al. 2015). Though the results of Chapter 3 suggest relatively small effects of cortisol on oxidative stress, they imply that our understanding of oxidative stress processes, and the factors that affect them remain poorly understood, and largely unpredictable. The results of Chapter 4 suggest that antioxidant capacity may be a component of smoltification in salmonids, and may help fish prepare for migration. Understanding the factors that affect antioxidant capacity may help us understand more general migration patterns and other population dynamics. My empirical studies suggest that individual oxidative status may play a role in survival to harsh conditions (i.e., winter conditions) and may promote migration. However, the mechanisms by which this occurs are not understood, and should be further studied given the implications of oxidative ecology.

Future Research Opportunities

This research has supported the recently proposed idea that oxidative stress is likely involved in a wide range of ecological phenomena (Beaulieu et al. 2013; Costantini 2014). In Chapter 2, oxidative stress was found to be largely understudied in the context of fish ecology. Further research in fish ecology, and biology more generally, should consider oxidative stress markers as factors that may underlie behaviors such as migration as well as individual variation in fitness.

Chapter 3 provided evidence that overwinter survival may be associated with oxidative stress markers, an area of research that remains largely unstudied. When investigating survival (and consequently mortality), oxidative ecology should be considered as a driving factor. While this study did not show evidence of any long-term effects of cortisol on oxidative stress levels, future research could study cortisol manipulations of different concentrations at varying times during smolt development. Furthermore, the relationship between oxidative stress and other forms of stressors (e.g., thermal, predation) would be an interesting avenue for future research.

Oxidative stress has been widely studied in the context of migration in birds, but there is a paucity of studies in the fish world. Future research should consider the involvement of oxidative stress levels and antioxidants in various migratory behaviors as observed in many fish species, especially those of high importance for fisheries management and ecosystem sustainability, since the results of such studies may provide insight as to what leads to successful migration. Additional experimental manipulations may include the manipulation of antioxidants (e.g., via N-acetylcysteine to increase glutathione, or buthionine sulfoxamine to completely inhibit glutathione). These types of experiments may provide a more mechanistic understanding of the physiological aspects of migration. For many salmonids (such as the brown trout, *S. trutta*, and the Atlantic salmon, *S. salar*), the consensus is that juveniles make the decision to migrate near the end of the summer, and subsequently begin the smoltification process. In Chapter 4, we demonstrated that migratory individuals had a higher antioxidant capacity in the fall preceding migration than resident individuals. However, our findings could not determine whether this increase in antioxidant was a result of the decision to migrate, or whether individuals with higher antioxidant capacity are the ones that decide to migrate. Future research should investigate the

temporal changes in oxidative status in order to answer this interesting question, which may provide insight into the determinants of migratory vs residency phenotypes.

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