

**Individual Variability and Fitness Consequences of Stress-induced
Cortisol in Wild Fish**

By

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Abstract

The adaptive glucocorticoid (GC) stress response exhibits substantial inter-individual variation that is thought to mediate behaviour and fitness but results remain inconclusive and sometimes contradicting. However, intra-individual variation in GC titres remains relatively unexplored and there exist few examples of links with direct fitness measures. I explored these issues in wild fish by measuring cortisol, the primary GC in fish, following exposure to experimental standardized stressors. In bluegill sunfish (*Lepomis macrochirus*) stress-induced cortisol is repeatable overall and intra-individual variation is influenced by individual size and condition. In pink salmon (*Oncorhynchus gorbuscha*), fish dying pre-spawn exhibited elevated stress-induced cortisol relative to spawning fish and variability in baseline cortisol defined behavioural traits. Significant repeatability of stress-induced cortisol validates the use of this metric as an individual trait in wild fish. Furthermore, the observed differences in reproductive success associated with stress-induced cortisol in a semelparous species confirm a relationship between GCs and fitness.

Dedication

I dedicate this thesis to my family for their constant support and encouragement and for sacrificing their kitchen tables to be used as my office for weeks on end.

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Co-Authorship

The presented work is a manuscript-based thesis. Chapters two and three have been prepared for submission to peer-reviewed journals and thus a degree of repeatability between chapters is to be expected. All presented material is a product of my own work but chapters two and three were conducted as a collaborative effort. As specified below, each co-author played a valuable role and provided comments and feedback on the manuscript. Co-author permission to include these manuscripts in my thesis can be found in the appendix.

Chapter 2: Condition Dependent Intra-individual Repeatability of the Stress-induced Cortisol in a Freshwater Fish

Cook, K.V., C.M. O'Connor, S.H. McConnachie, K.M. Gilmour, and S. J. Cooke

This manuscript is in preparation for submission to the Journal of Fish Biology. The project was conceived by Cooke, O'Connor and Cook with support for Gilmour.

Condition assessments were conducted based on those learned by McConnachie for the study species. Field work completed by Cook and McConnachie. All analysis conducted by Cook.

Chapter 3: Behaviour and Fitness Correlates of Baseline and Stress-Induced Plasma Cortisol Titres in Pink Salmon (*Oncorhynchus gorbuscha*) upon Arrival at Spawning Grounds

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The project was conceived by Cooke and Cook with support for Gilmour and Hinch. All field work conducted by Cooke, Cooke and McConnachie. All analyses completed by Cook.

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Acronyms and Abbreviations

ANOVA: analysis of variance

CV: Coefficient of Variation

FL: Fork Length

GC: Glucocorticoid

HAI: Health Assessment Index

HCT: Haematocrit

HPI: Hypothalamic-Pituitary Interrenal

HR: High responding

HSI: Hepato-somatic Index

K: Fulton's Condition Factor

LR: Low responding

RIA: Radioimmunoassay

RM ANOVA: Repeated measures Analysis of Variance

SEM: Standard error of the mean

SSI: Splenic-somatic Index

TL: Total Length

Chapter 1: General Introduction

The Stress Response in Fish

Wild fish are currently exposed to a multitude of stressors that are both anthropogenic (e.g. climate change, pollution, fisheries interactions, habitat alteration) and natural (e.g. food limitation, predator attacks, seasonal changes, hypoxia and social interactions). Understanding the basic biology underpinning how an individual will respond to these stress-inducing challenges is essential when considering the population effects of environmental change or disturbance. In the field of stress physiology, researchers quantify individual responses to challenges or stressors. The aim is to understand the mechanisms and factors underlying the individual's capacity to cope with stress, and the consequences of their responses to stress (Koolhaas *et al.*, 1999).

Defining what constitutes “stress” or a “stressor” has received considerable attention in the literature (Wendelaar Bonga, 1997). According to Barton and Iwana (1991), “stress in fish is a state caused by a stressor, that results in the deviation from a normal resting or homeostatic state”. Exposure to stress elicits a complex cascade of behavioural and physiological responses that are thought to be compensatory or adaptive mechanisms, enabling an animal to overcome a threat (Wendelaar Bonga, 1997; Barton, 2002). Therefore, when conducting physiological sampling, it is not stress that is quantified, but the responses to stressors which in turn reflect the degree or severity of the stress experienced (Barton, 2002). Primary responses to stress begin with a rapid increase in catecholamines, primarily epinephrine, from chromaffin tissue and the hypothalamic pituitary interrenal (HPI) axis is activated (Barton, 2002). Glucocorticoid

stress hormones (GCs), cortisol being the primary GC in fish, are then released from the interrenal cells of the head kidney and into the blood stream (Barton, 2002). These immediate physiological changes in hormones levels then induce further secondary responses at the blood and tissue levels including increases in cardiac output and oxygen uptake, mobilization of energy substrates, such as glucose, and disturbance of hydromineral balance (Wendelaar Bonga, 1997). Finally, tertiary responses extend to the level of the organism or population and involve changes in whole animal performance such as growth, disease resistance and overall survival (Wendelaar Bonga, 1997; Barton, 2002). Given this multitude of integrative responses, exposure to stress affects organismal biology at all levels of organization, from molecular and biochemical with the initial response and extending to responses at the population and community levels (Barton, 2002).

Cortisol is one of the most commonly measured indicators of stress in fish (Mommsen *et al.*, 1999). In aquaculture, cortisol levels are routinely quantified to understand the time course and magnitude of a stress response or to compare the severity of differing stressors. Unlike catecholamines, cortisol synthesis and release has a lag time of several minutes and therefore, proper sampling protocol can enable measurement of resting, baseline levels, as well as stress-induced concentrations (Wedemeyer *et al.*, 1990; Gamperl *et al.*, 1994). Baseline measurements of cortisol determine the current status of the individual prior to disturbance and should be measured in less than 3 minutes so to not see an effect of the stressor (Romero and Reed, 2005). The response, quantified as the change from baseline to stress-induced concentrations, quantifies the magnitude of perceived threat. Occasionally only a single sample is taken post-stress as a

measure of “stress-induced cortisol”. As this metric does not incorporate the change from baseline, it does not quantify the entire response to a stressor. However, some study specimens are too small to ethically obtain two samples or capture technique prohibits rapid sampling. In these cases, measurement of stress-induced cortisol must act as a proxy for the stress response with the assumption that baselines are near-zero, as expected for most fish species (see Barton, 2002 for typical baselines). Determining the response to a stressor is most common but Romero (2004) has suggested that given differing receptors, at least in mammals, using absolute stress-induced values of GCs may provide more robust data interpretation. Standard sampling protocol is to immediately sample for baseline physiology, apply a standardized stressor for a pre-determined amount of time (most often capture and restraint) and then sample for stress-induced cortisol once levels are known to peak in that species (~25 to 60 minutes).

Correlates of Glucocorticoid Concentrations

GC concentrations tend to rise with increased exposure to stress and thus are commonly used to quantify the magnitude of perceived stress (Barton and Iwama, 1991; Wendelaar Bonga, 1997). GCs are also frequently employed as a measure of ecosystem or individual health (Wikelski and Cooke, 2006; Busch and Hayward, 2009). Generally, elevated baseline levels are thought to indicate an individual in poorer condition or of decreased fitness (Bonier *et al.*, 2009a). This has led to a common misconception that stress is maladaptive. At chronic levels, GCs are indeed maladaptive and detrimental to organism function. Exposure to long-term and chronic stressors (e.g. poor habitat or low

social status) results in increased energy expenditure, reduced growth and disease resistance (Barton *et al.*, 1987), severe protein loss (Wingfield *et al.*, 1998), impaired cognitive abilities (Kitaysky *et al.*, 2003) as well as failure to reproduce or a delay in reproduction (Wingfield *et al.*, 1998; Wingfield and Sapolsky, 2003). However, all organisms adjust behaviour and physiology to accordingly conduct everyday processes. Non-chronically elevated baseline GCs are associated with coping mechanisms required for daily environmental demands, regulating behaviour and physiology to keep internal systems operational (Landys *et al.*, 2006).

On the other hand, additional and unexpected stressors, such as predator encounter, require immediate physiological and behavioural adjustments to overcome the challenge. The result is a short-term and acute stress response. Chronically stressed and acutely stressed are two states that differ enormously in their immediate physiological and behavioural responses as well as the consequences at an organismal level (Wingfield *et al.*, 1998; Buchanan, 2000; Wingfield and Sapolsky, 2003). The acute stress response and short term effects of elevated GCs are adaptive mechanisms, enabling an individual to re-establish a homeostatic state. The result is an increased activity, enhanced cognition and the mobilization of fat reserves and energy stores, a state described by Wingfield *et al.*, (1998) as the “emergency life history stage”.

The intensity of a response following an acute stressor and the subsequent elevation of circulating GCs can vary by more than two orders of magnitude among individuals following exposure to identical stressors (Barton, 2002). Despite this variability, laboratory research has shown this response to be consistent and heritable within captive individuals (Satterlee and Johnson, 1988; Pottinger *et al.*, 1992; Evans *et al.*, 2006). As

both behavioural and physiological responses to stress are controlled by common neuroendocrine signalling systems (Øverli *et al.*, 2000b), individual variability in the physiological response to stress also has consistent behavioural correlates that have been interpreted and categorized in various ways (Koolhaas *et al.*, 1999; Wingfield, 2003). With respect to fish, considerable behavioural work has been conducted on lines of high responding (HR) and low responding (LR) rainbow trout that have been created through selective breeding (Pottinger and Carrick, 1999). These studies demonstrate a tight coupling between stress physiology and behaviour in fish (Øverli *et al.*, 2007) and along with similar research in other taxa, has led to the theory that animals exhibit distinct coping styles being either “proactive” or “reactive” copers (Koolhaas *et al.*, 1999; Brelin *et al.*, 2005; Øverli *et al.*, 2005). The proactive coping style is characterised by a reduced GC response, high level of active avoidance, increased aggression, territorial control, and active attempts to counteract the stressful stimulus (Koolhaas *et al.*, 1999; Øverli *et al.*, 2005). Alternatively, reactive individuals respond to stress with low levels of aggression and immobility and have an increased response to stress (Koolhaas *et al.*, 1999; Øverli *et al.*, 2005). These behavioural traits also extend to social hierarchies as LR or proactive fish have been reported to consistently establish social dominance over HR fish (Pottinger and Carrick, 2001).

Individual variability in GC concentrations is also thought to mediate life history trade-offs whereby elevated levels result in the reduction of reproductive behaviour in favour of self-maintenance, promoting survival at the cost of reproduction (Ricklefs and Wikelski, 2002). Life history theory states that behaviour is modulated in order to maximize life-time fitness. The amount of energy available to an animal is finite and

coping mechanisms associated with stress are energetically demanding. Therefore, when in a stressed state, costly activities not required for immediate survival, such as reproduction, are suppressed or delayed until a more auspicious time (Wingfield and Sapolsky, 2003). This work is predominantly focused in avian studies due primarily to the extensive contributions of J.C. Wingfield (i.e. Wingfield *et al.*, 1995; Wingfield *et al.*, 1998; Wingfield and Sapolsky, 2003) and has more recently extended to reptiles (e.g. Moore and Jessop, 2003; Cote *et al.*, 2006) but remains largely unexplored in other vertebrate groups, especially fish (Wingfield, 2003). Although there has been continued interest in linking variation in both baseline and stress-induced GC titres with measures of individual fitness across several different taxa, direct fitness consequences of endogenous GC concentrations remain unclear (Breuner *et al.*, 2008; Bonier *et al.*, 2009a). Relationships between baseline GCs and fitness appear to fluctuate temporally, even within an individual, and are highly dependent on life history and ecological factors (Bonier *et al.*, 2009b). The theory stands that greater GC reactivity favours self maintenance (Ricklefs and Wikelski, 2002), but several studies have found elevated acute levels of GCs to decrease survival (Romero and Wikelski, 2001; Blas *et al.*, 2007; MacDougall-Shackleton *et al.*, 2009). Relationships between fitness and GCs are also driven by age, experience and reproductive investment. The presence of trade-offs between survival and reproduction are likely to be present in long-lived species with multiple reproductive opportunities (Angelier *et al.*, 2007a). However, in short-lived or semelparous species with minimal chances at reproduction, evolutionary theory predicts the stress response to be decoupled from behaviour as reproduction must occur despite stressful conditions (Wingfield and Sapolsky, 2003).

Variability in Glucocorticoid Concentrations

A common thread throughout the literature is that the relationships between fitness and GCs, for both baseline and the acute response, are highly context dependant. The strength and direction of correlations has been shown to be influenced by life history stage (Bonier *et al.*, 2009b), environmental stability (Angelier *et al.*, 2009), energetic constraints (Angelier *et al.*, 2010; Cote *et al.*, 2010), body condition (Romero and Wikelski, 2001) and reproductive investment (Bókony *et al.*, 2009). GCs are also dynamic and known to fluctuate naturally and consistently within an individual. Consistent variations in both baseline and stress-induced GC concentrations have been observed relative to geographic location and latitude (Silverin *et al.*, 1997), season (Wingfield *et al.*, 1994), photoperiod (Breuner *et al.*, 1999; Carere *et al.*, 2003; Lankford *et al.*, 2003), individual morph-type (Horton and Holberton, 2010) and human disturbances (French *et al.*, 2010). Despite this known variability, there has been little validation that GC concentrations are repeatable within an individual over time. Most studies therefore assume that a measured hormone value on one occasion is truly representative of the physiological phenotype of that individual.

Research assessing repeatability of GCs have yielded inconsistent results (e.g. Romero and Reed, 2008; Wada *et al.*, 2008) and there are only two examples of repeatability of GCs in free-ranging animals, both in colonial birds (Kralj-Fiser *et al.*, 2007; Cockrem *et al.*, 2009). However, in order to understand correlates of GCs at the individual level, we must also understand variation within an individual; if repeatability of hormone concentrations is apparent across all situations and determine what

environmental or physiological factors influence differing degrees of intra-individual variability.

