THE ECOLOGY OF STRESS:

A MULTIDISCIPLINARY PERSPECTIVE ON STRESS

IN WILD CENTRARCHID FISHES

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ABSTRACT

As anthropogenic challenges continue to affect our ecosystems, it is becoming increasingly important to understand the physiological and ecological impacts of stress in wild animals. This thesis presents a cohesive and multidisciplinary investigation of the 'ecology of stress'. Integrating tools from physiological, behavioural, and population ecology, I provide a comprehensive overview of the life-history mediators and individual- and population-level consequences of physiological stress in wild smallmouth bass (*Micropterus dolomieu*) and largemouth bass (*M. salmoides*). In an initial set of studies, I first demonstrated that in these parental care-providing fish species, regulation of the endogenous endocrine stress response during parental care is correlated with life-history traits. Specifically, larger, older, more experienced parents display an attenuated endocrine stress response when faced with a standardized stressor during parental care. I then demonstrated that all parents display an attenuated endocrine stress response when compared with the responses of non-parental fish. Using exogenous stress hormone implants, I experimentally determined that a chronic increase in circulating stress hormones during parental care is associated with premature nest abandonment and decreased immune function. The combination of results provides evidence that a robust endocrine stress response serves as a mechanism to reduce investment in current reproductive opportunities, and is influenced by current life-history stage as predicted by life-history theory. Expanding the scope of the thesis, I employed the same exogenous stress hormone implants and demonstrated that a transient endocrine stress response is

associated with long-term carryover effects. Specifically, fish treated with cortisol

hormone implants exhibited accelerated mortality during a natural challenge that

occurred 5 months after the cessation of the initial endocrine stress response. I further

determined that a transient endocrine stress response is energetically costly, and is

associated with long-term decreases in individual growth rates that are sufficient to cause

decreases in population growth rate. As a whole, this dissertation improves our

understanding of the ecology of stress in fish by demonstrating that life-history variation

underlies inter-individual variation in endocrine stress responses, and by providing

potential mechanisms underlying population-level consequences of stress.

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LIST OF ABBREVIATIONS

Abbreviation	Full name
11-KT	11-ketotestosterone
ACTH	adrenocorticotropic hormone
ANCOVA	analysis of covariance
ANOVA	analysis of variance
C	Celsius
CRF	corticotropin-releasing factor
CV	coefficient of variation
FSH	follicle-stimulating hormone
g	grams
g	gravity
GSI	gonadosomatic index
GnRH	gonadotropin-releasing hormone
ha	hectares
hrs	hours
HSD	honestly significant difference
HPA	hypothalamus-pituitary-adrenal
HPG	hypothalamus-pituitary-gonadal
HPI	hypothalamus-pituitary-interrenal

Abbreviation	Full name
J	joules
kg	kilogram
L	litres
LH	luetenizing hormone
m	metres
mg	milligrams
min	minutes
mL	millilitre
mm	millimetre
mmol	millimoles
ng	nanograms
PIT	passive integrated transponder
RIA	radioimmunoassay
s	seconds
SD	standard deviation
SEM	standard error of the mean
SMR	standard metabolic rate
T	testosterone
TL	total length
QUBS	Queen's University Biological Station

Abbreviation	Full name
yrs	years

PREFACE

Thesis format

I have opted to write this thesis in a manuscript-based format. For brevity, the chapter titles are modified slightly from the manuscript titles. The following are the five manuscripts that form the bases of this thesis:

1. O'Connor C.M., B.L. Barthel, K.M. Gilmour, G. Van Der Kraak, D.P. Philipp, S.J. Cooke. In review. The glucocorticoid stress response and circulating androgens during parental care are correlated with reproductive history and the timing of reproduction in a teleost fish. Physiol Biochem Zool PBZ-11019. Submitted March 3, 2011.

2. O'Connor C.M., C.Y. Yick, K.M. Gilmour, G. Van Der Kraak, G., S.J. Cooke. 2011. The glucocorticoid stress response is attenuated but unrelated to reproductive investment during parental care in a teleost fish. Gen Comp Endocrinol 170: 215-221.

3. O'Connor C.M., K.M. Gilmour, R. Arlinghaus, G. Van Der Kraak, S.J. Cooke. 2009. Stress and parental care in a wild teleost fish: Insights from exogenous supraphysiological cortisol implants. Physiol Biochem Zool 82: 709-719.

4. O'Connor C.M., K.M. Gilmour, R. Arlinghaus, D.P. Philipp, C.T. Hasler, S.J. Cooke. 2010. Seasonal carryover effects following the administration of cortisol to a wild teleost fish. Physiol Biochem Zool 83: 950-957.

5. O'Connor C.M., K.M. Gilmour, R. Arlinghaus, S. Matsumura, C.D. Suski, D.P. Philipp, S.J. Cooke. 2011. The consequences of short-term cortisol elevation on individual physiology and growth rate in wild largemouth bass (*Micropterus salmoides*). Can J Fish Aquat Sci 68: 693-705.

The manuscripts follow from one another in a logical progression, as will be explained in the general introduction and summarized in the general conclusion. In a manuscript-based thesis some amount of redundancy is inevitable. To reduce this redundancy, efforts were made to avoid repetitive information in the methods of the individual chapters, the acknowledgements were combined into a single section at the beginning of this thesis, and all literature cited was combined into a single section after the appendices.

Contribution of co-authors

This thesis represents the results of my own independent research. However, I have enormously benefited from the assistance of co-authors throughout my doctoral research. My co-supervisors Dr. Steven Cooke and Dr. Kathleen Gilmour are co-authors on all chapters, and have contributed to the design, execution, analysis and presentation of results for this research. Dr. Glen Van Der Kraak is a co-author on Chapters 2, 3, and 4, and contributed logistical and financial support for androgen assays, as well as input on

the presentation of results for these chapters. For Chapter 2, Dr. Brandon Barthel and Dr. David Philipp generously allowed me access to a long-term study population of fish, and provided me with long-term data on all individuals within the population. Both co-authors contributed to the execution and the presentation of results for this chapter. Dr. David Philipp also contributed financial support and provided input on the design and presentation of results for Chapter 5. For Chapter 3, Claire Yick contributed to the execution, analysis and presentation of results for the brood size manipulation experiment. Dr. Robert Arlinghaus is a co-author on Chapters 4, 5, and 6, and contributed financial support, as well as input on the design and presentation of results for these chapters. Caleb Hasler contributed to the analysis of telemetry data for Chapter 5, and provided input on the presentation of results for this chapter. Dr. Cory Suski provided the financial and logistical support necessary to measure metabolic rates of study animals in Chapter 6, while Dr. Shuichi Matsumura performed the derivations necessary to create the population model described in this chapter. Both co-authors also provided input on the presentation of results for this chapter. All co-authors granted permission to include these manuscripts as thesis chapters.

Selection of chapters

The chapters that comprise this thesis represent the majority of the research work I conducted during my tenure as a graduate student at Carleton University. However, I also conducted research during my time at Carleton University that was not included in this thesis, because it does not significantly contribute to the main research theme that I

will be developing throughout. A set of unpublished data provides a useful improvement

to the cortisol injection methodology presented in Chapters 4, 5, and 6, and is included as

Appendix I. One additional published manuscript presents data that contribute to the

interpretation of the overall results of the thesis, and is included as Appendix II.

GENERAL INTRODUCTION

Challenges are an inherent aspect of life for all organisms, and the ability of individuals to appropriately cope with both predictable challenges (e.g., parental care) and unpredictable challenges (e.g., extreme weather events) is a critical component of fitness. On the one hand, individuals must respond adequately, so that the challenge can be avoided or overcome. On the other hand, individuals must scale their response appropriately, or important time and energy resources will be squandered. Thus, both the extent (i.e., frequency and magnitude) of challenges and individuals' responses will influence long-term individual survival and viability. As anthropogenic changes continue to impact ecosystems, it is becoming imperative to understand how wild individuals cope with challenges at the physiological level, and how these individual responses translate to population-level responses. This thesis represents a comprehensive examination of the 'ecology of stress' in two wild fish species using an innovative framework, with multidisciplinary tools borrowed from physiology, behavioural ecology, and population ecology. In this general introduction, I will provide the physiological and ecological background necessary to lay the framework for the experimental data chapters that follow.

1.1. Stress: A physiological perspective. Historically, stress has been defined as the loss of homeostasis (Selye 1951), or the loss of the ability to maintain a constant internal state (Cannon 1929) owing to an extrinsic factor. A stressor is thus defined as a stimulus

that elicits a physiological stress response (Romero 2004). In brief, the primary vertebrate physiological stress response involves the release of catecholamines from the chromaffin tissues, and the activation of the hypothalamo-pituitary-adrenal (HPA) axis in tetrapods, or the homologous hypothalamo-pituitary-interrenal (HPI) axis in fish. HPA or HPI axis activation starts with the release of corticotropic-releasing factor (CRF) by the hypothalamus, stimulating the release of adrenocorticotropic hormone (ACTH) from the pituitary. ACTH then stimulates the adrenal glands in tetrapods, and the interrenal cells in fish, to produce and secrete glucocorticoids. Glucocorticoid production and secretion is controlled by a negative feedback loop, with circulating glucocorticoids serving to suppress the release of both CRF and ACTH. The primary glucocorticoid is cortisol in the case of teleost fish and most mammals, and corticosterone in amphibians, reptiles, birds and rodents (see reviews by Wendelaar-Bonga 1997; Mommsen et al. 1999; Sapolsky et al. 2000; Greenberg et al. 2002).

The release of catecholamines occurs within seconds. Elevation of catecholamines is associated with the classic "fight or flight" response: a rapid decrease in visceral activity and a shutdown of digestion, and a rapid increase in gas exchange efficiency, cardiovascular function, gluconeogenisis, and cognition (see reviews by Sapoksly et al. 2000; Romero and Butler 2007). The synthesis and release of glucocorticoids then takes minutes, and the genomic effects of elevated glucocorticoids can last for hours or days. The effects of elevated glucocorticoids are varied, and can serve to enhance or suppress the initial catecholamine response, or prepare the individual for future stress events (see review by Sapolsky et al. 2000). These effects can be highly

context-dependent, and have been widely explored across a variety of taxa. For example,

elevation of glucocorticoids has been shown to secondarily alter physiological processes

such as immune function (e.g., Barton 2002; Loiseau et al. 2008), reproduction (e.g.,

Schreck et al. 2001; Salvante and Williams 2003; Meylan and Clobert 2005), and

metabolism (e.g., Buttemer et al. 1991; Barton 2002; Miles et al. 2007). Elevation of

glucocorticoids also directly or indirectly affects tertiary whole-animal responses, such as

foraging (e.g., Wingfield et al. 1990; Gregory and Wood 1999; Cote et al. 2006), activity

levels (e.g., Astheimer et al. 1992; Cote et al. 2006), and territoriality (e.g., Wingfield and

Silverin 1986; DeNardo and Sinervo 1994). All of these glucocorticoid-induced changes

are initially adaptive, and serve to increase the probability of the individual surviving and

recovering from the stress event (Sapolsky et al. 2000; Barton 2002). This has

historically been termed the 'resistance' phase of the stress response (Selye 1951). The

'exhaustion' phase of the stress response occurs when the challenges persist, and the

physiological responses that had initially benefited the individual become detrimental

(Selye 1951).

1.2. Stress: A life history perspective. 'Life history' is normally taken to describe life

strategies shaped by natural selection, such as age and size at maturity, mortality rates,

and age-specific reproductive rates (Roff 2002). Throughout the year(s), an individual's

life history progresses along a predictable trajectory with states such as growth,

reproduction and migration usually being linked to seasonal processes. In life history

terms, Wingfield et al. (1998) suggested that the glucocorticoid stress response be termed

an 'emergency life history stage', and can be considered a distinct component of an individual's life history. The emergency life history stage can be entered from any predictable life history state, and is caused by unpredictable, transitory perturbations. Once the perturbation has passed, the individual can either return to the original state, or enter a new state appropriate to the season (Wingfield et al. 1998). The unstressed condition is considered allostasis, or 'maintaining stability through change' (McEwen and Wingfield 2003). Environmental perturbations add to the allostatic load of an individual, but the emergency life history stage is only entered when such perturbations exceed the capacity of the individual to maintain allostasis, such that 'allostatic overload' occurs (McEwen and Wingfield 2003). The stress response then serves to reduce allostatic load. Detrimental effects occur when allostatic load is persistently high.

Life history theory is based on the premise that individuals must allocate limited resources optimally among competing functions (e.g., growth and reproduction) in order to maximize lifetime fitness (Stearns 1989, 1992; Roff 2002). With the recent interest in the integration of physiological mechanisms with life-history theory, the physiological stress response has increasingly been put forward as a proximate mediator of such life-history trade-offs (Zera and Harshman 2001; Ricklefs and Wikelski 2002). This integration is known as the 'physiology/life-history nexus' (Ricklefs and Wikelski 2002) and provides a framework in which to examine the ecological consequences of physiological stressors. In short, the physiology/life-history nexus postulates that interactions between various aspects of the endocrine system limit the physiological responses that an individual can maintain at any given time. Trade-offs between such

incompatible endocrine states then drive whole-animal trade-offs, and can limit life-history variation in wild populations (Ricklefs and Wikelski 2002). The optimal physiological response to a given stressor should therefore vary depending on the life-history characteristics of the individual, as well as the characteristics of the surrounding environment (Wingfield and Sapolsky 2003).

1.3. Using trade-offs in reproductive investment to test the physiology/life-history nexus. Investment in current versus future reproduction is a classic example of a life-history trade-off. Traditionally, energy allocation towards current reproductive opportunities is thought to sequester resources from growth and survival, thereby decreasing future reproductive potential (Williams 1966). In endocrine terms, it has been established that suppression of reproductive function (e.g., Silverin 1986; Wingfield and Silverin 1986; Greenberg and Wingfield 1987; Schreck et al. 2001) and parental care behaviour (e.g., Silverin 1986; Clutton-Brock 1991) are a consequence of the glucocorticoid stress response. The underlying mechanisms are varied, and include actions of glucocorticoids within the hypothalamus-pituitary-gonadal (HPG) axis. Briefly, the HPG-axis involves secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus, which then stimulates the anterior pituitary to produce and release luteinizing hormone (LH) and follicle stimulating hormone (FSH). Once circulating, LH and FSH then regulate the production and release of sex steroids from the gonads. Sex steroids such as estradiol, progesterone, testosterone (T), and 11-ketotesterone (11-KT) influence important reproductive processes such as sexual

development, reproductive cycles, and reproductive behaviour. Glucocorticoids can interfere with reproduction at various levels within the HPG-axis (see reviews by Greenberg and Wingfield 1987; Moore and Jessop 2003; Fuzzen et al. 2011), as well as through actions that are independent of the HPG-axis (e.g., behavioural effects mediated through brain receptors; Sapolsky et al. 2000).

If interactions between endocrine agents mediate individual decisions regarding optimal investment in current versus future reproduction (Ricklefs and Wikelski 2002), then life-history theory predicts that circulating hormone levels should reflect current reproductive investment. Individuals with high investment in current reproduction should attenuate the glucocorticoid response and/or maintain hormones important for reproduction when faced with a stressor (Wingfield and Sapolsky 2003). Conversely, individuals with lower current reproductive investment should display a robust glucocorticoid response to a stressor, at the expense of maintaining reproductive hormones. Thus, the reproductive period, with well-elucidated endocrine mechanisms and with a long history of investigation from a life-history perspective, provides an ideal situation in which to test the premise of the physiology/life-history nexus.

1.4. Applying the framework of the physiology/life-history nexus. Studying physiological stress in wild animals within the framework of the physiology/life-history nexus as the potential to help us better understand and predict the consequences of stress at the population level (Young et al. 2006). Although the stress response is an adaptive mechanism, and can even be considered part of an individual's life history, severe or

prolonged stressors have the potential to negatively affect populations through both direct effects, and indirect effects. For example, a direct effect would include massive mortality caused by a severe weather event, as occurs in Galápagos iguanas during El Niño years (Romero and Wikelski 2001). An example of an indirect effect would be a reduction in reproductive success mediated through the re-allocation of resources from reproduction to survival (Wingfield 1988). While such effects of stress are well documented, it remains difficult to connect a stressor with population-level consequences. Integrating mechanistic physiological studies of stress with life history information has the potential to overcome many of these problems and improve our understanding of how and when stress will have long-term consequences. As an example how the framework of the physiology/life-history nexus can be applied, 'carryover effects' refer to situations where stress that occurs during one life history period affects the outcome of events during a subsequent life history period (Harrison et al. 2010). There is evidence, for example, that in migratory birds, overwintering conditions can affect subsequent breeding success (Saino et al. 2004; Norris et al. 2004; Studds and Marra 2005). Including carryover effects dramatically improves the ability of population models to predict long-term changes in population size in migratory birds (Webster et al. 2002; Norris 2005; Norris and Taylor 2006). Thus, applying the framework of the physiology/life-history nexus in wild animals (i.e., considering the interaction between physiological systems and life-history state) can provide critical information linking individual physiology to population dynamics.

1.5. An introduction to the black bass. The integration of life history and physiology (Ricklefs and Wikelski 2002) has been largely unexplored in taxa other than birds, where an extensive record of life history research preceded the addition of physiological data. There is a notable deficiency in the literature in research on this topic in fish (Wingfield and Sapolsky 2003). This dearth is surprising given that fish show a wide variety of life-history traits (Winemiller and Rose 1992; McCann and Shuter 1997; King and McFarlane 2003), and an extensive background exists on the physiology of stress responses in fish (see reviews by Schreck 1981; Barton and Iwama 1991; Wendelaar-Bonga 1997; Mommsen et al. 1999; Barton 2002). In the following dissertation chapters, I use the physiology/life-history nexus framework to investigate mediators and consequences of the cortisol stress response in teleost fishes. Specifically, my research focused on the black bass: smallmouth bass (*Micropterus dolomieu*) and largemouth bass (*M. salmoides*).

Black bass are long-lived fishes (~20 yrs) that, as with many animals, display increasing reproductive success with age (Suski et al. 2003). The adults of the species have few natural predators, but piscine predators prey heavily upon the young. As a result, offspring require diligent parental care during early development (Coble 1975). In both species, this parental care falls entirely to the male, who finds and defends a territory, scrapes a nest by digging in the substrate until a shallow depression is formed, and then attracts a single female, chasing her away once the eggs are deposited and fertilized (Brown 1984). The male then guards the eggs and developing offspring for approximately 30 days. In the early development stages, the male exhibits parental care

by fanning the eggs with his pectoral fins while swimming in place, to provide aeration and keep the nest free of silt; the male also removes debris from the nest. Throughout the parental care period, the male provides defence against potential predators (Brown 1984). Once offspring develop the ability to feed exogenously and evade predators, parental care is no longer necessary (Brown 1984). The parental care provided by black bass is energetically costly (Cooke et al. 2002a, 2006), and for black bass at the northern limit of their range (i.e., Ontario) seasonal timing compounds the challenges associated with reproduction. Specifically, black bass are quiescent during the overwintering period (Adams et al. 1982), and fish must spawn in the spring before they have had an opportunity to feed and restore energy reserves that were lost over the winter. These biological traits (i.e., parental care that is critical for reproductive success but costly for the parent; increasing reproductive success with age; distinct annual life-history cycle) provide the opportunity to study individual life-history trade-offs, and make black bass interesting and highly relevant fishes in which to test and apply the physiology/life-history nexus. Black bass are also an interesting model because they are among the most popular sport fishes in North America (Pullis and Laughland 1999), and are the focus of extensive stocking programs (e.g., Jackson 2002) and frequently subjected catch-and-release angling (e.g., Arlinghaus et al. 2007). Thus, research on the ecology of stress in black bass can provide immediate applied benefits.

The largemouth and smallmouth bass are similar with respect to important life history characteristics (Table 1.1). In Ontario, largemouth bass and smallmouth bass have similar growth rates (Mosindy 1998), age at maturity, and maxiumum lifespan

(Table 1.1). However, smallmouth bass have slightly larger eggs and frequently have fewer eggs than largemouth bass (Table 1.1), and tend to defend these larger eggs more aggressive than largemouth bass defend their nests (Cooke et al. 2006). In addition, largemouth bass show a preference for shallower, warmer water, and are found in shallow, weedy bays, while smallmouth bass show a preference for deeper, cooler water, and are found above rocky substrate (Coble 1975; Heidinger 1975). Study site, and the associated relative abundance of fishes, dictated the focal species in each of the thesis data chapters.

1.6. Synthesis. In this thesis, I combined field and laboratory techniques to provide a comprehensive investigation of stress in wild black bass using the framework of the physiology/life-history nexus. All dissertation chapters fall under two complementary themes. In the first two chapters, I explored inter-individual variation in the physiological stress response. These chapters tested the premise of the physiology/life-history nexus in a teleost fish, and help us better understand how natural stressors might differentially affect members of the population. For this work, I examined ecological and life-history correlates of post-stress circulating cortisol concentrations following a standardized stressor (3 min air emersion). In the final three chapters, I investigated the long-term consequences of stress, including direct and indirect effects, within an applied physiology/life-history framework. Using cortisol implants to elevate circulating cortisol concentrations in wild fish, I employed a variety of monitoring tools to determine how an application of cortisol affects individual physiology and behaviour. I further investigated

whether these individual effects can influence population dynamics. In summary, this thesis employs a combination of physiological and life history approaches to develop an understanding of whether individual fish modulate HPI-axis activation in response to life-history trade-offs, and explores the physiological, behavioural, and population-level consequences of doing so.

1.7. Tables

Trait	Largemouth bass	Smallmouth bass
Age at maturity (years)	3-4	3-4
Longevity (years)	10-18	8-16
Fecundity	17 000 - 21 000	2 000 - 21 000
Mean water temperature during care (˚C)	20.3	19.8
Egg diameter (mm)	2.09	3.11

Table 1.1. Life history traits of largemouth bass (*Micropterus salmoides*) and smallmouth bass (*M. dolomieu*) in Ontario. Primary sources were Scott and Crossman (1973), Carlander (1977), and Cooke et al. (2006).

CHAPTER 2

THE GLUCOCORTICOID STRESS RESPONSE AND CIRCULATING ANDROGENS DURING PARENTAL CARE ARE CORRELATED WITH REPRODUCTIVE HISTORY AND THE TIMING OF REPRODUCTION

2.1. Abstract

Despite a large body of literature exploring the ultimate factors influencing reproductive investment, the proximate mechanisms underlying differences in reproductive investment remain poorly understood. Using a long-term study population of wild smallmouth bass in a connected river-lake system, we investigated the potential roles of glucocorticoids (cortisol) and androgens (T, 11-KT) as proximate mechanisms underlying reproductive investment in fish providing nest-guarding paternal care. For all individuals, we examined correlations among endocrine, life history and environmental variables. We measured the cortisol response to a standardized stressor, and circulating T and 11-KT concentrations. We collected measures of parental quality, reproductive history, and the potential value of current parental care, assessed the timing of reproduction and nest location, and evaluated reproductive performance. We quantified the relationships among the variables using univariate statistics, multiple linear regression, and path analysis. Using univariate tests, we found that post-stress circulating cortisol concentrations were negatively correlated with water temperature, nesting date, parental age, and parental size. Post-stress circulating cortisol concentrations were also higher in fish nesting in riverine than lacustrine locations. Both circulating T and 11-KT concentrations were positively correlated with water temperature, nesting date, and number of previous nests, and testosterone was also positively correlated with parental size and age. When considering the interrelationships among all variables using the two multivariate approaches, the strongest direct relationships were between post-stress cortisol concentration and water temperature, post-stress cortisol and nesting location,

and 11-KT concentration and the number of previous nests. Collectively, the results

support a role for androgens as a signal influencing reproductive investment, and offer

insight into the proximate roles of both androgen and glucocorticoid steroid hormones

during parental care in a teleost fish.

2.2. Introduction

Life-history theory is based on the premise that individuals must allocate resources among competing functions (e.g., growth, immune function, reproduction). Individuals that make optimal allocations maximize lifetime fitness (Stearns 1989, 1992; Roff 2002). Investment in current versus future reproduction is one such trade-off because energy allocated towards current reproduction sequesters resources from growth and self-maintenance, thereby decreasing future reproductive potential (Williams 1966). Owing to its role in governing behavior, physiology and the responses of individuals to their environment, the endocrine system is increasingly put forward as a proximate mediator of such life-history trade-offs (Zera and Harshman 2001; Ricklefs and Wikelski 2002).

Potential endocrine mediators of the life-history trade-off between current and future reproduction are glucocorticoid and reproductive hormones. Glucocorticoid hormones serve many functions in vertebrates (Mommsen et al. 1999; Sapolsky et al. 2000), but are best known for initiating a suite of physiological and whole-animal changes, collectively termed a stress response, which serve to promote individual survival during and after exposure to a challenging event (Wingfield et al. 1998; Romero 2004). The stress response is therefore adaptive and critical for self-preservation, but it also can suppress other important functions, including reproduction (Sapolsky et al. 2000; Wingfield and Sapolsky 2003; Angelier and Chastel 2009). The underlying mechanism involves the actions of glucocorticoids at various locations within the HPG axis, which is responsible for the synthesis and secretion of reproductive hormones (Greenberg and Wingfield 1987; Moore and Jessop 2003; Fuzzen et al. 2011). As a result of the negative

relationship between the glucocorticoid stress response and reproductive hormones, an attenuation of the glucocorticoid stress response during key reproductive periods is thought to be an adaptive mechanism maintaining investment in current reproduction, at the potential expense of survival and future reproductive opportunities (Wingfield and Sapolsky 2003). Conversely, elevated levels of hormones important for reproduction (e.g., T, LH, oestradiol, prolactin) are considered indicative of increased reproductive investment. Elevated levels of these hormones are found to improve reproductive performance while reducing resource allocation to self-maintenance functions (Hegner and Wingfield 1987; Angelier and Chastel 2009). Elevated levels of reproductive hormones have also been found to suppress the glucocorticoid stress response itself (Pottinger et al. 1995, 1996; Fuzzen et al. 2011). The opposing relationships between glucocorticoids and the HPG axis at the endocrine level (Greenberg and Wingfield 1987; Fuzzen et al. 2011) thus result in trade-offs between competing system-level functions (Ricklefs and Wikelski 2002), and interactions between endocrine states may be a proximate mechanism mediating optimal resource investment between current and future reproduction (Wingfield and Sapolsky 2003; Angelier and Chastel 2009).

For many vertebrates, parental care represents a major reproductive investment (Clutton-Brock 1991). Life-history theory predicts that if the endocrine system is a proximate mechanism mediating investment between current and future reproduction, then endocrine activity during parental care should be correlated with the value of an individual's current reproductive opportunity relative to future opportunities. Thus, individuals providing care to valuable current offspring or with few opportunities for

future reproduction should display an attenuated glucocorticoid stress response when faced with a challenge during parental care relative to individuals with less valuable current offspring or more opportunities for future reproduction (Wingfield and Sapolsky 2003; Angelier and Chastel 2009). Correspondingly, individuals with higher current reproductive investment are expected to exhibit higher circulating levels of reproductive hormones relative to individuals with lower current reproductive investment (Wingfield and Sapolsky 2003; Goymann et al. 2007; Angelier and Chastel 2009). Previous research has provided empirical support for these scenarios (Heidinger et al. 2006; Angelier et al. 2006, 2007a, 2007b; Lendvai et al. 2007). However, examples remain restricted to studies in birds.

To address our objective, we conducted a population-level field-based study in the smallmouth bass. The smallmouth bass is a long-lived iteroparous freshwater fish common in North America. Males provide annual sole parental care to a single brood for up to 6 weeks during the spring; care consists of guarding eggs and then free-swimming larvae until the offspring have developed anti-predator behaviors (Cooke et al. 2006). This research was conducted on a population of smallmouth bass that has been the subject of long-term monitoring (>15 yrs), and the reproductive history for all nesting males is known (Barthel et al. 2008; B.L. Barthel, unpublished data). For all individuals, a suite of variables related to life history and reproductive investment was assessed: parental size, parental age, the number of previous nests, brood size, nesting date, water temperature and nest location. Larger smallmouth bass attract larger females (Hanson and Cooke 2009), obtain more eggs in their nest (Suski and Philipp 2004), and defend

offspring more successfully than smaller fish (Weigmann and Baylis 1995). Larger

individuals in the congeneric largemouth bass also have proportionally higher investment

in gonads (i.e., higher gonadosomatic index [GSI]) than smaller bass (Brown and Murphy

2004), and parental size was therefore considered a measure of parental quality and

parental investment. Parental age and the number of previous nests provided

reproductive history. For nest-guarding fish such as the smallmouth bass, larger broods

result in higher reproductive success per brood (Smith and Wootton 1995). Thus, brood

size was used as the measure of the potential value of current parental care. In

smallmouth bass, larger males tend to nest earlier in the year (Ridgway et al. 1991),

which is associated with higher offspring survival (Pine et al. 2000). The factors that

limit smaller males to nesting later in the year remain unclear, but evidence suggests that

smaller males need to feed for a longer period in the spring to build up the energy stores

necessary to maintain parental care behavior (Ridgway et al. 1991). Early nesting is also

associated with lower water temperatures, which are in turn correlated with lower cortisol

stress responses (e.g., Barton and Schreck 1987; Pottinger and Carrick 2000). Thus, the

timing of reproduction has the potential to influence both reproductive investment and

endocrine parameters, and we recorded the date that eggs appeared in the nest and water

temperature at the nest. Finally, smallmouth bass in this population exhibit high nest-site

fidelity throughout their reproductive lives to either a lake or a river section of the study

site, and males nesting in the two systems exhibit divergent life-history strategies

(Barthel et al. 2008). The river is a more variable environment, and males nesting in the

river have a lower mean age and size at maturity and lower nest success than males

nesting in the lake (Barthel et al. 2008). Nesting location (river or lake) was therefore included as an additional variable that could influence life-history trade-offs and therefore reproductive investment.

The cortisol stress response and circulating androgen concentrations were investigated as potential endocrine correlates of variation in reproductive investment. Circulating androgens, and in particular T and 11-KT are key steroids for reproduction in male fish. Levels generally decline during parental care relative to levels during courtship and mating, which suggests that these hormones may be more important in territory establishment and mating than during parental care (see review by Oliveira et al. 2002). However, the existing literature also supports a role for these hormones during parental care. Treatment with an androgen receptor antagonist decreased nest defense behavior in smallmouth bass (Dey et al. 2010). Furthermore, circulating T concentrations were found to be higher in individuals providing greater levels of parental care in a cichlid fish (*Neolamprologus pulcher*, Desjardins et al. 2008).

Conversely, cortisol is the primary glucocorticoid in teleost fish (Mommsen et al. 1999), and some evidence suggests that regulation of the cortisol stress response may negatively influence parental investment in smallmouth bass. The cortisol stress response is attenuated during parental care in smallmouth bass (see Chapter 3, Section 3.4), indicating either that activation of the HPG axis during parental care suppresses the cortisol stress response, or that a robust cortisol stress response is incompatible with the maintenance of parental care behaviors in this species. In either case, we predicted for the current study that within the parental care-providing fish, the extent of attenuation of

the cortisol stress response would correlate with reproductive investment. We predicted that a robust cortisol stress response and low circulating [T] and [11-KT] would be correlated with decreased reproductive investment, while an attenuated cortisol stress response and high circulating [T] and [11-KT] would be correlated with increased reproductive investment in our study population.

To summarize, if regulation of the cortisol stress response and circulating androgens underlies variation in reproductive investment, then the extent of the cortisol stress response should be negatively correlated with reproductive investment (Wingfield and Sapolsky 2003; Bokony et al. 2009), and circulating androgens should be positively correlated with reproductive investment (Angelier and Chastel 2009). We therefore predicted that larger, older males with more prior reproductive seasons, nesting in the lake earlier in the year and guarding larger broods, should display a greater attenuation of post-stress cortisol concentrations and higher circulating T and 11-KT concentrations than smaller, younger males with less experience, nesting later in the year and guarding smaller broods.