Knowledge Gaps

In stress physiology, there remains a need to assess the relationships between behaviour, fitness and GCs at the individual level, within single life history stages and under controlled conditions where social interactions can be accounted for. Given known intra-individual variations in GC concentrations, there is also need for controlled experiments in a laboratory setting followed by experimentation and observations in the wild to fully understand the consequences of individual variability in GCs. Laboratory studies act to unveil the mechanisms behind an individual's response to stress and studies of wild specimens allow for the incorporation of ecological interactions between hormones and individual behaviour or performance. This study will first comprise of a validation that measured values of stress-induced cortisol in wild fish are truly representative of that individual over time. Secondly, behavioural and fitness consequences of cortisol will be assessed in semelparous Pacific pink salmon in their natural environment.

Research Objectives and Predictions

This thesis will increase understanding of the consequences of intra- and inter-individual variability in cortisol concentrations in wild fish through two separate studies. The overall objectives are to determine 1) if stress-induced concentrations of cortisol are repeatable within an individual and 2) if varying levels of cortisol have fitness or behavioural correlates in a semelparous species.

Rationale and Hypotheses for Chapter 2

The currently acceptable sampling protocol in most physiological research is to take a single sample from each individual. However, given known fluctuations within an individual in GC concentrations, it is surprising the lack of studies validating repeatability of these hormones. Without such studies, we are assuming that a single sample is truly representative of that individual. Furthermore, given the variable nature of GCs, it is likely that some individuals exhibit greater intra-individual variation in hormone levels relative to others and that this differential variation within individual fish is related to other measures of physiology or condition. The objectives of chapter 2 were to assess overall repeatability of stress-induced cortisol in a wild fish and to explore the condition correlates of intra-individual variation in stress-induced cortisol concentrations. I hypothesized that individual condition influences intra-individual variability in stress-induced cortisol concentrations with the prediction that those individuals with a more variable response will show decreased condition.

Model Species for Chapter 2

Wild adult bluegill sunfish (*Lepomis macrochirus*) were used as a model species for chapter 2. Fish were captured from Lake Opinicon accessed from the Queen's University Biological Station in South-eastern Ontario, Canada. This species was chosen as they are extremely abundant, easily captured using non-invasive angling techniques and adapt quickly to holding facilities (Fobert *et al.*, 2009). Furthermore, bluegill have a common life history that is similar to many other fish species and are representative of the large and frequently studied centrarchid family.

Rationale and Hypotheses for Chapter 3

Relationships between individual variation in GC concentrations and direct fitness metrics remain inconclusive (Breuner *et al.*, 2008; Bonier *et al.*, 2009a). In chapter 3 these relationships were assessed using a semelparous wild fish. Baseline and stress-induced concentrations of cortisol were determined and assessed relative to reproductive behaviours such as aggression and mate interaction time as well as fitness metrics, including longevity and reproductive success. The role of cortisol in fish reproduction is also confounding as it is generally accepted that stress inhibits reproduction (Pankhurst and Van Der Kraak, 2000) but stress hormones also play a critical role in managing energy resources throughout the reproductive process (Milla *et al.*, 2009). It has also been predicted that reproductive behaviours would be decoupled from the GC response in semelparous species (Wingfield and Sapolsky, 2003). This chapter will provide an assessment of these hypotheses by providing a direct link between fitness and both

baseline and acutely elevated GCs, increasing the understanding of the role of cortisol in reproduction in a semelparous species. By sampling both sexes from different time periods throughout the migration, changes in physiology across time and between sexes were explored. The experimental design results in the test of several hypotheses:

- 1) The magnitude of the stress response differs between sexes and with sexual maturity
- 2) Variability in cortisol concentrations influence reproductive behaviours on the spawning grounds
- 3) Variability in cortisol concentrations influence reproductive success in females and hence, fitness

Model Species for Chapter 3

Pacific pink salmon (*Onchorynchus gorbuscha*) on the spawning grounds will be used as a model species for this research. Pacific salmon are an excellent model, especially on the spawning grounds. Spawning behaviours are dynamic, consistent within an individual, easily quantified, and fish are highly territorial (Healey *et al.*, 2003). Being semelparous and having only one chance to reproduce, we are provided with an opportunity to directly measure individual fitness as failure to reproduce results in zero fitness. By determining the percentage of eggs dropped for each individual, we are able to directly quantify individual fitness for females. Specifically pink salmon were used as high numbers consistently return to the spawn every second year and thus large sample

sizes were obtained for all analyses. Work was conducted from a controlled spawning channel in which all fish experience the same conditions.

Chapter 2: Condition Dependent Intra-individual Repeatability of Stress-induced Cortisol in a Freshwater Fish

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Abstract

The glucocorticoid (GC) stress response is thought to mediate life-history trade-offs and behavioural strategies. Studies exploring such correlates and consequences of GC variability typically assume the stress response to be repeatable within an individual. Although repeatability has been demonstrated under laboratory conditions, results have been more variable for wild animals, and the reasons underlying these inconsistencies remain unknown. We first tested whether stress-induced circulating cortisol concentrations are repeatable within individual wild teleost fish. We then tested the hypothesis that intra-individual variability in the cortisol stress response is correlated with body condition. Wild-caught bluegill sunfish (*Lepomis macrochirus*) were held under controlled conditions in individual experimental chambers and subjected to repeated standardized stressors (3 times over 6 days) to sample for stress-induced circulating cortisol concentrations. At the end of 6 days, various indicators of fish condition were assessed for all individuals. We found that overall, stress-induced circulating cortisol concentrations for wild bluegill held in captivity were repeatable. We also document considerable intra-individual variation in stress-induced cortisol concentrations across the three trials that was related to Fulton's condition factor and fish size (eviscerated mass). The findings provide evidence that the magnitude of intra-individual variation in stress-induced circulating cortisol concentrations is condition- and

size-dependent, having important implications for the interpretation of studies that examine correlates of GC concentrations.

Introduction

Although once regarded merely as sampling error and/or annoying variance, it is becoming more common to explore the significant inter-individual variation that exists in physiological characteristics (Bennett, 1987; Williams, 2008). Physiological diversity has been particularly well studied in the context of glucocorticoid (GC) stress hormones. GCs serve a range of functions, and are best known for their role during the physiological stress response (Sapolsky *et al.*, 2000; Landys *et al.*, 2006). Baseline, or non-stressed GCs, as well as stress-induced concentrations of GC hormones exhibit considerable variation among individuals and are thought to be individual traits with consistent consequences and correlates at the individual level (Breuner *et al.*, 2008; Bonier *et al.*, 2009)

In fish, exposure to an acute stressor stimulates the hypothalamic-pituitary-interrenal axis (HPI axis; the teleost homolog of the mammalian HPA axis) and results in the synthesis and release of GC hormones into circulation (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999; Barton, 2002). Cortisol is the primary GC in fish and along with glucose, a secondary stress response, is the most commonly measured hormone used to quantify the magnitude of perceived threat from a stressor (Mommsen *et al.*, 1999). During the stress response, GCs act to mobilize fatty acids and liver glycogen which provide the energy required to meet challenges associated with the stressor (Wingfield *et*

al., 1998; Barton, 2002). However, with the energy available to an animal being finite, the GC response affects resource allocations within an individual, and energy directed towards a stress response occurs at the cost of other energy-demanding processes. Accordingly, GCs are thought to mediate life history trade-offs (Ricklefs and Wikelski, 2002), individual fitness (Breuner *et al.*, 2008) survival (Romero and Wikelski, 2001; Blas *et al.*, 2007) and stress coping strategies (Koolhaas *et al.*, 1999; Øverli *et al.*, 2007).

To assess physiology in relation to evolutionary theories and biology (e.g. Ketterson and Nolan Jr, 1999; Zera and Harshman, 2001), the measured physiological trait must be consistent and repeatable within an individual. However, studies exploring individual traits influenced by GC variability usually rely upon measurements of GCs in a single sample from each specimen, especially for wild populations. Underlying this methodology is the assumption that the stress response is repeatable within an individual. Although repeatability of the stress response has been confirmed in captive populations of fish (Pottinger *et al.*, 1992; Schjolden *et al.*, 2005), it has never been explored in wild fish. With respect to wild populations, only two studies have confirmed repeatability, both in colonial birds (Kralj-Fiser *et al.*, 2007; Cockrem *et al.*, 2009). Results of repeatability are often variable. For example, Romero and Reed (2008) reported that the repeatability of baseline GC concentrations in captive house sparrows (*Passer domesticus*) was dependent on the experimental conditions (e.g. short day vs. long day). Similarly, Wada and colleagues (2008) found that stress responsiveness was repeatable in female zebra finches (*Taeniopygia guttata*) from nestling to adult, but not in males. In gilted sea bream (*Sparus aurata*), the magnitude of intra-individual variability is known to differ among individuals with some individuals exhibiting almost identical

concentrations of stress indicators across trials and others displaying a high degree of variation (Tort *et al.*, 2001).

The reasons underlying inconsistency in an individual's response to an identical stressor remain unclear. The hypothesis that irregularity in hormone titres within an individual is influenced by measures of behaviour (While *et al.*, 2010) and condition (Collier *et al.*, 2010) has recently emerged in the literature, primarily in the field of biomedical research. Understanding the significance of intra-individual variation in GC levels is becoming increasingly important as the literature exploring correlates of hormone titres at the individual level rapidly expands (e.g. Angelier *et al.*, 2010; Hau *et al.*, 2010). Assessing intra-individual variability in GC concentrations with respect to measures of condition is a novel approach that may yield explanations for inconsistencies in repeatability.

Most often research assessing correlates of GCs quantifies the stress response, the change from baseline to stress-induced concentrations, for analyses. However, baseline samples should ideally be collected in under 2 minutes (Romero and Reed, 2005) but when working with wild populations, it can often be difficult to rapidly sample individuals. Some collection methods do not allow for immediate sampling or capture multiple individuals at once. Additionally, some study specimens are too small to ethically sample both pre- and post- stress. Therefore, as measuring the stress response is not always an option, understanding the biology of absolute cortisol concentrations is equally important. Furthermore, recent literature has argued that absolute stress-induced GC concentrations may actually provide more accurate data interpretation than the overall response (Romero, 2004). Here we quantified individual condition and

repeatedly measured both stress-induced cortisol and glucose concentrations in bluegill sunfish (*Lepomis macrochirus*). We had two objectives: first, to assess the repeatability of the stress-induced cortisol and glucose concentrations in a wild fish; second, to explore condition correlates of differential intra-individual variability within an individual. We tested the hypothesis that intra-individual variability in stress-induced cortisol is related to condition. Based on results from captive populations, we predicted stress-induced cortisol and glucose to be repeatable across trials and that intra-individual variation in stress-induced cortisol would be predicted by condition whereby fish with lower condition indices would exhibit more variable stress-induced cortisol concentrations across repeated sampling periods. The study was conducted using bluegill because they are abundant, adapt easily to holding conditions, and tolerate repeated sampling (Fobert *et al.*, 2009). Each individual fish was sampled three times for peak cortisol concentrations following exposure to a standardized stressor. Because peak GC response to an acute stressor varies according to species (Romero, 2004), a time course analysis of stress-induced cortisol concentrations was also conducted.

Materials and methods

Study site and study animals

Research was conducted at Queen's University Biological Station in south-eastern Ontario, Canada (44°34'N, 76°19'W) in accordance with CCAC guidelines under an animal care permit (#100446) obtained from Carleton University. All fish were collected from Lake Opinicon in mid-May 2010 by rod and reel angling using barbless circle hooks. In this lake, bluegill begin to form breeding colonies in mid-May and breed

through to July (Cargnelli and Neff, 2006). While fish were showing signs of sexual maturity and colonies had begun to form at the time of collection, fish used in the current study had not yet spawned. Both sexes were sampled and only fish over 170 mm (total length; TL) were used, up to 215 mm.

Study 1: Time course of cortisol response

To determine a time course of cortisol elevation in bluegill, fish were captured and then subjected to a standardized stressor consisting of 3 min of air exposure during which they were placed in a small, covered container lined with moistened padding to prevent desiccation and injury. Following air exposure, fish were held individually in 20 L buckets that were regularly refreshed with lake water. A blood sample was collected on individual fish after 10 (n=7), 20 (n= 8), 30 (n=7), 40 (n=8), or 50 (n=8) min.

Approximately 0.5 mL of blood was collected via caudal puncture using a 1 mL luer-lock sodium-heparinized (10,000 USP Units/ML, Sandoz, QC., Canada) syringe with a 25 gauge 1 ½” needle (BD, Franklin Lakes, NJ). Blood samples were collected within 2 min of approaching the fish and placed in a water-ice slurry for no more than 1 h. Samples were then centrifuged for 5 min at 2000 g (Fisher Scientific Micro-fuge), and the resultant plasma was stored at -80°C until analysis of plasma cortisol concentrations.

Study 2: Repeatability of stress-induced circulating cortisol concentrations

Fish were landed within 10 s to minimize capture stress, immediately placed in large coolers filled with lake water, and transported by boat to the laboratory where they were held in 100 L fibreglass tanks supplied with flowing lake water for a 24 h recovery period. Following the 24 h period of recovery from angling, individual fish (n=35) were exposed to the standardized stressor of 3 min of air exposure as described above, and then placed individually into 20 L buckets that were regularly refreshed with lake water. Based on the results of the time course experiment, a blood sample was collected at 45 min. Due to small size of the study specimen and repeated sampling, baseline cortisol was not measured. The total blood drawn (~0.2 mL) was minimal and only as much as required for assays. Fish were then placed into individual sensory deprivation chambers (115 cm x 22 cm x 22 cm) made of opaque black Perspex and supplied with a constant flow of lake water. The experimental chambers were located in an entry-controlled room that ensured fish were not exposed to external stimuli. After 48 h in the black boxes they were sequentially removed, again exposed to the standardized stressor and a blood sample was collected. This process was repeated a third time after another 48 h, resulting in 3 measures of stress-induced cortisol concentration for each fish, all 48 h apart and in response to identical stressors. In all cases, blood samples were collected within 2 min of approaching the fish and placed in a water-ice slurry for no more than 1 h prior to analysis.

Blood glucose concentration was measured using a drop (~ .05 mL) of whole blood with a hand-held glucose meter (ACCU-CHEK glucose meter; Roche Diagnostics, Basel, Switzerland), a device previously validated for use in fish (Cooke *et al.*, 2008).

Haematocrit (HCT), a measure of the oxygen carrying-capacity of blood (Evans, 1993), was determined using micro-haematocrit capillary tubes centrifuged for 5 min in a haematocrit centrifuge (CritSpin-Micro-Haematocrit Centrifuge). Blood was then centrifuged (Fisher Scientific Micro-Fuge) at 2000 *g* for 5 min. Plasma protein concentration, a measure of general nutritional health (Adams *et al.*, 1993) was assessed using a hand-held protein refractometer. The remaining plasma was frozen immediately in liquid nitrogen and stored at -80 °C for further analysis.

Following collection of the third blood sample, fish were euthanized by cerebral percussion and dissected to obtain measures of body condition including hepato-somatic index (HSI), splenic-somatic index (SSI), Fulton's condition factor (K), and several other physiological and body condition indices combined to form a health assessment index (HAI) modified from (Adams *et al.*, 1993). HSI (the ratio of liver mass to body mass, %) is associated with the nutritional state of the fish and provides an estimate of its energy status (Chellappa *et al.*, 1995). SSI (the ratio of spleen mass to body mass, %) is used to evaluate splenic enlargement, an indicator of disease (Adams *et al.*, 1993). Fulton's condition factor ($K = \text{mass} \cdot \text{length}^{-3} \cdot 10^6$, where mass is expressed in g and length in mm) was calculated as an additional metric that has been reported to be a good indicator of individual energetic state and overall quality for bluegill in Lake Opinicon (Neff and Cargnelli, 2004). The HAI is a rapid and inexpensive method of evaluating the effects of stress on the health of fish populations. It is a quantitative index in which necropsy observations are scored numerically so that statistical comparisons can be made (Adams *et al.*, 1993). This protocol been used in numerous studies to relate overall condition to environmental stress (i.e. Schleiger, 2004; Yeom and Adams, 2007). We modified the

index to focus on six main variables that were expected to reveal differences in health and immune function among our treatments: average haematocrit and plasma protein concentration across the three trials (see Adams *et al.*, 1993) for details on the rating system used), skin condition (an average of external parasite load and % coverage of the fish's skin by fungus [10=low, 20=moderate, 30=high]), gill condition (an average of level of gill fray [10=low, 20=moderate, 30=high], presence of necrotic gill tissue [10=low, 20=moderate, 30=high], and parasite load [10=low, 20=moderate, 30=high]), liver colour (0=normal, 10=cream coloured, 30=completely discoloured) and internal parasite load (an average of parasite load from the liver, kidney, heart and intestine [0=none, 10=low, 20=moderate, 30=high]). All six variables were added together to yield the total HAI (a value out of 60, with higher numbers representing individuals in poorer condition). All condition assessments were conducted by the same person.

Cortisol analysis

Plasma cortisol concentration was measured using a commercial radioimmunoassay kit (immunoChem Cortisol ¹²⁵I RIA Kit, MP Biomedicals, Orangeburg, NY) and a Cobra Auto-Gamma counter (Hewlett-Packard, Palo Alto, CA) following the methods outlined by Gamperl *et al.*, (1994). All samples were measured in a single assay, and intra-assay variability (% CV) was 9.7%.

Statistical analyses

Statistical analyses were conducted using SPSS Statistics 19.0 (2010). Residuals were tested for deviations from a normal distribution using Shapiro-Wilk goodness-of-fit tests, and variables were log-transformed where necessary. If log-transformation did not achieve a normal distribution, non-parametric statistics were used. A Levene's test was used to check for homogeneity of variances and in repeated-measures analyses, sphericity was tested using a Mauchly's test. The level of significance for all tests (α) was assessed at 0.05 unless otherwise noted. Data are reported as mean values \pm 1 standard error of the mean (SEM).

For the time course experiment, Kruskal-Wallis tests were conducted to determine whether fish differed in size among sampling times, and to assess the statistical significance of differences in cortisol concentrations (ng ml^{-1}). The Kruskal-Wallis test was chosen because of the small sample sizes in each group. Post-hoc Mann-Whitney tests compared the subsequent time period to the previous following a significant test.

Sex differences between condition parameters were explored using student's *t*-tests. For variables measured after each sampling period, mixed design (MD) analysis of variance (ANOVA) tests effects of sampling period, sex and the interaction of these two factors. Post hoc pairwise comparisons were conducted following a significant model. Repeatability of stress-induced cortisol and glucose was assessed using the repeatability statistic (*r*) according to Lessells and Boag, (1987) on ranked cortisol data. We chose to rank the data as few individuals from the third trial had exceptionally high cortisol values that influenced all statistical models. To determine *r*, individuals are ranked according to

the magnitude of stress-induced cortisol concentration for each trial. A one-way ANOVA is then used to assess rank consistency within an individual across sampling periods. Individual identity is the independent variable and rank is the dependant variable. A non-significant ANOVA would support the null hypothesis that individual rank is not consistent and therefore, that the measured value is not repeatable (Romero and Reed, 2008). The value of r is calculated according to the formula $r = s^2_A / (s^2 + s^2_A)$ where s^2_A is the among-group variance and s^2 is the within-group variance (Lessells and Boag, 1987).

Intra-individual variability of stress-induced cortisol concentrations was determined by calculating the standard deviation of all three measures of cortisol for each individual, and was termed ‘cortisol variability’. As we are mainly interested in cortisol, ‘glucose variability’ was not explored. We aimed to assess condition and physiological correlates of cortisol variability. To gain a full understanding of the relationships between physiology and condition, we also assessed condition correlates of stress-induced cortisol concentrations. Only stress-induced cortisol from the first trial was used as it was collected before the holding period and thus is most representative of a natural stress response. We used two separate backward stepwise multiple regression models (p to enter <0.05 and p to remove >0.1) to determine condition predictors of cortisol variability and absolute stress-induced cortisol from the first trial. Models were tested for multicollinearity by assessing variable variance proportions. If predictor variables share high variance proportions on the same eigenvalue, the assumption of multicollinearity is broken. From initial saturated models comprising all dependent variables, the least significant terms were sequentially eliminated until obtaining a model where all retained

variables had a significant effect on the independent variable. Condition predictors included K, HAI, HSI, SSI, plasma protein and HCT. Sex and size (eviscerated mass) were included as predictors in each model. For variables quantified following each blood sample (HCT, plasma protein and glucose), only those from initial capture were included in the models. All variables except sex (coded as 0=males, 1=females) were log-transformed.

Results

Study 1: Time course of stress-induced cortisol concentrations

The mean total length across all fish was 155.4 ± 4.2 mm and size did not differ significantly among sampling times (Kruskal-Wallis: $H_4 = 1.1$, $p = 0.892$). A significant effect of sampling time on stress-induced cortisol concentration was detected (Kruskal-Wallis: $H_4 = 21.82$, $p < 0.001$; Fig. 1). Post-hoc tests revealed a significant increase from 30 to 40 min (Mann-Whitney: $U = 5.00$, $p = 0.014$), but not between any other time period (Mann-Whitney: $U = 40.00$, $p = 0.17$ for 10 to 20 min; $U = 11.00$, $p = 0.093$ for 20 to 30 min; $U = 20.00$, $p = 0.355$ for 40 to 50). Therefore, maximal cortisol levels were not reached until ~40 min after the standardized stressor (Fig. 1) and 45 min was selected as the time to sample blood for detection of maximal cortisol concentrations post-stress.

Study 2: Repeatability of stress-induced cortisol concentrations

Of all parameters measured, sex differences were only apparent in stress-induced cortisol and glucose, both being greater in females (Table 2-1). In both sexes, repeated sampling

influenced individual physiology. There was an effect of sampling period on both stress-induced cortisol concentrations and haematocrit (Mixed Design ANOVA: $F_{(2, 66)} = 19.2$, $P < 0.0001$ and $F_{(2, 66)} = 25.1$, $P < 0.0001$, respectively; Fig. 2-2). Planned contrasts revealed that in the third trial stress-induced cortisol to increased ($F_{(1, 33)} = 28.7$, $P < 0.0001$) but haematocrit decreased ($F_{(1, 33)} = 29.4$, $P < 0.0001$; Table 2-2). Glucose and plasma protein did not change across sampling periods (P 's > 0.3). For stress-induced cortisol, there was a significant interaction effect between sampling period and sex ($F_{(2, 66)} = 5.2$, $P = 0.008$) meaning sexes responded to repeated stress differently; males showed a steady increase where females remained consistent until the third trial (Fig. 2-2). Regardless of this sampling effect, repeatability analyses according to Lessells and Boag (1987) determined that ranked stress-induced cortisol concentrations were consistent among individuals across all trials (ANOVA: $F_{34, 70} = 3.195$, $p < 0.001$; Fig. 2-3) with a calculated repeatability statistic of $r = 0.432$. This was not true of stress-induced glucose rankings (ANOVA: $F_{34, 70} = 0.771$, $p = 0.796$; Fig. 2-3). Although stress-induced cortisol was repeatable, there was considerable intra-individual variation (Fig. 2-3) which was explained by both condition factor (K) and eviscerated weight (multiple regression: $F_{3, 31} = 11.62$, $R^2 = 0.53$, $p < 0.0001$; Table 2-3). Smaller fish with decreased condition as determined by Fulton's condition factor had greater intra-individual variability in stress-induced cortisol concentrations. Weight was the only predictor of stress-induced cortisol (multiple regression: $F_{2, 32} = 20.95$, $R^2 = 0.57$, $p < 0.0001$; Table 2-3) whereby smaller fish had greater circulating stress-induced cortisol.

Discussion

We tested for the repeatability of stress-induced cortisol concentrations in bluegill sunfish. A significant repeatability statistic confirmed intra-individual consistency of stress-induced cortisol (Lessells and Boag, 1987) and was comparable to that of studies in other taxa (Romero and Reed, 2008; Wada *et al.*, 2008; Cockrem *et al.*, 2009). However, we observed a considerable degree of intra-individual variation in stress-induced cortisol concentrations or ‘cortisol variability’. We predicted fish in poor condition to have more variable stress-induced cortisol following repeated stress and consistent with this prediction, smaller fish (eviscerated weight) in relatively poor condition (Fulton’s condition factor) exhibited greater cortisol variability.

Condition predicts intra-individual variation

The observed negative relationship between condition and cortisol variability implies the existence of feedback between an individual fish’s ability to respond to stress and its current condition. The results could be explained by a variety of factors. Most intuitively, that poor condition alters the normal functioning of the HPI-axis resulting in erratic and irregular reactivity. Indeed, there is a strong association between condition and stress physiology across taxa, including in fish (Barton *et al.*, 1987; Barton and Iwama, 1991). Interacting stressors in a short amount of time or repeated stress are known to alter HPA/HPI axis reactivity and do result in unpredictable individual variability in GC responsiveness (Schreck, 2000; Romero, 2004). This unpredictability of responses to acute stress may be exemplified with poor condition. However, responses to multiple simultaneous, or repeated stressors are poorly understood (Schreck, 2000).

Following exposure to repeated stress, fish have been found to both accumulate (Barton *et al.*, 1986; Maule *et al.*, 1996) and attenuate (Pickering and Stewart, 1984; Barton *et al.*, 1987; Jentoft *et al.*, 2005) their response. With an increase in stress-induced cortisol and a decrease in haematocrit in the third trial, we did see an effect of the experimental protocol (i.e. sampling and holding) on the condition of our study specimens. It is quite possible that the smaller, poor condition fish were affected more severely by experimental conditions making the effects of repeated stress cumulative. On the other hand, larger and healthier individuals were able to maintain stress-induced cortisol levels within a normal range.

A negative relationship between condition factor and size is a consistent trend for bluegill from Lake Opinicon (Neff and Cargnelli, 2004) and common with Fulton's condition factor in fish (Sutton *et al.*, 2000). That both variables very similarly predicted cortisol variability and no other condition measures were included in the model suggests that inclusion of condition factor is perhaps mostly attributed to its relationship with size. Size was also the only predictor of absolute stress-induced cortisol concentrations; smaller individuals displayed higher concentrations. Magee *et al.*, (2006) found a similar relationship with size and baseline cortisol concentrations in male bluegill at the end of the parental care period, indicating that larger males pay a lower physiological cost for providing parental care. Although our study was conducted prior to the parental care period, fish had formed colonies. The observed relationship between size and stress-induced cortisol could similarly be explained by reproductive trade-offs mediated by physiological mechanisms. Although we did not quantify age, it can be assumed that larger fish are also older. Larger individuals of the *Lepomis* genus are also known to

have greater reproductive success (Dupuis and Keenleyside, 1988; Neff and Cargnelli, 2004) and in general, older parents are more successful (Mauck *et al.*, 2004). The endocrine response is thought to be a mechanism underlying age-specific reproductive performance (Heidinger *et al.*, 2006; Angelier *et al.*, 2007). When the value of current reproduction is high relative to the value of future reproduction, as it is expected in older adults, the stress response should be attenuated to ensure reproduction is not inhibited (Wingfield and Sapolsky, 2003). Therefore, it is possible that larger and more experienced bluegill have a suppressed stress-induced cortisol relative to smaller fish to favour reproduction and that this suppression is maintained across repeated or cumulative stressors. Alternatively, smaller fish in poor condition invest more energy into current survival than reproduction (Ricklefs and Wikelski, 2002) and thus are perhaps more likely to accumulate responses under repeated stress, increasing intra-individual variation in concentrations in these individuals.

Stress-induced glucose did not show overall repeatability. There is considerably less information on the hyperglycaemic stress response. However, as glucose reflects the metabolic response of the fish and is affected by diet content and blood sugar concentration (Barton *et al.*, 1988), being held without any food resources could differentially affect their abilities to launch a glucose response. Furthermore, the time-course validation was conducted to determine the peak time for cortisol and thus may not represent the peak time for glucose. Although glucose is a reliable indicator of stress in fish (Silbergeld, 1974), it seems to not be a consistent trait within an individual and thus should not be used as a correlate or predictor of evolutionary and ecological traits in fish, as is cortisol.