2.3. Materials and methods

2.3.1. Study site and animals. This study was conducted in a short reach of the Mississippi River of Ontario (Frontenac County, Ontario, Canada; 44°57 N; 76°43 W). For a full description of the study site, see Barthel et al. (2008). In brief, the study site is composed of distinct upstream riverine (Mississippi River) and downstream lacustrine (Miller's Lake) habitats separated by a <1 m waterfall that is not a barrier to smallmouth

bass movement. The study site is bordered upstream by a >2 m waterfall that is a barrier to smallmouth bass, and downstream by a series of rapids and waterfalls. Radio telemetry studies have not detected fish movement out of the study site in either direction (Barthel et al. 2008). Although smallmouth bass show high nest-site fidelity to either the river or the lake during the reproductive period, most fish overwinter in the lake (Barthel et al. 2008).

2.3.2. Field data collection. During May and June 2009, the study site was surveyed by snorkelling at least every 48 h. For the long-term dataset, all nests were identified as in previous years. Because androgens decline across parental care (i.e., androgens are highest while guarding eggs, and decline as offspring develop) in smallmouth bass (Chapter 3, Section 3.4), only parental fish guarding nests with fresh eggs (<24 hrs old) were used in the current study. The date that eggs were laid was recorded. All nests were marked with a numbered tile, and assigned an egg score, which is a standard measure of the number of eggs within a nest and ranges from 1 (<1000 eggs) to 5 (>4000 eggs; Philipp et al. 1997). Focal parental males were then captured by targeted rod-and-reel angling. For standardization, the duration between hooking and landing the fish was always <15 s. Once on the boat, fish were then subjected to a standardized 3 min air emersion stressor. Following the standardized stressor, males were held singly for 25 min in 48 L coolers filled with fresh water, to allow circulating cortisol concentrations to rise (Mommsen et al. 1999). A single post-stress 1 ml blood sample was then withdrawn by caudal puncture into lithium-heparinized 3 ml vacutainer-style syringes (B.D.,

Franklin Lakes, NJ). As part of the long-term dataset, all fish in this system are fitted

with an intracoelomic passive integrated transponder (PIT) tag (12.5 x 2.0 mm) when

they are first captured. For the current study, as in previous years, all fish were scanned

for a PIT tag using a reader (Biomark, Boise, ID), and a new tag was inserted in first-time

nesting males. Fish were measured for total length (TL), scales were collected for aging

(Jearld 1983; Barthel et al. 2008), and fish were fin clipped so that snorkelers could

visually confirm that the parental fish was correctly captured from each nest. Handling

time for PIT tagging, TL measurement, and collection of scales was always <2 min. Fish

were released at their nest, and water temperature at the nest was recorded. Snorkelling

surveys were continued until the end of the reproductive season to assess reproductive

success. When a male raised a brood until the offspring were able to feed exogenously

and evade predators, the nesting attempt was considered successful (Philipp et al. 1997).

In total, 255 parental males were found guarding nests at various developmental stages in

the system and included as part of the long-term dataset. Of these, 131 fish were

identified and captured on fresh eggs, and were included in the current study.

2.3.3. Hormone analysis. Plasma cortisol concentration was determined using a

commercial kit (ImmunoChem Cortisol ^{125}I RIA kit; MP Biomedicals, Orangeburg, NY)

previously validated for teleost fish (Gamperl et al. 1994). All plasma samples were

measured in a single assay, and intra-assay variability (% CV) was 3.6%. Measurements

of T and 11-KT followed the methods outlined in McMaster et al. (1992). T and 11-KT

content were measured in duplicate by ^{3}H-radioimmunoassay using antibodies provided

by Medicorp (Medicorp Inc., Montréal, QC), and Helix Biotech (Helix Biotech Corporation, Vancouver, BC), respectively. Cross-reactivity of the T antibody with 11-KT was 7.5%, while cross-reactivity of the 11-KT antibody with T was 4.8%. For T, inter-assay variability was 5.2% while intra-assay variability was 4.4%. For 11-KT, inter-assay variability was 8.7% while intra-assay variability was 4.8%.

2.3.4. Statistical analyses. Data were examined using both univariate and multivariate methods. First, the relationships among all variables were explored using appropriate univariate pair-wise tests. Pearson's correlations were used when both variables were continuous, while analysis of variance (ANOVA) models were employed where one variable was continuous and the other was discontinuous. A Chi-square goodness-of-fit test was used for the one pair-wise test between two discontinuous variables. Residuals were tested, and total length, [cortisol], [T] and [11-KT] were log-transformed to meet the assumptions of normal distribution and homogeneity of variances. Two variables (the number of previous nests, and parental age) could not be transformed to meet the assumption of normal distribution, and non-parametric equivalents (Spearman's correlations or Wilcoxon rank-sums test) were therefore employed. Because multiple comparisons were carried out for all variables, a Bonferroni correction for multiple tests was employed. With 11 pair-wise comparisons for each variable, $\alpha=0.0045$. Although other techniques to control false discovery rates would be less conservative than the Bonferroni correction, we opted to use this conservative technique given the expectation for a high degree of correlation among variables.

Following univariate analysis, multivariate analyses were used to explore the network of interrelationships among variables. Two approaches were employed. With this dataset, a number of the measured variables were correlated (e.g., older fish are also larger and more experienced). Such correlation among independent variables reduces the power to determine which among the correlated variables are explaining the variation in the dependent variables. Therefore, we also used both multiple regression analysis and a path analytical approach to explore relationships within the dataset. Both methods have advantages and disadvantages, and the combination of methods provides a more complete picture. First, traditional multiple linear regression analysis was used. Forward stepwise regression was used to select variables. Linear regression models were then built using the independent variables selected during forward stepwise regression, and used to determine which of the life history and environmental variables significantly explained the variation in the endocrine variables. For the multiple regression approach, $\alpha=0.05$.

Path analysis presents multiple correlated variables as a network of pathways (Li 1975; Mitchell 1993; Sinervo and De Nardo 1995), and is based on calculating the total pair-wise relationship between each potential pair of variables, and then calculating what proportion of this total relationship is direct, or is influenced by indirect relationships through other correlated variables using partial regression coefficients (Li 1975; Mitchell 1993). Regression coefficients were first obtained for all pairs of variables using linear regression models, or using a nominal logistic model in the one case where both variables were discontinuous (the relationship between nest location and nest success). Constructing the path analysis then followed the process outlined by Mitchell (1993).

The pair-wise regression coefficients represent the total relationship between two variables. The direct relationship between two variables is then calculated as the total relationship minus the sum of indirect pathways.

All statistical analyses were conducted using JMP 7 (SAS Institute, Cary, NC) and Excel 2008 (Microsoft Corporation, Redmond, WA).

2.4. Results

2.4.1. Univariate results. The data are summarized in Table 2.1. Table 2.1 compares all parameters between parents guarding fish in the lake and in the river, and then compares all parameters between parents that were ultimately successful or unsuccessful in their reproductive attempts.

Univariate analyses of pair-wise relationships among all variables revealed many significant relationships (statistics presented in Tables 2.1, 2.2 and 2.3). As expected, there were strong correlations among life-history traits. Older fish were more successful at raising their broods to independence (Table 2.1), were larger and more experienced, and guarded larger broods (Table 2.2). Strong correlations also occurred among life-history traits and variables associated with the timing and location of nesting. Males nesting in the lake were larger (Table 2.1). Larger, more experienced males nested earlier in the year at cooler water temperatures (Table 2.2). Finally, correlations also occurred among endocrine parameters, life-history traits, and variables associated with the timing and location of nesting. Post-stress circulating [cortisol] was found to be higher in fish nesting in the river than in the lake (Table 2.1; Figure 2.1A). Post-stress

circulating [cortisol] was also negatively correlated with parental size (Table 2.2; Figure 2.1B) and age (Table 2.2; Figure 2.1C), and positively correlated with water temperature and the date that eggs were laid (Table 2.2). Circulating [T] was positively correlated with parental size (Table 2.2; Figure 2.2A), parental age (Table 2.2; Figure 2.2B) and the number of previous nests (Table 2.2; Figure 2.2C), and negatively correlated with water temperature and the date that eggs were laid (Table 2.2). Circulating [11-KT] was positively correlated with the number of previous nests (Table 2.2; Figure 2.3) and negatively correlated with water temperature and the date that eggs were laid (Table 2.2).

There were no differences in mean endocrine parameters between fish that were ultimately successful or unsuccessful in their nesting attempt (Table 2.2). Also, the ratio of successful to unsuccessful fish did not differ between the two nesting locations (Table 2.3).

2.4.2. Multiple linear regression approach. Forward stepwise linear regression selected water temperature, nesting location, nest success, and number of previous nests as variables that significantly improved the fit of a multiple linear regression model predicting post-stress circulating [cortisol]. Of these variables, water temperature and location were significant individual variables in the final model (Table 2.4). For [T], nesting date, brood size, number of previous nests, total length, age, and water temperature were selected as variables that significantly improved the fit of the model, with brood size and total length being significant variables in the final model (Table 2.4). Finally, water temperature, brood size, number of previous nests, age, nesting location,

and nesting date were selected as variables that significantly improved the fit of the

multiple linear regression model predicting [11-KT], and number of previous nests and

age were significant in the final model (Table 2.4).

2.4.3. Path analytical approach. Regression coefficients (R values) were generated for

all pair-wise relationships (Table 2.5), and from these regression coefficients (i.e., total

relationships), a path diagram was constructed (Figure 2.4). Partial regression

coefficients (i.e., direct relationships) generated from the path analysis are also presented

for all pair-wise relationships (Table 2.5). The path analysis does not support direct

relationships between circulating post-stress [cortisol] and parental age, or between

[cortisol] and parental size (Figure 2.1B and 2.1C), indicating that these relationships can

be accounted for indirectly through the strong positive relationship between [cortisol] and

water temperature, and the strong negative relationships between parental size and

nesting date. For the androgen variables, the path analysis also does not support direct

relationships between circulating [T] and parental size, or between [T] and parental age

(Figure 2.2A and 2.2B), indicating that these relationships can be accounted for indirectly

through the strong negative relationship between [T] and nesting date, and the strong

negative relationships between parental size and nesting date. The path analysis supports

direct relationships between post-stress [cortisol] and water temperature, and between

post-stress [cortisol] and nesting location; the path analysis also supports direct

relationships between [T] and [11-KT] and nesting date and previous nesting experience,

and between [11-KT] and water temperature (Table 2.5; Figure 2.4). Post-stress

[cortisol] is higher with higher water temperatures, and higher in fish nesting in the river (Table 2.5; Figure 2.4). Circulating [T] and [11-KT] are higher in fish nesting earlier in the year, and higher in more experienced fish; [11-KT] is lower with higher water temperatures (Table 2.5; Figure 2.4).

2.5. Discussion

The results of this study demonstrate that post-stress circulating cortisol and androgen concentrations are correlated with measures of nest location and the timing of reproduction as well as reproductive history in smallmouth bass providing care to fresh eggs. We discuss insights gained from this dataset on the potential roles of cortisol and androgen regulation during parental care in smallmouth bass.

2.5.1. Selection of sampling protocol.
The fish used in the current study were important as individuals within a long-term (>15 y) study population. To minimize the impact of our sampling protocol, we were limited to collecting only a single blood sample per fish. In order to understand the information provided by a single blood sample, we first obtained both baseline and post-stress [cortisol] and [T] data from a separate population of fish. These fish were originally captured as part of a separate study; for full details, please refer to Chapter 3. For the purposes of the current study, baseline and post-stress [cortisol] and [T] data were reanalyzed using linear regression models to assess the relationship between baseline and post-stress [steroid], and the relationship between post-stress [steroid] and the steroid response to the standardized stressor (i.e., the difference between post-stress and baseline

concentrations). Only data from a subset (n=49) of appropriate fish were used (i.e., only

values obtained from unmanipulated, control smallmouth bass providing care to eggs <24 h

old).

These data demonstrate that in smallmouth bass providing parental care to fresh eggs,

baseline circulating cortisol concentrations were consistently low, with little variation among

values (Figure 2.5A). Post-stress cortisol concentration was unrelated to baseline circulating

cortisol concentration (Figure 2.5A), but was an accurate measure of the cortisol stress

response (Figure 2.5B). On the other hand, post-stress androgen concentrations more closely

reflected baseline androgen levels (Figure 2.5C) than the ability of parents to maintain

androgen concentrations following a stressor (Figure 2.5D). Thus, being limited to a single

sample, we opted to use only the post-stress sample, since it provides an accurate measure of

the cortisol stress response (Figure 2.5B), and a reasonable measure of baseline circulating

androgen concentration (Figure 2.5C).

2.5.2. Limitations of a correlative dataset. In the current study, many variables were

highly correlated. Older males were also large and more experienced, guarded more

eggs, nested earlier in the year at colder water temperatures, and were more successful

than younger males. Univariate statistics provide a measure of which variables in

isolation will influence steroid concentrations, but may overestimate relationships, since

they do not take into account potential correlated variables. On the other hand, multiple

linear regression models and path analysis have low power to resolve individually

significant variables when the independent variables are so highly correlated, and

therefore underestimate relationships. Indeed, there is no statistical method that can entirely disentangle these variables to reliably identify which among the variables are the driving factors influencing post-stress cortisol and circulating androgen concentrations. By exploring the data using multiple statistical methods, we can draw some general conclusions (Table 2.6). Using all three methods, post-stress cortisol concentration is positively correlated with water temperature, and higher in fish nesting in the river than in the lake. Using all three methods, 11-KT concentration is positively correlated with previous nesting experience. Conservatively, we can conclude from our data that these relationships are significant and direct. Using two of the three methods, T concentration is positively correlated with total length and previous nesting experience, and negatively correlated with nesting date, while 11-KT concentration is negatively correlated with water temperature and nesting date. While these results are more liberal, our data strongly suggests that total length, age, previous nesting experience, water temperature and nesting date all are important factors correlated with endocrine parameters during parental care. As with all correlative datasets, experimental manipulation is necessary to determine causal relationships.

2.5.3. What can we learn about the role of cortisol and androgens during parental care? Using various statistical approaches, we found that post-stress circulating cortisol concentration was consistently correlated with water temperature, while circulating androgen concentrations often correlated with water temperature and nesting date. Nesting earlier in the season provides clear advantages, since it improves offspring

survival through their first summer (Pine et al. 2000), gives offspring a longer growing season prior to their first winter (Wiegmann et al. 1992), and in turn increases first-winter survival (Biro et al. 2004). The results of the current study suggest that the cooler water temperatures faced by early nesters may also facilitate the physiological capacity of fish to provide parental care. There is evidence that increased androgen concentrations and a decreased cortisol stress response may benefit parental care-providing smallmouth bass. In fish, circulating T concentration is higher in individuals providing greater levels of parental care in both laboratory (Desjardins et al. 2008) and field settings (Appendix II, Section ii.v). Parental care-providing smallmouth bass show a lower cortisol stress response for a given temperature than non-parental fish (Chapter 3, Section 3.4). Based on the available information, it is likely that fish gain multiple benefits by nesting at cooler temperatures, both in terms of increased offspring survival, and in terms of increased physiological capacities. Mechanistic studies detailing the downstream effects of the relationship between physiological parameters and water temperature are necessary to draw definitive conclusions regarding the potential benefits of lower water temperature on parental care behavior in smallmouth bass.

Factors beyond water temperature are also likely to affect endocrine regulation during parental care. In particular, 11-KT concentration was consistently positively correlated with the number of previous nests, while circulating T concentration was positively correlated with total length and previous nests using two of the three statistical approaches. These results are consistent with previous literature showing increased reproductive hormone levels in older parents (Angelier et al. 2007a; Angelier and Chastel

2009). These endocrine factors may likely contribute to the pattern of increased

reproductive success at cooler water temperatures for older, larger smallmouth bass.

Three hypotheses have been put forward to explain the consistently demonstrated

positive relationships among reproductive investment, parental age, and reproductive

hormone levels. 'Parental constraint' posits that young animals are physiologically

incapable of investing heavily in offspring; 'parental restraint' suggests that older animals

increasingly invest in offspring as future reproductive opportunities become limited; and

'selection' proposes that lower quality animals succumb earlier in life than higher quality

animals, and the result is a higher quality group of older parents (Curio 1983; Forslund

and Pärt 1995). All three hypotheses make many of the same predictions, and drawing

conclusions regarding ultimate drivers or correlations therefore becomes challenging.

However, the lack of relationship between endocrine parameters and brood size in the

current study can be viewed as indirect support for the 'parental constraint' or 'selection'

hypotheses, rather than the 'restraint' hypothesis (Curio 1983). Collectively, these results

suggest that androgen parameters may reflect an increased physiological capability

(either as a result either of increased capacity with age, or as a result of selection) in

larger, older parents, rather than sensitivity to current reproductive potential.

2.5.4. Why is the cortisol stress response higher in river-nesting fish? In this

connected river-lake study system, males show high nest-site fidelity, and there is

divergence in life-history traits between the two locations. Males nesting in the river are

younger and smaller at maturity than fish nesting in the lake, and have lower reproductive

success (Barthel et al. 2008). In the current study, post-stress cortisol concentrations were higher in fish nesting in the river than in the lake. This difference in cortisol responsiveness may reflect differences in environmental variables between the two locations. Additional research that examines more specifically the many variables that differ between the two locations (e.g. water flow, predation rates, turbidity) is necessary to fully understand differences in endocrine regulation between the two locations.

An alternate explanation is that there may be differences in the characteristics of the fish themselves between the two locations. Differences in stress coping styles have been documented in fish (Pottinger and Carrick 1999; reviewed by Schjolden and Winberg 2007). Proactive individuals are more active, more aggressive, routine-forming, and exhibit higher sympathetic activity in response to a stressor, whereas reactive individuals freeze and exhibit higher HPI-axis activity in response to a stressor, but are more responsive to environmental changes and exhibit more behavioral flexibility (Coppens et al. 2010). Thus, it is possible that reactive fish (high cortisol response to stress) nest in the more unpredictable riverine environment at higher rates than proactive fish (low cortisol response to stress). Further research investigating whether the differences in cortisol responsiveness are maintained year-round, including during winter when all fish congregate in the lake (Barthel et al. 2008), would be helpful as a first step in investigating this possibility.

2.5.5. Summary. The results of this study demonstrated that the timing of reproduction and nest location are correlated with the cortisol stress response, while the timing of

reproduction and reproductive history are correlated with androgen concentrations during parental care in a population of wild teleost fish. The resultant relationships contribute to a pattern of higher reproductive success in more experienced males that reproduce earlier in the year. Thus, the current study provides support for increased androgen concentration as a signal of increased reproductive investment, and for increased androgen concentration as a potential mechanism driving increasing reproductive success with age. The current study suggests that while the cortisol stress response varied among the parental care-providing fish, it was correlated more strongly with environmental variables than with life-history traits.

This study identified total length, age, previous nesting experience, water temperature and nesting date as important factors correlated with endocrine parameters during parental care. The results provide the framework and ecological relevance for further experimental work in which life-history and environmental variables are manipulated to better understand the driving factors influencing patterns of hormone regulation in relation to reproductive investment.

2.6. Figures
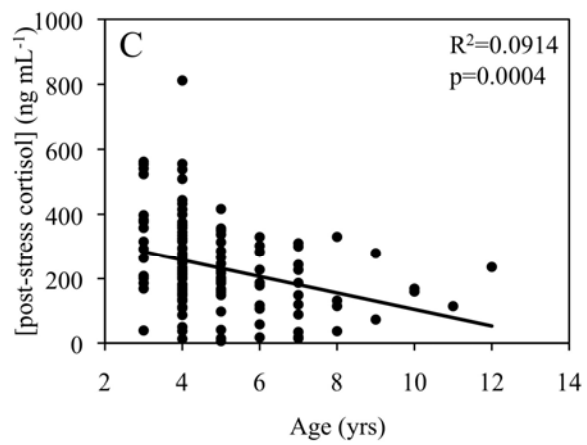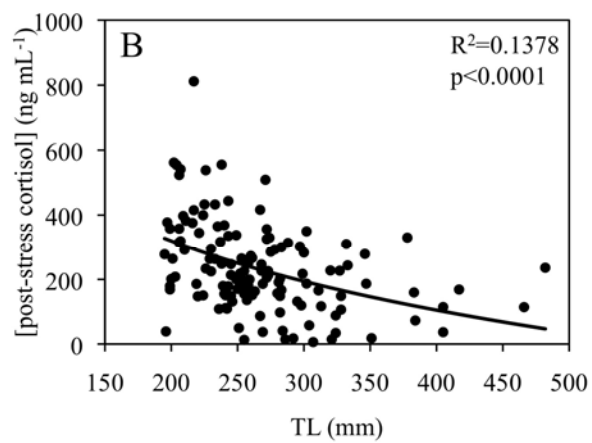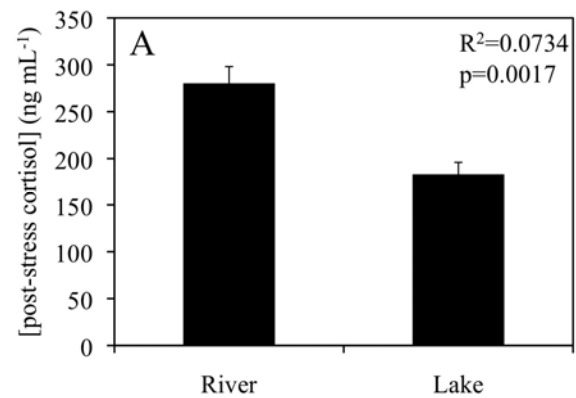
Figure 2.1. Significant (α=0.0045) pair-wise relationships between post-stress circulating cortisol concentration and the life-history variables (A) nest location, (B) TL, and (C) age for 131 male smallmouth bass captured from the Miller's Lake/Mississippi River system while guarding fresh eggs in May-June 2009. The explained variance (R^2) and significance (p) are noted on each graph. For (A) values are presented as mean ± SEM.
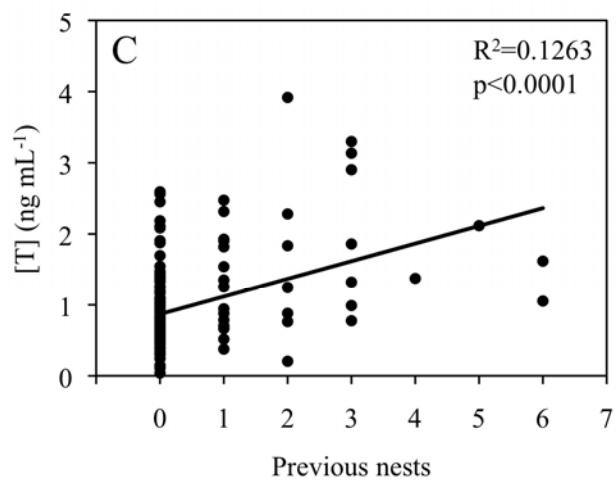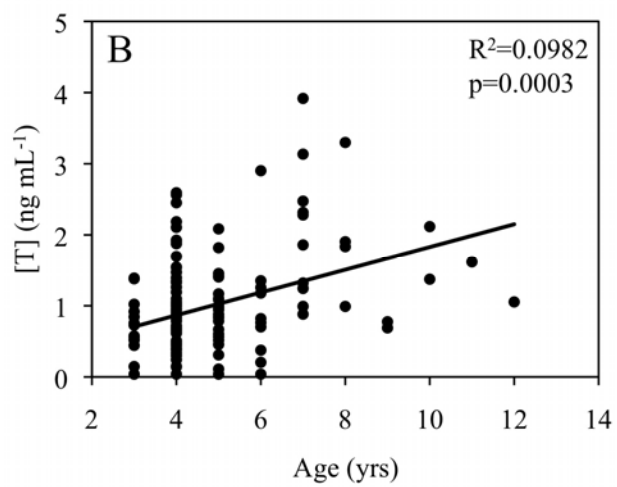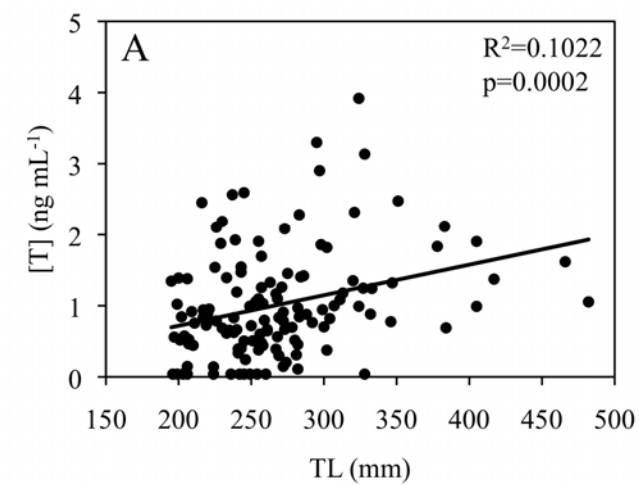
Figure 2.2. Significant (α=0.0045) pair-wise relationships between circulating T

concentration and the life-history variables (A) TL, (B) age, and (C) the number of

previous nests for 131 male smallmouth bass captured from the Miller's Lake/Mississippi

River system while guarding fresh eggs in May-June 2009. The explained variance (R^2)

and significance (p) are noted on each graph.
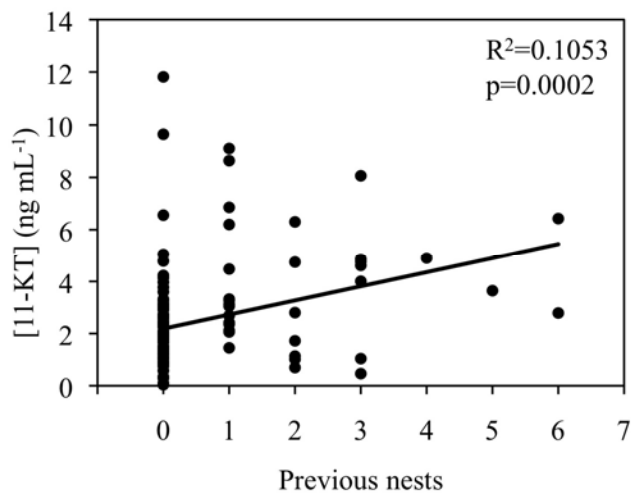
Figure 2.3. The significant (α=0.0045) pair-wise relationship between circulating 11-

11-KT concentration and the number of previous nests for 131 male smallmouth bass

captured from the Miller's Lake/Mississippi River system while guarding fresh eggs in

May-June 2009. The explained variance (R^2) and significance (p) are noted on the graph.
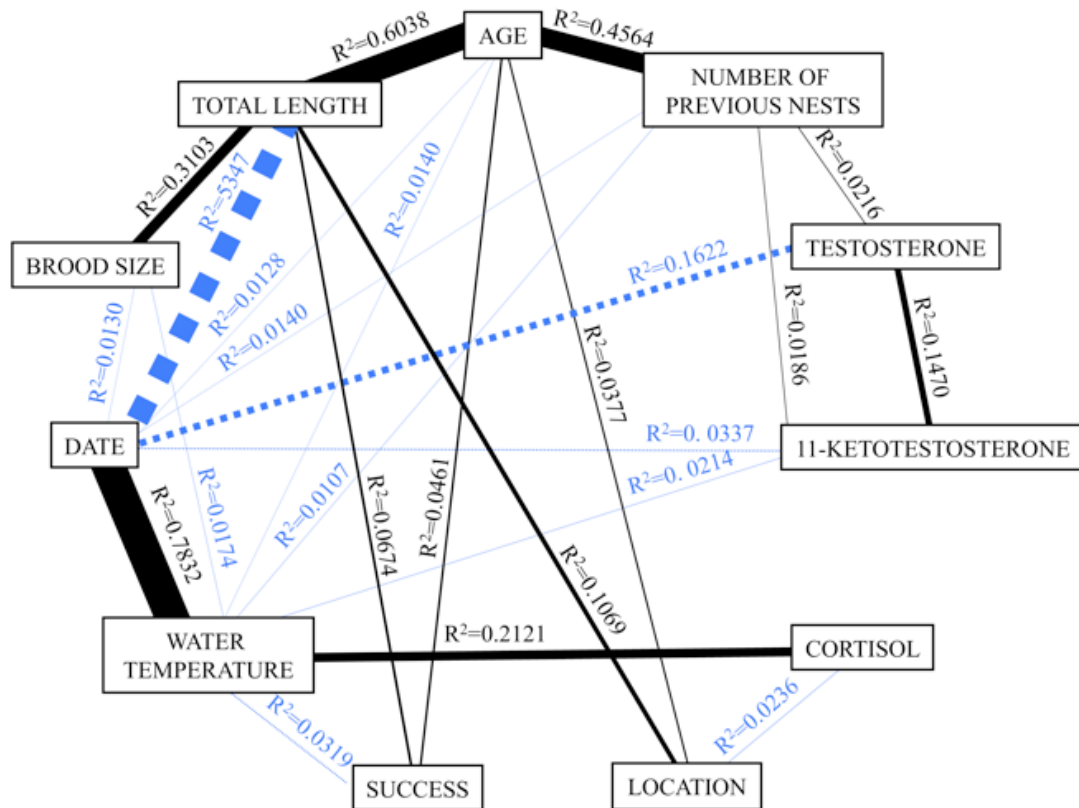
Figure 2.4. Path analysis diagram showing direct relationships ($R^2 > 0.01$) among measured life-history variables and endocrine parameters for 131 male smallmouth bass (*Micropterus dolomieu*) captured from the Miller's Lake/Mississippi River system while guarding fresh eggs in May-June 2009. The width of each line is proportional to the strength of the direct relationship (see Table 2.5). Solid lines indicate positive relationships, while dashed lines indicate negative relationships.
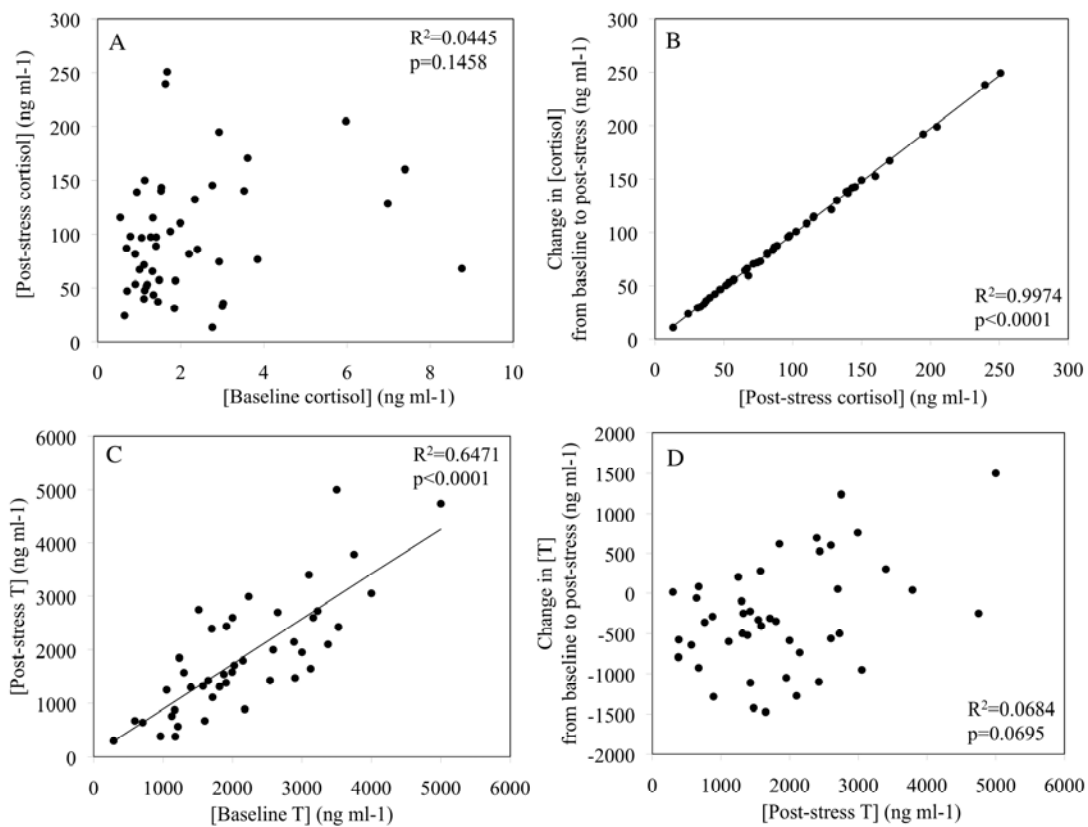
Figure 2.5. The relationship between baseline and post-stress circulating steroid concentration (A, B), and the relationship between post-stress circulating steroid concentration and the response following a standardized stressor (i.e., the difference between post-stress and baseline circulating hormone concentrations; C, D). Relationships are depicted for cortisol (A,C) and testosterone (T; B, D). The explained variance (R^2) and significance (p) are noted on each graph.