Study limitations

Study fish were exposed to multiple stressors simultaneously and our results do show an effect of holding and/or repeated sampling. Changes in stress-induced cortisol throughout the experimental period could be attributed to the cumulative effects of exposure to repeated acute stressors, the increasing intensity of stressors relating to holding throughout the experimental period, and/or hyperreactivity due to effects of chronic stress. Inability to distinguish among these factors is a major impediment to data interpretation. It is probable that the captive holding of wild fish coupled with repeated sampling in this experiment considerably raised baseline cortisol concentrations. However, the cortisol values measured in this study are stress-induced concentrations rather than the cortisol response, the latter being the difference between stress-induced and pre-stress (baseline) cortisol concentrations. Without quantifying baseline cortisol, we cannot quantify the impact of experimental procedures on individual physiological condition or confirm the presence of chronic stress. Measuring only stress-induced concentrations does also present difficulties in comparing these results to studies in which the stress response has been assessed, as is most common. Owing to chronic stress or elevated baselines, the stress response could have actually decreased in this study, as observed in other species, while stress-induced concentrations rose. Furthermore, the present study involved bringing wild fish into the lab which likely does not provide the same information as measuring wild fish in the field. As such, we cannot use these results to directly draw conclusions regarding the repeatability of stress-induced cortisol concentrations in wild fish.

Conclusions and implications

This study reported repeatability of stress-induced cortisol concentrations in a wild fish. Our findings also suggest that intra-individual variation in stress-induced cortisol as a result of repeated sampling is related to individual condition. We specifically revealed that larger fish in better condition exhibit less intra-individual variability in stress-induced cortisol concentration. We proposed several explanations for this finding. Poor condition could alter the regular functioning of the HPI axis or simply that fish in poor condition respond more severely to the detrimental effects of holding and repeated sampling and thus accumulate their responses over time. Lastly, that the observed relationship is explained by age-specific reproductive trade-offs as mediated by the endocrine response. Larger and older fish with greater reproductive investment suppress their response to acute stress compared to smaller individuals invested into current survival that exhibit relatively enhanced stress-induced cortisol concentrations (Wingfield and Sapolsky, 2003; Angelier *et al.*, 2007). In this scenario, the inclusion of condition factor as a predictor of intra-individual variation is due to its consistent relationship with size in fish (Sutton *et al.*, 2000; Neff and Cargnelli, 2004).

Findings are most likely attributed to a combination of the aforementioned explanations. Given a poor understanding of repeatability of cortisol concentrations in wild fish, we suggest caution in assuming a measured value is truly representative of that individual, especially if in a deteriorated condition. Research assessing correlates of individual GC responsiveness, especially in degraded environments or in populations where fish may be in poor condition, should ideally sample each individual more than once. Since we acknowledge that capturing the same individual multiple times is often

difficult, the results of our study highlight, at the very least, the importance of considering individual condition and size together with GC concentrations measured from a single blood sample

Tables

Table 2-1: Differences in physiological and condition metrics between male and female bluegill sunfish (*Lepomis macrochirus*) sampled following three exposures to a standardized stressor. Indicated blood parameters were quantified following each stress exposure while condition measures only upon termination of the experimental period. P-values represent differences between sexes (Student's *t* test for single samples and Mixed design ANOVAs for repeated samples). Bold text represents statistically significant differences ($P < 0.05$). The grand mean is presented for those variables not differing between sexes ($P > 0.1$).

	Mean \pm SE		P-value
	Males	Females	
Stress-induced Cortisol [†]	260.5 \pm 34.7	545.6 \pm 58.4	<0.0001
Stress-induced Glucose [†]	4.6 \pm 0.4	6.0 \pm 0.4	0.014
Cortisol Variability [‡]	163.9 \pm 29.3	304.9 \pm 80.9	0.06
HSI	1.3 \pm .06	1.6 \pm .07	0.08
	Grand Mean \pm SE		
Condition Factor	1.7 \pm .02		0.60
SSI	0.1 \pm .02		0.94
Plasma Protein [†]	54.9 \pm 1.1		0.16
Haematocrit [†]	31.9 \pm 1.1		0.46
HAI	42.1 \pm 2.3		0.51

[†] Mean from all three sampling periods for each fish

[‡] Standard deviation of stress-induced cortisol for each individual fish across three sampling periods

Table 2-2: Changes in physiological parameters across time periods for bluegill sunfish (*Lepomis macrochirus*) sampled following three exposures to a standardized stressor. Bold text is used to indicate a statistically significant main effect of sampling period (Mixed design ANOVA; $P < 0.05$). Glucose and plasma protein (not shown) did not differ across trials.

	Mean \pm SEM			P-Value
	Trial 1	Trial 2	Trial 3	
Stress-induced Cortisol (ng mL ⁻¹)	267.1 \pm 35.4	295.0 \pm 29.2	537.1 \pm 73.8	<0.001
Haematocrit	31.9 \pm 1.1	32.5 \pm 1.0	24.6 \pm 1.1	<0.001

Table 2-3: Results of backward stepwise multiple regression (P to enter < 0.05, P to remove > 0.1) testing the effects of physiological and condition parameters on 1) intra-individual variability in stress-induced cortisol concentrations and 2) stress-induced cortisol prior to holding in bluegill sunfish (*Lepomis macrochirus*) repeatedly sampled following exposure to a standardized acute stressor. Variables are listed in the order they were excluded from the model. Main effects retained in the final model are indicated in bold. Non-significant results presented are the p-values just before removal from the model

Model 1			
Variable	Dependent Variable: Cortisol Variability [‡]		
	B	P-value	F
HSI	277.24	0.54	4.26
Sex	-66.10	0.35	5.52
HAI	211.85	0.32	7.11
Haematocrit	380.42	0.25	8.33
Plasma Protein	-928.89	0.10	9.94
SSI	-192.26	0.07	
Weight	-642.43	0.04	11.62
Condition Factor	-2675.79	0.01	
Model 2			
Variable	Dependent Variable: Stress-induced Cortisol [†]		
	B	P-value	F
Condition Factor	-166.88	0.88	5.04
Plasma Protein	-209.38	0.73	5.97
HAI	157.47	0.44	7.17
Haematocrit	215.70	0.49	8.60
Sex	-70.37	0.35	10.80
SSI	-82.66	0.38	14.15
HSI	550.63	0.06	
Weight	-1368.49	0.0001	20.95

Model 1: $R^2 = 0.53$, $F_{3,31}=11.62$, $P<0.0001$, Model 2: $R^2 = 0.57$, $F_{2,32}=20.95$, $P<0.0001$

All variables except sex (0=males, 1=females) are log-transformed

[‡] Cortisol variability calculated as the standard deviation of stress-induced cortisol for each individual fish across three sampling periods

[†] Values from the first trial were used to eliminate sampling effects seen in subsequent measurements

Figures

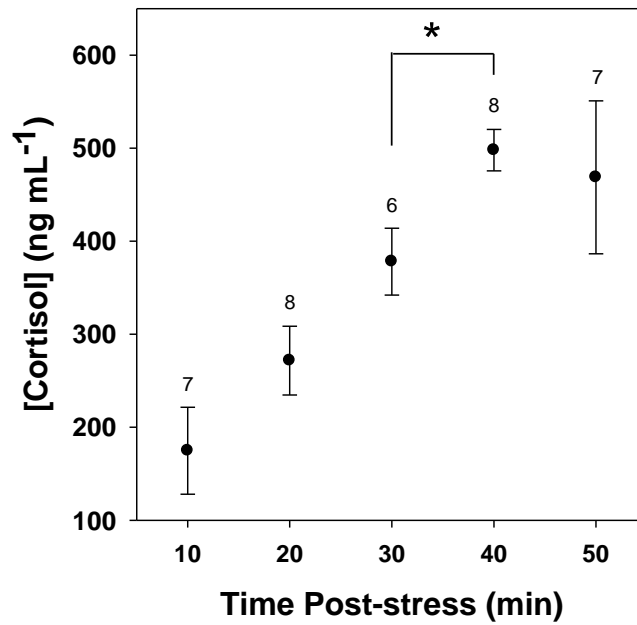


Figure 2-1: Plasma cortisol concentrations measured 10-50 min following exposure to a standardized acute stressor (3 min of air exposure) in bluegill sunfish (*Lepomis macrochirus*). Values are means \pm 1 SEM. An asterisk (*) indicates a significant difference between the indicated points (Mann-Whitney U: P = 0.014). Sample sizes indicated above each point.

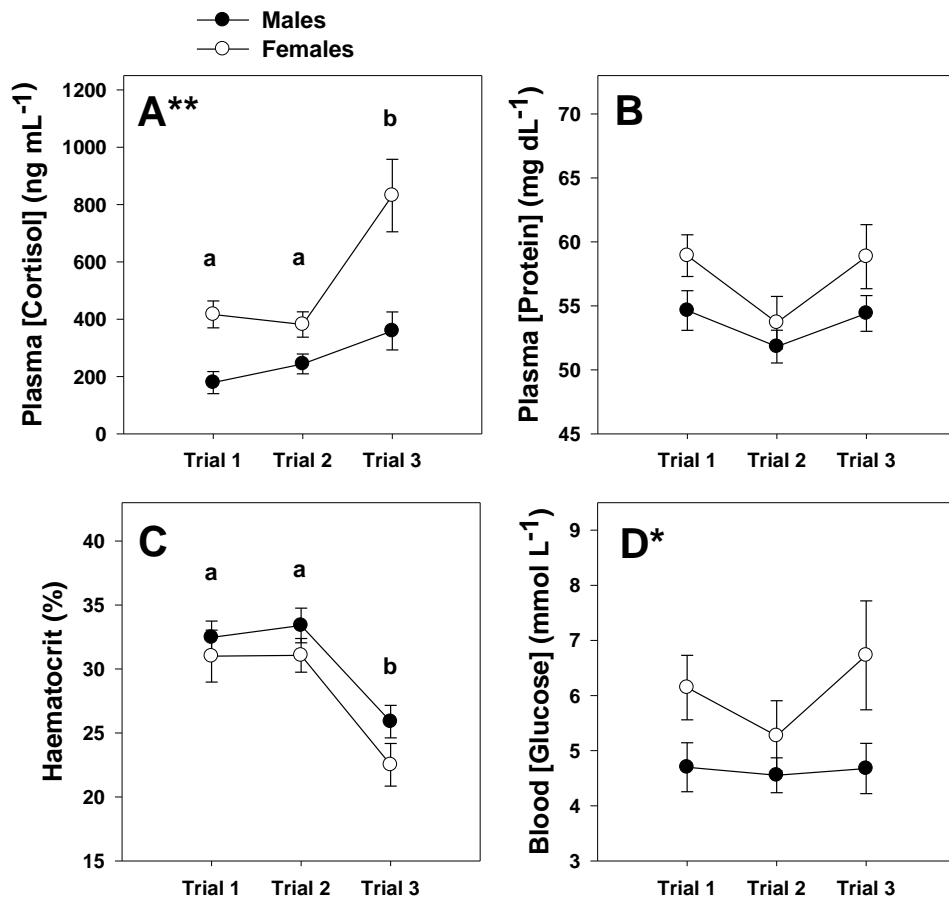


Figure 2-2: Change in mean A) stress-induced cortisol concentrations (ng ml⁻¹), B) plasma protein (mg dL⁻¹), C) haematocrit (%) and D) stress-induced glucose concentrations (mmol L⁻¹) between sampling periods for both sexes of bluegill sunfish (*Lepomis macrochirus*) exposed to a standard stressor of air emersion on three separate occasions (n=35). Significant differences across sampling groups are denoted by lower case letters, a double asterisk (**) denotes a significant main effect of sex as well as a significant interaction effect of sex and sampling period and a single asterisk (*) denotes a significant effect of sex only (Mixed design ANOVA: P < 0.05). Values are means + 1 SEM.

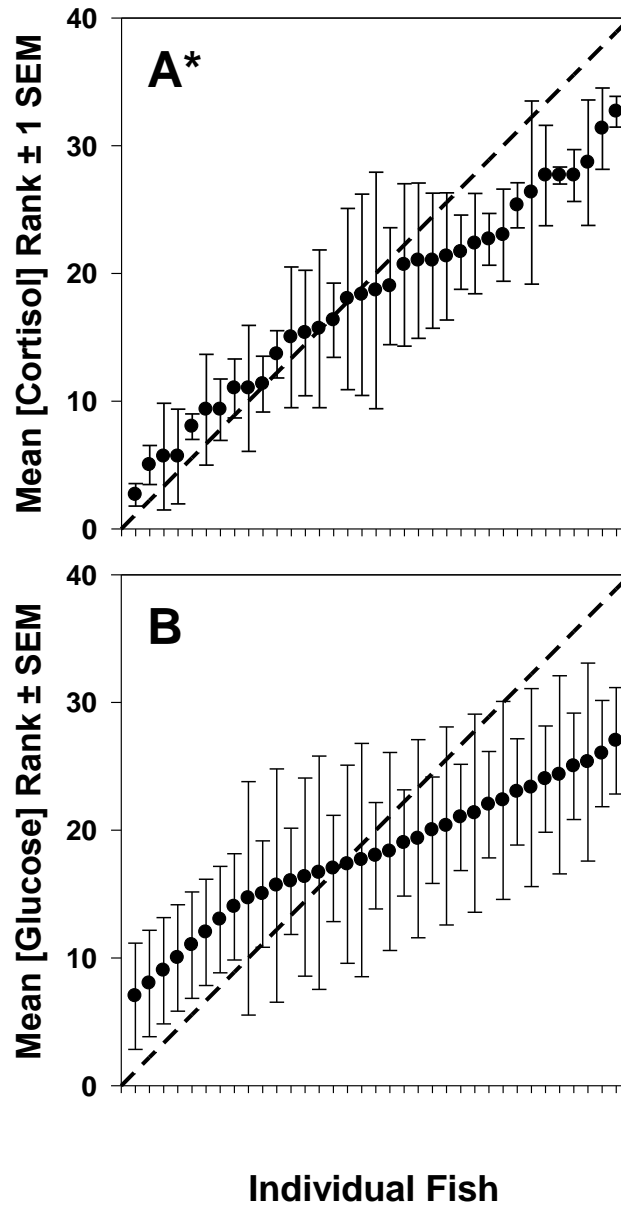


Figure 2-3: Repeatability of ranked A) stress-induced cortisol concentrations (ng mL^{-1}) and B) stress-induced glucose concentrations for bluegill sunfish (*Lepomis macrochirus*) sampled on three occasions following exposure to a standardized stressor of 3 minutes of air exposure ($n=35$). An asterisk (*) represents significant repeatability overall (One-way ANOVA: $p < 0.05$). Each point represents mean rank \pm 1 SEM for an individual fish, ordered on x-axis from smallest to greatest. The dotted line represents perfect repeatability where every fish has identical ranks across all trials (Romero and Reed, 2008). Fish with greater error have a low consistency of measured values and fish with very little error have highly consistent responses to acute stress across the three trials.

Chapter 3: Behaviour and Fitness Correlates of Baseline and Stress-Induced Plasma Cortisol Titres in Pink Salmon (*Oncorhynchus gorbuscha*) upon Arrival at Spawning Grounds

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Abstract

Semelparous Pacific salmon (*Oncorhynchus spp.*) serve as an excellent model for examining the relationships between fitness, behaviour and individual variation in glucocorticoid (GC) stress hormone levels as reproductive behaviours are highly variable among individuals and failure to reproduce results in zero fitness. Pink salmon (*O. gorbuscha*) were intercepted upon arrival at the spawning grounds at three time periods. Both baseline and stress-induced plasma cortisol concentrations were assessed in relation to behaviour, longevity and reproductive success. Results revealed differences between sexes and with increasing maturity. The study period marked a year of high reproductive success and only nine of all sampled females (12%) failed to spawn. Female pre-spawn mortalities were characterized by significantly elevated stress-induced cortisol concentrations and decreased longevity as well as baseline cortisol above the normal range. Interestingly, reproductive behaviours were associated with baseline but not stress-induced cortisol levels. In females there was a negative relationship between baseline cortisol concentrations upon arrival and both aggression and mate interaction time. In males a similar trend was observed but only with mate interaction time. The observed behavioural correlations are likely a factor of social status where dominant individuals, known to have higher reproductive success, are characterized by lower cortisol levels relative to subordinate conspecifics. Findings show both elevated baseline

and stress-induced cortisol concentrations at arrival to the spawning grounds have negative fitness consequences in pink salmon.

Introduction

Individual variation in glucocorticoid (GC) stress hormones are thought to mediate life history trade-offs (Ricklefs and Wikelski, 2002) and behaviour (Koolhaas *et al.*, 1999). However, fish remain an underused model to assess these relationships (Mommsen *et al.*, 1999; Wingfield, 2003) despite wide variation in reproductive strategies and behaviour as well as a thorough understanding of their stress physiology (Wendelaar Bonga, 1997; Mommsen *et al.*, 1999; Barton, 2002). When teleost fish are exposed to an acute stressor, the hypothalamic-pituitary-interrenal (HPI) axis is activated resulting in an increase in circulating cortisol concentrations. Cortisol is the primary GC hormone in fish and it acts to mobilize energy stores which allow the fish to cope with the stressor (Wendelaar Bonga, 1997; Barton, 2002). As cortisol is synthesized and then secreted upon demand, levels begin to rise several minutes after exposure to a stressor. It is therefore possible to measure pre-stress concentrations (baseline) as well as stress-induced values (the absolute post-stress value) and the total response (change from baseline to stress-induced concentrations) within an individual (Romero, 2004). Both baseline and response GCs exhibit variability that is generally consistent within an individual (Pottinger *et al.*, 1992; Romero and Reed, 2008) but have differing consequences (Romero, 2004).

There has been recent interest in linking variation in GC titres with measures of individual fitness across a range of taxa (Breuner *et al.*, 2008; Bonier *et al.*, 2009a). The

“Cort-Fitness hypothesis” has been proposed whereby elevated baseline cortisol levels are assumed to indicate an individual or population of reduced relative fitness. On the other hand, the acute GC response is an adaptive mechanism that facilitates escape from challenging situations (Wingfield *et al.*, 1998). Intuitively, it would seem that a greater stress response would have positive fitness effects. However, launching a stress response is energetically demanding and consequently results in the reallocation of costly behaviours, such as reproduction, to self-maintenance (Ricklefs and Wikelski, 2002). Studies of correlations between both measures of GCs and direct measures of fitness have yielded inconsistent results (Breuner *et al.*, 2008; Bonier *et al.*, 2009a). A common thread throughout the literature is that these relationships are highly context dependent, being influenced by a variety of factors including life history stage (Bonier *et al.*, 2009b), environmental stability (Angelier *et al.*, 2009) and energetic constraints (Angelier *et al.*, 2010; Cote *et al.*, 2010).

Pacific salmon (*Oncorhynchus spp.*) serve as an interesting and practical model for exploring performance correlates of GCs. Fitness metrics such as spawning behaviour and reproductive success can be easily quantified (Quinn, 1999; Healey *et al.*, 2003; Mehranvar *et al.*, 2004) and lifetime reproductive output is compressed into a single breeding effort allowing for direct quantification of fitness. Cortisol has both stimulatory and inhibitory effects in Pacific salmon. Stress indicators (e.g. osmolality, lactate, plasma cortisol and glucose) markedly increase in the transition to freshwater (Cooke *et al.*, 2006a; Cooke *et al.*, 2006b; Crossin *et al.*, 2009) and throughout the migration (Hendry and Berg, 1999; Crossin *et al.*, 2003). These elevated concentrations assist with natal stream homing (Carruth *et al.*, 2002) and can accelerate ovulation (Schreck, 2010)

but also cause tissue degeneration (Hendry and Berg, 1999; Maldonado *et al.*, 2000) and suppress immune function (Maule *et al.*, 1996). Once in freshwater, Pacific salmon cease feeding and depend entirely on endogenous energy reserves. Over 50% of body energy is required to reach the spawning grounds (Crossin *et al.*, 2004) and upon arrival, highly aggressive behaviour and territoriality pose additional stress and energetic costs (Healey *et al.*, 2003). Females compete for access to quality nest sites and defend their territories (Essington *et al.*, 2000) and in males, aggression determines access to mates (Healey *et al.*, 2003). Spawning behaviours in Pacific salmon have been quantified in relation to energy allocation (Healey *et al.*, 2003), reproductive success (Mehranvar *et al.*, 2004) and baseline stress indicators (Hruska *et al.*, 2010), but not to our knowledge in relation to acute GC concentrations.

In this study we characterized baseline and stress-induced cortisol levels in male and female pink salmon across three different arrival times to the spawning grounds, in relation to behaviour, longevity (total time in the channel) and reproductive success (in females only, as determined by percentage of eggs released). Longevity has fitness consequences for both sexes. For males, the longer they sustain reproductive maturity in the channel, the more likely they are to mate with multiple females. The longer a female defends a nest, the less likely it is to be dug up by subsequent females (Quinn and Foote, 1994). Our first hypothesis was that cortisol concentrations differ with arrival time. Animals with a high investment in current reproduction are expected to have elevated baseline GC levels (Bókony *et al.*, 2009) but an attenuated stress response (Wingfield and Sapolsky, 2003). Therefore we predicted baseline cortisol levels to increase but stress-induced cortisol to decrease with arrival to the spawning grounds. Our second hypothesis

was that cortisol concentrations influence fitness. Maintaining elevated GCs is energetically costly (Wingfield and Sapolsky, 2003) and spawning fish have very limited energy resources (Crossin *et al.*, 2004). We predicted that those fish with elevated levels of baseline and stress-induced cortisol would have decreased longevity (males) and lower reproductive success (females). Lastly we hypothesized that cortisol influences reproductive behaviour within an individual. In juvenile salmonids, subordinate social status is a chronic stressor characterized by elevated baseline cortisol levels (Gilmour *et al.*, 2005) and in populations of rainbow trout (*O. mykiss*), individuals with a consistently low cortisol response to standardized stressors become socially dominant (Øverli *et al.*, 2000a). We therefore predicted that more aggressive, dominant individuals would have decreased baseline and stress-induced cortisol, but we also expected an effect of size, as larger fish are known to hold dominant positions in some species of Pacific salmon (Foote, 1990).

Methods

All work was conducted in accordance with CCAC guidelines under an animal care permit obtained from Carleton University and Fisheries and Oceans Canada. Research was carried out in October 2009 at the Weaver Creek Spawning Channel, located in the lower Fraser valley in British Columbia, Canada (Fig. 3-1). The 2930 m long and 6.1 m wide artificial spawning channel is approximately 100 km from the mouth of the Fraser River and 2 km downstream of Harrison Lake (Quinn 1999; Fig. 3-1). The spawning channel was constructed from a small tributary of the Fraser River to enhance salmon production, particularly sockeye (*O. nerka*), but chum (*O. keta*) but pink salmon are also

present and abundant. In this closed system, gravel substrate is of appropriate size for spawning (1.2-7.6 cm), depth is consistent (25-30 cm), flow conditions are controlled and stable (0.4 m/s), and fish densities are monitored (Quinn 1999).

Physiological Sampling and Determination of Reproductive Success

Upon leaving the natural river system, fish enter a concrete holding area from which they are passed through a counting fence into the spawning channel (see Fig. 3-1). Fish were collected by dip-netting individuals from the holding area. It was assumed that all fish at one sampling time had an equal likelihood of entering the channel and were of similar maturity. Immediately after capture, a standard stressor of two minutes air exposure was applied. This stressor is known to increase cortisol concentrations (Arends *et al.*, 1999), and ensured that all individuals were exposed to an equivalent magnitude of stress.

During air exposure, fish were held in a moistened, foam-lined, V-shaped trough, sampled for approximately 1.5 mL of blood by caudal puncture using 3 mL lithium-heparinized vacutainers (B.D. Vacutainer, Franklin Lakes, NJ) with 21-gauge, 1.5” needles and tagged with a Peterson disk or cinch tag. Post sample collection, pressure was applied to the sampling site to facilitate clotting. A second blood sample was collected using the same procedure after 25 minutes to measure stress-induced cortisol. Between sampling times, individuals were placed in black nylon fish bags (1 m x 0.2 m) with mesh ends oriented into the water flow in the channel. The second blood sample was collected while fish remained in the fish bags. Fish were released into the channel once the sample had been collected. Blood samples were held in a water-ice slurry for no

more than 45 minutes and centrifuged for 5 minutes at 10,000 g (Compact II Centrifuge, Clay Adams, Parsippany, NJ). Plasma samples were flash frozen in liquid nitrogen and stored at -80°C until analysis. Plasma cortisol concentration was determined using a commercial radioimmunoassay kit (ImmunoChem Cortisol ¹²⁵I RIA; MP Biomedicals, Orangeburg, NY) and a Cobra Auto-Gamma counter (Hewlett-Packard, Palo Alto, CA), a procedure commonly used to measure cortisol in fish plasma (Gamperl *et al.*, 1994). Intra-assay variability was $9.3 \pm 0.7\%$ and all samples were assayed together.

The sampling and tagging process was repeated on three different groups during the run representing early, middle and late timing groups from the population. Thirty fish of each sex were processed in each group resulting in a grand total of 180 fish. The early and late fish were tagged with Peterson disk tags, and individuals were located in the channel by walking its length twice-daily and reading the tags using binoculars. Each fish was observed for ~30 s to determine the status of redd establishment and calculate the total number of days on a redd (for females). The point at which each fish left pooling aggregations to create or defend territories was noted. As behavioural observations were carried out at the same time on a separate group of fish (see below), fish from the middle group were not tagged to be located in the channel due to time constraints. Instead these fish were tagged with cinch tags from which ID could be read upon retrieval. Dead fish were collected daily and longevity was calculated as the number of days between initial sample and death. Following the methods of Hruska *et al.* (2010), reproductive success was determined (on carcasses) as the percentage of eggs remaining relative to the expected number of eggs as determined by a fork-length-to-egg-mass relationship constructed from unripe females. It was not uncommon for small

numbers of eggs to be lost during processing or transportation to the lab. To definitively separate females that reproduced successfully from those that did not, females that dropped between 51 and 89 % of their eggs (n=10) were excluded from analyses of reproductive success. Although these fish may have spawned a proportion of their eggs, it was rare for only a proportion but not all eggs to be spawned and it can be assumed that these fish have reduced fitness relative to those release all eggs. We justify excluding these fish as we are interested in comparing those successful to those not successful. Five fish were never recovered from the channel and two were scavenged by gulls (*Larus spp.*) and thus were also excluded. Females that released >90% of eggs were considered spawners, and those that released <50 % of eggs, non-spawners.

Behavioural Assessments

An additional group of 13 fish of each sex from the middle timing group was used to conduct detailed behavioural observations in an experimental arena. These fish were held in the spawning channel in an area enclosed with Vexar rigid mesh netting (approximately 5 m x 2 m; Masternet, Mississauga, Ontario). Each individual was observed from shore for 10 minutes daily until death. The order of observation was randomly chosen each day. For both sexes, behavioural metrics included aggression score and mate interaction time. Aggressive behaviours included chases, charges, and bites as described by Healey et al. (2003). Aggression score was calculated for each fish using an approach adapted from Mehranvar *et al.*, (2004) as the total number of attacks given minus the number of attacks received, divided by the total observation time for

each individual. A positive score indicates a dominant individual and a negative score a subordinate. Mate interaction time was calculated as total time spent interacting with the opposite sex divided by total observation time and multiplied by 100 to represent the percentage of total observation time spent with a mate. If a female was courted by multiple partners during one observation period, times with all mates were included in the score. For females, number of days on a redd were also quantified.

Statistical Analyses

Statistical analyses were conducted using SPSS Statistics 19.0 (2010). Residuals were examined for normal distributions using the Shapiro-Wilk goodness-of-fit test and heterogeneity of variance was assessed using Levene's test. Variables were log-transformed to meet assumptions of normality where necessary. If log-transformation did not yield normality, non-parametric statistical tests were used. All regression models were conducted with a stepwise forward selection procedure (P to enter = 0.05, P to remove = 0.10) and tested for multicollinearity by assessing variable variance proportions. If predictor variables share high variance proportions on the same eigenvalue, the assumption of multicollinearity is broken. Accuracy of the logistic regression model was determined by analyzing residual statistics according to Field (2009). The level of significance for all tests was assessed at 0.05 (α). Means are reported \pm standard error of the mean (SEM).