2.7. Tables

Variable	Mississippi River (n=71)				Miller's Lake (n=60)				R^2	F statistic	Z statistic	p value
	Mean	Median	SE	Range	Mean	Median	SE	Range				
Total length (mm)	**252.2**	**239.0**	**5.29**	**195.0 – 405.0**	**284.5**	**267.5**	**7.61**	**196.0 - 482.0**	**0.0890**	**12.727**	---	**0.0005**
Age (years)†	4.6	4.0	0.16	3.0 - 8.0	5.2	4.0	0.26	3.0 - 12.0	---	---	1.7553	0.0729
Number of previous nests†	0.7	0.0	0.15	0.0 - 5.0	0.8	0.0	0.22	0.0 - 7.0	---	---	0.4545	0.6494
Brood size (egg score)	2.3	2.0	0.10	1.0 - 4.0	2.5	2.0	0.10	1.0 - 5.0	0.0077	0.9966	---	0.3200
Water temperature (ºC)	15.5	15.5	0.08	14.5 - 17.0	15.4	15.5	0.09	14.0 - 16.5	0.0045	0.5873	---	0.4449
Date (days)	May 30	June 2	0.77	May 17 - June 14	May 30	June 1	0.85	May 17 - June 7	0.0024	0.3116	---	0.5777
Post-stress [cortisol] (ng ml^{-1})	**280.3**	**283.5**	**17.77**	**14.4 - 811.7**	**183.3**	**183.9**	**12.7**	**6.1 - 508.7**	**0.1251**	**18.4414**	---	**<0.0001**
[T] (ng ml^{-1})	1.0	0.8	0.09	0.4 - 3.2	0.9	0.8	0.09	0.4 - 3.9	0.0009	0.1180	---	0.7318
[11-KT] (ng ml^{-1})	2.3	2.0	0.22	0.4 - 11.8	2.6	2.2	0.29	0.4 - 9.6	0.0085	1.1147	---	0.2930

Variable	Successful (n=93)				Unsuccessful (n=38)				R^2	F statistic	Z statistic	p value
	Mean	Median	SE	Range	Mean	Median	SE	Range				
Total length (mm)	274.3	267.0	5.50	199.0 - 482.0	249.0	238.0	8.50	195.0 - 466.0	0.0455	6.1587	---	0.0144
Age (years)†	**5.1**	**4.0**	**0.18**	**3.0 - 12.0**	**4.3**	**4.0**	**0.24**	**3.0 - 11.0**	---	---	**3.1771**	**0.0015**
Number of previous nests†	0.9	0.0	0.16	0.0 - 7.0	0.4	0.0	0.23	0.0 - 7.0	---	---	2.5279	0.0115
Brood size (egg score)	2.4	2.0	0.08	1.0 - 5.0	2.3	2.0	0.14	1.0 - 4.0	0.0023	0.3016	---	0.5838
Water temperature (ºC)	15.5	15.5	0.08	14.0 - 17.0	15.5	15.5	0.11	14.0 - 16.5	0.0004	0.0494	---	0.8244
Date (days)	May 29	May 30	0.70	May 17 - June 14	May 31	June 2	0.96	May 17 - June 7	0.0125	1.6560	---	0.2004
Post-stress [cortisol] (ng ml^{-1})	241.0	224.9	15.4	6.1 - 811.7	223.3	204.7	16.66	38.9 - 399.6	0.0034	0.4456	---	0.5056
[T] (ng ml^{-1})	1.0	0.9	0.1	0.4 - 3.9	1.0	0.8	0.13	0.4 - 2.9	0.0015	0.2037	---	0.6525
[11-KT] (ng ml^{-1})	2.5	2.0	0.3	0.4 - 9.6	2.3	2.0	0.32	0.4 - 11.8	0.0019	0.2584	---	0.6121

Table 2.1. Summary of data collected for 131 male smallmouth bass (*Micropterus dolomieu*) captured while guarding fresh eggs in Miller's Lake and upstream river section of the Mississippi River, Ontario, Canada in May-June 2009. The results of statistical analyses comparing differences in mean values between the two locations are presented. The results of statistical analyses comparing differences in mean values between successful and unsuccessful fish are also presented. Bass were deemed to be successful in their nesting attempt when they remained with their offspring until the offspring were able to feed exogenously and evade predators and

unsuccessful when they abandoned their offspring prematurely. For summary data, the mean, median, standard error and range of

values are presented for each variable. For statistical analyses, analysis of variance (ANOVA) models were performed unless

otherwise noted. Bold underlined text indicates statistically significant difference following a Bonferroni correction for multiple tests

(α=0.0045). †Data were not normally distributed and non-parametric Wilcoxon rank sums tests were employed.

	Total length	Age†	Previous nests†	Brood size	Water temperature	Date	Post-stress [cortisol]	[T]	[11-KT]
Total length (mm)	1.0000								
Age (years)†	**0.9031**	1.0000							
Number of previous nests†	**0.6027**	**0.6648**	1.0000						
Brood size (egg score)	**0.5412**	**0.4946**	**0.4462**	1.0000					
Water temperature (^{o}C)	**-0.6775**	**-0.6034**	**-0.5557**	**-0.4925**	1.0000				
Date (days)	**-0.7291**	**-0.6538**	**-0.6185**	**-0.5262**	**0.8824**	1.0000			
Post-stress [cortisol] (ng ml^{-1})	**-0.3943**	**-0.3524**	-0.1237	-0.1982	**0.4783**	**0.3948**	1.0000		
[T] (ng ml^{-1})	**0.3084**	**0.3347**	**0.3981**	0.1018	**-0.4183**	**-0.4119**	-0.0776	1.0000	
[11-KT] (ng ml^{-1})	0.2424	0.1443	**0.4088**	0.0941	**-0.4057**	**-0.3556**	-0.1318	**0.5931**	1.0000

Table 2.2. Correlation coefficients for all pair-wise relationships between continuous data collected for 131 male smallmouth bass (*Micropterus dolomieu*) captured from the Miller's Lake/Mississippi River system while guarding fresh eggs in May-June 2009. Pearson's correlations were performed and Pearson's correlation coefficients presented unless otherwise noted. Bold underlined text indicates a statistically significant difference following a Bonferroni correction for multiple tests (α=0.0045). †Data were not

normally distributed and non-parametric Spearman's correlations were performed, with the Spearman's correlation coefficients presented.

	Successful (n=93)	Unsuccessful (n=38)
Mississippi River (n=71)	46	25
Miller's Lake (n=60)	47	13

Table 2.3. The number of male smallmouth bass (*Micropterus dolomieu*) captured while guarding fresh eggs in Miller's Lake and upstream river section of the Mississippi River, Ontario, Canada in May-June 2009 that were either successful (i.e. remained with their offspring until the offspring were able to feed exogenously and evade predators) or unsuccessful (i.e. abandoned their offspring prematurely) in their nesting attempt. A Chi-square test indicated that there was no difference in the ratio of successful and unsuccessful parents between the two locations (χ^2=2.9410, p=0.0864).

Endocrine parameter	Variable	Step	Cumulative R^2	F statistic	p value
Post-stress [cortisol] (ng ml^{-1})	**Water temperature (^{o}C)**	**1**	**0.2270**	**36.148**	**<0.0001**
	Location (lake or river)	**2**	**0.3270**	**21.702**	**<0.0001**
	Success (successful or unsuccessful)	3	0.3435	2.375	0.1257
	Number of previous nests†	4	0.3534	1.971	0.1628
[T] (ng ml^{-1})	Date (days)	1	0.1623	2.331	0.1293
	Brood size (egg score)	**2**	**0.1830**	**4.070**	**0.0458**
	Number of previous nests†	3	0.2072	1.491	0.2243
	Total length (mm)	**4**	**0.2175**	**4.728**	**0.0315**
	Age (years)†	5	0.2325	2.620	0.1080
	Water temperature (^{o}C)	6	0.2426	1.691	0.1958
[11-KT] (ng ml^{-1})	Water temperature (^{o}C)	1	0.1993	2.623	0.1077
	Brood size (egg score)	2	0.1382	2.178	0.1425
	Number of previous nests†	**3**	**0.1619**	**11.767**	**0.0008**
	Age (years)†	**4**	**0.2078**	**10.459**	**0.0015**
	Location (lake or river)	5	0.2281	3.594	0.0603
	Date (days)	6	0.2362	1.357	0.0246

Table 2.4. Results of forward stepwise regression to select parameters that explain variation in circulating steroid hormone concentration for 131 male smallmouth bass (*Micropterus dolomieu*) captured from the Miller's Lake/Mississippi River system while guarding fresh eggs in May-June 2009. Bold underlined text indicates statistically significant relationships (α=0.05) in the final linear regression model. †Data were not normally distributed and tests were performed on rank data.

	Total length	Age†	Previous nests†	Brood size	Water temperature	Date	Location	Success	Post-stress [cortisol]	[T]
Age (years)†	**0.6037**									
	0.6037									
Number of previous nests†	**0.1850**	**0.4564**								
	0.0000	0.4564								
Brood size (egg score)	**0.3102**	**0.2395**	**0.0737**							
	0.3102	0.0032	0.0000							
Water temperature (°C)	*0.4805*	*0.3858*	*0.1962*	*0.2424*						
	0.0021	*0.0140*	*0.0107*	*0.0174*						
Date (days)	*0.5347*	*0.4644*	*0.2521*	*0.2719*	**0.7832**					
	0.5347	*0.0128*	*0.0140*	*0.0130*	0.7832					
Location (lake or river)	**0.1069**	0.0036	0.0000	0.0084	*0.0092*	*0.0061*				
	0.1069	0.0377	0.0000	0.0000	*0.0000*	*0.0000*				
Success (successful or unsuccessful)	**0.0674**	**0.0652**	0.0076	0.0030	*0.0022*	0.0180	0.0233			
	0.0674	0.0461	0.0000	0.0000	*0.0319*	*0.0000*	0.0046			
Post-stress [cortisol] (ng ml⁻¹)	*0.1258*	*0.0672*	0.0019	*0.0170*	**0.2119**	**0.1653**	*0.0630*	0.0031		
	0.0032	*0.0008*	*0.0000*	*0.0000*	0.2121	0.0000	0.0236	0.0000		
[T] (ng ml⁻¹)	**0.0917**	**0.0884**	**0.0909**	**0.0247**	*0.1026*	*0.1622*	*0.0001*	0.0151	*0.0073*	
	0.0001	0.0047	0.0216	0.0000	*0.0000*	0.1622	0.0000	0.0022	*0.0000*	
[11-KT] (ng ml⁻¹)	0.0291	0.0187	**0.0383**	0.0045	*0.0800*	*0.1143*	*0.0043*	0.0003	*0.0050*	**0.1470**
	0.0033	0.0024	0.0186	0.0000	*0.0214*	*0.0337*	0.0008	0.0000	*0.0001*	0.1470

Table 2.5. The total and direct relationships between each pair of variables for 131 male smallmouth bass (*Micropterus dolomieu*) captured from the Miller's Lake/Mississippi River system while guarding fresh eggs in May-June 2009. The top value for each pair of

variables represents the total relationship. Bold underlined text indicates statistically significant relationships (α=0.05) as determined by linear regression models for all pairs of variables except in the case of location and success where a nominal logistic model was used. Italicized text indicates negative relationships. The bottom value for each pair of values represents the direct relationships as calculated using a path analytical approach (Li 1975; Mitchell 1993). Path analysis was performed on regression coefficients, but for consistency, squared regression coefficients (R^2 values) are presented in this table. †Data were not normally distributed and tests were performed on rank data.

Endocrine parameter	Variable	Statistical approach		
		Univariate	Multiple regression	Path analysis
Post-stress [cortisol] (ng ml-1)	Total length (mm)	**Significant**	Non-significant	Indirect
	Age (years)	**Significant**	Non-significant	Indirect
	Number of previous nests	Non-significant	Non-significant	Indirect
	Brood size (egg score)	Non-significant	Non-significant	Indirect
	Water temperature (^{o}C)	**Significant**	**Significant**	**Direct**
	Date (days)	**Significant**	Non-significant	Indirect
	Location (lake or river)	**Significant**	**Significant**	**Direct**
	Success (successful or unsuccessful)	Non-significant	Non-significant	Indirect
[T] (ng ml-1)	Total length (mm)	**Significant**	**Significant**	Indirect
	Age (years)	**Significant**	Non-significant	Indirect
	Number of previous nests	**Significant**	Non-significant	**Direct**
	Brood size (egg score)	Non-significant	**Significant**	Indirect
	Water temperature (^{o}C)	**Significant**	Non-significant	Indirect
	Date (days)	**Significant**	Non-significant	**Direct**
	Location (lake or river)	Non-significant	Non-significant	Indirect
	Success (successful or unsuccessful)	Non-significant	Non-significant	Indirect
[11-KT] (ng ml-1)	Total length (mm)	Non-significant	Non-significant	Indirect
	Age (years)	Non-significant	**Significant**	Indirect
	Number of previous nests	**Significant**	**Significant**	**Direct**
	Brood size (egg score)	Non-significant	Non-significant	Indirect
	Water temperature (^{o}C)	**Significant**	Non-significant	**Direct**
	Date (days)	**Significant**	Non-significant	**Direct**
	Location (lake or river)	Non-significant	Non-significant	Indirect
	Success (successful or unsuccessful)	Non-significant	Non-significant	Indirect

Table 2.6. A summary table outlining the relationships between life history and endocrine variables for 131 male smallmouth bass (*Micropterus dolomieu*) captured from the Miller's Lake/Mississippi River system while guarding fresh eggs in May-June 2009, as determined by three separate statistical approaches. For univariate and multiple regression statistical approaches, significant or non-significant relationships are noted. For path analysis, direct or indirect relationships are noted. Bold text indicates a

significant or direct relationship. Bold underlined text indicates variables found to be

consistently significant and direct using all three statistical approaches.

CHAPTER 3

THE GLUCOCORTICOID STRESS RESPONSE DURING PARENTAL CARE

IS ATTENUATED BUT UNRELATED TO REPRODUCTIVE INVESTMENT

3.1. Abstract

We investigated whether circulating glucocorticoids and androgens are correlated with reproductive investment in smallmouth bass, a teleost fish with sole paternal care. Circulating cortisol and androgens prior to and 25 min following a standardized 3 min emersion stressor were quantified for non-reproductive and parental fish across the parental care period. To experimentally investigate the influence of reproductive investment on endocrine parameters, we manipulated brood size (reduced, enlarged, sham-treated, or unmanipulated) 24 hrs prior to sampling parental fish. We predicted that fish guarding offspring would exhibit increased androgens and baseline cortisol levels, and an attenuated cortisol response to the stressor when compared with non-reproductive individuals. We further predicted that these effects would scale with reproductive investment. As predicted, parental care-providing fish exhibited lower post-stress plasma cortisol concentrations than non-reproductive fish. This difference was strongest early during parental care. However, no differences in baseline or post-stress cortisol concentrations were detected among parents guarding offspring with varying brood sizes. There was, however, a trend for parental fish to exhibit an increased cortisol response following brood manipulation, regardless of the direction of change in brood size, a response that likely reflected disturbance. No differences were found in baseline cortisol concentrations. Circulating androgens were found to be highest during early parental care, and no differences were found among parents guarding manipulated broods. Collectively, these findings demonstrate that the endocrine stress response is affected by reproductive status, but the response in this species does not appear to be scaled

according to reproductive investment as predicted by life-history theory.

3.2. Introduction

The premise of life-history theory is that individuals should make optimal trade-offs

among competing functions in order to maximize their fitness (Williams 1966; Pianka

1970, 1972; Charnov and Krebs 1974). Investment in current versus future reproduction

is one such trade-off because energy allocated towards current reproductive opportunities

sequesters resources from growth and survival, thereby decreasing future reproductive

potential (Williams 1966; Trivers 1972a, 1972b). Recently, the endocrine system and in

particular the glucocorticoid hormones have attracted interest as potential mediators of

these trade-offs (Zera and Harshman 2001; Ricklefs and Wikelski 2002; Denver et al.

2009). The glucocorticoid hormones are best known for their rapid elevation in response

to a challenge, initiating a suite of physiological changes collectively termed a stress

response (Mommsen et al. 1999; Sapolsky et al. 2000). In the short-term, this response is

considered adaptive because it initiates a suite of system-level and whole-organism

responses that promote survival of the individual through a challenge (Wingfield et al.

1998; Sapolsky et al. 2000; Greenberg et al. 2002). However, these physiological

changes also interrupt other important functions including reproduction (e.g., Silverin

1986; Wingfield and Silverin 1986; Greenberg and Wingfield 1987; Schreck et al. 2001)

and parental care behaviour (e.g., Silverin 1986; Clutton-Brock 1991). Baseline levels of

glucocorticoids also serve important biological functions, and may play a role in

preparing an organism to appropriately respond to a future challenge (Sapolsky et al.

2000; Romero 2004). Whereas elevation of glucocorticoids in response to stress inhibits

reproduction, increases in baseline glucocorticoid levels may benefit reproductive activity

by mobilizing energy reserves for the challenges associated with mating or parental care (Moore and Jessop 2003; Romero 2004). Therefore, both baseline glucocorticoid levels and the extent of glucocorticoid elevation during a challenge are thought to mediate the trade-off between current and future reproduction, and may function in a complementary fashion (Romero 2004). Whereas increases in baseline levels during reproductive periods are associated with increased investment in current reproduction (Moore and Jessop 2003; Bokony et al. 2009; Bonier et al. 2009), augmented glucocorticoid levels when faced with a challenge during reproduction are thought to shift resources away from current reproductive investment and towards survival and future reproduction (Wingfield and Sapolsky 2003; Breuner et al. 2008). Thus, a pattern of increase baseline glucocorticoids and an attenuated glucocoirticoid response to stress is thought to be adaptive during important reproducitve periods (Wingfield and Sapolsky 2003).

If interactions between endocrine agents mediate individual decisions regarding optimal investment in current versus future reproduction (Ricklefs and Wikelski 2002), then life-history theory predicts that circulating hormone levels should reflect current reproductive investment. Individuals with high investment in current reproduction should display higher baseline glucocorticoid levels, while attenuating the glucocorticoid response and/or maintaining hormones important for reproduction when faced with a challenge (Wingfield and Sapolsky 2003). Conversely, individuals with lower current reproductive investment should display low baseline glucocorticoid levels and a robust glucocorticoid response to a challenge during parental care, at the expense of maintaining reproductive hormones. In the current study, we investigated the influence of

reproductive investment on the glucocorticoid (cortisol) and reproductive hormone (androgen) concentrations of smallmouth bass, a teleost fish with annual sole-male parental care. In teleost fish, cortisol is the primary glucocorticoid (Mommsen et al. 1999), and androgens are implicated in male parental care (see review by Oliveira et al. 2002). We used a standardized stress protocol that involved capture and 3 min of air exposure, and we collected blood samples prior to and 25 min following the stressor (a period of time that corresponds to the peak of cortisol elevation following a stressor for this species; Cooke et al. 2002b). We first tested whether baseline and post-stress cortisol and androgen levels varied in parental care-providing males compared to non-parental fish. We then investigated the possibility that baseline and post-stress cortisol and androgen levels varied with offspring age or brood size during parental care. Life-history theory predicts that parental investment should increase as offspring develop and the probability of offspring survival increases, but then decrease again as offspring achieve independence (Sargent and Gross 1985). Supporting this prediction, activity levels in smallmouth bass (indicative of nest defence and fanning, and thus of reproductive investment) peak when the offspring are approximately 2 weeks old (Cooke et al. 2002a). Thus, we evaluated baseline and post-stress circulating cortisol and androgen concentrations at three offspring development stages. For nest-guarding fish, larger broods result in higher reproductive success per brood (Smith and Wootton 1995). Therefore, male smallmouth bass should have higher investment in larger broods. Indeed, males with larger broods expend more energy defending the nest than do males with smaller broods (Ridgway 1989; Suski et al. 2003). Thus, for an experimental

measure of reproductive investment, we manipulated brood size (reduced, enlarged, sham-treated, or unmanipulated) 24 hrs prior to evaluating baseline and post-stress circulating cortisol and androgen concentrations among treatment groups.

We predicted that parental care-providing smallmouth bass would display higher baseline cortisol levels and attenuate the cortisol response in comparison to non-parental fish. Within parental care-providing fish, we predicted that males would exhibit the highest baseline cortisol levels and the greatest attenuation of the cortisol stress response in the middle of parental care, when energetic demands are highest (Cooke et al. 2006). We predicted that androgen levels would be high during early parental care, following nest establishment (Kindler et al. 1989; Magee et al. 2006). We further predicted that males guarding enlarged brood sizes would display the highest baseline cortisol levels, the greatest attenuation of the cortisol response, and the highest androgen levels when compared with males guarding control, sham, or reduced broods.

3.3. Materials and methods

3.3.1. Series 1: Brood age. During May and June 2008, male smallmouth bass guarding nests at various stages of development were identified by snorkelling on Charleston Lake, a public lake in eastern Ontario that is part of the Gananoque River system (44°32'N, 75°59"W). For all males, an egg or fry score was assigned (see Chapter 2, Section 2.3.2). Fry scores are based on a similar relative scale. For standardization, only males with an egg or fry score of 3 or 4 were included. For the purposes of this study, offspring development stages were as outlined in (Cooke et al. 2002a). The 'egg' stage denotes

freshly laid and fertilized eggs. 'Egg-sac fry' appear approximately 1 week post-fertilization, when the yolk sac has been partially absorbed, and the larvae have developed tails and are able to wriggle within the nest. At this stage, the larvae are still feeding endogenously, and are unable to swim. The 'free-swimming fry' stage occurs approximately 3 weeks post-fertilization, when the offspring have almost entirely absorbed the yolk sac, and have gained the ability to swim. Larvae at this stage subsist on a combination of endogenous and exogenous feeding, and can be found in the water column above the nest. The parental male is still required to defend the brood, since free-swimming fry have not yet fully developed predator avoidance tactics (Brown 1984).

On May 22, 25, and 26, 2008 (water temperature 14 - 16ºC), 25 parental male smallmouth bass (TL 350 - 466 mm) guarding nests with fresh eggs (0 to 1 day old) were identified. On June 2 and 5, 2008 (water temperature 17 - 18ºC), a different group of 23 males (TL 339 - 493 mm) guarding nests that contained egg-sac fry was identified. Finally, on June 12 and 13, 2008 (water temperature 20 - 21ºC), a final group of 23 males (TL 273 - 465 mm) guarding free-swimming fry was identified. Individual fish were sampled at only one stage. Once a nest was identified, the parental male was captured using standard rod-and-reel angling and a rubber mesh landing net, and immediately placed in a foam-lined trough filled with fresh lake water, a procedure that took less than 30 s from hooking the fish. A blood sample (~1 mL) was withdrawn by caudal puncture as described previously (Chapter 2, Section 2.3.2). Fish were then subjected to a standardized stressor consisting of 3 min of air exposure, as described previously (Chapter 2, Section 2.3.2). In this case, fish were placed on a damp foam mat within a 35

x 60 cm plastic tub covered by a loosely-fitted lid during air exposure. After the stressor, fish were placed in individual 50 L coolers filled with fresh lake water for a 25 min recovery period, before a second blood sample was withdrawn as previously described. TL was measured and the fish was released.

To provide a comparison group of non-nesting control fish, 53 mature fish of reproductive size (TL 273 - 502 mm) were captured in May and June 2009, from several lakes in eastern Ontario across a range of water temperatures that corresponded to the water temperatures during parental care (see Table 3.1). Identifying the sex of smallmouth bass that are non-reproductive is not reliable without dissecting the animals, so these non-reproductive fish were likely a mixture of males and females. Upon capture, all fish were subjected to the sampling regime and standardized stressor described above.

In all groups, sampling took place throughout the day (between 9:00 and 19:00). Blood samples were processed and stored as described previously (Chapter 2, Section 2.3.2). Blood samples were excluded when more than 90 s elapsed between the start and end of the blood sampling procedure. This resulted in the exclusion of 3 males guarding free-swimming fry and 1 non-reproductive fish.

3.3.2. Series 2: Brood size. Between May 12 and 14, 2008 (water temperature 14ºC) male smallmouth bass guarding nests with fresh eggs (0 - 1 days old) were identified by snorkelling on Sand Lake, a public lake that is part of the Rideau River system in eastern Ontario (44°30'N, 76°20'W). All nests were individually marked with a numbered tile, and the brood size of each nest was classified according to egg score. Again for

standardization, only males guarding nests with egg scores of 3 and 4 were included in

this study. In total, 57 parental fish were randomly assigned to one of four treatment

groups: controls (n=14), shams (n=14), enlarged nests (n=14), and reduced nests (n=15).

Eggs were removed from reduced nests to reduce the egg score to 1 (<500 eggs). Fresh

eggs from other nests were added to enlarged nests to double the brood size, resulting in

an egg score of 5 (>4000 eggs). Eggs were removed from and then returned to sham

nests, with no net gain or loss of eggs, while control nests were not manipulated. Turkey

basters and mason jars filled with fresh lake water were used for all egg transfers

(Ridgway 1989). Between 18 and 30 hrs following brood size manipulation, all male

parental fish were captured, subjected to the standardized stress protocol and sampled for

blood as described above. Sampling for all groups took place throughout the day.

To determine whether brood manipulation itself was affecting the endocrine

response of parental males to the standardized stressor, we conducted a follow-up study

that used sham brood size manipulation (i.e., nest disturbance) of varying duration. On

May 26, 2009 (water temperature 16ºC) 32 male smallmouth bass guarding nests with

fresh eggs with egg scores of 3 or 4 were identified by snorkelling on Charleston Lake.

Nests were individually identified with numbered tiles, and fish were randomly assigned

to one of three treatment groups: control (n=11), short nest disturbance (n=8), and long

nest disturbance (n=9). Nest disturbance involved the snorkeler using a turkey baster to

mimic egg transfer, but without any direct contact between the turkey baster and the eggs,

for 5 min (long) or 1 min (short). Control nests were not disturbed. All fish were

captured 18 to 30 hrs following nest disturbance, subjected to a stress protocol and

sampled using the methods described above. Sampling for all groups took place throughout the day. As before, fish were excluded if more than 90 s elapsed between the start and end of the blood sampling procedure. This resulted in the exclusion of 1 short disturbance fish.

3.3.3. Hormone analysis. Cortisol was determined on non-extracted plasma samples using the same methods as described in Chapter 2 (Section 2.3.4). Intra-assay variability was 7.3%. Inter-assay variability was 6.9%. Androgens were determined using the same methods as described for T in Chapter 2 (Section 2.3.4). All samples for each experiment were run in single assays. Intra-assay variability ranged from 3.7 - 14.3%. Since cross-reactivity of the T antibody with 11-KT is high (7.5%, see Chapter 2, Section 2.3.4), the values obtained from this T assay reflect both circulating T and 11-KT concentrations, and are termed 'androgens' throughout the manuscript.

3.3.4. Statistical analysis. Because male and female non-reproductive fish were used as a comparison group for parental care-providing fish, the distribution of hormone levels for non-reproductive fish was examined for evidence of bimodality. Baseline, post-stress, and change in cortisol concentrations were compared among parental and non-reproductive fish using analysis of covariance (ANCOVA) tests. Parental care status (egg stage, egg-sac fry stage, free-swimming fry stage, or non-reproductive) was the independent variable, with water temperature and sampling time of day as covariates. Androgen concentrations were compared among parental fish using similar ANCOVA

tests; non-reproductive fish were excluded from these analyses. Finally, we compared baseline to post-stress hormone levels using paired Student's t-tests.

To examine the effect of brood size, baseline, post-stress and change in cortisol and androgen concentrations were compared among treatment groups (control, sham, enlarged nest, reduced nest) using ANCOVA tests. Sampling time of day was used as a covariate. To examine the effect of nest disturbance on the steroid parameters, ANOVA tests were run among treatment groups (control, short disturbance, long disturbance). Sampling time of day was not available for this experiment. As each experiment occurred in a single day, water temperature was not included in any of these statistical models. As before, we also compared baseline to post-stress hormone levels using paired Student's t-tests. Following a significant ANCOVA, the relationships between circulating steroid concentrations and main effects were explored more fully using separate ANOVAs and Tukey's honestly significant difference (HSD) post-hoc tests for significant discontinuous main effects, and separate regressions for significant continuous main effects.

In all cases, residuals were tested for normal distribution using Shaprio-Wilks goodness-of-fit tests, and for homogeneity of variance by visual inspection (Zar 1999). All analyses were performed using the statistical package JMP, version 7.0.1 (SAS Institute Inc., Cary, NC). The level of significance for all tests (α) was 0.05. All results are presented as mean $\pm$ SEM.

3.4. Results

3.4.1. Series I: Brood age. Neither baseline nor post-stress cortisol concentrations for non-reproductive fish deviated from a normal distribution (Shaprio-Wilks goodness-of-fit tests, $p > 0.05$). There was no evidence of bimodality. This observation suggests that non-parental male and female fish did not differ with respect to cortisol stress responses, and validates our use of these fish as a comparison group for the parental males.

There were no differences in baseline cortisol concentrations with reproductive status or stage, nor was there an effect of temperature or sampling time (baseline ANCOVA whole model $F_{5,110} = 2.064$, $p = 0.075$; Figures 3.1A and 3.2A). However, cortisol levels increased significantly following the standardized stressor ($t_{109} = 15.521$, $p < 0.001$), and both parental care status (post-stress status effect $F_{3,3} = 5.086$, $p = 0.002$; Figures 3.1B and 3.2B) and water temperature (post-stress temperature effect $F_{1,1} = 17.451$, $p < 0.001$; Figure 3.1B) significantly affected post-stress cortisol concentrations (post-stress ANCOVA whole model $F_{5,108} = 11.256$, $p < 0.001$; Figures 3.1B and 3.2B). There was no effect of sampling time (post-stress timing effect $F_{1,1} = 1.557$, $p = 0.215$). Post-stress cortisol concentrations increased with water temperature (Figure 3.1B) and were lowest in fish guarding fresh eggs, returning to non-reproductive levels in fish guarding free-swimming fry (Figures 3.1B and 3.2B). Similarly, the change in cortisol levels also increased with water temperature and was lower in the fish providing care (change ANCOVA whole model $F_{5,104} = 10.169$, $p < 0.001$; status effect $F_{3,3} = 4.861$, $p = 0.003$; temperature effect $F_{1,1} = 12.785$, $p < 0.001$; timing effect $F_{1,1} = 1.329$, $p = 0.252$).

Reproductive stage marginally affected baseline androgen concentrations

(baseline ANCOVA whole model $F_{4,63}$=8.398, p<0.001; status effect $F_{2,2}$=3.093, p=0.052; temperature effect $F_{1,1}$=0.241, p=0.625; timing effect $F_{1,1}$=1.71 p=0.679; Figure 3.2C) and significantly affected post-stress androgen concentrations (post-stress ANCOVA whole model $F_{4,61}$=12.990, p<0.001; status effect $F_{2,2}$=4.694, p=0.013; temperature effect $F_{1,1}$=0.060, p=0.807; timing effect $F_{1,1}$=0.645, p=0.425; Figure 3.2C), with androgen levels declining across parental care (Figure 3.2C). However, while androgens declined in all groups following the standard stressor (t_{64}=-2.015, p=0.048), there was no effect of reproductive stage or any other measured parameter on the extent of the change in androgen levels following the stressor (change ANCOVA whole model $F_{4,61}$=0.312, p=0.869).

3.4.2. Series II: Brood size. We found no differences in baseline, post-stress, or change in cortisol concentrations among males in the brood manipulation experiment (baseline ANCOVA whole model $F_{4,51}$=0.626, p=0.646; post-stress ANCOVA whole model $F_{4,51}$=1.592, p=0.191; change ANCOVA whole model $F_{4,51}$=1.649, p=0.177; Figures 3.3A and 3.3B). We again documented an increase in cortisol concentrations following the standard stressor (t_{54}=12.065, p<0.001). There was a marginally non-significant effect of treatment group on baseline androgen concentrations (baseline ANCOVA whole model $F_{4,52}$=2.873, p=0.032; treatment effect $F_{3,3}$=2.654, p=0.058; timing effect $F_{1,1}$=2.579, p=0.115; Figure 3.3C). Although we again documented a decrease in androgen levels following the standardized stressor (t_{54}=-2.225, p=0.030), there was no difference among treatment groups in the extent of the change in androgen concentrations

(change ANCOVA whole model $F_{4,51}=1.034$, p=0.399), or in post-stress androgen concentrations (post-stress ANCOVA whole model $F_{4,51}=1.144$ p=0.347; Figure 3.3C).

As in the brood manipulation experiment, we found no differences in baseline cortisol concentrations among males in the nest disturbance experiment (baseline ANOVA $F_{2,25}=2.192$, p=0.256; Figure 3.3D). Cortisol levels again increased following the standard stressor ($t_{26}=10.585$, p<0.001), and in this case both post-stress cortisol concentrations and the change in cortisol concentration were significantly different among treatment groups (post-stress ANOVA $F_{2,25}=7.161$, p=0.004; change ANOVA $F_{2,25}=7.161$, p=0.004). The cortisol response to a standard stressor increased with the extent of previous nest disturbance (Figure 3.3E). However, we found no differences in baseline, post-stress, or change in androgen concentration among these treatment groups (baseline ANOVA $F_{2,26}=2.819$, p=0.078; post-stress ANOVA $F_{2,26}=1.866$, p=0.176; change $F_{2,26}=0.561$, p=0.578; Figure 3.3F), and in this case, androgen levels did not decline following the standardized stress protocol ($t_{54}=-0.503$, p=0.619).

3.5. Discussion

3.5.1. Did the cortisol stress response vary with reproductive investment? A key finding of the current study was the attenuation of the cortisol response to a standardized stressor during parental care in smallmouth bass. This finding is consistent with previous work in avian (reviewed by Wingfield and Sapolsky 2003), reptilian and amphibian systems (reviewed by Moore and Jessop 2003), and is the first study to note this phenomenon in a teleost model. We hypothesize that attenuation of the cortisol stress

response during parental care may be an adaptive mechanism to avoid elevating cortisol titres beyond a threshold where parental care behaviours can no longer be maintained, in agreement with the 'resistance to stress' hypothesis (Wingfield and Sapolsky 2003). The attenuation of the cortisol response during parental care documented in this study may be an adaptive mechanism to avoid or at least postpone negative effects of elevated cortisol during parental care.

Our results provided only weak evidence that post-stress cortisol values in smallmouth bass vary according to reproductive investment within the parental care period. That is, the cortisol stress response of males guarding young at the free-swimming fry stage was no longer significantly different from that of non-reproductive fish, suggesting that the attenuation of the cortisol response disappears as the brood reaches independence. However, with respect to brood size there were no differences among males guarding enlarged, reduced, sham, or control broods. This finding contrasts with that of a previous study showing that in house sparrows (*Passer domesticus*), birds with enlarged broods exhibited a greater attenuation of the glucocorticoid stress response than birds with reduced broods (Lendvai et al. 2007). These results are also in contrast to previous studies in which, at a behavioural level, smallmouth bass defend enlarged broods more vigorously than smaller broods (Ridgway 1989; Suski et al. 2003). Taken as a whole, these results suggest that the cortisol stress response in bass is coarsely tuned to reproductive state but not finely tuned to investment within the reproductive period.

3.5.2. Other factors influencing the cortisol stress response. We documented an

increase in the cortisol stress response with increasing water temperature in smallmouth

bass. This relationship is consistent with findings from previous studies in teleost fish

demonstrating a rise in the rate and/or magnitude of cortisol elevation following a

stressor with increasing water temperature (e.g., Barton and Schreck 1987; Pottinger and

Carrick 2000; Davis et al. 2001; Lyytikainen et al. 2002; King et al. 2006; Lima et al.

2006). The relationship is interesting given that smallmouth bass provide annual parental

care that lasts for 4 to 6 weeks in the early spring, during which water temperatures

increase from approximately 13 to 20°C (Ridgway 1988; Cooke et al. 2006). As noted in

Chapter 2 (Section 2.5.2), the relationship between water temperature and the cortisol

stress response may contribute to the observed decrease in reproductive success in

smallmouth bass as water temperature increases (Suski and Ridgway 2007).

We also demonstrated that recent exposure to stressors has a significant effect on

the cortisol response during a subsequent challenge. Previous studies on smallmouth bass

demonstrated that multiple repeated stressors are cumulative, with fish showing a higher

peak cortisol response to the final disturbance than to the initial disturbance (Carmichael

et al. 1983). Similar findings have been reported in rainbow trout (*Oncorhynchus mykiss*;

Flos et al. 1988), and in chinook salmon (*O. tshawytscha*; Barton et al. 1986; Maule et al.

1988). It is likely that the parental smallmouth bass in this study perceived nest

disturbance as a stressor. Although the fish had recovered from the stress of nest

disturbance by the time of capture (based on the low baseline cortisol concentrations

measured), the capture-and-standardized-stress likely acted as a repeated stressor, and the

cortisol values following air exposure therefore reflected a cumulative effect. Although further research is necessary to understand the nature of the relationship, our results emphasize the importance of considering an individual's recent history when interpreting endocrine data.

3.5.3. Did androgens vary with reproductive investment? We documented a decline in mean androgen concentrations across parental care, a result that is consistent with previous work in the confamilial bluegill (*Lepomis macrochirus*; Magee et al. 2006), where mean androgen levels were highest during nest establishment stages, and declined during care stages. These findings suggest that androgens may be more important during nest and territory establishment than during parental care (Kindler et al. 1989; Knapp et al. 1999; Magee et al. 2006). In agreement with this pattern, androgen levels exhibited little evidence of varying according to reproductive investment within the parental care period, and did not differ significantly among males guarding manipulated broods.

3.5.4. Study limitations. Baseline cortisol levels were not affected by any measured parameter, a finding that contrasts with previous studies in the bluegill, where baseline cortisol levels were reduced in males with reduced brood sizes (Magee et al. 2006). However, in the current study baseline cortisol levels in all fish were uniformly low, and frequently near the 3 ng mL^{-1} detection limit of the radioimmunoassay kit, making it difficult to detect non-random variation that may have existed.

Androgen concentrations declined following the standardized stressor in only two of the three studies presented, and did not decline following the standardized stressor in the nest disturbance experiment. The inconsistency of the decline in androgen levels as a result of the standardized stress protocol suggests that the timescale of measurement may be inappropriate. The 25 min post-stress hormone measurement may reflect declining androgen levels, with increased variation as a result of variation in both rate and magnitude of the decline.

3.5.5. Summary. The key finding of the current study was the attenuation of the stress response during parental care in a teleost fish. The cortisol stress response was not, however, related to reproductive investment within individual smallmouth bass providing parental care to developing offspring. These findings suggest that resistance to stress during parental care in this teleost fish may function at a broad-scale level rather than showing fine-scale modulation based on offspring value.

3.6. Figures
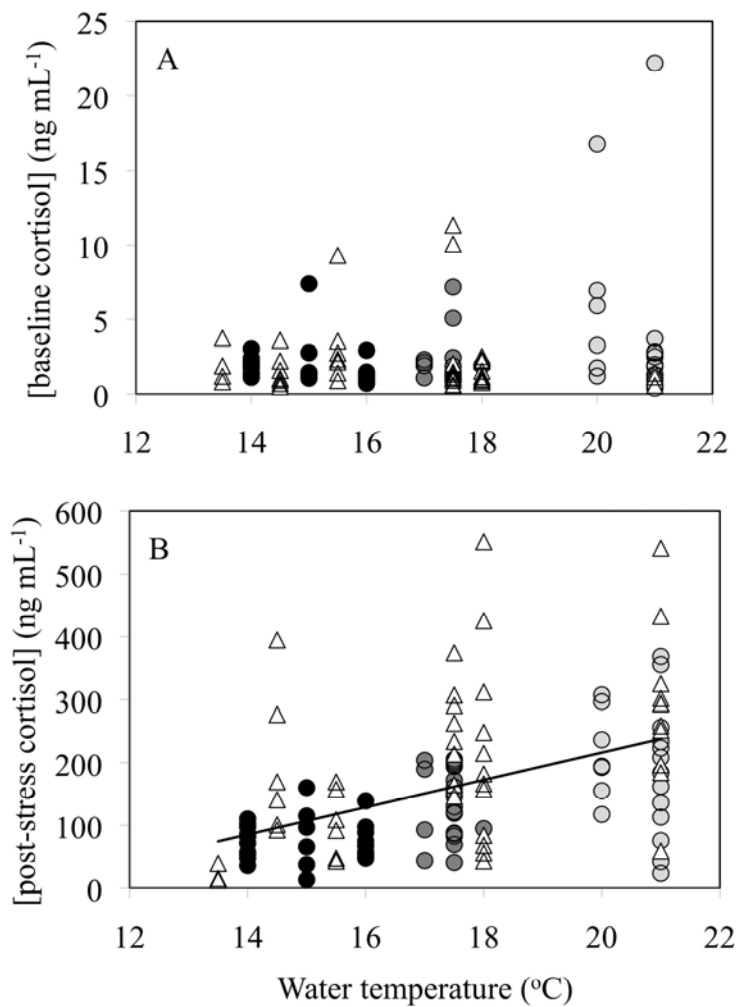
Figure 3.1. The relationship between (A) baseline and (B) post-stress circulating cortisol

concentrations and water temperature for smallmouth bass guarding offspring at various

development stages, and for non-parental fish. Fish providing care to eggs are

represented by closed black circles (●), fish providing care to egg-sac fry are represented by closed dark grey circles (●), fish providing care to free-swimming fry are represented by closed light grey circles (○), and non-reproductive fish are represented by open triangles ($\triangle$). Neither offspring development stage nor water temperature affected (A) baseline cortisol concentrations, but both factors had a significant effect on (B) post-stress circulating cortisol concentrations ($p < 0.05$; see text for statistical details).
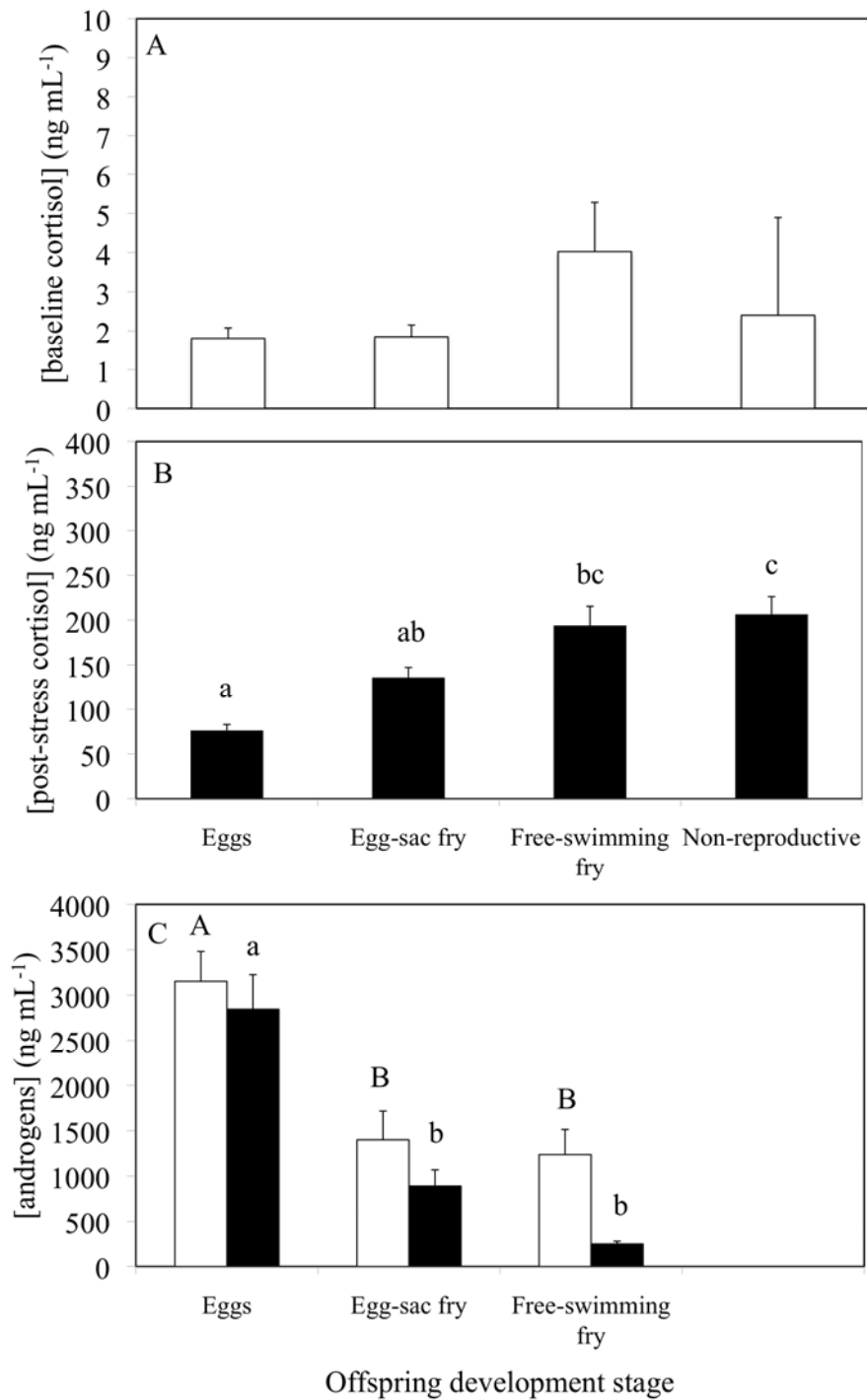
Figure 3.2. Circulating (A) baseline and (B) post-stress cortisol and (C) androgen

concentrations are depicted across the parental care period in smallmouth bass. Baseline

values are indicated by open bars, while post-stress values are indicated by closed black

bars. Both (B) post-stress cortisol and (C) androgen levels varied significantly among

offspring development stages ($p<0.05$; see text for statistical details). Note that

androgens were not measured in non-reproductive fish. Values are presented as mean ±

SEM. Different capital letters indicate statistical differences among baseline groups,

while different lower-case letters indicate statistical differences among post-stress groups

as indicated by post-hoc tests ($p<0.05$; see text for statistical details).
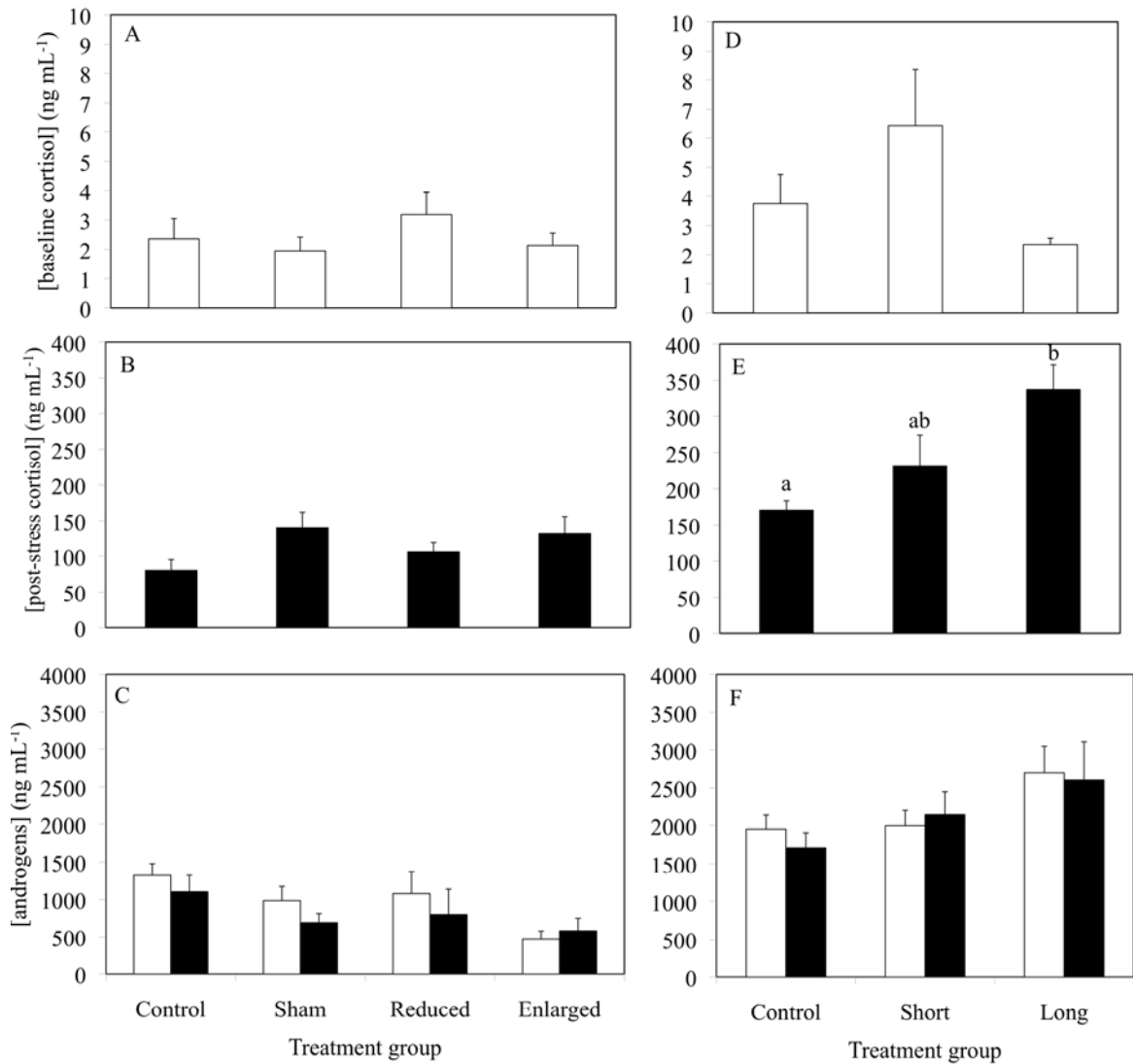
Figure 3.3. Circulating (A,D) baseline and (B,E) post-stress cortisol and (C,F) androgen concentrations are depicted among male smallmouth bass treatment groups in a (A-C) brood manipulation study, and a (D-F) nest disturbance study. All values are presented as mean ± SEM. All brood manipulations and disturbances occurred approximately 24 hrs prior to capture and sampling of the fish. Post-stress cortisol concentrations varied

among fish in the nest disturbance treatment groups. Different lower-case letters indicate statistical differences among post-stress groups (p<0.05; see text for statistical details). Differences between the results of the two experiments (i.e., across panels) should be interpreted with caution, as water temperatures varied between the two experiments, and androgen assays for the two experiments were run separately and without an inter-assay control.

CHAPTER 4

STRESS AND PARENTAL CARE: INSIGHTS FROM CORTISOL IMPLANTS

4.1. Abstract

Male largemouth bass provide sole parental care over a 4 - 6 week period to a single

brood, fanning the eggs to keep them oxygenated and free of silt and defending the brood

until the offspring develop anti-predator tactics. During this period fish are highly active

and have few opportunities for feeding, so this activity is energetically costly. To

understand some of the consequences of stress during this challenging period, we injected

fish with cortisol suspended in coconut oil to experimentally raise circulating cortisol in

parental males for the first week of the parental care period. We compared parental care

behaviour between control, sham-treated (injected only with coconut oil) and cortisol-

treated parental males. We further compared physiological parameters associated with

metabolism and reproductive function between cortisol-treated and control males. The

cortisol injections resulted in supraphysiological levels of circulating plasma cortisol,

giving us insight into potential maximal effects of stress during parental care. At these

supraphysiological levels, the cortisol-treated fish displayed higher concentrations of

circulating glucose and cholesterol and lower concentrations of circulating triglycerides

when compared to control fish, with no change in plasma concentrations of total protein.

Plasma concentrations of androgen were similarly unaffected by cortisol treatment. In

the short-term (initial 1 - 2 weeks), parental care of eggs and egg-sac fry was maintained

by all groups, with no differences observed in behaviour (e.g., tending, vigilance,

defence) among the groups. However, the cortisol-treated fish abandoned their offspring

at a higher rate than in the control or sham groups. The fish treated with cortisol also

tended to develop external *Saprolegnian* infections, indicative of compromised immune

function. These data demonstrate that exogenous cortisol elevation during parental care results changes in energy use, and a decrease in immune function. Interestingly, the data also suggest resistance to stress during parental care in largemouth bass, with no changes in parental care behaviour prior to abandonment.

4.2. Introduction

Many studies have investigated the effects of stress on reproductive function in vertebrates. Most of this work has focused on functions associated with ovarian development, such as stress during ovarian follicle maturation through to ovulation (e.g., Campbell et al. 1992; Pankhurst and Van Der Kraak 1997; Pankhurst et al. 1999; Pottinger 1999), or on maternal effects during offspring development (e.g., Schreck et al. 2001; Salvante and Williams 2003; Meylan and Clobert 2005). The effects of stress on functions associated with reproductive behaviour are less well-studied. In some animals, behaviours such as territory acquisition and parental care are essential components of reproduction. Research on birds has demonstrated that elevations of plasma corticosterone reduce the rate at which parents feed their offspring (Silverin 1986) while research in lizards (DeNardo and Sinervo 1994) and birds (Wingfield and Silverin 1986) has demonstrated that elevated plasma corticosterone reduces the ability of a male to maintain a territory. There is a notable shortage of studies on the effects of stress on reproductive behaviour in fish (Wingfield and Sapolsky 2003). To date, only a handful of studies have examined how endogenous cortisol and androgen levels fluctuate naturally throughout parental care in fish (Knapp et al. 1999; Pankhurst et al. 1999; Buchner et al. 2004; Magee et al. 2006; Bender et al. 2006, 2008) and we are not aware of any studies that manipulate cortisol titres in wild fish before or during parental care. This dearth is surprising given that parental care is relatively common, appearing in approximately 20% of fish families (Gross 2005), and an extensive background exists on the physiology of the stress response in fish (see reviews by Schreck 1981; Barton and

Iwama 1991; Wendelaar-Bonga 1997; Mommsen et al. 1999; Barton 2002), making them an excellent comparative model for understanding the physiology of parental care behaviour.

Resource limitation is an often-cited basis for trade-offs in reproductive function (Calow 1985; Webb et al. 2002). A stress response may reduce the scope available for other functions (Davis and Schreck 1997), including the reproductive effort prior to spawning (Priede 1985). In fish, this is particularly interesting, since growth is indeterminate and fecundity is correlated with body size (Heino and Kaitala 1999). Stress during reproduction should carry the same energetic costs as stress during non-reproductive periods, but how this will affect the parental care behaviour of a fish, and the ultimate reproductive success of a fish, has never been examined in wild fish.

Unlike avian or mammalian parental care (dominated by bi-parental care and female-only care, respectively) parental care in fish is frequently male-only (Gross 2005). There is evidence that the role of androgens in male parental-care providing teleosts may differ from avian and mammalian systems (Hirschenhauser et al. 2004; Desjardins et al. 2005; Magee et al. 2006). While androgens are cited as important in territorial and reproductive behaviour in many vertebrates (Wingfield et al. 1990), changes in behaviour following corticosterone elevation in birds and lizards appear to be unrelated to decreases in testosterone (Wingfield and Silverin 1986; DeNardo and Sinervo 1994). Comparative work on stress during parental care in fish could prove useful in elucidating mechanisms through which cortisol exerts its effect on reproductive behaviour mediated by androgens, but no studies exist on this topic.

The present study investigated the impact of exogenous cortisol elevation during parental care in the largemouth bass in freshwater lakes in eastern Ontario, Canada. Largemouth bass are a long-lived (~20 yrs) top predator in many temperate lentic systems. The adults of the species have few natural piscine predators, but the young are heavily preyed upon by both conspecifics and other fish, and as a result require diligent parental care during early development (Coble 1975). Otherwise, brood predators will decimate an undefended nest within minutes (Philipp et al. 1997). During the parental care period, the offspring develop through a series of predictable stages. We use the same terminology as outlined previously (Chapter 3, Section 3.3.1) to describe these stages. Parental care entails fanning the eggs to provide oxygen and remove silt, removing debris, and defending the offspring against potential brood predators (Coble 1975). This parental care is energetically costly (Cooke et al. 2002a; Cooke et al. 2006), and combined with reduced feeding opportunities, can result in males losing significant mass during the parental care period (Cooke et al. 2002a).

Here we focus on the behaviour (brood tending, vigilance, and defence) and nest success of largemouth bass in which circulating cortisol concentrations were elevated by means of an intraperitoneal injection of cortisol in coconut oil, compared to sham controls which were administered the vehicle without cortisol, and untreated controls. Such exogenous manipulations of cortisol titres are extremely common in the study of fish comparative physiology (see review by Gamperl et al. 1994). To date, however, these manipulations have been largely restricted to the laboratory environment (see review by Mommsen et al. 1999). We further investigated potential physiological

mechanisms of behavioural change by comparing indicators of energy use (glucose,

cholesterol, triglycerides and total protein) and androgen between cortisol-treated

largemouth bass and reference control parental males with offspring at the same

developmental stage.

4.3. Materials and Methods

4.3.1. Animals, treatments, and collection of blood samples. From May 10 to 28,

2007, a total of 52 largemouth bass guarding nests with fresh eggs (<24 hrs old) were

identified by snorkelling in Long Lake, a private research lake managed by the Queen's

University Biological Station (QUBS) in eastern Ontario (44º31'N, 76º20'W). No

angling or other disturbance was permitted during the study period so there is no

possibility that fish were captured or harvested during this period. Mean daily water

temperatures (~1 m below the surface) increased from 15 to 21ºC across parental care.

The nests were individually marked with a numbered tile, and the fish were randomly

assigned to one of four treatment groups: control (n=15), sham treatment (n=8), low dose

cortisol treatment (n=14), and high dose cortisol treatment (n=15). The control animals

were neither captured nor handled in any way. Treatment fish were captured by rod-and-

reel angling, landed with a rubber mesh net within 10 s, and placed in a foam-lined

trough filled with fresh lake water. Fish were then uniquely identified by intracoelomic

implantation of a PIT tag (2 mm x 9 mm). The fish were briefly air-exposed (~10 s)

while mass was determined using a portable electronic balance, and returned to the water-

filled trough for administration of an intraperitoneal injection of coconut oil (*Cocos*

nucifera; Sigma C1758, Sigma-Aldrich Inc., St. Louis, MO) containing cortisol

(hydrocortisone; Sigma H4001, Sigma-Aldrich Inc., St. Louis, MO). This method is an

established means of raising plasma cortisol in fish, and creates a slow-release cortisol

implant that will elevate plasma cortisol for approximately 5 - 6 days (see Gamperl et al.

1994). All fish were injected with 0.005 mL of coconut oil per g of fish body weight.

Sham treatment fish (TL=358 ± 12 mm [mean ± SEM]) were injected with pure coconut

oil. Low dose cortisol treatment fish (TL=365 ± 7 mm) were injected with 10 mg mL^{-1}

cortisol in coconut oil, while high dose cortisol treatment fish (TL=377 ± 10 mm) were

injected with 40 mg mL^{-1} cortisol in coconut oil. These doses were chosen based on

literature values used to induce exogenous stress responses in teleost fish (see Gamperl et

al. 1994). During treatment, a snorkeler was deployed to protect the eggs from nest

predators, and remained with the nest until the parental male returned following release.

All techniques were performed without anesthesia (see Cooke et al. 2005a for rationale),

and total treatment time was typically <90 s.

A subset of the low dose cortisol (n=7; TL=363 ± 10 mm) and high dose cortisol

(n=9; TL=363 ± 16 mm) fish were recaptured between May 19 and May 28, 2007, 5 - 6

days after the initial cortisol treatment, at the egg-sac fry offspring development stage.

After capture, these fish were placed in a water-filled foam-lined trough, non-lethally

sampled for ~1.5 mL of blood as previously described (Chapter 2, Section 2.3.2), and

scanned with a PIT-tag reader to confirm identity. All fish were then released. As with

the cortisol treatment, a snorkeler remained with the nest until the parental male returned,

and all techniques were performed without anesthesia, with a total handling time <90 s.

Mean daily water temperatures (~1 m below the surface) increased from 16 to 21ºC

during this period. The physiological parameters (plasma concentrations of androgen,

triglycerides, cholesterol, total protein, and glucose) of these animals were compared to

reference values obtained using the same sampling method from parental fish over 3

years, from 2 nearby lakes. Long Lake, our focal study lake, is a relatively small lake

(~20 ha), and the fish used for the behavioural component of the study comprised the

entire population of nesting males in the lake. We chose to capture size-matched nesting

males from nearby water bodies over multiple years to obtain reference values for the

physiological component of this study, rather than reduce the sample size of untreated

controls for the behavioural data. Since none of the reference groups (i.e., across years or

lakes) were different from one another in any of the parameters examined (see Table 4.1),

we are confident that the differences we observed in the cortisol-treated group were a

result of the exogenous cortisol implants, rather than a year or lake effect. Largemouth

bass with offspring at the egg-sac fry stage (n=18; TL=365 ± 19 mm) were obtained May

15 to 16, 2006 (water temperature ~18ºC) from Lake Opinicon, a nearby public lake that

is part of the Rideau River system in eastern Ontario (44°30'N, 76°20'W). Largemouth

bass with offspring at the egg-sac fry offspring development stage were also sampled on

June 2, 2007 (water temperature ~20ºC; n=6; TL=376 ± 18 mm), and June 4 to 11, 2008,

(water temperature ~20ºC; n=9; TL=368 ± 13 mm) from Charleston Lake, another lake in

close proximity that is part of the Gananoque river system in eastern Ontario (44°32'N,

75°59"W).