Two-way ANOVAs were used to determine the effect of sampling period and sex on baseline cortisol concentrations, stress-induced cortisol concentrations and fish size

(FL). As longevity could not be transformed to meet assumptions of normality, separate non-parametric tests assessed differences between sex (Mann-Whitney U) and across sampling periods (Jonckheere-Terpstra). Regression analyses were conducted to determine predictors of spawning success (binary logistic; spawners vs. non-spawners), days on a redd and longevity (multiple regressions). Preliminary analyses revealed longevity to decrease with arrival time, likely due to a strong correlation with individual maturity upon arrival, and was thus included as a predictor of reproductive success and days on a redd along with cortisol measures in females. However, longevity is the only fitness metric for males and is known to have fitness consequences in both sexes. To use longevity as a fitness metric, we assumed fish from each sampling group were of equal maturity and conducted multiple regressions within each sampling group with baseline and stress-induced cortisol as predictors along with sex (binary-coded).

In the enclosure experiment, multiple regression was also used to determine behavioural predictors (aggression score, mate interaction time) of success (females: number of days on a redd; males: longevity) while including size (FL) as a predictor. To determine relationships between behaviour and cortisol, simple regressions analyses were conducted in both sexes between behaviour metrics and both baseline and stress-induced cortisol as well as fork length.

We excluded the cortisol response (change from baseline to stress-induced) from statistical analyses. In mature Pacific salmon, as exceptionally high circulating cortisol concentrations are maintained, stress-induced cortisol rather than the calculated response may be more comparable to analyses of the response in iteroparous species with near-zero baselines. Furthermore, preliminary exploratory analyses revealed no trends with

the cortisol response and a recent review has suggested that absolute stress-induced concentrations of GCs are more likely to provide correct interpretations of data (Romero, 2004).

Results

In total, we retrieved 98 females and 91 males (Table 3-1). Fish size (fork length; FL) did not differ across the sampling periods (one-way ANOVA: $F_{(3, 93)} = 1.088$, $p = 0.358$; Table 3-1) and was greater in males (52.6 ± 0.38) than females (50.7 ± 0.20 ; Mann-Whitney U test: $U = 5696.0$, $z = 4.279$, $P < 0.0001$). FL was not correlated with either measure of cortisol or reproductive success in either sex. Two-way ANOVAs revealed an effect of sex for both baseline and stress-induced cortisol (baseline $F_{(1, 181)} = 100.1$, $p < 0.0001$; stress-induced $F_{(1, 178)} = 51.9$, $p < 0.0001$) and sampling period (baseline $F_{(2, 181)} = 7.7$, $p < 0.001$; stress-induced $F_{(2, 178)} = 14.8$, $p < 0.0001$) but no interaction (baseline $F_{(2, 181)} = 1.9$, $p = 0.143$; stress-induced $F_{(2, 181)} = 0.6$, $p = 0.549$). In both measures and in both sexes, cortisol did not differ between the early and middle run but was decreased in the late run. In all cases, females had higher cortisol levels than males (Baseline: males 147.56 ± 11.2 , females 333.78 ± 17.7 ; Stress-induced: males 249.69 ± 13.9 , females 496.93 ± 22.0 ; Fig. 3-2).

Fitness Metrics and Relationships with Cortisol

Fitness metrics for females included percentage of eggs dropped, number of days on a redd and longevity. Percentage of eggs dropped did not differ between sampling groups

(Kruskal-Wallis: $H_{(3)} = 37.2$, $p < 0.0001$). Females were grouped into spawners ($n=73$) or non-spawners ($n=9$). A significant binary logistic model ($X^2_{(2)} = 21.99$, $P < 0.0001$; Table 3-2) revealed stress-induced cortisol ($Z_{(1)} = 9.61$, $P = 0.002$) and longevity ($Z_{(1)} = 6.94$, $P = 0.008$) to predict spawning success. Number of days on a redd ranged from 0 to 10 days and was similar between the early (4.0 ± 0.5 days) and late (4.2 ± 0.3 days) groups (Student's t -test: $t_{42}=0.424$, $p=0.674$) and was significantly greater in successful fish (Mann-Whitney U test: $U = 358.5$, $z = 3.71$, $P < 0.0001$; Table 3-3), confirming this variable as a fitness metric. Longevity was the only significant predictor of number of days on a redd (Multiple regression: $R^2 = 0.160$, $F_{(1,66)} = 12.599$, $P = 0.001$). Logically, the more days a female spends in the channel overall, the longer she protects a redd (data not shown).

Longevity did not differ between sexes (Mann-Whitney U test: $U = 4861.0$, $z = 1.360$, $P = 0.174$). In both sexes there was a significant decreasing trend with arrival date (Jonckheere-Terpstra: males $J = 477.0$, $z = -6.5$, $p < 0.0001$; females $J = 712.0$, $z = -5.8$, $p < 0.0001$; Figure 3-3), emphasizing its probable relationship with maturity upon arrival. Assuming fish from each sampling period were of similar maturity, multiple regression analyses were conducted within each arrival group separately but models did not yield significance.

Behavioural Metrics and Cortisol

Behaviour predicted success in females as aggression was positively related to number of days on a redd (Multiple regression: $R^2 = 0.59$, $F_{(1,11)} = 15.71$, $P = 0.002$). There was also

a non-significant positive association with mate interaction time but no effect of size (FL). Baseline cortisol predicted individual behaviour (Fig. 3-4). In females, baseline cortisol was negatively associated with both aggression and mate interaction time (Simple regression: $R^2=0.40$, $F_{(1,10)} = 6.54$, $P = 0.029$ and $R^2 = 0.58$, $F_{(1,10)} = 13.80$, $P = 0.004$, respectively). Neither stress-induced cortisol nor FL were significant factors. Similarly in males, there was a negative relationship between baseline cortisol and mate interaction time (Simple regression: $R^2 = 0.59$, $F_{(1,11)} = 15.71$, $P = 0.002$). There were no significant associations between aggression and cortisol concentrations, or between aggression and FL.

Discussion

A recent review has suggested the need for more direct measures of fitness in relation to the acute stress response (Breuner *et al.*, 2008). We accomplished this by linking reproductive success in a semelparous species to individual variability in circulating cortisol titres following exposure to acute stress. Although other studies exploring fitness correlates of the stress response have emerged since Breuner and colleague's review (i.e. Angelier *et al.*, 2009; MacDougall-Shackleton *et al.*, 2009), conclusions remain inconsistent. The present study is unique as individuals are fully invested in reproduction and baseline cortisol values are exceptionally high. Our results found cortisol concentrations to be associated with performance measures and fitness. While stress-induced cortisol predicted spawning success, behavioural metrics were correlated with baseline cortisol.

Characteristics of the Stress Response

Both sexes exhibited baseline and stress-induced cortisol levels that were well above values reported for non-reproductive salmonids (see Barton, 2002). The higher cortisol concentrations of females compared to males parallels previous research on several species of Pacific salmon (Hane *et al.*, 1966; Donaldson and Fagerlund, 1972; Kubokawa *et al.*, 1999; Kubokawa *et al.*, 2001) and is consistent with *in vitro* experiments on HPI axis reactivity in sexually mature chinook salmon (*O. tshawytscha*; McQuillan *et al.*, 2003). As predicted and consistent with evolutionary theory (Wingfield and Sapolsky, 2003), stress-induced cortisol levels declined with sexual maturity. Pottinger *et al.* (1995) observed a similar pattern in rainbow trout and suggested that as rates of increase were similar in mature and immature fish, the cortisol/ACTH feedback equilibrium was modified in mature fish to a lower “set point”. Although the stress response is a mechanism allowing for increased individual survival, it is also energetically consuming (Ricklefs and Wikelski, 2002). An attenuated stress response is therefore expected in animals with limited opportunities to reproduce (Wingfield and Sapolsky, 2003). When reproduction is of primary value, the stress response loses importance and suppression acts to conserve energy.

Cortisol increases in senescent salmonids (Hruska *et al.*, 2010; Barry *et al.*, 2010) and in birds, with reproductive investment (Bonier *et al.*, 2009b). The finding of decreased baseline cortisol concentrations in later arriving and more mature fish was therefore surprising and contrary to our predictions. No differences between the early and middle sampling groups are likely because cortisol was already at maximal levels upon arrival. A decrease in baseline cortisol has been observed in mature sockeye

salmon followed by a steady increase until senescence (Kubokawa *et al.*, 1999). Perhaps Pacific salmon maintain an ability to suppress baseline cortisol levels when fully mature and in the earlier stages of breeding in an attempt to conserve energy. Earlier-arriving individuals therefore have high cortisol relative to mature fish as they are not yet fully invested in reproduction. As condition deteriorates, and cortisol metabolism is inhibited (Barry *et al.*, 2010), cortisol peaks again, ultimately leading to senescence.

Fitness Correlates of Cortisol Concentrations

Pre-spawn mortality is common across all species of Pacific salmon and the causes of this phenomenon remain unclear (Cooke *et al.*, 2004; Quinn *et al.*, 2007). Several theories exist. The physiological stress hypersecretion hypothesis suggests that fish with higher levels of cortisol die earlier (Barry *et al.*, 2010; Hruska *et al.*, 2010) and the energy exhaustion hypothesis suggests that fish die once a critical minimal energy threshold is breached (Hruska *et al.*, 2010). Finally, it has been predicted that successful fish will arrive early and have greater longevity (Hruska *et al.*, 2011). These theories do not however extend to consequences of stress-induced cortisol or the stress response.

We show support for several hypotheses regarding pre-spawn mortality.

Unsuccessful females were characterized by elevated stress-induced cortisol, fewer days on a redd, and decreased longevity. Correspondingly, Hruska *et al.*, (2011) reported that spawned fish lived approximately 2 days longer than non-spawners. Similar relationships of decreased fitness with elevated stress-induced GCs have been found in other taxa. An increased stress response corresponded with a lower return probability in song sparrows

(*Melospiza melodia*; MacDougall-Shackleton *et al.*, 2009) and decreased survival to adulthood in juvenile stork nestlings (*Ciconia, ciconia*; Blas *et al.*, 2007). Romero and Wikelski (2001) found stress-induced corticosterone in marine iguanas (*Amblyrhynchus cristatus*) to determine survival in El Niño years with little to no food availability. However, although results are consistent with previous findings, the unique physiological characteristics of Pacific salmon argue for caution in extending the application of evolutionary theories regarding fitness and cortisol established in iteroparous species.

While differences between groups in baseline cortisol are not statistically significant, we would argue for biological significance. Average baseline cortisol for spawners were within the range of levels typical of female pink salmon at Weaver Creek (250-350 ng mL⁻¹; McConnachie 2010) whereas those for non-spawners were considerably higher (from 413.1-546.2 ng mL⁻¹) which does provide support for the cortisol hypersecretion hypothesis. However, with only 9 fish (12 % of sample) failing to spawn, the power of our results is low. The Weaver Creek spawning channel is an artificial habitat that has been optimized for successful spawning. Densities are much lower than in the wild, and both substrate and flow are ideal. More variability in reproductive success might occur in a natural setting.

Reproductive success was only detectable in females. As males continually generate sperm, we lacked a direct measure of fitness. Males also adopt several different reproductive strategies which further obscures quantifying fitness. Longevity is thought to provide an indirect measure of fitness for both sexes (Quinn and Foote, 1994). Our results suggest that longevity is a better predictor of maturity upon arrival than fitness as we observed no behavioural or physiological predictors of longevity, as with other fitness

measures (females only), and along with cortisol, longevity decreased with sampling period. It seems that to use longevity as a measure of fitness, level of maturity between individuals must be controlled for.

Behavioural Correlates of Cortisol Concentrations

For both sexes, behaviour was associated with baseline but not stress-induced cortisol. Consistent with physiological correlates of social status (Gilmour et al., 2005), females with elevated baseline cortisol concentrations upon arrival had lower aggression scores. Of interest is that cortisol and aggression were not related in males. Male aggression is highly dependent on overall density and the operational sex ratio (OSR). The even OSR used in this study was much lower than in natural settings as generally the proportion of males is higher (Quinn *et al.*, 1996). In Pacific salmon, the dominant male monopolizes access to a female and subordinate males take on satellite positions surrounding this male (Foote, 1990). According to Quinn (1996), males may only be able to monopolize access to a female at low OSR's. Generally we observed the dominant male to hold position with a female and increased aggression amongst satellites which provides a potential explanation for the lack of relationship between aggression score and baseline cortisol, as predicted for social salmonids. The significant negative relationship between baseline cortisol and mate interaction time in males however does imply a role for baseline cortisol in determining social status. In all cases, results are consistent with physiological characteristics of dominance hierarchies whereby the dominant individual (greater mate interaction time in males, aggression in females) has relatively lower baseline cortisol levels upon arrival compared to subordinates who exhibit elevated cortisol and behavioural inhibition (Gilmour *et al.*, 2005).

In contrast to our predictions and contrary to previous work (Foote, 1990), we saw no associations between aggression and fork length. However, most research of physiology and behaviour in mature Pacific salmon has focused on sockeye salmon. Therefore, species-specific differences in behaviour and physiology could account for observed inconsistencies with previous research. We also predicted behaviour to be correlated with stress-induced cortisol. However, this prediction was based on previous work conducted in iteroparous species (Øverli *et al.*, 2005). In semelparous species, it has been theorized that reproductive behaviour would be decoupled from the stress response (Breuner *et al.*, 2008) as reproduction must occur despite environmental or social stressors (Wingfield and Sapolsky, 2003).

Conclusions

Our results find both baseline and stress-induced cortisol levels in pink salmon to be correlated with direct and indirect fitness measures. With respect to baseline cortisol, non-spawners had baseline cortisol concentrations beyond the normal range for pink salmon of the study population. Additionally, concentrations were associated with individual behaviour. We observed individuals showing signs of dominance to have relatively lower baseline cortisol. Dominant fish are known to have greater reproductive success (Chebanov *et al.*, 1983; Quinn and Foote, 1994) and indeed, more aggressive females in this study spent more days protecting a redd, a proxy for fitness.