To validate the cortisol dose for this species, and to verify the time course of cortisol elevation, 36 size-matched largemouth bass were captured using the same rod-and-reel methods as described above from Lake Opinicon between May 9 and May 13, 2007, and transported in aerated coolers to fish holding facilities at QUBS. These fish were assigned to a high dose cortisol treatment (n=18), low dose cortisol treatment (n=6), sham treatment (n=6), or control treatment (n=6) group and treated as described above. After injection, the fish were placed in individual opaque experimental chambers (~12 L) supplied with a constant flow of fresh water from Lake Opinicon until blood sampling. To avoid biasing the data, 5 blood samples (3 high dose cortisol, 1 low dose cortisol, and 1 control fish) that took more than 3 min between opening the experimental chamber and completing the blood sample were discarded. To verify the cortisol doses, a sub-sample of the high dose cortisol treatment fish (n=5; TL=369 ± 12 mm), and all fish from the low dose cortisol treatment (n=5; TL=357 ± 14 mm), sham treatment (n=6; TL=368 ± 13 mm), and control treatment groups (n=5; TL=370 ± 23 mm) were removed after 3 days from the experimental chambers, and sampled for blood as described above. To verify the time course for cortisol elevation, a second sub-sample of the high dose cortisol treatment fish (n=6; TL=381 ± 12 mm) was sampled for blood after 6 days, and the remaining group (n=4; TL=348 ± 22 mm) was sampled after 9 days.

For a comparison of the exogenous cortisol elevation to endogenous cortisol elevation caused by stress in this species, an additional group of size-matched fish (n=6; TL=354 ± 17 mm) was captured from Lake Opinicon between September 24 and 27,

2007 (water temperature ~18ºC), transported back to QUBS as described above, and individually placed in a 100 L tank where they were chased to exhaustion (loss of equilibrium) by tail pinching (3 min). They were then held in aerated coolers for 30 min, and blood was sampled as described above. Blood samples were handled and stored as described previously (Chapter 2, Section 2.3.2).

4.3.2. Biochemical and endocrine analysis. Nutritional status of cortisol-treated and reference control groups was evaluated by quantifying plasma concentrations of triglycerides, cholesterol, total protein and glucose (Congleton and Wagner 2006) using a Roche Hitachi 917 analyzer (Basal, Switzerland) and were based upon the International Federation of Clinical Chemistry and Laboratory Medicine standard reference model. All techniques followed procedural guidelines for standardization and quality assurance established by the Veterinary Laboratory Association Quality Assurance Program and the Canadian Food Inspection Agency External Proficiency Panel.

Plasma cortisol was determined using the methods described in detail in Chapter 2 (Section 2.3.4). Intra-assay variability was 11.1%, while inter-assay variability was 12.9%. Androgens were determined using the methods previously described in Chapter 3 (Section 3.3.3). Intra-assay variability was 3.3%, and inter-assay variability was 15.2%.

4.3.3. Behavioural data collection. All nesting bass identified in Long Lake were included in the behavioural analysis (control n=15, sham treatment n=8, low dose cortisol treatment n=14, and high dose cortisol treatment n=15) representing the entire male

reproductive effort in the lake for that year. The day after treatment (the day after nest identification for the control animals), all fish were observed according to a behavioural protocol by one of three trained snorkelers. These observations were repeated in the same order every other day, for up to 2 weeks, to collect data at the egg stage, and the egg-sac fry and free-swimming fry offspring developmental stages. Behavioural metrics entailed:

1.   Tending score. Fish were observed at a distance of approximately 3 m for 5 min, and fish were scored with respect to behaviour every 15 s. Score 0 indicated that the fish was greater than 2 m from the nest, score 1 indicated that the fish was within 2 m of the nest, score 2 indicated that the fish was directly above the nest, while score 3 indicated that the fish was engaged in nest defence. These scores were summed for each 5-minute observation period, and the tending score was calculated as the sum of all of the individual scores. This tending score is a measure of brood care. Brood care in largemouth bass consists of fanning eggs to provide oxygen, removing debris from the nest, and chasing away predators (Coble 1975). Diligent parents that spend a high proportion of time within the vicinity that allows them to provide necessary brood tending behaviours received a higher tending score.

2.   Turning score. Fish were observed at the same 3 m distance for 3 separate 1 min periods. The fish was required to be directly above the nest for the entirety of each 1 min period. During each 1 min period, the number of ¼ turns made above the nest was recorded. The numbers of ¼ turns from each of the 3

separate 1 min periods were averaged to calculate the turning score. Turns
above the nest are a measure of vigilance, as the parental male monitors the
surrounding area for nest predators; more vigilant parents will receive higher
turning scores (Hinch and Collins 1991).

3.   Aggression score. The snorkeler then placed a jar containing a nest predator
(a small [TL 125-150 mm] bluegill sunfish) both 0.5 m away from the nest, and
directly in the nest, and recorded the number of aggressive behaviours towards
the nest predator during a 60 s period, a method frequently used to evaluate
parental care behaviour in fish (e.g., Fitzgerald and Caza 1993). An aggressive
behaviour was identified as a hit (when the fish made physical contact with the
jar), a charge (when the fish approached the jar rapidly, but did not make
physical contact), or a yawn (when the fish faced the jar and opened its mouth
rapidly in a gulping gesture, a threatening behaviour that precedes charges or
hits). All aggressive behaviours were weighed equally, and the aggression
score was calculated by summing the aggressive gestures at both distances.
This is a measure of nest defence, with more aggressive parents receiving
higher scores.

Monitoring of the nest by snorkelling continued every other day for up to 2 weeks to
record presence of the parental fish, and developmental stage of the offspring. Permanent
departure of the parental fish or total predation of the brood prior to the free-swimming
fry stage was considered a failed reproductive attempt, while reaching the free-swimming
fry stage was considered a proxy of nest success (Philipp et al. 1997). Any evidence of

Saprolegnian infection was also noted during this monitoring, with a record of where the infection occurred (head, body, fins), and the approximate percentage of the body surface area affected.

4.3.4. Statistical analyses. Prior to subsequent ANOVA tests, plasma cortisol concentrations between the high and low dose cortisol treatment groups were compared. Normal distribution for all parameters was tested using the goodness-of-fit test, and for homogeneity of variance using Levene's test. Meeting these assumptions, plasma cortisol levels between the low and high cortisol treatment groups were compared using a Student's t-test. No difference in circulating plasma cortisol levels between the groups was detected (Student's t-test, $t[14]=0.59$, $p=0.56$; Figure 1), and so these groups were combined into a single cortisol treatment group for all subsequent analysis (n=16; TL=371 ± 6 mm).

Plasma androgen, triglyceride, cholesterol, plasma protein, and glucose concentrations were compared between the cortisol and control treatment groups. Data for triglyceride concentration did not meet the assumptions of normality, and were log-transformed before further analysis (non-transformed data are presented in Figure 4.2). Data for glucose concentration did not meet the assumptions of equality of variances, and so a Welch's ANOVA was performed (Zar 1999). For all other variables, concentrations of plasma constituents were compared among the groups using ANOVAs. Tukey's post-hoc HSD tests were performed to identify the source of differences where significant differences were detected by ANOVA.

Behavioural metrics were compared among the cortisol, sham, and control treatment groups, and between offspring development stages. However, high abandonment rates in some of the treatment groups compromised sample size for repeated measures when data from parents with free-swimming fry were included. This offspring development stage was dropped from the analysis, and only individuals with data at both the egg stage and the egg-sac fry stage were retained. This approach resulted in a final sample size of n=13 for the control group, n=6 for the sham treatment group, and n=16 for the combined cortisol treatment group. When data for a single individual included multiple behavioural observations at a single offspring development stage, as occurred for 6 fish at the egg stage, and 2 fish at the egg-sac fry stage, the mean of the observations from the individual were taken for that stage. Data for the turning score and the aggression score did not meet the assumptions of normality, and were log-transformed before further analysis (non-transformed data are presented in Figure 4.3). Relationships among the treatment groups and across egg stages were tested using two-way repeated-measures ANOVAs using treatment group and offspring development stage as independent variables. Behavioural metrics at the egg stage were also compared between fish that ultimately abandoned their offspring, and fish that were successful in their reproductive effort using Student's t-tests. As above, data for the turning score and the aggression score did not meet the assumptions of normality, and were log-transformed before further analysis (non-transformed data are presented in Figure 4.4).

Abandonment and *Saprolegnian* infection rates were compared among the cortisol, sham, and control treatment groups using likelihood ratio tests.

All analyses were performed in the statistical packages JMP, version 7.0.1 (SAS Institute Inc., Cary, NC). The level of significance for all tests (α) was assessed at 0.05. All results are stated mean $\pm$ SEM.

4.4. Results

4.4.1. Cortisol implant effectiveness. The high and low doses selected for this study were combined into a single cortisol treatment group for all subsequent analyses, with a supraphysiological plasma cortisol concentration of 2457 ± 375 ng mL^{-1} 5 - 6 days after initial cortisol injection (Figure 4.1). In the parallel laboratory study, sham treatment also resulted in an elevation of plasma cortisol, with plasma cortisol concentrations of 269 ± 66 ng mL^{-1} 3 days after initial coconut oil injection, comparable to the levels seen during the apparent endogenous cortisol stress response 30 min following exhaustive exercise (Student's t-test, t[10]=0.78, p=0.45; Figure 4.1).

4.4.2. Physiological parameters. Of the nutritional parameters examined, only total protein concentration was insensitive to supraphysiological cortisol treatment (ANOVA, $F_{3, 45}$=1.12, p=0.35; Table 4.1). Cholesterol was significantly higher in the cortisol treated group than in control fish (ANOVA, $F_{3,45}$=3.86, p=0.02; Table 4.1), with a similar pattern for glucose (Welch's ANOVA, $F_{3,45}$=43.85, p<0.01; Table 4.1). Conversely, triglycerides were significantly lower in the cortisol-treated group (ANOVA, $F_{3,45}$=2.89, p<0.05; Table 4.1). There was insufficient plasma to run androgen analyses on 11 samples from the reference controls in 2006, leaving a sample size of n=7; other

reference control group sample sizes and the cortisol treatment group sample size remained the same for androgen as for the nutritional indices. Androgen levels were unaffected by cortisol treatment (ANOVA, $F_{3,34}$=0.20, p=0.90; Table 4.1).

4.4.3. Whole-animal parameters. Parental care behaviour was not affected by supraphysiological cortisol treatment in this study. There were no differences in the tending score (two-way ANOVA with repeated measures, $F_{3,34}$=0.81 p=0.45), turning score (two-way ANOVA with repeated measures, $F_{3,34}$=0.69, p=0.51), or aggression score (two-way ANOVA $F_{3,34}$=2.89, p=0.07) at any offspring development stage among the three treatment groups (Figure 4.2). There was, however, a difference in the rates of early abandonment among the groups (likelihood ratio test, $\chi^2_{2,N=52}$=8.25, p=0.02), and in the rates of *Saprolegnian* infections among the groups (likelihood ratio test, $\chi^2_{2,N=52}$=9.82, p=0.01; Figure 3). These *Saprolegnian* infections were mild, isolated to the head or fins, and covered <30% of the affected region. Abandonment was low for the control and sham-treated groups, and behavioural data at the egg stage for fish that abandoned were available for only a small number of control (n=3) and sham-treated fish (n=1). As a result, behaviour between fish that abandoned and fish that were successful were analysed using only cortisol-treated fish. There were no differences in tending score (Student's t-test, t[24]=0.21, p=0.83), turning score (Student's t-test, t[24]=1.33, p=0.19), or aggression score (Student's t-test, t[24]=1.58, p=0.13) between fish that abandoned their offspring, and fish that were ultimately successful (Figure 4.4).

4.5. Discussion

Parental care in largemouth bass involves a suite of behaviours that are critical to ensure the success of a reproductive attempt. In this study, we found that exogenous supraphysiological elevations of plasma cortisol in parental males resulted in physiological changes consistent with a change in energy mobilization and carbohydrate catabolism. Observationally, we noted some evidence of immune suppression. We found that ultimately, supraphysiological elevations of plasma cortisol were associated with a decrease in nest success, with fewer cortisol-treated males guarding the offspring until the free-swimming fry stage when compared to the control or sham-treated groups. We did not observe a change in androgen, important reproductive hormones, or in parental care behaviour prior to nest abandonment. We also did not observe a difference in behaviour of cortisol-treated fish between fish that ultimately abandoned and cortisol-treated fish that were successful. These results indicate that while supraphysiological cortisol elevation during parental care decreases nest success, this is not a direct affect of cortisol on reproductive hormones or on behaviour (e.g., reduction in vigilance resulting in depredation and subsequent abandonment). Instead, the decrease in nest success is likely associated with longer-term secondary and tertiary affects of cortisol on other functions, such as metabolism or immune function.

4.5.1. Limitations of intraperitoneal cortisol injections. The use of cortisol in coconut oil to raise plasma cortisol created some confounding factors associated with elevated

cortisol as a result of the capture and handling stress of administering injections (see

Gamperl et al. 1994). The use of a sham treatment group does not entirely control for

these factors, since the sham treatment itself can be a stressor that can raise plasma

cortisol, perhaps chronically. Indeed, the sham treatment fish in the laboratory

verification study showed plasma cortisol levels approaching those of the exhaustively

exercised fish (Figure 4.1). We do not believe that this methodological limitation reduces

the validity of the study, but it does require acknowledgement. Our cortisol-treated fish

were subjected to handling stress, and received an additional treatment of a cortisol-

impregnated vehicle, while the sham treatment fish were subjected to handling stress and

injection with the vehicle alone. The control and reference control groups were untreated

and unhandled in any way. Our results represent the comprehensive effects of handling

stress and extended cortisol elevation during parental care, rather than an isolated effect

of elevated plasma cortisol.

The field setting for this study precluded a pilot study on the effectiveness of the

cortisol dose, with water temperatures dictating a simultaneous laboratory study. As a

result, the doses selected based on literature from rainbow trout (Gamperl et al. 1994)

resulted in plasma cortisol concentrations an order of magnitude higher than desired in

largemouth bass (Figure 4.1). This could be a result of inter-specific differences, or it

could be a result of warmer water temperatures, and therefore a softer coconut oil implant

and faster release of cortisol; studies in rainbow trout are generally conducted at water

temperatures of ~12°C, while water temperatures during parental care in largemouth bass

can exceed 21°C. Despite the pharmacological dose, we observed a typical glucose

response to elevated plasma cortisol (see reviews by Schreck 1992; Mommsen et al. 1999), and the fish were able to maintain parental care behaviour at control levels for ~1 - 2 weeks. Therefore, we believe that our results are still interesting, and valid in their direction and overall message. Although limited by methodology, this is the first study to have examined elevated plasma cortisol during parental care in a fish and provides unique insight into the physiological mechanisms that drive whole-animal consequences of stress during parental care, and a framework for further studies on this topic.

4.5.2. Changes in energy mobilization. The results of this study showed a significant effect of supraphysiological cortisol treatment on three of the four nutritional parameters examined: glucose, cholesterol, and triglycerides (Figure 4.2). The plasma hyperglycaemic effects of cortisol in fish have been well documented (see reviews by Schreck 1992; Mommsen et al. 1999), and despite the supraphysiological dose of cortisol in this study, the increase in plasma glucose is consistent with previous work indicating that elevations of plasma cortisol increase carbohydrate catabolism (Axelrod and Reisine 1984). The relationships between cortisol elevation and protein and lipid metabolism are less clear-cut, and studies to date have yielded varied and often conflicting results (see reviews by Van Der Boon et al. 1991; Mommsen et al. 1999). In this particular study, feeding may also be a confounding effect. In largemouth bass, foraging is reduced during parental care, and only occurs opportunistically (Cooke et al. 2006). Cortisol treatment in juvenile rainbow trout causes anorexia and loss of condition even when animals are supplied with ample food (Gregory and Wood 1999). It is possible that cortisol treatment

of parental male largemouth bass caused a similar rejection of opportunistic meals. Particularly for total plasma protein, any potential increases in plasma protein as a result of an increase in protein catabolism (e.g., Mommsen et al. 1999) might be countered by decreases in plasma protein as a result of fasting (Congleton and Wagner 2006). Similarly, potential feeding differences complicate the interpretation of results for plasma lipids. Increases in cholesterol seen in the cortisol-treated fish could be an indication of increased catabolism of stored lipid reserves, a widely-accepted effect of cortisol (Mommsen et al. 1999), but in this case may be confounded by a decrease in lipid availability as a result of fasting, or by the use of coconut oil as a vehicle for the cortisol. Although extensive inter-specific variation requires caution when applying studies from rainbow trout to largemouth bass, studies in juvenile rainbow trout have also shown that triglycerides decrease during periods of starvation (Congleton and Wagner 2006). Taken together, the increase in cholesterol and decrease in triglycerides suggests a change in lipid metabolism that could be a direct result of cortisol effects on lipid catabolism, an indirect effect as a result of changes in feeding behaviour, or a combination of these effects. It is worthwhile to note at this point that the increase observed in plasma cholesterol in cortisol-treated bass could also indicate a decrease in steroidogenesis, consistent with a decrease in endogenous cortisol production as a result of negative feedback (Wenderlaar-Bonga 1997). In summary, the direct and indirect effects of exogenous cortisol elevation on metabolism are difficult to disentangle; what is clear is that exogenous supraphysiological cortisol elevation alters the mobilization of carbohydrates and lipids from the tissues. Further study is necessary to determine

whether stressors during parental care deplete overall energy reserves, which could result in reductions in the life-time fitness if male largemouth bass are unable to acquire sufficient energetic resources prior to the next reproductive period such that they have to forego reproduction in one or more seasons (e.g., Wiegmann et al. 1992) or could result in overwinter mortality, a common phenomena associated with low energetic reserves in bass (Hurst 2007).

4.5.3. Maintenance of plasma androgen and parental care behaviour, and a reduction in nest success. The cortisol-treated group in this study showed no differences in behaviour during the egg and egg-sac fry offspring development stages when compared to the sham-treated or control groups. We also observed no differences in behaviour of cortisol-treated fish between fish that ultimately abandoned, and fish that were successful, although it is important to note that the low sample size of control and sham-treated fish that ultimately abandoned precluded a comparison using those treatment groups. The lack of a behavioural response was surprising given results from other taxa during parental care, and from fish during non-reproductive periods. This result was also surprising given the extent of plasma cortisol elevation found in this study. Previous studies in birds have shown a decrease in aggression and tending as a result of corticosterone elevation during parental care (Silverin 1986; Wingfield and Silverin 1986). Non-reproductive rainbow trout show decreases in feeding behaviour and swimming behaviour when treated with cortisol (Gregory and Wood 1999). Similar to our fish model, however, a study in the Lapland longspur bird (*Calcarius lapponicus*)

showed that during severe winter storms, the parental birds were able to resist stress for several days before abandoning when the storms persisted (Astheimer et al. 1995). In that case, endogenous corticosterone was elevated when the birds abandoned their broods. In our study, abandonment occurred past the time course for exogenous cortisol elevation in many individuals. This observation, combined with the ability to maintain parental care behaviour at controls levels until abandonment, suggests a secondary or tertiary cause of ultimate brood abandonment in fish, rather than an immediate or direct response to the supraphysiological cortisol elevation.

We observed no changes in plasma androgen as a result of cortisol treatment. This also differs from the situation in bluegill sunfish, where endogenous cortisol and androgen levels appear to be negatively correlated during the reproductive period (Magee et al. 2006), and sockeye salmon (*Oncorhynchus nerka*), where stress during the reproductive migration causes reproductive hormones, including testosterone, to plummet (Kubokawa et al. 1999; Hinch et al. 2005). It is possible that our androgen assay was not sensitive enough to detect differences among the very low androgen levels seen in our study treatment groups. More research is necessary to fully elucidate the role of androgens in parental care in largemouth bass, and the influence of elevated plasma cortisol on androgens.

4.5.4. Synthesis: Resistance to stress and life-history trade-offs. The stress response is a plastic trait, and individuals will modulate their stress response depending on their life history, and their probability of current and future reproductive success (Stearns and

Koella 1986). Resistance to stress during reproduction is generally considered a trade-off

in favour of current reproductive opportunity, at the expense of survival probability and

potential future reproductive opportunities (Wingfield et al. 1998). Animals with few

future reproductive opportunities, or valuable current reproductive opportunities, should

be more resistant to stress during parental care (Wingfield and Sapolsky 2003). This

hypothesis has been supported through various intraspecific studies in birds. For

example, as mentioned above, older birds are better able to resist stress during

reproduction than younger birds (Angelier et al. 2007a, 2007b). Birds at higher latitudes,

with fewer annual reproductive opportunities, attenuate their stress response during

reproduction when compared to birds in more temperate climates (Silverin et al. 1997).

Largemouth bass, with a long lifespan and limited annual reproductive opportunities,

display a life-history strategy that is consistent with the model of stress resistance during

parental care (Wingfield and Sapolsky 2003). In the current study, the ability of

largemouth bass to temporarily maintain parental care despite handling stress,

supraphysiological increases in plasma cortisol, and *Saprolegnian* infections, suggests

that they are resistant to the effects of elevated plasma cortisol at the whole-animal level

during the reproductive period. Although it is always possible that other, unstudied

variables were involved, the changes in nutritional indices detected in this study suggest

that temporary resistance to stress is limited by energetic resources. For fish, the

energetic costs to stress may be even more important than in other taxa, since growth in

fish is indeterminate, and fecundity is related to body size (Heino and Kaitala 1999).

4.5.5. Summary. This study represents an initial investigation of stress during parental care in a teleost fish. This is the first study that examines how elevated plasma cortisol affects parental care behaviour and nest success in fish, with an introductory investigation of some of the energetic and endocrine factors that could be mediating the whole-animal effects. Although our results reflected supraphysiological rather than environmental elevation of plasma cortisol during parental care, some general conclusions can still be drawn. These levels of plasma cortisol elevation caused changes in mobilization or carbohydrates and lipids from the tissues. Whether these effects were a direct result of elevated plasma cortisol or an indirect result of feeding effects is unclear. Cortisol-treated fish were able to temporarily maintain the same quality of parental care as untreated controls, with no change in tending, vigilance, or nest defence, until prematurely abandoning their broods. There was also no change in plasma androgen as a result of cortisol treatment. This temporary maintenance of parental care behaviour suggests that fish are sensitive to life history trade-offs in their whole-animal stress responses, and are unwilling to immediately abandon valuable reproductive opportunities. Future studies would greatly benefit from the inclusion of measures of body condition and energy use, to determine whether resistance to stress is limited by energetic stores in this system. Further research that explicitly incorporates life history (see Wingfield and Sapolsky 2003) into studies of stress and resistance to stress during parental care is necessary to develop a comprehensive understanding of the relationship between stress and reproduction, and the driving mechanisms behind these relationships. Collectively, this type of research on wild fish has the potential to yield information on

the role of stress in ecological and evolutionary processes.

4.6. Figures
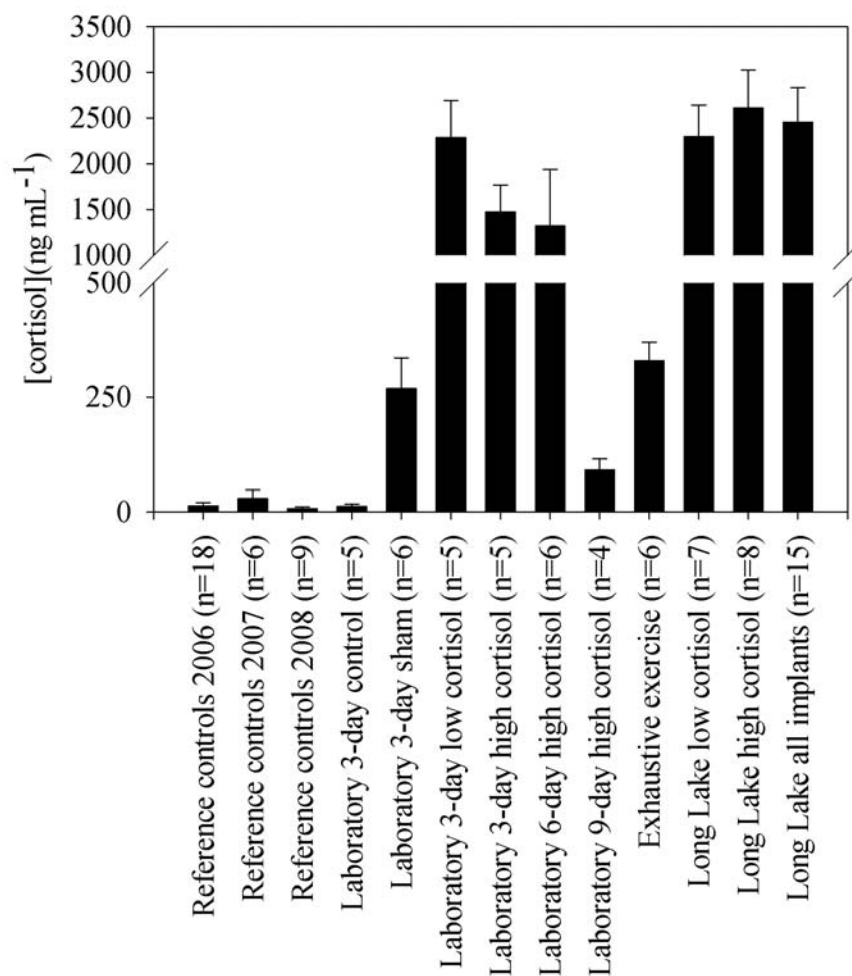
Figure 4.1. Plasma cortisol concentrations (mean ± SEM) achieved by the exogenous cortisol treatment and sham treatment, and the approximate time course of cortisol elevation in largemouth bass. This can be compared with the endogenous cortisol elevation as a result of exhaustive exercise, and reference levels of plasma cortisol in control largemouth bass.
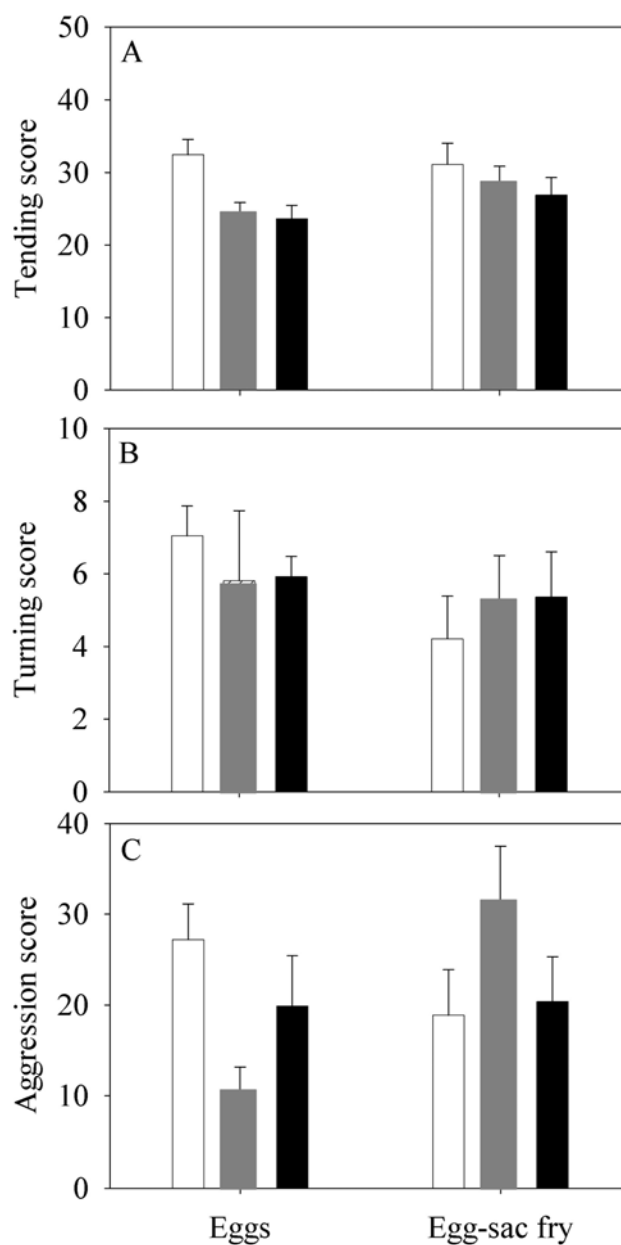
Figure 4.2. Measurements of behaviour in control, sham-treated, and cortisol-treated

parental largemouth bass at the two earliest offspring development stages; the egg stage,

and the egg-sac fry stage. All values are presented as mean ± SEM. Open bars indicate

control fish, while grey bars indicate sham-treated fish, and filled bars indicate cortisol-

treated fish. (A) Tending score is a measure of brood care, while (B) turning score is a

measure of vigilance, and (C) aggression score is a measure of brood defence. Two-way

ANOVAs were performed for all differences in behavioural measures for both treatment

differences, and offspring stage differences. None of the behavioural measures were

significant ($p > 0.05$; see text for statistical details).
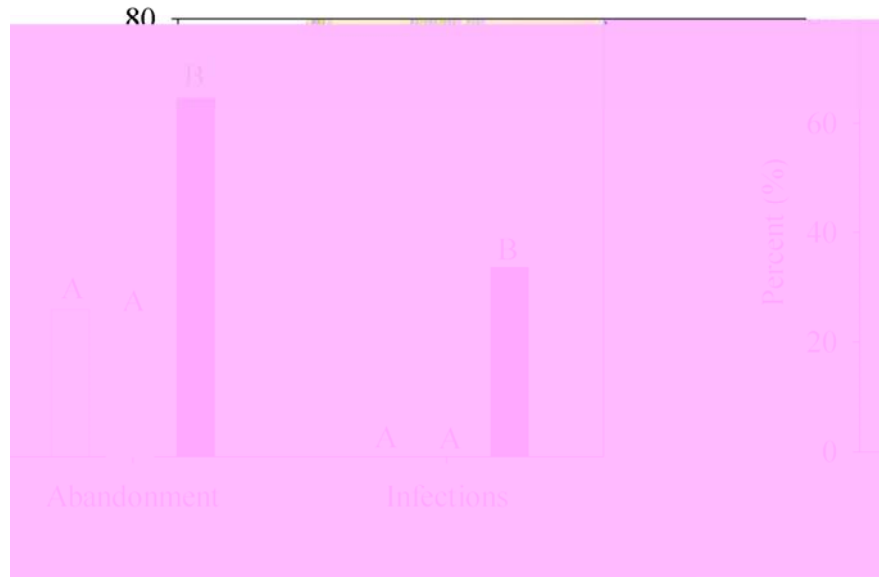
Figure 4.3. Proportion of control, sham-treat, and cortisol-treated parental largemouth bass that permanently left the nest area prior to the swim-up fry stage, resulting in a reproductive failure for the nesting attempt; and that displayed evidence of minor (<30% of either the head or fins affected) *Saprolegnian* infections. Open bars indicate control fish, grey bars indicate sham-treated fish, and filled bars indicate cortisol-treated fish. Different letters among treatment groups indicate statistical difference (p<0.05; see text for statistical details).
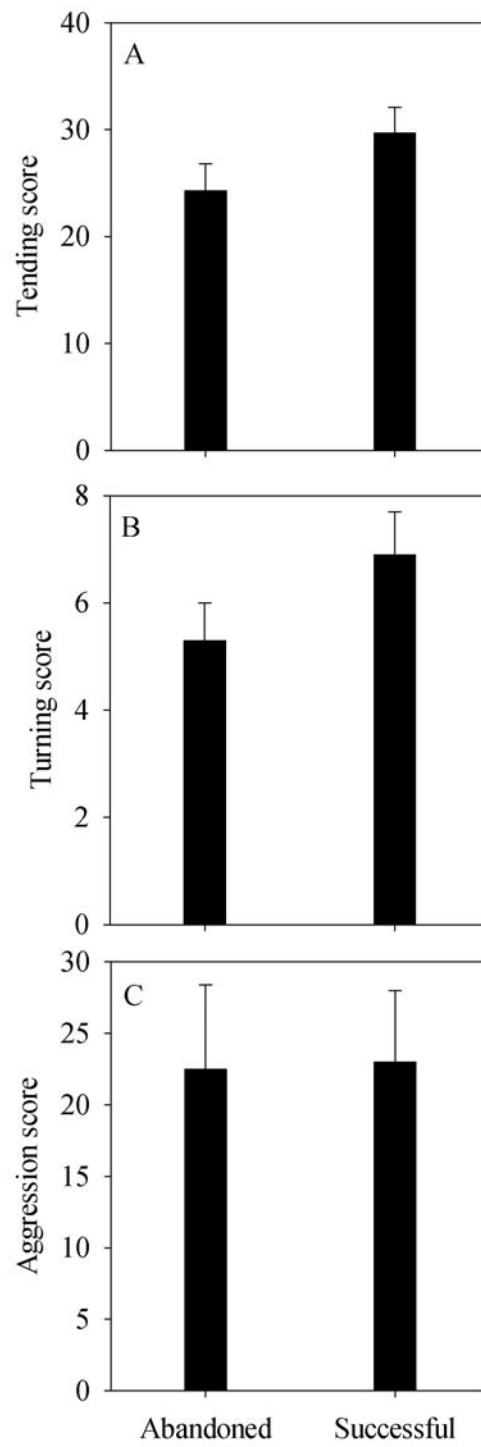
Figure 4.4. Measurements of behaviour in cortisol-treated parental males at the egg

stage, comparing fish that ultimately abandoned their broods with fish that were

ultimately successful in their reproductive effort. All values are presented as mean ±

SEM. (A) Tending score is a measure of brood care, while (B) turning score is a measure

of vigilance, and (C) aggression score is a measure of brood defence. Student's t-tests

were performed between the fish that abandoned and fish that were successful, and none

of the behavioural measures were significant ($p>0.05$; see text for statistical details).

4.7. Tables

	Physiological parameters				
	Androgens (ng mL^{-1})	Total protein (mmol L^{-1})	Cholesterol (mmol L^{-1})	Triglycerides (mmol L^{-1})	Glucose (mmol L^{-1})
Reference controls 2006 (n=18)*	0.178 ± 0.059	38.7 ± 0.93	14.1 ± 0.81^{AB}	0.725 ± 0.06^{A}	2.32 ± 0.09^{A}
Reference controls 2007 (n=6)	0.140 ± 0.031	40.5 ± 1.65	14.7 ± 0.74^{AB}	0.650 ± 0.21^{AB}	2.86 ± 0.29^{A}
Reference controls 2008 (n=9)	0.141 ± 0.022	37.0 ± 1.07	12.9 ± 1.01^{A}	0.660 ± 0.14^{AB}	2.24 ± 0.15^{A}
Cortisol (n=16)	0.168 ± 0.038	39.5 ± 1.01	16.8 ± 0.81^{B}	0.471 ± 0.06^{B}	7.34 ± 0.58^{B}

*n=7 for androgen only

Table 4.1. Physiological parameters (mean ± SEM) for parental male largemouth bass. Plasma from cortisol-treated parental males was taken at the egg-sac fry stage, 5 - 6 days after cortisol injection. Plasma from reference control males was taken at the equivalent egg-sac fry stage, from untreated control fish in nearby lakes over 3 years. Different letters indicate statistical difference ($p<0.05$; see text for statistical details).

CHAPTER 5

SEASONAL CARRYOVER EFFECTS FOLLOWING THE

ADMINISTRATION OF CORTISOL

5.1. Abstract

Stress can have sub-lethal effects that are manifested either immediately, or at spatial or temporal scales that are removed from the stress event (i.e., carryover effects). We tested whether a short-term elevation of plasma cortisol would result in seasonal carryover effects in wild largemouth bass. Using exogenous hormone implants, we raised circulating cortisol concentrations in a group of wild fish for approximately 5 days in October 2007. We then compared activity (velocity, distance travelled) of cortisol-treated to sham-treated and control animals throughout the winter using an automated acoustic telemetry array. Immediately following treatment, the cortisol-treated fish showed increased activity relative to controls. However, this difference disappeared following the cessation of the elevation of circulating cortisol. During the winter of 2007 to 2008, the lake experienced a near-complete winterkill event, providing insight into how a transient stress response can influence the response of wild animals to subsequent challenges. Most fish carrying acoustic transmitters succumbed during this winterkill event, but cortisol-treated fish died earlier than fish in other groups, and showed a decrease in activity relative to controls and sham-treated fish prior to mortality. This study provides preliminary evidence of seasonal carryover effects in wild fish, and yields insight into the ecological consequences of stress across broad temporal scales.

5.2. Introduction

Carryover effects refer to situations where conditions during one period affect the

outcome of events during a subsequent period (Harrison et al. 2020). Carryover effects

may significantly influence population abundance, and our ability to predict changes in

population size (Webster et al. 2002; Norris 2005; Norris and Taylor 2006), but are rarely

examined in wild populations owing to the logistical difficulties associated with

quantifying effects that are removed from their cause by large temporal or spatial scales.

Therefore, there have been few previous studies of carryover effects, and these have

focused primarily on migratory bird populations. In migratory birds, overwintering

conditions can affect the subsequent breeding success of barn swallows (*Hirundo rustico*)

following their migration (Saino et al. 2004). The quality of tropical overwintering

habitat affects arrival date of American redstarts (*Setophaga ruticilla*) to their temperate

summer breeding grounds, which then affects reproductive success (Norris et al. 2004).

Similarly, improving the quality of overwintering habitat improves return rates and long-

term survival in American redstarts (Studds and Marra 2005). Intra-generational

carryover effects have been investigated to a limited extent in other systems, including

evidence that carryover effects can occur across metamorphosis. For example, the

lifetime mating success of adult damselflies (*Lestes viridis*) is influenced by carryover

larval constraints (De Block and Stoks 2005), while tadpole food resources can affect

subsequent adult performance in northern red-legged frogs (*Rana aurora aurora*,

Chelgren et al. 2006). However, carryover effects have been virtually unexplored in wild

populations without a distinct migratory or metamorphic process to delineate life history

stages.

Wild fish inhabiting highly seasonal environments (environments with large differences between summer and winter water temperatures) provide a logistically tractable complementary model system to study carryover effects. In this case, the winter period constitutes a significant natural challenge that punctuates the annual life history cycle of fish in northern latitudes (Conover 1992), with high condition-dependent winter mortality (Biro et al. 2004). However, little is known about the overwinter ecology of freshwater fish. Recent advances in telemetry technology have revealed that largemouth bass remain active in winter, although at reduced levels relative to the warmer summer season (Hanson et al. 2007). There is some evidence that brown bullhead (*Ictalurus nebulosus*) and largemouth bass will cease feeding below 10°C (Crawshaw 1984), and it is no surprise that even with abundant food supplies, the condition of largemouth bass declines over the winter in northern areas (Fullerton et al. 2000). Smallmouth bass and largemouth bass appear to aggregate in areas with favourable conditions (e.g., high oxygen, Gent et al. 1995; warm temperature, Karchesky and Bennett 2004; presence of macrophytes, Karchesky and Bennett 2004; proximity to conspecifics, Hasler et al. 2007). However, beyond these preliminary studies, nothing is known about how environmental conditions during the favourable summer and fall seasons affect overwintering behaviour or survival of wild freshwater fish.

Freshwater fish constitute an interesting model system to study carryover effects not only owing to biological characteristics and logistical considerations, but also because freshwater fish are increasingly exposed to a variety of anthropogenic stressors. These

stressors are both chronic (e.g., habitat degradation, Cooke and Suski 2008; climate change, Portner and Farrell 2008) and acute (e.g., catch-and-release angling, Cooke and Schramm 2007; release of bycatch in commercial fishing operations, Davis 2002). Although most of these stressors do not immediately kill fish, they may cause long-term physiological changes that impair the ability of fish to perform essential activities, such as finding food, evading predators, reproducing successfully, and resisting disease (Barton 2002). Thus, the quantification of carryover effects is a critical step in managing populations of fish routinely exposed to these anthropogenic activities.

The aim of this study was therefore to determine whether experience of a transient stressor resulted in carryover effects using a wild population of largemouth bass at the northern limit of their range as the model system. We used an exogenous pharmacological implant of cortisol, which is the primary glucocorticoid in fish, and of wide interest owing to its role in the physiological stress response (Wendelaar-Bonga 1997; Mommsen et al. 1999). Cortisol implants mimic the physiological effects of exposure to a stressor by inducing an elevation of circulating cortisol concentrations (Gamperl et al. 1994). In the present study, we experimentally induced a transient 5-day elevation of cortisol in the fall, and then compared the subsequent behaviour and mortality rates of cortisol-treated fish to sham-treated and control fish throughout the winter using a remote acoustic 2-dimensional whole-lake telemetry array. During the winter, the study system experienced a winterkill event with near-complete mortality, a severe ecological challenge often associated with hypoxia (Greenbank 1945; Hurst 2007). This winterkill event provided a unique opportunity to assess the impact of transient

cortisol elevation on the behaviour and mortality during a subsequent extreme environmental challenge.

5.3. Materials and methods

5.3.1. Study site. Warner Lake is a small (8.2 ha) private research lake in eastern Ontario (44º31'N, 76º22'W). In 2003, Warner Lake was equipped with a whole-lake acoustic telemetry array that allows near-real time positioning of fish in the wild (fully described in Cooke et al. 2005b). Briefly, thirteen hydrophones are positioned strategically throughout the lake so that the location of fish equipped with transmitters can be tracked with sub-meter precision in near-real time. This system is unique in the world in terms of coverage of an entire lake to assess the behaviour and fate of wild freshwater fish. Warner Lake is a closed system for fish with no potential for immigration or emigration, and is closed to public fishing.

5.3.2. Study animals and treatments. From October 10 to 13, 2007, largemouth bass were captured by rod-and-reel angling from Warner Lake and transported to shore in coolers of lake water. On shore, fish were weighed, and fish greater than 450 g were deemed suitable to carry the intraperitoneal acoustic transmitters (Model MD-11-18; Lotek Wireless, Newmarket, ON; 8.4 g in the air), according to the accepted '2%' rule of biotelemetry (where the transmitter must be no greater than 2% of the animal's total mass, Winter 1983). Suitable fish (n=25) were assigned to one of three treatment groups while deliberately ensuring that all groups contained fish with the same size distributions:

control (n=8; 638.5 ± 125.6 g [mean ± SEM]), sham treatment (n=8; 637.6 ± 72.3 g) or

cortisol treatment (n=9; 693.6 ± 113.0 g). All fish were anaesthetized in an induction

bath containing 50 mg L^{-1} clove oil (10% clove oil emulsified in ethanol; Prince and

Powell 2000) in fresh lake water. After loss of equilibrium, fish were placed on a surgery

table that allowed the gills to be irrigated with an aerated solution of fresh lake water

containing 10 mg L^{-1} clove oil. The fish were implanted with transmitters following

procedures described in Cooke et al. (2003). Cortisol-treated fish were injected

intraperitoneally with 10 mg mL^{-1} of cortisol (hydrocortisone; Sigma H4001, Sigma-

Aldrich Inc., St. Louis, MO) emulsified in coconut oil (*Cocos nucifera*; Sigma C1758,

Sigma-Aldrich Inc., St. Louis, MO), at 0.005 mL g^{-1} body weight, following the dosage

previously described for 'low dose' fish (Chapter 4, Section 4.3.1). Sham treatment fish

were given intraperitoneal injections of pure coconut oil at 0.005 mL g^{-1} body weight.

Control fish were not injected. All fish were immediately released from the same

location upon regaining equilibrium.

5.3.3. Behavioural data collection and analyses. The acoustic transmitters are

programmed to relay positional information to the hydrophones every 59.5 s.

Information from the hydrophones is then relayed through submerged cables to receivers

on the shore, where the information is stored on flash cards. Information was collected

continuously throughout the study period, excluding November 28, 2007 through to

December 7, 2007, when the receiver was not functioning due to a power outage. Raw

position solutions are generated by triangulating acoustic signals received from a single

tag at a single time-point by three or more hydrophones within the array using the two-dimensional positioning engine within the program BioMAP (Lotek Wireless, Newmarket, ON). Erroneous positions are removed through BioMAP's filtering program (for full description, see Hanson et al. 2007). Filtered raw position data are then queried to generate tables of activity information (distance travelled, and velocity) for each fish. The instantaneous distance travelled (m) between each signal transmission is calculated using the Pythagorean Theorem, assuming that each fish travels between positions while maintaining the same depth. Fish movement is restricted to a depth between 6.5 and 5.5 m once the lake is ice-covered (Hanson et al. 2007), and the limited change in depth throughout the winter months minimizes the impact of this variable on measurements of horizontal movements during this period. Instantaneous velocity (m s^{-1}) is determined by dividing the instantaneous distance moved by the time between signal transmissions. Mortality of individuals was determined by importing the raw position data to ArcMAP version 9 (ESRI Inc., Redlands, CA) and viewing daily positional tracks. Fish were deemed to be dead once tracks indicated no directional pattern, since dead fish still show limited and non-directional movement due to water movement. Death dates were confirmed using minimum convex polygons (Rogers and White 2007).

We sought to assess both the short- and long-term behavioural consequences of a short-term elevation of circulating cortisol. As such, we examined the behavioural data at two scales. First, to assess short-term behavioural consequences, we examined activity and spatial behaviour during the first 5 days following transmitter implantation. Second, to assess the long-term behavioural consequences, we first plotted general activity trends

for all treatment groups over the winter (running 5-day means). Using water temperature

profiles (collected using the telemetry array from n=8 fish implanted with transmitters

equipped with temperature sensors for a different study; Hasler et al. 2009) we then

selected three representative 5-day periods to examine the data in more detail (fall,

November 8-12; early winter, December 26-30; late winter, February 13-17). These days

represented periods of stable water temperature with environmental conditions typical of

the periods of interest. During all 5-day periods, we examined activity by calculating

mean daily distance travelled (m) and mean velocity (m s^{-1}) over the entire 5-day period.

5.3.4. Statistical analyses. A product-limit log-rank survival analysis was conducted to

determine if the timing of mortality differed among the treatment groups. This test

compares the mean length of time that fish in each group survived following the date of

transmitter implantation. ANOVA tests were conducted to determine whether there was

a difference in mean daily distance travelled and mean velocity among treatment groups

during the first 5 days following treatment. For the long-term data, two-way ANOVA

models with repeated measures were run for distance travelled and mean velocity using

the three representative 5-day periods. Following a significant two-way ANOVA models

with repeated measures, we ran separate one-way ANOVAs at each selected 5-day time

period to better understand the relationships among treatment groups. Post-hoc Tukey's

HSD tests were employed following all significant one-way ANOVAs to determine

where among groups the differences lay. It is important to note that only fish alive

during all representative periods were used for the repeated measures ANOVAs. The

assumptions of equality of variances and normal distribution were tested and met for all

analyses. All analyses were performed in the statistical packages JMP, version 7.0.1

(SAS Institute Inc., Cary, NC). The level of significance for all tests (α) was assessed at

0.05. All results are presented as mean ± SEM.

5.4. Results

All fish in all treatments survived the tagging and manipulation procedures, with no

mortality observed in any treatment group until late December, more than two months

after tagging. Interestingly, the timing of mortality differed significantly among the

treatment groups. The cortisol-treated individuals succumbed significantly earlier in the

winter than individuals in the other groups (log-rank survival analysis, χ^2=11.71, df=2,

p<0.01; Figure 5.1).

There was a significant effect of treatment on swimming activity during the first

five days after treatment and transmitter implantation (Figure 5.2). There was no

difference in mean velocity of fish among treatment groups ($F_{2,22}$=1.51, p=0.24, Figure

5.2A). However, fish treated with cortisol travelled greater daily distances than fish in

the control group ($F_{2,22}$=3.76, p=0.04, Figure 5.2B).

In the long term, there was a slight trend for cortisol-treated fish to display

increased activity throughout the winter. There was a large divergence between the

cortisol-treated fish and the other treatment groups during the late winter, with activity in

the sham-treated and control groups increasing in the late winter, while activity decreased

slightly in the cortisol group (Figure 5.3). Using representative 5-day periods to examine

these relationships in detail, a two-way ANOVA with repeated measures revealed significant differences for both mean velocity (Figure 5.4A) and mean daily distance travelled (Figure 5.4B). For both mean velocity and mean daily distance travelled, there was an interaction between seasonal period and treatment group (mean velocity Roy's Max Root $F_{2,16}$=5.51, p=0.02, Figure 5.4A; mean daily distance travelled Roy's Max Root $F_{2,16}$=5.93, p=0.01, Figure 5.4B). Separate one-way ANOVAs at each time period revealed that while there was a trend for cortisol-treated fish to display increased activity during the early winter, differences in activity were only significant during the late winter period, when the activity of control and sham-treated fish increased significantly, and the activity of cortisol-treated fish decreased (mean velocity $F_{2,18}$=3.874, p=0.043, Figure 5.4A; mean daily distance travelled $F_{2,18}$=4.091, p=0.037, Figure 5.4B).

5.5. Discussion

We present evidence that a transient elevation of plasma cortisol during the fall can result in carryover effects across seasons. Wild largemouth bass that experienced cortisol treatment during the fall suffered accelerated mortality and displayed altered behaviour when compared to sham-treated or control fish during a subsequent winterkill event. While these results may not be typical of a more benign winter, they provide novel insight into how stress effects may operate across seasons and multiple challenges in wild populations.

5.5.1. Study limitations. It is important to note that the cortisol administered in this

study generated supraphysiological concentrations of circulating cortisol (see Chapter 4, Section 4.4.1). In the current study, the field setting precluded analysis of pilot samples prior to initiating the full experiment, and the result was that the doses selected, which were based largely on literature for salmonid fish (Gamperl et al. 1994), elicited plasma cortisol concentrations in largemouth bass that were an order of magnitude higher than desired. Despite the pharmacological dose, fish exhibited a typical glucose response to elevated plasma cortisol (see Chapter 4, Section 4.4.2), and we observed no immediate mortality in the cortisol-treated group. While acknowledging this methodological limitation, our examination of potential carryover effects in a fish provides unique insight into the physiological mechanisms that drive whole-animal consequences of stress across a broad temporal scale, and generates a framework for further studies on this topic. As noted in Chapter 4 (Section 4.5.1), the use of exogenous cortisol administration (particularly of a pharmacological dose) is a first step in testing the possibility of carryover effects in a wild fish population. Further research is necessary to understand whether a physiological stress response would cause similar long-term carryover effects.

5.5.2. Evidence of carryover effects. A winterkill event is a rapid phenomenon associated with hypoxic conditions under ice and is common in small northern lakes dominated by centrarchids (Greenbank 1945; Hurst 2007). While we did not measure dissolved oxygen throughout the winter, the extraordinarily heavy snowpack during the winter of 2007 to 2008 caused hypoxic conditions and subsequent winterkills in many small lakes in eastern Ontario (Scott Smithers, Ontario Ministry of Natural Resources,

personal communication). It is noteworthy that the majority of the fish in the sham-treated and control groups died within a very short period of time, between March 2 and 14, 2008, also consistent with a rapid winterkill event. The cortisol-treated fish, however, suffered a steady rate of mortality throughout the winter. This mortality pattern suggests that cortisol treatment during the fall was associated with accelerated mortality throughout the winter, and may have been unrelated to the winterkill event. Further research is needed to determine whether differences in overall mortality among the treatment groups would occur under more typical winter conditions.

As well as treatment effects on survival, we found differences in behaviour among the treatment groups. During the first 5 days after treatment, the cortisol-treated fish displayed elevated activity when compared to the control treatment group. Our data documenting elevated activity as a result of cortisol treatment in a wild, free-swimming fish complement those of previous laboratory studies. In rainbow trout, chronic cortisol elevation is associated with a decrease in aggressive locomotor activity when faced with a conspecific, but no difference in locomotion when undisturbed (Øverli et al. 2002). In the same species, cortisol elevation is also associated with a decrease in aggression and social status (Gilmour et al. 2005), but no differences in swimming performance were found (Gregory and Wood 1999). Taken together, these results demonstrate that laboratory studies may not capture the full range of behaviours displayed by wild, free-swimming fish during an endocrine stress response. Interestingly, the activity difference in the present study was manifested as an increase in distance travelled, without an increase in mean velocity. This suggests that fish subjected to the cortisol treatment

decreased the time spent resting, rather than increasing swimming velocity while moving. This pattern is consistent with previous studies on Gambel's white-crowned sparrows (*Zonotrichia leucophrys gambelii*), where corticosterone treatment decreased resting behaviour (Breuner et al. 1998). It is possible that the short-term response to an elevation of circulating cortisol is an increase in avoidance behaviour, as the animal attempts to remove itself from adverse stimulus (Wingfield et al. 1998).

The activity levels of cortisol-treated fish were reduced when compared to control and sham-treated fish during the late winter, more than 4 months after circulating cortisol levels would have returned to control levels. Typically, largemouth bass in the Warner Lake system decrease activity as water temperatures drop in the fall, and then increase activity slowly through the winter as the animals acclimatize to the consistent cold temperatures (Hanson et al. 2007; Hasler et al. 2009). In this study, all treatment groups displayed the expected pattern until the final weeks prior to the winterkill event, when the sham-treated and control individuals increased their activity while the cortisol-treated fish further reduced their activity. A physiological explanation of these behavioural differences may be related to energetics. Elevation of cortisol is known to reduce feeding and body condition in laboratory studies (Gregory and Wood 1999; Mommsen et al. 1999). Winter is energetically demanding, and causes a loss of body condition even under favourable circumstances (Fullerton et al. 2000). Any loss of body condition in the fall as a result of transient cortisol elevation likely carried a cost throughout the winter for the cortisol-treated animals. It is also possible that cortisol-treated fish expended more energy through the winter months, with the non-significant trend for increased activity

until late winter. Poor body condition and low energetic stores may have limited the behavioural responses of the animals, and also resulted in the observed accelerated mortality.

5.5.3. Summary. In summary, this study provides initial evidence that an elevation of circulating cortisol in the fall is associated with accelerated mortality and differences in behaviour during a subsequent winter, which included a winterkill event. In this case, the elevation of circulating cortisol and the winter were severe. Further research is necessary to understand and quantify seasonal carryover effects across a range of conditions. Research of this kind will increase our understanding of stress-induced physiological changes at the organismal level, and directly benefit our knowledge of the population biology of wild animals (Calow and Sibly 1990), as well as our understanding of the ecological and evolutionary consequences of stress (Calow and Forbes 1998).

5.6. Figures
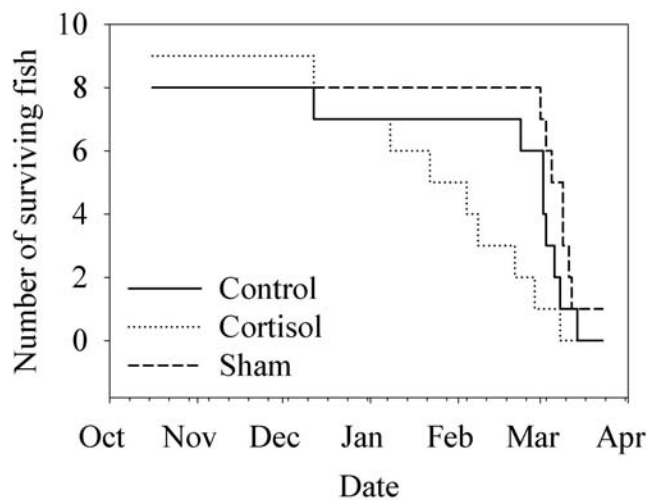
Figure 5.1. Surviving largemouth bass from all treatment groups between October 18, 2007 and March 15, 2008. There is a highly significant effect (p<0.01) of treatment group on survival rate, with cortisol-treated fish succumbing earlier in the winter than fish in the other treatment groups (see text for statistical details).
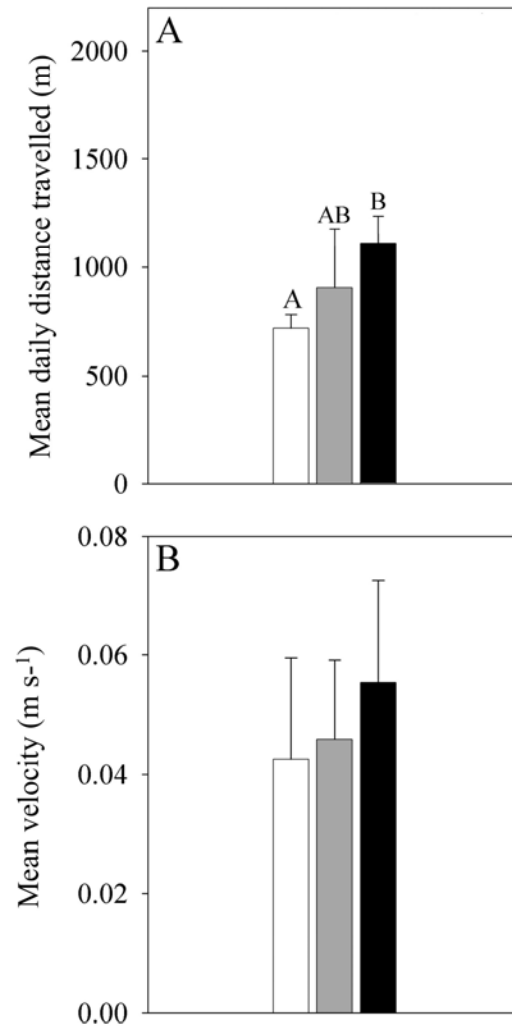
Figure 5.2. Short-term activity of largemouth bass from all treatment groups (i.e., activity during the first five days after transmitter implantation). Open bars indicate control fish, grey bars indicate sham-treated fish, and closed black bars indicate cortisol-treated fish. Values are presented as mean ± SEM. There was a significant difference in (A) mean daily distance travelled among the groups, with higher activity in the cortisol-

treated group (p<0.05; see text for statistical details).
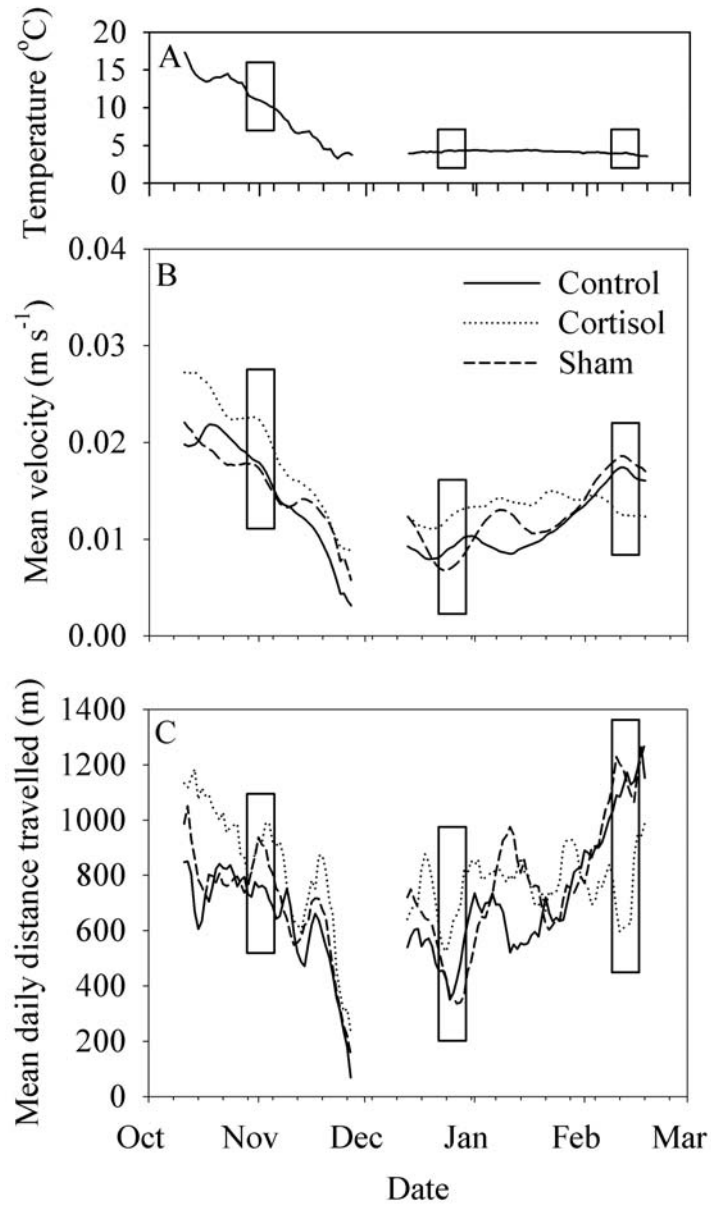
Figure 5.3. Water temperature (A) and long-term activity (B, C) of largemouth bass

from all treatment groups between October 18, 2007 and February 20, 2008. Five-day

running means for (B) daily distance travelled and (C) mean velocity show general activity trends through the winter. A power outage occurred from November 28 to December 7, and because data are plotted as 5-day running means, the first data point following the power outage occurs December 12. Boxes indicate the 5-day periods used for further statistical analysis.
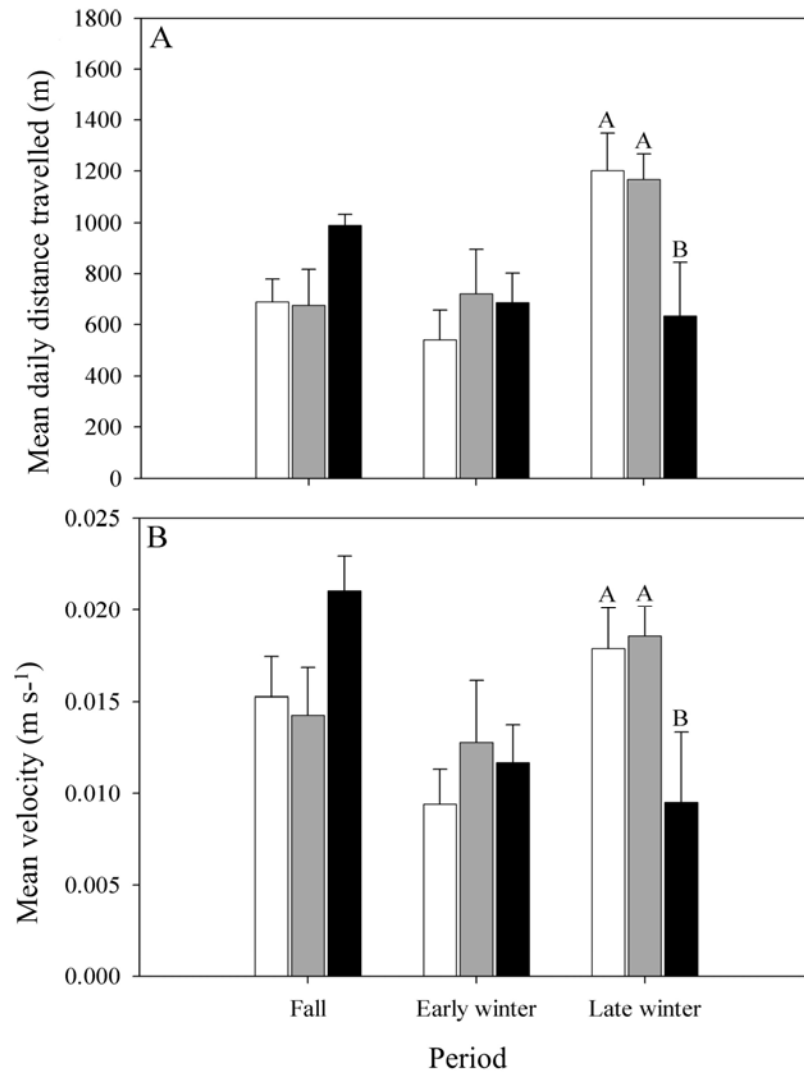
Figure 5.4. Long-term activity of largemouth bass from all treatment groups (i.e.,

activity during representative 5-day periods in the fall [November 8 to 12], early winter

[December 26 to 30], and late winter [February 13 to 17]). Open bars represent control

fish, while closed grey bars represent cortisol-treated fish, and closed black bars represent

cortisol-treated fish. All values are presented mean ± SEM. There is a significant interaction between time period and treatment group for both (A) mean daily distance travelled and (B) mean velocity, driven by differences between the cortisol-treated fish and other treatment groups during the late winter ($p<0.05$; see text for statistical details).