Unsuccessful female fish exhibited elevated stress-induced cortisol concentrations and decreased longevity. In reptiles, elevated GCs are known to increase energy

expenditure (Cote *et al.*, 2006). As pink salmon have a minimum energetic threshold (Crossin *et al.*, 2003), increased energy expenditure attributed with this elevated cortisol in response to acute stress could likely influence longevity on the spawning grounds and reproductive success. Romero and Wikelski (2001) suggested that when in exceptionally poor condition with little chance of survival, a surge in the physiological response to stress may act to mobilize energy as a final effort of survival. The few non-spawning individuals arriving with abnormally high stress-induced cortisol in response to acute stress may have arrived to the spawning grounds in an advanced state of deterioration, perhaps due previous stress exposure. Severe conditions act as a mechanism of natural selection (Brown and Brown, 1998), thus exerting selective pressure on physiological traits. Individual differences in responses to acute stress, especially in challenging situations or when body condition is low, could therefore be a substrate for natural selection for Pacific salmon as suggested by Romero and Wikelski (2001) for Galapagos marine iguanas during El Nino years of low food availability.

That so few individuals (12% of sample) failing to spawn had distinctly elevated stress-induced cortisol implies that the relationship between acutely-elevated cortisol and fitness is not linear, as described by Busch and Hayward (2009). Abnormally elevated GCs are perhaps indicative of a poor quality individual unable to cope with the physiological demands of an extremely challenging reproductive strategy. Furthermore, indirect measures of fitness (behaviour, longevity, days on a redd) were unrelated to stress-induced cortisol indicating that detrimental effects of stress-induced cortisol are primarily physiological rather than behavioural. We also observed an effect of baseline cortisol on fitness. In females, baseline cortisol was elevated in non-spawners and

negatively correlated with aggression (a predictor of days on a redd) providing support for both the cortisol hypersecretion hypothesis specific to Pacific salmon (Hruska et al., 2010) and the cort-fitness hypothesis, proposed for all taxa (Bonier et al., 2009a). In a system with greater variability or in a year of lower success when behavioural differences would perhaps play a more prominent role in defining success, we would expect to see a greater effect of baseline cortisol in addition to that of stress-induced cortisol. Although our findings do confirm a negative relationship between fitness and cortisol in pink salmon, there remains much to be learned regarding the evolutionary consequences of individual variability in the mechanistic capacity to respond to stress in semelparous species.

Tables

Table 3-1: Fork lengths (FL; mean \pm SE) and sample sizes for pink salmon (*Onchorynchus gorbuscha*) sampled across 3 time periods from the Weaver Creek spawning channel.

	Early		Middle		Late		Enclosure	
	Oct. 5 th , 2009		Oct. 9 th , 2009		Oct. 13 th , 2009		Oct. 9 th , 2009	
	FL	n	FL	n	FL	n	FL	n
Males	52.7 \pm 0.7	28	50.2 \pm 1.8	22	53.4 \pm 0.7	28	51.8 \pm 0.7	13
Females	50.7 \pm 0.36	30	50.5 \pm 0.4	26	50.8 \pm 0.3	29	50.1 \pm 0.5	13

Table 3-2: Results from a binary logistic regression model assessing predictors of reproductive success in female pink salmon (*Onchorynchus gorbuscha*) sampled at three different times at the Weaver Creek Spawning Channel. Significant differences between spawners (released >90% of eggs) and non-spawners (released < 50% of eggs) are indicated in bold type and with an asterisk.

		B \pm SE	P-value
Step 1	Constant	5.26 \pm 1.5	<0.0001
	Stress-induced Cortisol (ng mL⁻¹)	-.05 \pm .02	0.01
R ² = 0.11 (Cox & Snell), 0.23 (Nagelkerke), X ² ₍₁₎ = 9.78, P = 0.002			
Step 2	Constant	2.92 \pm 1.7	0.08
	Stress-induced Cortisol (ng mL⁻¹)	-.078 \pm .03	0.002
	Longevity (days)	.49 \pm 0.19	0.008
	Baseline Cortisol (ng mL⁻¹)		0.135
R ² = 0.24 (Cox & Snell), 0.47 (Nagelkerke), X ² ₍₂₎ = 21.99, P < 0.0001			

Table 3-3: Means of physiological parameters (baseline and stress-induced plasma cortisol concentrations) and fitness proxies (longevity and number of days on a redd) for reproductively successful (spawners) and unsuccessful (non-spawners) female pink salmon (*Onchorynchus gorbuscha*) sampled from the Weaver Creek Spawning Channel

	Means \pm SE	
	Spawners (n=9)	Non-spawners (n=73)
Stress-induced Cortisol (ng mL⁻¹)	481.59 \pm 21.2	712.85 \pm 68.8
Baseline Cortisol (ng mL⁻¹)	317.19 \pm 18.0	436.12 \pm 78.1
Longevity (days)	9.10 \pm 0.4	6.56 \pm 1.2
Days on a Redd (days)	4.65 \pm 0.29	0.93 \pm 0.5

Figures

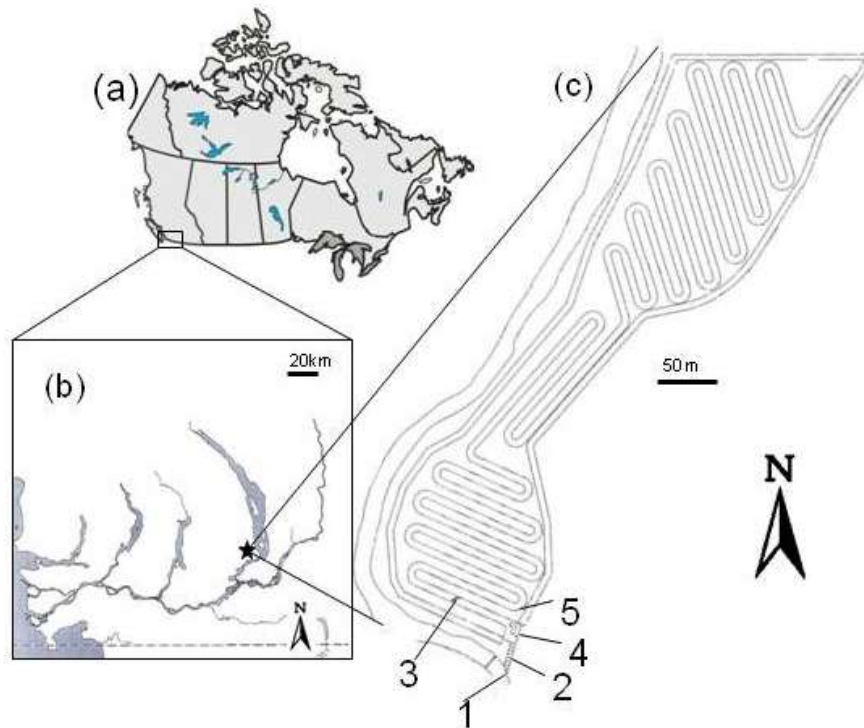


Figure 3-1: Map detailing the study site. The Weaver Creek Spawning Channel (c) is located in the lower Fraser River (b) in British Columbia, Canada (a). The artificial channel is part of a natural creek system (1). Salmon leave the creek, swim up a fish ladder (2) and hold below the splitter shed (3) before entering the holding pool from which fish were sampled (4). After sampling, fish were released to the channel to spawn (5). Figure extracted with permission from Hruska *et al.* (2011)

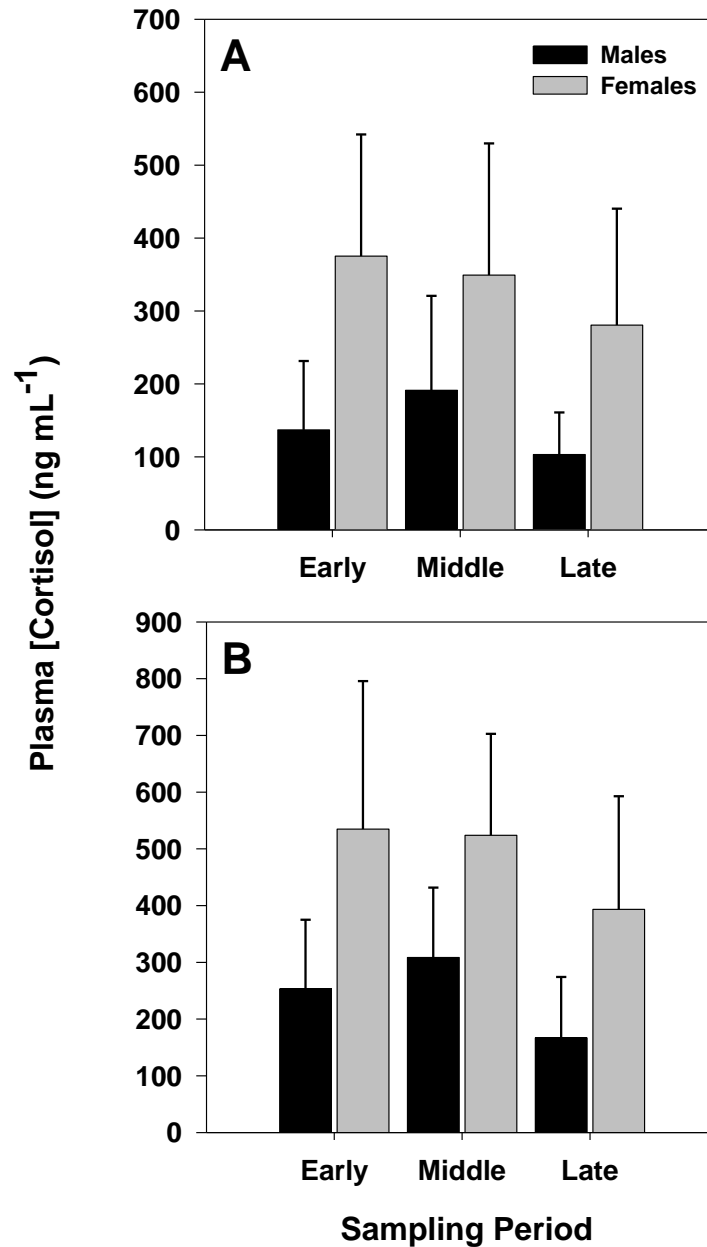


Figure 3-2: Mean \pm SE baseline (A) and stress-induced (B) cortisol concentrations between sexes and as a function of arrival time for pink salmon (*Onchorynchus gorbuscha*) sampled from the Weaver Creek Spawning Chanel in 2009. Both measures of cortisol significantly differed between sexes and across sampling times (Two-way ANOVA: P's < 0.05). An * denotes a mean within that sex that is significantly different from the previous timing group

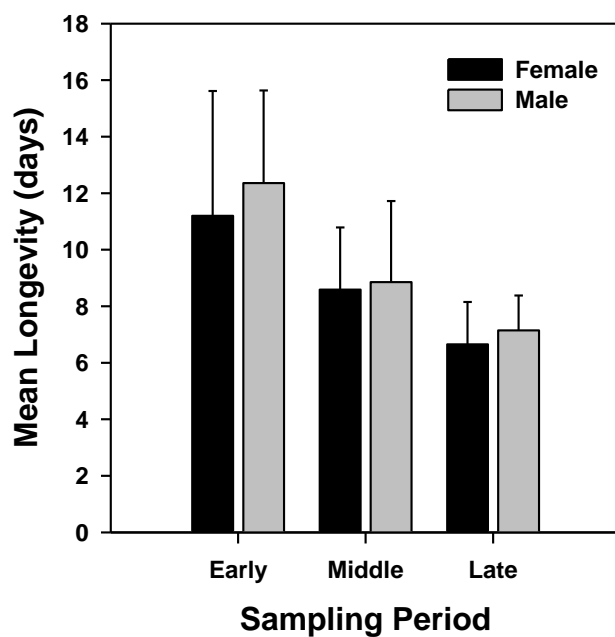


Figure 3-3: Longevity, i.e. the number of days in the spawning channel from sampling to death, for both sexes of pink salmon (*Onchorynchus gorbuscha*) as a function of sampling time. There were no differences between sexes (Mann-Whitney: $P > 0.05$) and when combined, longevity significantly decreased with sampling period (Jonckheere-Terpstra: $P < 0.05$). Differing letters indicate statistical differences between sampling periods. Fish were sampled from the Weaver Creek Spawning Channel.

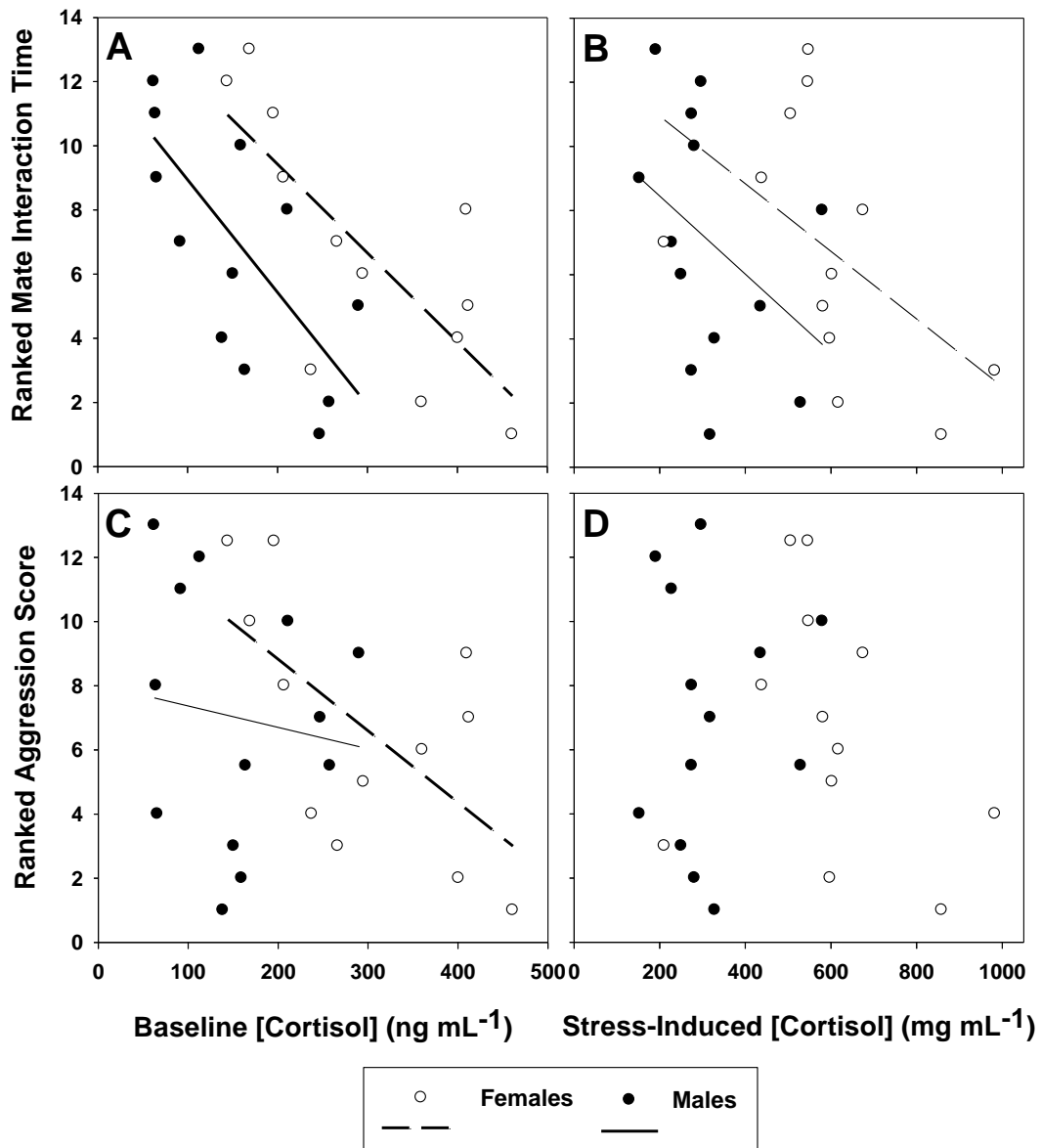


Figure 3-4: Correlations between ranked behavioural metrics and cortisol concentrations for both sexes of pink salmon (*Onchorynchus gorbuscha*) sampled from the Weaver Creek Spawning Channel and held in an experimental area within the channel. Panels are regression of the following in both sexes: mate interaction time and baseline [cortisol] (A), mate interaction and stress-induced [cortisol] (B), aggression score and baseline [cortisol] (C), aggression and stress-induced [cortisol] (D). Bold lines are significant whereas lines not in bold represent non-significant trends. Greater mate interaction time and aggression are characteristics of dominant fish.

Chapter 4: General Discussion

Introduction

This thesis is a compilation of two distinct studies that help to elucidate how both the intra-individual variation (chapter 2) and inter-individual variation (chapter 3) of stress responsiveness is associated with performance measures, condition and fitness in wild fish. I brought wild fish into a laboratory setting (chapter 2) and observed them in their natural environment (chapter 3). By using a standardized stressor for all individuals in both studies, I was able to compare and contrast differences in the magnitude of the stress response between and within individuals. Although variability in GCs is thought to mediate ecological and evolutionary trade-offs in animals on the individual level (see Ketterson and Nolan Jr, 1999; Zera and Harshman, 2001; Ricklefs and Wikelski, 2002), it is still rare for studies to present, let alone formally analyze individual variability in physiological or endocrine traits (Bennett, 1987; Williams, 2008).

Physiological research is typically conducted under controlled laboratory settings and with captive populations. A benefit of conducting laboratory research is that it is possible to control for many factors that cannot be controlled for in the wild (i.e. rapid environmental fluctuations, predator-prey interactions, habitat variability) but one can never be certain that results truly translate to wild animals in their natural environment (Cooke and O'Connor, 2010). The majority of research assessing correlates of GCs uses birds or reptiles while fish remain relatively unexplored in this regard (Mommsen *et al.*, 1999; Wingfield, 2003). Additionally, explorations of how stress affects reproduction are generally focused on iteroparous species (Silverin *et al.*, 1997; Schreck *et al.*, 2001;

Angelier *et al.*, 2007b) despite the unique life history of semelparous species that, as they have only one lifetime opportunity to reproduce, provide a direct and quantifiable measure of fitness.

Ecological correlates of inter-individual variation in GC titres have been explored quite extensively in wild animals (Breuner and Hahn, 2003; Wingfield, 2003; Cockrem, 2007; Ball and Balthazart, 2008; Sloman *et al.*, 2008). However, these studies adopt the rarely-tested assumption of temporal repeatability of hormone titres. In captive populations and to a lesser extent wild populations, the GC response has shown a degree of repeatability, but not always consistently. Reasons for this inconsistency remain unknown and recently the idea has emerged that intra-individual variability in hormone titres may also play a role in defining individual traits. In chapter 2 I determined repeatability of stress-induced cortisol in wild bluegill brought into controlled laboratory settings and attempted to explain intra-individual variation with measures of individual condition. In chapter 3 I sought to clarify the relationship between GCs and fitness. Although GCs are known to mediate individual traits (Wingfield, 2003; Cockrem, 2007; Øverli *et al.*, 2007), links with direct fitness metrics have been inconclusive. Most research, however, does not directly quantify fitness but instead performance measures and proxies of individual fitness (Breuner *et al.*, 2008). By using a semelparous species as a model, where failure to reproduce results in zero fitness, I was able to directly assess fitness in relation to the magnitude of an individual's GC response by intercepting fish prior to entering the spawning grounds.

Findings and Implications

The research I conducted revealed condition correlates of intra-individual variation in stress-induced cortisol titres (chapter 3) and behavioural and fitness correlates of inter-individual variation in baseline and stress-induced cortisol concentrations (chapter 2). Results from this research fill a void in the literature exploring repeatability of stress-induced cortisol and the effects of varying concentrations at the individual level on survival and reproduction. The significant repeatability of stress-induced cortisol in Chapter 2 validates the use of this metric as a mediator of individual traits but emphasizes the importance of size and condition in analyses. A consistent finding between both studies is that elevated stress-induced cortisol is associated with negative consequences. Smaller bluegill in poor condition exhibited elevated stress-induced cortisol and female pink salmon failing to spawn also had relatively greater stress-induced cortisol relative to spawning females. These results are not intuitive as it is well established that the acute response is adaptive (Wingfield *et al.*, 1998). However, I am not the first to show reduced survival with a high stress response (Romero and Wikelski, 2001; Blas *et al.*, 2007; MacDougall-Shackleton *et al.*, 2009). We are not able to determine from the bluegill data whether elevated stress-induced cortisol or intra-individual variability is a cause or consequence of poor condition. In the salmon study, very few individuals (12% of sample) failed to spawn. Romero and Wikelski (2001) suggested that when in exceptionally poor condition with little chance of survival, a surge in the physiological response to stress may act to mobilize energy as a final effort of survival. The few non-spawning individuals arriving with abnormally high stress-induced cortisol in response to acute stress may have arrived to the spawning grounds in an abnormally deteriorated

condition, perhaps due previous stress exposure. Similarly, increased intra-individual variation of stress-induced cortisol in bluegill that were perhaps already in poor condition could be a consequence of their further declining condition throughout the sampling period relative to those collected in good condition. In chapter 3 I also failed to show an association between behaviour and stress-induced cortisol. Spawning fish have exceptionally elevated cortisol levels and are in a rapidly declining condition. Perhaps increased non-repeatability of stress-induced cortisol titres in these fish, due to poor condition, masks any relationships with behaviour.

There are both theoretical and applied implications of this research. In chapter 2, I tested whether a measured value of stress-induced cortisol is truly repeatable and explored potential explanations for non-repeatability at the individual level. Overall the stress response was a repeatable characteristic but smaller fish in poor condition and with relatively higher concentrations of stress-induced cortisol exhibited greater intra-individual variation in response to repeated standardized stressors. These results have important implications for further research assessing the correlates of the GC response.

1. It cannot always be assumed that measured value of stress-induced GCs is truly representative of that individual across all situations and it is recommended that when possible an individual is sample twice.
2. In degraded environments when fish may be in poor condition or when having elevated levels of stress-induced cortisol, measured physiological responses to stress may not be repeatable.
3. It is important that variables such as size, sex and individual condition are taken into account when assessing correlates of the GC response.

In chapter 3, I tested the validity of evolutionary theory surrounding trade-offs associated with baseline and acute GC concentrations using a semelparous model. Results provide support for theories proposed for semelparous species and others determined in iteroparous species that had been previously unexplored in semelparous species.

1. In species with limited reproductive opportunities, reproductive behaviours will likely be decoupled from the stress response.
2. The magnitude of stress-induced cortisol can predict survival and reproductive success.
3. In fish with hierarchal social structures, a subordinate status is characterized by elevated baseline cortisol.

Future Directions and Further Questions

The results of this research show that intra- and inter-individual variation in the magnitude of response to an acute stressor in wild fish has fitness and performance consequences affecting reproductive success and condition. However results do lead to further potential questions to be explored in this field both in semelparous and iteroparous species.

1. The stress response is an adaptive mechanism that promotes immediate survival (Wingfield *et al.*, 1998). Why are we repeatedly seeing reduced survival with elevated acute GCs? Is the relationship between acute GCs and survival perhaps quadratic and not linear? Is it just the very few poor

quality individuals in the population driving these relationships?

Intuitively, the lack of a stress response should also be maladaptive.

2. Pink salmon dying pre-spawn had high stress-induced cortisol and decreased longevity but results do not determine whether failure to reproduce is due to poor quality of the individual or to factors associated with elevated stress-induced cortisol. As the stress response is known to be energetically demanding, to clarify observed results, one could explore whether the magnitude of stress-induced cortisol upon arrival is correlated with energetic status.
3. Is elevated stress-induced cortisol a consequence of poor condition or a cause? Is an elevated stress response a mechanism of selection in certain environments or a result of an individual in poor quality?
4. Did I fail to see behavioural correlates of stress-induced cortisol in Chapter 3 because the responses of mature Pacific salmon to acute stress are not repeatable due to their exceptionally poor condition? Or is it because the stress response becomes decoupled from reproductive activities as has been hypothesized for animals with few breeding opportunities (Wingfield and Sapolsky 2003)?
5. Chapter 2 revealed stress-induced cortisol to increase with repeated stress. In migrating salmon, does previous stress history affect subsequent responses? Could the elevated stress-induced cortisol on the spawning grounds in non-spawning fish be a consequence of greater exposure to acute stress earlier in the migration compared to those fish able to spawn?

With current technology we cannot control potential stressful interactions prior to arrival at the spawning grounds. The questions could be experimentally explored by intercepting and sampling fish prior to entering the spawning grounds, applying varying levels of stress and recapturing and re-sampling the same fish on the spawning grounds. This method would also provide a further understanding of intra-individual variability of GCs in fish in poor condition.

6. The loss of the stress response is common in mature semelparous species. Fish must overcome many stressors throughout the migration that require escape behaviour but as they mature and reproduction becomes of primary importance, it is logical that they forgo the costly response to acute stress. However, at which point in the migration is the stress response lost? Is it adaptive to maintain a stress response until fully prepared to spawn? Or is it lost slowly with increasing maturity? Does it correspond with ovulation in females or spermiation in males?

Overall conclusions

The role of glucocorticoids in ecology and evolution is complex. We are beginning to understand these relationships with laboratory research and results from the biomedical field. However we must persist with explorations of animals in their natural habitats. The two biggest challenges currently in the field of stress physiology are:

1) Determine why GC titres are sometimes repeatable and other times not. To continue research assessing correlates of GCs, this is valuable information.

2) Determine at which point a response to acute stress becomes maladaptive.

Perhaps this relationship is not linear and recent research showing decreased survival as a consequence of elevated stress-induced cortisol is attributed to poor individual quality whereby the stress response no longer acts to re-establish homeostasis but elicits additional stress.

There has been continued interest in using physiological sampling as a tool for conservation. However before this can be done accurately, we must first understand the mechanisms by which individuals respond to stress. Exploring repeatability and direct fitness consequences of GCs is a start in this regard.

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Appendices

Appendix 1: Permission from Co-authors

From: Kathleen Gilmour <Kathleen.Gilmour@uottawa.ca>

To: Katrina Cook <katrina.vcook@gmail.com>

Hi Katrina;

You have my permission to include both of the papers listed below in your thesis.

Best wishes,
Katie Gilmour

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From: Hinch, Scott <shinch@mail.ubc.ca>

Mon, Mar 14, 2011 at 2:03 PM

To: Katrina Cook <katrina.vcook@gmail.com>

Hi Katrina

I give permission for Katrina Cook to include in her thesis a paper that I am co-author on entitled, "Behaviour and Fitness Correlates of Baseline and Stress-Induced Plasma Cortisol Titres in Pink Salmon (*Oncorhynchus gorbuscha*) upon Arrival at Spawning Grounds".

Scott Hinch
Professor
Forest Sciences Dept., UBC
2424 Main Mall,
Vancouver, BC Canada

From: Sarah McConnachie <smcconna@connect.carleton.ca>

**Tue, Mar 15, 2011 at
3:01 PM**

To: Katrina Cook <katrina.vcook@gmail.com>

Hi Katrina,

You have my permission to include the co-authored manuscripts "Condition dependent intra-individual repeatability of the cortisol stress response in a freshwater fish" and "Behaviour and fitness correlates of baseline and stress-induced plasma cortisol titres in pink salmon (*Onchorhynchus gorbuscha*) upon arrival at the spawning grounds" as chapters in your M.Sc. thesis.

Sincerely,
Sarah McConnachie

Sarah McConnachie, M.Sc.
Fish Ecology and Conservation Physiology
Carleton University
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From: Connie O'Connor <constanceoconnor@gmail.com>

**1, Mar 14, 2011 at 1:40
PM**

To: Katrina Cook <katrina.vcook@gmail.com>

Hi Katrina,

You have my permission to include the co-authored manuscript "Condition dependent intra-individual repeatability of the cortisol stress response in a freshwater fish" as a chapter in your M.Sc. thesis.

Cheers,
Connie

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