CHAPTER 6

THE CONSEQUENCES OF SHORT-TERM CORTISOL ELEVATION ON

INDIVIDUAL PHYSIOLOGY AND GROWTH RATE

6.1. Abstract

In this study, we explored growth, survival, and potential population-level effects of short-term experimentally-induced stress in largemouth bass. Cortisol implants (50 mg kg^{-1} body mass) were used to increase circulating stress hormones in a group of wild fish in a research lake for ~6 days in June 2007. Through mark-and-recapture, we compared survival, growth, and plasma biochemistry of cortisol-treated, sham-treated and control fish at liberty until October 2007. Cortisol-treated fish displayed persistent growth rate depression compared to other groups. However, neither plasma biochemistry nor mortality rates differed among treatments. In a complementary study, we found that the standard metabolic rates (SMR) of cortisol-treated was higher than control fish ~56 hrs following treatment. Bioenergetics modelling revealed that a transient elevation in SMR alone was insufficient to explain the observed growth depression. Finally, we constructed a simple population model to explore potential consequences of growth depression. We found that a 10% reduction in population growth rate is conceivable when 39% of the population experiences a stress causing the growth rate depression documented in this study. Our study is novel in highlighting that individual and potentially population-level growth depression can result from a single stress event of short duration.

6.2. Introduction

The endocrine stress response is an adaptive mechanism that promotes the survival and

recovery of individuals during and after challenging events (Sapolsky et al. 2000;

Greenberg et al. 2002). This response, characterized by the production and release of

glucocorticoid hormones (Axelrod and Reisine 1984), is associated with a suite of

secondary system-level and tertiary whole-animal changes that range from increases in

carbohydrate catabolism to complex behavioural changes (Barton 2002). One of the

primary adaptive roles of cortisol is the mobilization of energy reserves in response to

stress (Van der Boon et al. 1991; Schreck et al. 1997; Wendelaar-Bonga 1997). Elevated

cortisol is also associated with increases in aerobic and anaerobic metabolism (Morgan

and Iwama 1996; De Boeck et al. 2001) and an increase in standard metabolic rate

(Lankford et al. 2005). These changes serve to increase the energy immediately available

for individuals to adequately respond to abiotic or biotic challenges.

      Elevated plasma cortisol is associated not only with the mobilization of energy

reserves, but also with a reduction in feeding (Gregory and Wood 1999; Lankford et al.

2005). The relationship between foraging behaviour and stress has been explored in a

wide variety of taxa using a range of stressful stimuli, and consistently links stress to a

reduction in foraging (see reviews by Schreck et al. 1997; Carr 2002; Greenberg et al.

2002). For example, simulated trawling (Olla et al. 1997), environmental toxicants

(McGeer et al. 2000), and salt stress (De Boeck et al. 2000) have been reported to reduce

feeding behaviour in salmonids. In particular, food-searching behaviour is reduced in

response to stress (Beitinger 1990), and in response to increased levels of predation

(Gilliam and Fraser 1987; Abrahams and Sutterlin 1999). The proximate mechanism underlying the organismal changes in response to stress is uncertain, but evidence strongly supports the involvement of the hypothalamus-pituitary-interrenal axis (Bernier and Peter 2001), specifically cortisol and corticotropin-releasing factor (Bernier 2006). For example, administrations of exogenous cortisol (Gregory and Wood 1999) and corticotropin-releasing factor (De Pedro et al. 1993; Bernier and Peter 2001) have both been shown to decrease feeding in fishes in the laboratory. However, whether such mechanisms also operate in wild fish populations *in situ* has not yet been studied.

Although the cellular and organismal changes associated with a stress response are adaptive in the short term, these changes become maladaptive and are associated with decreases in somatic growth rate when circulating cortisol concentrations are chronically elevated (Gregory and Wood 1999; Edeline et al. 2009). For example, in goldfish (*Carassius auratus*), elevated cortisol has also been shown to reduce growth despite normal feeding behaviour (Bernier et al. 2004) indicating that there is a metabolic cost of stress. For wild fish, where animals rely on foraging success to recover energy stores lost during exposure to an acute stressor, and where anti-predator behaviour must be maintained to survive, reductions of individual growth rates may have important implications for survival and reproduction. For example, in males, larger individuals may be better competitors and attract more females (Foote 1988; Dunlop et al. 2007), and indeed larger male smallmouth bass are able to obtain more eggs to fertilize and guard than smaller males (Dunlop et al. 2007; Hanson and Cooke 2009). In females, fecundity is exponentially correlated with body size (Sargent and Gross 1986; Birkeland and

Dayton 2005). Furthermore, fish size for both sexes can be correlated with overwinter survival in northern latitudes (Biro et al. 2004). Therefore, reductions in individual somatic growth rate can have a negative impact on reproduction and survival. While there have been laboratory studies that have confirmed costs to stress and extrapolated the results to wild animals (Edeline et al. 2009), it is unclear if laboratory-assessed impacts of stress are relevant to wild fish populations.

In this study, we investigated the potential long-term (5 month) consequences of a transient (6 day) elevation of circulating cortisol in wild, free-swimming fish. We employed a mark-and-recapture approach to compare survival, growth rates, and plasma biochemical indices among largemouth bass treated with an implant that raises circulating cortisol for ~6 days, sham-treated fish, and control fish at liberty over a 5-month period. SMR measurements and bioenergetics modelling were employed to understand whether changes in metabolic rate could account for changes in growth rate. Finally, we constructed a simple population model to explore potential population-level consequences of short-term stress. The combination of experiments was aimed at exploring the long-term consequences of exposure to a transient cortisol elevation, identifying some of the potential regulating mechanisms, and exploring how these changes might affect wild fish populations.

6.3. Materials and methods

6.3.1. Long-term effects of transient plasma cortisol elevation in free-swimming wild fish. To study the long-term (5 month) costs of a short-term (6 day) cortisol

elevation, we captured 207 mature (>250 mm) largemouth bass by rod-and-reel angling

from Warner Lake, a small (8.2 ha surface area) private research lake in eastern Ontario

(44º31'N, 76º22'W). To avoid any confounding effects of reproduction, initial treatment

occurred between June 24 and 28, 2007, after the cessation of all spawning and parental

care activities in this lake. Warner Lake is closed to immigration and emigration for fish

and fishing (including recreational angling) is prohibited aside from research purposes.

All captured fish were landed within 20 s, and placed in a foam-lined trough that exposed

the ventral side while keeping the gills submerged in fresh lake water. To establish

baseline plasma biochemical parameters indicative of feeding and fasting (Congleton and

Wagner 2006), approximately 1.5 mL of blood was withdrawn by caudal puncture into

lithium-heparinized 3 mL vacutainer-style syringes (B.D., Franklin Lakes, NJ) from a

random subset of animals. All fish were then scanned for a PIT tag (12.5 x 2.0 mm)

using a PIT tag reader (Biomark, Boise, ID), and given a unique intracoelomic PIT tag if

necessary. Warner Lake is a research lake, and fish have been PIT-tagged since 1993 for

routine population monitoring. Of the 207 fish captured, 55 fish already carried PIT tags.

Fish were measured (TL), and size-matched by TL into three treatment groups: cortisol,

sham, or control (Table 6.1). Fish requiring new PIT tags were also distributed evenly

among treatment groups. Fish were treated with cortisol as described previously

(Chapter 4, Section 4.3.1). For all fish, body mass was estimated from a mass-TL

relationship, (mass in g)=$(1.359 \ 10^{-4})$(TL in mm)$^{2.996}$. This relationship (R^2=0.949) was

previously developed from 68 mature largemouth bass captured between 2003 and 2006

in Warner Lake during the summer post-spawning period (i.e., June and July). All fish

were released following treatment. Sexing largemouth bass externally is unreliable out of

the breeding season, and so fish were not sexed, and were likely a mixture of males and

females.

To monitor the fate and growth of the three treatment groups, between August 20

and 25 (i.e., 54 to 63 days following initial treatment), 206 mature largemouth bass were

captured by rod-and-reel angling from Warner Lake, sampled for blood, measured (TL),

and scanned with a PIT tag reader as described above. The process was repeated between

October 10 and 15 (i.e., 105 to 114 days following initial treatment), when 151 mature

largemouth bass were captured, sampled for blood, measured (TL) and scanned with a

PIT tag reader.

All blood samples were handled and stored as described previously (Chapter 2,

Section 2.3.2).

Water temperatures were stable between June 24 and August 25, ranging from

23°C to 25°C, with the highest temperatures occurring between July 20 and August 10.

After August 25, water temperatures declined steadily through the fall, reaching 11°C by

October 15.

6.3.2. Biochemical indices of fasting. To better appreciate the relevance of circulating

plasma biochemical parameters assessed in the wild, 12 largemouth bass were captured

by rod-and-reel angling from Lake Opinicon, a nearby public lake that is part of the

Rideau River system in eastern Ontario (44°30'N, 76°20'W) from June 15 to 19, 2007.

These fish were transported to QUBS (on Lake Opinicon) in aerated coolers, where they

were measured (TL), weighed, and size-matched by mass into two groups; fed and fasted

fish (Table 6.1). Fish were anchor-tagged for individual identification, and then placed in

two 750 L holding tanks with flow-through lake water, in mixed treatment groups (n=3

from each treatment group in each tank; n=6 fish per tank).

Starting on the day of capture, and every other day thereafter, fish in the fed

treatment group were force-fed 2% of their body mass with a 1 g mL^{-1} mixture of blended

trout pellets (Purina Aquamax Grower, Purina Mills Ltd., MO) and fresh lake water,

using a 50 mL syringe attached to a piece of flexible plastic tubing that placed the food

manually in the stomach. Fish in the fasted treatment group were handled in an identical

fashion except that no food was injected. Fish were held singly in aerated coolers of

fresh lake water for approximately 10 min post-feeding to ensure that no fish regurgitated

the mixture. After 7 days (approximately 8 hrs following the fourth feeding), fish were

caught individually from the group tanks and blood samples were withdrawn within 2

min. Plasma was separated and stored as described above. Water temperatures were

maintained at 20.0 ± 0.9ºC (mean $\pm$ standard deviation [SD]) throughout the experiment.

6.3.3. Biochemical analysis. As indicators of recent feeding (Congleton and Wagner

2006), plasma activity of aspartate transaminase and plasma concentrations of

cholesterol, glucose, magnesium, total protein, and triglycerides were quantified using a

Roche Hitachi 917 analyser (Roche, Basal, Switzerland) as previously described for

triglycerides, cholesterol, total protein and glucose (Chapter 4, Section 4.3.2). For the

fish used in the Warner Lake long-term monitoring, pre-treatment (June) plasma samples

were not available for all fish recaptured in August and October 2007, and a repeated

measures approach was thus not possible. Therefore, if multiple plasma samples (i.e., at

multiple capture periods) existed for an individual fish, a subset of plasma samples was

analysed such that only a single plasma sample was analysed for each individual. From

the June sampling period, 22 samples were analysed as pre-treatment values (n=8

cortisol-treated, n=8 sham-treated, n=6 control). From the August sampling period,

samples from 15 cortisol-treated, 15 sham-treated, and 14 control fish were analysed.

From the October sampling period, samples from 7 cortisol-treated, 10 sham-treated, and

10 control fish were analysed. All plasma samples from the force-feeding experiment in

the laboratory were analysed.

6.3.4. Metabolic effects of cortisol elevation. To determine whether metabolic rate was

increased by the short-term elevation of circulating plasma cortisol, 12 largemouth bass

were captured by rod-and-reel angling from Lake Opinicon from June 21 to 23, 2008.

Fish were transported to QUBS in aerated coolers, where they were measured (TL),

weighed, and size-matched by mass into two groups; cortisol-treated and control (Table

6.1). Over the 3-day measurement period, 4 fish were captured per day, and 2 fish

assigned to each treatment group per day. As described above, cortisol-treated fish were

injected within 1 hr of capture with 10 mg mL^{-1} cortisol in a coconut oil vehicle at a dose

of 0.005 mL g^{-1}, while control fish were not injected. In this case, we were interested in

quantifying a change in metabolic rate as a result of our cortisol treatment, relative to a

control animal. Since access to the respirometer was limited, and the objective of this

portion of our study was to determine the short-term metabolic consequence of our cortisol treatment rather than to disentangle the specific effect of the cortisol elevation from the handling stress of injecting the implant, sham treatments were not employed for this aspect of the study (see DiBattista et al. 2005 for further rationale). All fish were housed singly in 40 L holding tanks with flow-through lake water for 48 to 52 hrs post-capture both to ensure that metabolic measurements were carried out at peak circulating cortisol concentrations for the cortisol-treated fish (Chapter 4, Section 4.4.1), and to ensure that fish were in post-absorptive digestive state (Alsop and Wood 1997).

Metabolic measurements were carried out using the Loligo AutoResp intermittent flow-through respirometry equipment and software (Loligo Systems ApS, Tjele, Denmark) with an 11.4 L chamber (see Gingerich et al. 2009 for full description) from June 23 to 25, 2008. With this system, dissolved oxygen (DO) levels inside the closed system are measured every second for 15 min. The system is then opened for a 10 min flush period, and metabolic recordings for the next period begin following a 1 min lag after the system is closed. Blank tests were run for 2 to 3 hrs prior to placing a fish in the respirometry chamber, and oxygen consumption values were corrected accordingly. Fish (2 cortisol-treated and 2 control fish per night) were then placed in the respirometry chamber between 7 pm and 8 pm in the evening for a 12 hr period. The 6 lowest recordings were averaged to calculate the SMR for each fish (Steffensen et al. 1994). Mean temperature during the metabolic rate measurements was 23.8 ± 0.6°C (mean $\pm$ SD).

6.3.5. Statistical analysis. For each individual study described above, ANOVA models were used to ensure that fish were appropriately size-matched. Chi-square goodness-of-fit tests were used to test that the fish receiving new PIT tags were evenly distributed across treatment groups for the long-term monitoring aspect of the study.

To analyse mortality patterns of cortisol-implanted versus other treatment groups in the wild, recapture rates (ratios of fish recaptured once, recaptured twice, and never recaptured) were compared among treatment groups using Chi-square goodness-of-fit tests. Given low sample size for ratio estimators, a post-hoc power analysis was conducted to determine the power of this test.

Differences in mean growth rates among the treatment groups over the monitoring period were determined using an ANCOVA with treatment group (cortisol, sham, control) as the independent variable, and initial TL as the covariate. The interaction term was included in the model. Growth rate across the monitoring period was the dependent variables, and was determined for each individual by subtracting the total length upon recapture from the initial total length, and dividing by the number of days between captures. For the fish captured more than once, only the final capture was used to calculate that individual's growth rate. Following a significant ANCOVA, post-hoc Tukey's HSD tests on the treatment effect were used to determine where among the treatment groups the differences lay.

All biochemical parameters in the field were compared among cortisol-treated, sham-treated and control wild largemouth bass in June prior to treatment, in August (54 to 63 days following treatment) and in October (105 to 114 days following treatment)

using ANOVAs. Tukey's HSD post-hoc tests were employed following significant ANOVAs to determine group differences in mean biochemical parameters. In the complementary laboratory analysis, all plasma biochemical parameters were compared between the fed and fasted fish from the feeding experiment using Student's t-tests. To ensure that all potentially important biochemical indices were identified, a liberal uncorrected $\alpha=0.05$ was employed.

To determine whether cortisol treatment resulted in short-term changes in metabolic rate, treatment group were used as the dependent variables in an ANCOVA, with whole-animal SMR as the independent variable, and mass as a covariate. The interaction term was included in the model.

All residuals were tested for normal distribution using goodness-of-fit tests, and for homogeneity of variances using Levene's test, and assumptions were met. All statistical analyses were conducted using JMP 7 (SAS Institute, Cary, NC). All results are reported as mean $\pm$ SEM, and $\alpha=0.05$, unless otherwise specified.

6.3.6. Bioenergetics modelling. To explore the relative contribution of increase in SMR to growth rate depression in response to the cortisol injection, a series of simulations of fish growth were conducted using the largemouth bass model developed by Rice et al. (1983) using Fish Bioenergetics software (Version 3.0, University of Wisconsin-Madison, Madison, WI). In a parallel study, field activity rates were found to be similar among the different treatment groups following the cessation of cortisol elevation (see Chapter 5, Section 5.4), and therefore activity was assumed to be equal among treatment

groups in all bioenergetic models. We also assumed piscivory (a reasonable assumption

for largemouth bass of the sizes used in the present experiment; Heidinger 1975; Keast

1985), and therefore, that food would provide 4000 J of energy g^{-1} (wet mass). Because

the bioenergetics model requires a fish mass variable, mean initial and final masses for

control and cortisol-treated fish in the long-term study were estimated using a mass-TL

relationship described above. Taking into account the increase in SMR found in our

study (see Section 6.4.4), we assumed that SMR remained increased for only the 6-day

period of cortisol elevation, and used the bioenergetics model to determine whether an

increase in SMR would alone be sufficient to cause the growth depression documented in

this study between June and August. This 60-day period was selected rather than the

entire 114-day monitoring period so that water temperature could be set as a constant

24˚C in the model.

6.3.7. Population modelling. To explore the potential demographic costs of endocrine

stress in terms of individual growth depression as assessed in the empirical portion of our

study, we constructed a deterministic Leslie-matrix population model (Caswell 2001) and

compared model runs with and without potential growth depression resulting from

cortisol treatment. We also explored the fraction of the population that would need to

experience a stress-induced growth depression in order to see reductions in the population

growth rate. Population modelling was conducted as a theoretical experiment and was

not indented to provide precise predictions for Warner Lake. The population model was

first run with a standard growth model, and then contrasted to a model run in which

somatic growth was depressed in fish for a single growing season by the empirical

estimate of percentage growth depression measured in the field. We assumed that fish of

all age classes (from age 1 to age 10) are influenced by the same percentage of growth

depression for the single growing season. The approach followed the work by Edeline et

al. (2009).

Largemouth bass length (TL, mm) at age a (years) was modelled using a von

Bertalanffy growth model as:

(1) $L_a = L_\infty \left(1 - \exp\left[-0.19(a + 0.024)\right]\right).$

The default parameter values for the growth model in equation (1) were taken from the

Ontario population of largemouth bass reported in Beamesderfer and North (1995).   For

simplicity, we assumed no differences in growth among males and females. To account

for density-dependence in growth, a negative relationship between population abundance

and individual growth estimated for smallmouth bass (Dunlop et al. 2007) was

considered as:

(2) $L_\infty = L_{\infty,\max} / (1 + 0.37 D^{0.29}),$

where D is the population density (the number of fish of age 1 or older per ha). The

value of $L_{\infty,\max}$ (mm) was determined so that $L_\infty = 560$ mm (an empirical value L_∞ for

the Ontario population, Beamesderfer and North 1995) at an intermediate level of

population abundance (D = 3.5 (/ha)). We varied D between 0.5 and 10.0 to check the

sensitivity of the model to the population density and found little effect on the outcome.

Based on empirical data for maturation of largemouth bass reported by Carlander (1977),

the relationship between fish length and proportion of sexually mature female fish at age

class a was represented using a sigmoid function as:

(3) $p_a = 1 / \left(1 - 0.1366 \exp\left[-(L_a - 208.2) \right] \right)$.

We assumed that the sex ratio of the population is 50:50, and the number of recruits was

determined by the number of eggs produced by females, since there is no information on

density-dependence in spawning stock size-recruitment relationships reported for

largemouth bass. To this end, size-dependent fecundity (egg number produced by a

female of L_a) $f_{m,a}$ was defined according to Laarman and Schneider (1985) as:

(4) $\log_{10} f_{m,a} = -0.4254 + 3.2857 \log_{10} L_a$

where the subscript m represent mature. We assumed there is no sex difference in natural

mortality rates but defined size-dependent natural mortality for immature and mature fish

separately. Size-dependent annual survival for immature largemouth bass $s_{i,a}$ was based

on the study of Gutreuter and Anderson (1985) as:

(5) $\quad s_{i,a} = \left[1 - 1.2318\exp(-0.01933 L_a)\right]^{12}$

where the subscript i represent immature. A fixed annual survival rate (0.73) was

assumed for immature fish of 200 mm or larger. Size-dependent annual survival for

mature fish was defined according to Dunlop et al. (2007) reporting in smallmouth bass.

We took this relationship based on the finding that mortality rates of largemouth bass and

smallmouth bass do not differ significantly (Beamesderfer and North 1995). Based on

Dunlop et al. (2007) we defined an upper limit of annual survival rates (0.46) for very

large fish based on their description of background mortality as:

(6) $\quad s_{m,a} = \exp(-0.00938 L_a + 4.3572)$

where the subscript m represent mature. Using the population model defined by

equations (1) – (6) we computed the population's finite rate of increase λ as the

dominant eigenvalue of the resulting Leslie matrix M (Caswell 2001). The form of the

Leslie matrix we used was:

$$(7) \qquad M = \begin{pmatrix} s_0 f_1 & s_1 f_2 & \cdots & s_{a_{max}-2} f_{a_{max}-1} & s_{a_{max}-1} f_{a_{max}} \\ s_0 & 0 & \cdots & 0 & 0 \\ 0 & s_1 & & 0 & 0 \\ \vdots & & \ddots & \vdots & \vdots \\ 0 & 0 & \cdots & s_{a_{max}-2} & 0 \end{pmatrix}.$$

The age-specific survival and fecundity (egg number per female at age a) in the matrix

was represented as:

$$(8) \qquad s_a = p_m S_{m,a} + (1 - p_m) S_{i,a}$$

and

$$(9) \qquad f_a = p_m f_{m,a}.$$

The survival from eggs to age 1 fish, s_0 , was determined by the method of Vaughan and

Saila (1976) on the assumption that the population is at equilibrium without individual

growth depression. We assumed a_{max} =11, and sensitivity analyses showed that

increasing a_{max} caused negligible changes in the population growth rate λ.

6.4. Results

6.4.1. Standardization among treatment groups. There were no differences in initial TLs among the three treatment groups from Warner Lake in June (Table 6.1). There were also no differences in initial TLs detected among the subsets of each treatment group recaptured only in August, only in October, or at both sampling periods (Table 6.1). There were no differences among the treatment groups in the proportion of fish given new PIT tags relative to fish already carrying PIT tags (χ^2=2.53, df=2, p=0.28). Similarly, there were no differences in mass or TL between the force-fed and fasted treatment groups in the complementary laboratory study examining biochemical indices of fasting (Table 6.1), and there were also no differences in mass or TL between the cortisol-treated and control fish used for metabolic rate measurements (Table 6.1).

6.4.2. Long-term effects of transient plasma cortisol elevation in wild largemouth bass. In total, 86 fish were recaptured once: 24 cortisol-treated fish, 29 sham-treated fish, and 33 control fish. Of these, 58 were recaptured once in August: 19 cortisol-treated, 16 sham-treated, and 23 control fish. Twenty-eight were recaptured once in October: 5 cortisol-treated, 13 sham-treated, and 10 control fish. An additional 8 fish were recaptured at both sampling periods: 2 cortisol-treated, 2 sham-treated, and 4 control fish (Table 6.2). While fewer cortisol-treated fish over the study period were recaptured than control and sham-treated fish, the ratios of fish recaptured once, recaptured twice, or never recaptured did not differ among the three groups (χ^2=3.77, df=4, p=0.44). This result indicates mortality rates did not differ significantly among the groups during the

summer months. However, a power analysis revealed that with only 24 fish per treatment group recaptured across the monitoring period (the minimum number of fish per treatment group recaptured once in our study), the power of our study to detect biologically relevant levels of mortality (relative to the control group) was low (Table 6.3).

Mean growth rate (mean increase in TL per day for each fish) over the monitoring period was significantly lower for the cortisol-treated fish than for the control and sham-treated fish (model $F_{5,\,84}$=9.69, p<0.001; treatment group $F_{2,\,2}$=9.94, p<0.001; Figure 6.1). As expected, initial TL was also important in predicting growth over the season (initial TL $F_{1,\,1}$=26.89, p<0.001). The interaction effect was not significant (interaction $F_{2,\,2}$=0.01, p=0.98). The mean growth rate for control fish was 0.15 ± 0.02 mm day^{-1} (mean $\pm$ SEM), while the growth rate for the sham-treated fish was 0.09 ± 0.06 mm day^{-1}, and the mean growth rate for the cortisol-treated fish was 0.01 ± 0.02 mm day^{-1}, which amounts to a 93% decrease in growth rate for the cortisol-treated group relative to the control group, and an 89% decrease in growth rate relative to the sham-control group. Note that for fish recaptured twice, only the data from October was used in the statistical analysis.

6.4.3. Biochemical indices of fasting. Among fish included in the long-term study from Warner Lake, no differences in biochemical variables were detected among the treatment groups in June, prior to treatment (Table 6.4). Chloride varied significantly among the treatment groups in August (54 to 63 days following treatment), with sham-treated fish

exhibiting significantly higher plasma chloride concentrations than cortisol-treated fish (Table 6.5). Similarly, sham-treated bass exhibited significantly higher plasma glucose concentrations than cortisol-treated bass in October (105 to 114 days following treatment; Table 6.6). No other differences among treatment groups were detected (Tables 6.5 and 6.6).

Individuals that were force-fed exhibited significantly lower plasma sodium concentrations than bass that were fasted for 7 days, and significantly higher plasma concentrations of triglycerides (Table 6.7). No other differences were detected between fed and fasted fish in the laboratory (Table 6.7).

6.4.4. Short-term metabolic effects of cortisol elevation. Both cortisol treatment and mass significantly impacted mean SMR measured ~56 hrs after treatment with cortisol (model $F_{3,8}$=46.07, p<0.01; Figure 6.2). Mass was the main effect (mass $F_{1,1}$=98.67, p<0.01), but treatment group was also a significant effect (treatment $F_{1,1}$=20.20, p<0.01), as was the interaction of these two factors (interaction $F_{1,1}$=12.54, p<0.01). Mean mass-corrected SMR for control fish was 79 ± 2 mg O_2 kg^{-1} h^{-1} as compared to 93 ± 2 mg O_2 kg^{-1} h^{-1} for cortisol-treated fish; this represented an 18% increase in SMR, on average, as a result of cortisol elevation.

6.4.5. Bioenergetics modelling. During the 60-day growth period between June and August, at a mean water temperature of 24ºC, the control fish grew from an initial mean mass of 459.1 g (calculated using the mass-TL relationship from the TL of 320.1 mm) to

a mean final mass of 497.7 g (calculated from the TL of 329.2 mm). To achieve this

level of growth, it was estimated in the bioenergetics model that individuals would have

to have consumed on average 373.6 g of fish prey, with 33% of consumed food allocated

to growth. Applying the same growth and consumption rates over the same period to the

cortisol-treated fish revealed that these fish (with an initial mean mass of 529.2 g

calculated from the TL of 333.5 mm) would have needed to consume on average 410.9 g

of prey. Assuming piscivory, food would provide 4000 J g^{-1}. Taking into account the

increase in SMR, and assuming that SMR remained increased for only the 6-day period

of cortisol elevation, the model predicted that the cortisol-treated fish should have

achieved a final mass of 568.7 g. From our growth data, we calculated from the mass-TL

relationship that the control fish achieved a final mass of only 530.9 g (from the TL of

334.3 mm). Thus, the bioenergetics modelling revealed that either the metabolic rate of

the cortisol-treated fish must have remained elevated beyond the 6-day period of cortisol

elevation, or there must have been an additional mechanism causing the growth

depression, such as a reduction in foraging, or sustained metabolic costs as a result of the

transient stressor.

**6.4.6. The potential population-level consequences of stress-induced growth

depression**. Decreased somatic growth resulting from short-term cortisol implants

affects age-specific survival and age-specific fecundity, which is predicted to result in a

decrease in population growth rate λ of a prototypical largemouth bass population

(Figure 6.3A). For example, in our model, a 50% decline in somatic growth rate of all

individuals of the population in a single growing season is predicted to result in a 14%

decrease in population growth rate, while a 99% decline in somatic growth rate is

predicted to result in a 37% decrease in population growth rate. In the present

experiment, cortisol-treated fish showed an 89% depression in growth rate over the

growing season compared to sham-treated fish (Figure 6.1). When all the fish of the

population would experience a similar level of stress, the decline of population growth

rate as a result of such growth depression over a single growing season would be 23%.

Since most stressors do not affect all individuals equally, we also modelled the

percentage of the population that would need to experience growth depression to see

biologically interesting reductions in population growth rate. We found that to

experience a 5% and 10% reduction in population growth, 19% and 39% of the

largemouth bass population, respectively, would need to experience stress causing the

growth depression documented in the empirical component of this study (Figure 6.3B).

6.5. Discussion

We found that a short-term cortisol implant resulted in changes in the long-term growth

rate of wild largemouth bass in a natural lake. In the field, we found few long-term

changes in plasma biochemistry, and no changes in plasma biochemistry that were

consistent with complete fasting, as indicated by the complementary laboratory study. In

exploring other potential mechanisms of the stress-induced growth rate depression, we

found in the laboratory that metabolic rate was elevated during the period of cortisol

elevation. However, bioenergetics modelling suggested that this increased metabolic rate

was not sufficient to explain the observed growth depression in the field if metabolic rate elevation is maintained only while circulating cortisol is elevated. Therefore, growth depression is likely to be explained by other mechanisms such as energy loss resulting from coping with the stressor (i.e., metabolic costs are sustained beyond the elevation of plasma cortisol), or a reduction in foraging that was not captured using our measured biochemical indices. While we found no differences in recapture rates among the various treatment groups, suggesting no differences among groups in mortality across the summer, the power of our study was low, and results were thus inconclusive. However, even without differences in mortality rate among the groups, growth depression resulting from short-term stress was found to potentially alter long-term population growth rate as indicated by a complementary theoretical population modelling exercise. For a reduction in population growth rate to occur, a substantial fraction of the largemouth bass population would need to experience a stress event causing individual growth depression.

6.5.1. Mechanisms of long-term growth suppression. The results of our study demonstrate that a cortisol hormone implant constitutes a challenge from which fish are unable to fully recover during a single growing season. We also found that the elevation of circulating cortisol was associated with an increase in SMR. This result was consistent with previous studies on laboratory rainbow trout, where elevations of plasma cortisol have been associated with an increase in SMR (Morgan and Iwama 1996). Furthermore, this is consistent with previous studies examining chronic stressors, where both social stress in rainbow trout (Sloman et al. 2000) and chronic stress in green sturgeon

(*Acipenser medirostris*) have been shown to increase SMR (Lankford et al. 2005), and is consistent with the prevalent notion that elevated cortisol in fish is associated with an increase in catabolic activity and energy use (De Boeck et al. 2001). However, our bioenergetics modelling suggested that a short-term increase in SMR (i.e., an increase during only the 6-day period of cortisol elevation) would not be sufficient to account for the observed long-term differences in growth rate among treatments without some concurrent additional mechanism such as a reduction in feeding.

Stress in largemouth bass reduces feed intake (Siepker et al. 2006), and administration of exogenous cortisol hormone implants has also been shown to reduce appetite in rainbow trout (Gregory and Wood 1999). We did look at fasting and its biochemical correlates, but the most noteworthy finding was that there were very few changes either between the fed and fasted fish in the laboratory or among the cortisol-treated, sham-treated, and control fish over the 114-day monitoring period in the wild. These data suggested that wild fish defend physiological homeostasis in the measured plasma parameters despite fasting. However, stability of biochemical profiles does not exclude the possibility that feed intake of cortisol-treated fish was reduced relative to control fish, potentially explaining the growth depression observed in the field. Further studies that incorporate different response metrics of body condition and feeding history, and more detailed behavioural studies are necessary to understand the potential long-term changes in foraging behaviour as a result of elevated circulating plasma cortisol in largemouth bass and other fish in the wild.

The combination of results from the present study revealed that transient elevations of cortisol carry both immediate energetic costs and long-term growth costs. In addition to the possibilities investigated in the present study of cortisol-induced changes in feeding behaviour and metabolic rate, other factors may have contributed to the long-term impairment of growth by transient cortisol elevation. Both stress and experimental elevations of plasma cortisol are associated with reductions in immune function and increases in disease susceptibility (Wendelaar-Bonga 1997; Barton 2002; Greenberg et al. 2002), and it is possible that compensating for the long-term challenges associated with disease constitutes a persistent energetic cost that could also help to explain the differences in growth detected among the treatment groups in our study. Stress and experimental elevations of plasma cortisol are also associated with complex behavioural changes including changes in social dominance (Barton 2002; Greenberg et al. 2002). Previous research looking at behavioural metrics found rapid resubmission of normal behavioural patterns after a single stress event in wild fish (e.g., resumption of normal behaviour in northern pike, *Esox lucius*, following a catch-and-release angling event; Klefoth et al. 2008; Arlinghaus et al. 2009). However, cortisol-treated fish in the current study may have been altering their behaving on a fine scale in a way that also increased their energetic expenditure beyond the cessation of the cortisol hormone implant. Further research that examines the long-term metabolic costs of short-term cortisol elevation is necessary to fully understand the energetic costs of a short-term stressor in wild, free-swimming animals. Particularly useful would be research that incorporates detailed behavioural data to more thoroughly explore the feeding behaviour

and activity rates of fish that have been exposed to a transient stressor or to transient cortisol elevation.

6.5.2. Potential consequences of individual physiological stress for the population. The results of the present study did not provide any evidence of differing mortality rates among the treatment groups. However, the power to detect biologically meaningful differences in mortality rates among the groups was low. Yet the long-term energetic costs, particularly the persistent growth costs, might have implications for the long-term survival and reproductive output of fish, and even population growth rates, without an immediate increase in mortality. Mortality rates in this species are size-dependent, and female fecundity and female preferences scales with male size (Dunlop et al. 2007). Our population modelling was used to explore potential scenarios of how individual growth depression might affect population growth rate, and suggested that somatic growth depression caused by endocrine stress could result in a substantial decrease in population growth. This theoretical modelling exercise suggests that the experience of sub-lethal stress in wild fish has the potential to reduce long-term survival and lifetime reproductive out, and affect population growth rate, as has been shown in laboratory populations (Edeline et al. 2009). It is important to note that our population model indicated that a substantial fraction (39%) of the population would need to be exposed to a single stress event to cause a 10% reduction in population growth rate. Such wide-scale stress could conceivably occur as a result of environmental challenges such as oxygen depletion, or anthropogenic activities such as intense boating activity, or catch-and-release angling. It

is important to also consider that in the model, growth was assumed to return to normal pre-stress levels after the single season of growth suppression. In reality, compensatory growth might occur (see Ali et al. 2003), and future studies monitoring fish over a longer period of time would be useful in determining when and to what degree this occurs.

6.5.3. Study limitations. The cortisol administered in our study generated supraphysiological concentrations of circulating cortisol ($\sim$2000 ng mL^{-1}) as reported previously in detail (see Chapter 4, Section 4.4.1). However, despite the pharmacological dose, the increase in SMR with cortisol treatment was comparable in magnitude to previous studies where chronic stressors initiated the physiological response (Sloman et al. 2000; Lankford et al. 2005). As noted previously, largemouth bass exhibited a typical glucose response to elevated plasma cortisol (see Chapter 4, Section 4.4.2). While acknowledging the methodological limitation of a supraphysiological dose, our examination of the long-term effects of cortisol elevation in a wild free-swimming fish provides unique insight into the physiological mechanisms that drive whole-animal consequences of stress. The current study provides a first step in elucidating the nature of long-term consequences of stress in a wild fish population, and may inspire future research aimed at understanding the magnitude of effect caused by a single stressor of interest.

Another limitation arises due to our use of TL as a measure of fish size, and the increases in TL per day over the monitoring period were used as a measure of fish growth. The use of TL as a single measurement of growth is limited, because it does not

take into account any measure of body condition (i.e., relative mass or girth per TL), which can also be affected by cortisol treatment (e.g., Barton et al. 1987). Furthermore, in our study there was no specific assessment of measurement error associated with taking measurements of total length (i.e., measuring the same fish multiple times during a single sampling period), or with other sources of variation (e.g., males versus females, or single versus double recapture). Given that decreases in TL were documented across the monitoring period in some individuals, it is clear that there was a level of error associated with taking TL measurements. However, we have no reason to suppose that sources of measurement error differentially affected a specific treatment group, and therefore we are confident that the differences among treatment groups reflect true differences, and not an anomaly caused by measurement error. Nonetheless, future studies should certainly include measurements of both TL and body mass and condition, as well as assessments of measurement error. Measurements of body condition would also yield more detailed insight into the potential for compensatory growth in a cortisol-treated fish (Ali et al. 2003).

A final limitation of our study relates to the population modelling exercise. Although our model illustrated the potential population-level effects of a single stress event affecting a portion of the target population, there are limitations to this model. We only used one process of density-dependence (i.e., density-dependence in growth), and we did not include density-dependent fecundity and mortality in the model because no data was available for largemouth bass. We also did not explicitly include size-dependent mate choice of males by females, because again, the data was not available for

this species. Our model nevertheless served as a test of the impact of growth depression by holding the general population model constant and varying only the growth sub-model. Thus, our predictions of population level effects of growth depression should hold qualitatively, although we cannot claim precise predictive power. Another limitation arises from modelling a single fish population closed to immigration, emigration and fishing, and ignoring inter-species interactions. Inter-specific interactions might accelerate or suppress the magnitude of the reduction of population growth rate in the target population. Finally, as the experimental data provided no information about the difference in growth depression between males and females, we assumed that both males and females experienced the same level of growth rate depression. The reduction of population growth rate shown in the present study essentially resulted from a reduction of lifetime reproductive success in females through decreased fecundity and increased mortality due to individual growth depression. Male largemouth bass provide nest-guarding parental care, and if the quality of the care depends on males' size (see Hanson and Cooke 2009), the reduction in male size might result in a further reduction of population growth rate.

6.5.4. Summary. To conclude, despite the limitations mentioned above, our study is the first to show growth rate depression after a single stress event in a wild free-swimming fish. Bioenergetics modelling suggests that mechanisms such as long-term metabolic costs or reduced feeding would account for this growth rate depression. Simple population modelling exercises suggest that if a substantial proportion of the population

experiences such a stress event, the population-level growth rate will be affected. Across a range of taxa, there have been few studies that have studied or documented instances in which stress at the level of the individual cascades to influence population-level processes (Calow and Forbes 1998). Understanding the relationships between sub-lethal stressors and ecologically-relevant measures such as individual or even population-level growth rates is critical to further understand the 'ecology of stress' in wild fish, particularly given the level of environmental change and disturbance occurring in aquatic ecosystems.

6.6. Figures
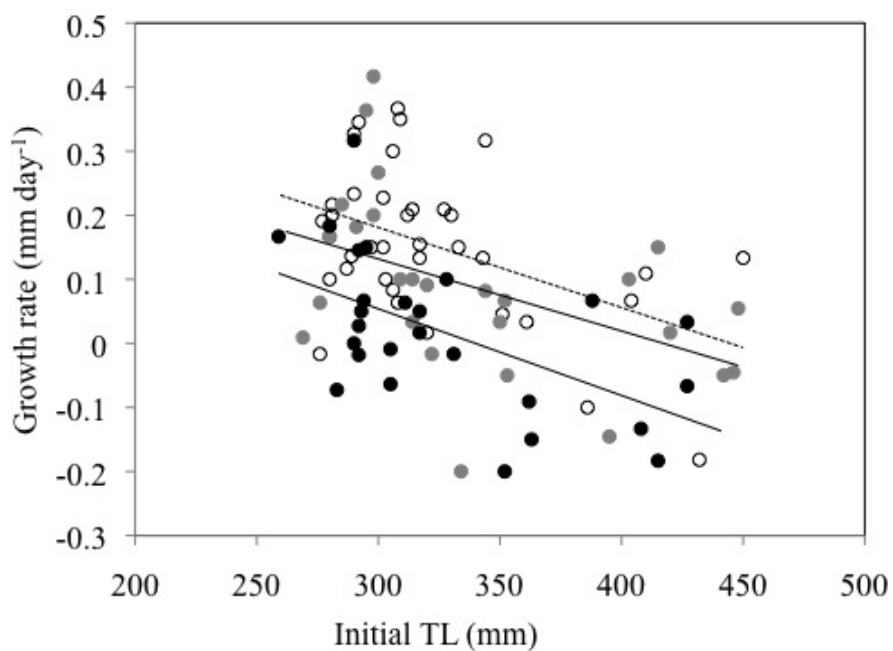
Figure 6.1. Growth rate (change in TL in mm per day) plotted as a function of initial TL

for cortisol-treated (n=26), sham-treated (n=31), and control (n=37) largemouth bass.

Cortisol-treated fish are represented by closed black circles (●), sham-treated fish are

represented by the closed grey circles (●), and control fish are represented by the open

circles (○). The mean growth rate for the cortisol-treated fish was lower than the mean

growth rate for the sham-treated and control fish (p<0.05; see text for statistical details).
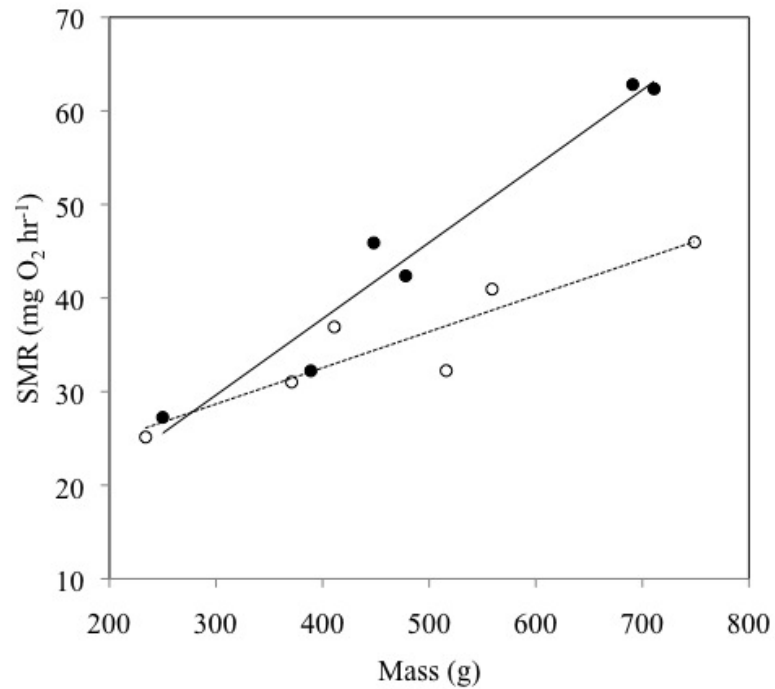
Figure 6.2. Whole-animal standard metabolic rates depicted as a function of mass and treatment group (control and cortisol-treated fish; n=6 in each group). Cortisol-treated fish are represented by closed circles (●), while control fish are represented by open circles (○). Mass, treatment group, and the interaction effect were all significant ($p < 0.05$; see text for statistical details).
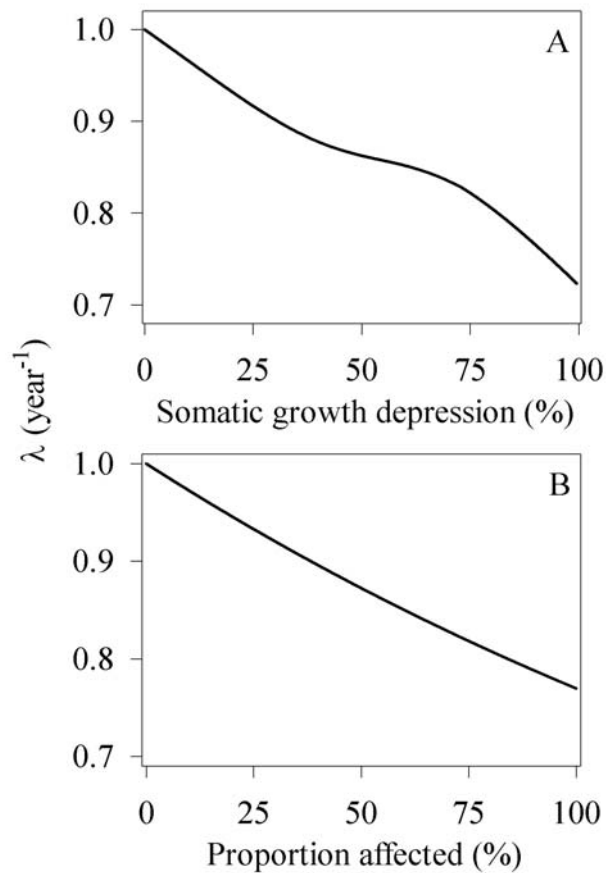
Figure 6.3. The demographic cost of endocrine stress estimated by Leslie matrix projection modelling. (A) Estimated population rates of increase (λ) plotted against hypothetical somatic growth depression, with all individuals of the population being assumed to experience the same level of growth depression. (B) Estimated population rates of increase (λ) plotted against the proportion of population experiencing an 89% somatic growth depression for a single growing season, which corresponds to the empirical estimate.

6.7. Tables

Study component	Cortisol	Sham	Control	Fed	Fasted	F-statistic	t-ratio	df	p-value
Full group caught in June (TL)	326.7 ± 47.8 (69)	331.6 ± 50.1 (69)	325.4 ± 44.0 (69)	---	---	0.33	---	204	0.72
Subset recaptured in August (TL)	337.4 ± 12.9 (19)	336.2 ± 13.8 (16)	322.7 ± 9.0 (23)	---	---	0.58	---	55	0.58
Subset recaptured in October (TL)	307.5 ± 6.5 (5)	339.4 ± 19.1 (13)	331.3 ± 16.4 (10)	---	---	0.67	---	25	0.51
Subset recaptured twice (TL)	298.5 (2)	317.0 (2)	299.7 ± 5.6 (4)	---	---	3.12	---	4	0.15
Laboratory feeding study (TL)	---	---	---	310.0 ± 17.6 (6)	296.7 ± 23.3 (6)	---	0.21	10	0.66
Laboratory feeding study (mass)	---	---	---	381.7 ± 69.8 (6)	373.3 ± 65.6 (6)	---	0.09	10	0.93
Metabolic rate measurements (TL)	330.2 ± 17.9 (6)	---	324.3 ± 17.5 (6)	---	---	---	0.24	10	0.82
Metabolic rate measurements (mass)	494.5 ± 72.7 (6)	---	473.3 ± 72.3 (6)	---	---	---	0.20	10	0.84

Table 6.1. TL (in mm; mean ± SEM; sample sizes are listed in parentheses) and mass (in g; mean ± SEM; sample sizes are listed in parentheses) measurements for fish included in the study. Shown are the TLs of cortisol-treated, sham-treated and control largemouth bass in June; initial TLs of fish recaptured only in August, only in October, and in both August and October; TLs and masses of fish used to obtain fasted and fed biochemical indices; and TLs and masses of fish used for metabolic rate measurements. Within each study, there were no statistical differences among the treatment groups (p>0.05; see text for statistical details).

Recaptured subset	Cortisol	Sham	Control
Fish recaptured once	24	29	33
Fish recaptured twice	2	2	4
Fish never recaptured	43	38	32

Table 6.2. Number of fish recaptured once, twice, or never recaptured in the cortisol-treated, sham-treated, and control groups of fish. There were 69 fish treated in each group in June. There are no differences among the treatment groups in the ratios of fish captured once, fish recaptured twice, and fish never recaptured among treatment groups ($p > 0.05$; see text for statistical details).

Theoretical % mortality (relative to the control group)	Power (1-ß)
5	0.05
10	0.10
20	0.18
30	0.46
50	0.86
90	0.99

Table 6.3. Results of a post-hoc power analysis to determine the power of detecting a decline in recapture rate in the sham-treated or cortisol-treated group relative to the control group, using a conservative estimate of 24 fish recaptured once per treatment group across the monitoring period. Power analysis reveals that biologically relevant declines would be difficult to detect given the small sample size.

Parameter	Control	Sham	Cortisol	F-statistic	Error df	p-value
Aspartate transaminase (U L^{-1})	40 ± 12.2	46 ± 10.6	38 ±10.6	0.14	19	0.87
Ca^{2+} (mmol L^{-1})	3.4 ± 0.08	3.5 ± 0.07	3.5 ± 0.07	0.67	19	0.52
Cl^{-} (mmol L^{-1})	112 ± 3.3	123 ± 2.9	119 ± 2.9	3.08	19	0.07
Cholesterol (mmol L^{-1})	15 ± 1.8	13 ± 1.6	17 ± 1.6	1.57	19	0.23
Glucose (mmol L^{-1})	2.5 ± 0.10	2.6 ± 0.09	2.4 ± 0.09	1.25	19	0.31
Mg^{2+} (mmol L^{-1})	1.2 ± 0.05	1.3 ± 0.04	1.2 ± 1.04	1.79	19	0.19
P^{3-} (mmol L^{-1})	2.4 ± 0.19	2.6 ± 0.16	2.4 ± 0.16	0.41	19	0.67
K^{+} (mmol L^{-1})	3.0 ± 0.3	3.4 ± 0.2	3.5 ± 0.2	0.82	19	0.45
Na^{+} (mmol L^{-1})	167 ± 3.8	178 ± 3.3	174 ± 3.3	2.45	19	0.11
Total protein (g L^{-1})	40 ± 2.0	42 ± 1.7	42 ± 1.7	0.24	19	0.79
Triglycerides (mmol L^{-1})	3.9 ± 0.63	2.8 ± 0.55	2.6 ± 0.55	1.22	19	0.32

Table 6.4. Plasma biochemistry (mean ± SEM) of cortisol-treated (n=8), sham-treated (n=8), and control (n=6) wild largemouth bass prior to treatment in June. There are no statistical differences in plasma biochemistry among the groups (α=0.05).

Parameter	Control	Sham	Cortisol	F-statistic	Error df	p-value
Aspartate transaminase (U L^{-1})	37 ± 6.4	56 ± 6.4	37 ± 6.7	2.97	41	0.06
Ca^{2+} (mmol L^{-1})	3.2 ± 0.06	3.3 ± 0.06	3.3 ± 0.06	0.36	41	0.70
Cl^{-} (mmol L^{-1})	**114 ± 2.9^{AB}**	**120 ± 2.9^{A}**	**109 ± 3.0^{B}**	**3.57**	**41**	**0.04**
Cholesterol (mmol L^{-1})	12 ± 0.8	12 ± 0.8	12 ± 0.8	0.02	41	0.98
Glucose (mmol L^{-1})	2.4 ± 0.12	2.7 ± 0.12	2.6 ± 0.12	1.67	41	0.20
Mg^{2+} (mmol L^{-1})	2.3 ± 0.11	2.5 ± 0.11	2.4 ± 0.11	0.63	41	0.54
P^{3-} (mmol L^{-1})	2.3 ± 0.11	2.5 ± 0.11	2.4 ± 0.11	0.63	41	0.54
K^{+} (mmol L^{-1})	2.6 ± 0.31	2.6 ± 0.31	2.4 ± 0.32	0.10	41	0.90
Na^{+} (mmol L^{-1})	166 ± 1.8	170 ± 1.8	167 ± 1.9	1.41	41	0.26
Total protein (g L^{-1})	39 ± 1.0	39 ± 1.0	41 ± 1.1	1.96	41	0.15
Triglycerides (mmol L^{-1})	2.7 ± 0.40	1.9 ± 0.40	1.9 ± 0.42	1.19	41	0.31

Table 6.5. Plasma biochemistry (mean ± SEM) of cortisol-treated (n=15), sham-treated (n=15), and control (n=14) wild largemouth bass 54 to 63 days after treatment, in August. Bold text indicates variables for which statistically significant differences were detected; treatment groups that differed are indicated with different letters (α=0.05).

Parameter	Control	Sham	Cortisol	F-statistic	Error df	p-value
Aspartate transaminase (U L^{-1})	30 ± 11.9	57 ± 11.9	40 ± 14.2	1.26	24	0.30
Ca^{2+} (mmol L^{-1})	3.5 ± 0.12	3.6 ± 0.12	3.3 ± 0.14	1.51	24	0.24
Cl^{-} (mmol L^{-1})	114 ± 4.7	100 ± 4.7	125 ± 5.7	3.04	24	0.07
Cholesterol (mmol L^{-1})	13 ± 1.4	14 ± 1.4	12 ± 1.7	0.67	24	0.52
Glucose (mmol L^{-1})	**2.2 ± 0.19^{AB}**	**2.8 ± 0.19^{A}**	**2.0 ± 0.23^{B}**	**4.55**	24	**0.02**
Mg^{2+} (mmol L^{-1})	1.4 ± 0.05	1.4 ± 0.05	1.3 ± 0.06	0.24	24	0.79
P^{3-} (mmol L^{-1})	2.4 ± 0.12	2.3 ± 0.12	2.4 ± 0.14	0.09	24	0.91
K^{+} (mmol L^{-1})	2.1 ± 0.37	2.0 ± 0.37	2.6 ± 0.45	0.66	24	0.52
Na^{+} (mmol L^{-1})	173 ± 1.9	170 ± 1.9	171 ± 2.3	0.56	24	0.58
Total protein (g L^{-1})	39 ± 1.4	40 ± 1.4	38 ± 1.7	0.66	24	0.53
Triglycerides (mmol L^{-1})	2.5 ± 0.71	2.1 ± 0.71	3.6 ± 0.84	1.00	24	0.38

Table 6.6. Plasma biochemistry (mean ± SEM) of cortisol-treated (n=7), sham-treated (n=10), and control (n=10) wild largemouth bass 104 to 115 days after treatment, in October. Bold text indicates variables for which statistically significant differences were detected; treatment groups that differed are indicated with different letters (α=0.05).

Parameter	Fed	Fasted	t-ratio	df	p-value
Aspartate transaminase (U L^{-1})	37 ± 19	57 ± 19	0.73	8	0.48
Ca^{2+} (mmol L^{-1})	3.2 ± 0.1	3.2 ± 0.1	0.09	8	0.92
Cl^- (mmol L^{-1})	75 ± 5	88 ± 5	1.89	8	0.09
Cholesterol (mmol L^{-1})	12 ± 1	15 ± 1	1.74	8	0.11
Glucose (mmol L^{-1})	9.9 ± 1.0	6.8 ± 1.0	-2.13	8	0.06
Mg^{2+} (mmol L^{-1})	1.3 ± 0.0	1.3 ± 0.0	-0.66	8	0.53
P^{3-} (mmol L^{-1})	1.6 ± 0.1	1.9 ± 0.1	1.79	8	0.11
K^+ (mmol L^{-1})	2.8 ± 0.3	3.2 ± 0.3	0.85	8	0.42
Na^+ (mmol L^{-1})	**145 ± 2**	**153 ± 2**	**2.86**	**8**	**0.02**
Total protein (g L^{-1})	49 ± 2	47 ± 2	-0.77	8	0.46
Triglycerides (mmol L^{-1})	**2.6 ± 0.4**	**0.6 ± 0.4**	**-3.59**	**8**	**<0.01**

Table 6.7. Plasma biochemistry (mean ± SEM) of fed and fasted wild largemouth bass in the laboratory. Bold text indicates statistical differences (α=0.05).

GENERAL CONCLUSIONS

In this thesis, I have integrated theoretical insights from life-history literature with physiological mechanisms to explore the mediators and consequences of physiological stress in wild fishes, specifically in black bass. I first focused on the interactions among life-history traits and endocrine regulation during parental care to explore how life-history traits may mediate the physiological stress response. I then expanded the scope of the thesis to investigate longer-term individual and population-level consequences of sub-lethal physiological stress.

7.1. Synthesis. In summary, the results of Chapter 2 indicated that the glucocorticoid stress response and circulating androgens during parental care are correlated with life-history variation within a long-term study population of fish. In particular, endocrine parameters are correlated with measures of reproductive history and parental quality, indicating that regulation of the endocrine system may serve as a mechanism increasing reproductive success with age in teleost fish. However, endocrine parameters are not correlated with a measure of the value of the current reproductive opportunity (i.e., brood size). In Chapter 3, I expanded upon these findings to investigate whether endocrine parameters are influenced by current life-history stage, and I conducted an experiment to confirm the correlative finding that endocrine parameters are not correlated with brood size. The results of Chapter 3 demonstrated that the glucocorticoid stress response is attenuated during parental care, and confirmed that the glucocorticoid stress response is

not influenced by measures of the value of the current reproductive opportunity.

Together, the results of Chapters 2 and 3 demonstrated that attenuation of the

glucocorticoid stress response during parental serves as a broad-scale mechanism

maintaining investment in parental care behaviours.

In Chapter 4, I expanded upon these results, and conducted an experimental

manipulation of circulating cortisol during parental care. This experiment explored how

a sustained glucocorticoid stress response may divest reproductive investment away from

parental care. I found that a sustained elevation of circulating cortisol during parental

care is associated with increased nest abandonment. However, parents maintain parental

care behaviours prior to abandonment. At the mechanistic level, results suggest that a

sustained glucocorticoid stress response divests investment from parental care through

indirect mechanisms (e.g., an energetic cost) rather than by directly influencing parental

behaviours.

Chapter 5 and 6 moved beyond the parental care period, and explored the longer-

term consequences of a sustained glucocorticoid stress response within the framework of

the physiology/life-history nexus. The results of Chapter 5 demonstrated that a

glucocorticoid stress response is associated with long-term carryover effects, even after a

period of apparent recovery. In this case, a 5-day elevation of circulating cortisol was

associated with accelerated mortality during a challenge that occurred 5 months

following the cessation of the experimentally induced physiological stress response. In

Chapter 6, I explored potential mechanisms to explain this phenomenon, and investigated

the long-term energetic consequences of physiological stress at the individual level. I

found that the physiological stress response is associated with a long-term decrease in individual somatic growth rate. Furthermore, I explored whether such individual consequences can translate to population-level changes. I demonstrated through a modelling exercise that the documented decreases in somatic growth are sufficient to decrease the population growth rate. These impacts are likely an underestimate, given that the population model does not take into account the documented reduction in reproductive success from Chapter 4, or the accelerated mortality from Chapter 5.

Taken together, the results of the thesis establish that life-history stage and individual life-history variation influence the primary (i.e., endocrine) stress response, as well as the secondary and tertiary (i.e., physiological and whole-animal) consequences of endocrine stress in black bass. The secondary and tertiary consequences of endocrine stress can be long lasting, and have consequences for population dynamics of wild populations. These results emphasize the value in integrating life-history measurements into the study of stress in wild fish.

7.2. Future directions. The results of this thesis lead to new questions. In particular, many questions arise regarding the mechanisms underlying some of the documented patterns. For example, in Chapters 2 and 3, I found a positive relationship between water temperature and post-stress circulating cortisol concentrations. This finding is in many respects unsurprising given that fish are ectothermic animals, and that there is a well-documented positive relationship between water temperature and the rate and/or magnitude of cortisol increase following a challenge (e.g., Barton and Schreck 1987;

Pottinger and Carrick 2000; Davis et al. 2001; Lyytikainen et al. 2002; King et al. 2006; Lima et al. 2006). What remains unclear is whether secondary and tertiary effects (i.e., physiological or behavioural responses) downstream of the primary (i.e., endocrine) stress response also increase proportionally with the increase in water temperature. Further mechanistic studies investigating secondary and tertiary stress effects across a range of temperatures are necessary in order to understand the functional significance of the positive relationship between water temperature and post-stress circulating cortisol concentrations. Similarly, it would be beneficial to explore other aspects of the HPI axis in response to temperature. Factors such as down-regulation of cortisol receptors or cortisol binding proteins with changing water temperature may make the documented changes in circulating cortisol concentrations less biologically relevant. These results also emphasize the importance of integrating water temperature into any future research concerned physiology and life history in an ectothermic animal living in a highly seasonal environment.

Why did endocrine regulation during parental care not reflect brood size in Chapters 2 and 3? Previous research has demonstrated that smallmouth bass are sensitive to brood size, in that manipulating brood size affects aggressive nest defence behaviour (Ridgway 1989; Suski et al. 2003). The results of Appendix II and Chapter 4 may provide a clue. The results of Appendix II demonstrate that smallmouth bass do not have a cortisol response when faced with a simulated brood predator, while in Chapter 4 a cortisol implant did not influence parental care behaviours. From these results, it appears that cortisol does not play a role in mediating fine-scale parental care behaviour in

smallmouth bass. Fine-scale adjustments in aggressive parental care behaviour in response to brood size (i.e., Ridgway 1989; Suski et al. 2003) are likely mediated by different physiological mechanisms (e.g., androgens, prolactin) than adjustment of circulating cortisol titres. This finding is interesting because birds with enlarged broods display an attenuated corticosterone response to a standardized stressor during parental care (Lendvai et al. 2007). Further mechanistic studies are necessary in order to understand whether this is difference in consistent between the two taxa, and to understand the importance of this divergence.

One of the limitations of Chapters 4, 5, and 6 was the use of a supraphysiological dose of cortisol, a consequence of the field-based nature of these experiments. However, I developed a modification to the cortisol implant approach that yields physiologically relevant concentrations of circulating cortisol. This dose is outlined in Appendix I, and was successfully employed by Dey et al. (2010) to elevate circulating cortisol in parental smallmouth bass. Dey et al. (2010) also provide partial support for the results obtained with the supraphysiological cortisol dose employed in Chapters 4, 5, and 6. Parental smallmouth bass treated with a physiological dose of cortisol displayed the same transient behavioural resistance and early nest abandonment as the largemouth bass given a supraphysiological dose in Chapter 4. At least in this case, the downstream effects of physiological and pharmacological doses were consistent with one another. However, one unanswered question from Chapter 5 would certainly be better addressed using a physiological cortisol dose. In Chapter 5, the cortisol-treated fish displayed lower activity rates when faced with a secondary challenge than control and sham-treated fish.

The question remains, was this lower activity the result of energetic constraints, or the result of a change in the functioning of the HPI-axis (e.g., long-term changes to receptor sensitivity)? And if is the latter, would such a change in the HPI-axis occur following a physiological dose? Further research using a physiological dose of cortisol is necessary to understand the mechanisms underlying the documented behavioural changes.

A final mechanistic question that arises from the results of Chapter 3, 4, and 5 involves the specific mechanism driving the energetic cost to stress. The results of Chapter 3 confirm that elevated cortisol levels carry an immune cost in wild fish, as has been shown in previous laboratory studies (see reviews by Barton 2002; Loiseau et al. 2008). The results of Chapter 5 confirm that elevated cortisol levels carry at least a short-term metabolic cost in wild fish, again, as has been shown in laboratory populations (e.g., Lankford et al. 2005). Finally, previous laboratory studies have suggested that elevated cortisol levels are associated with reduced long-term feeding in fish (e.g., Barton et al. 1987; Gregory and Wood 1999), although we could not confirm this result using biochemical indices in wild fish in Chapter 5. It would be interesting and valuable to disentangle how long-term changes in immune function, metabolic rate, and feeding behaviour are interacting to result in the growth rate depression documented in Chapter 5. For example, does metabolic rate remain elevated beyond the duration of the cortisol elevation? And how do the costs associated with immune function change during recovery from a stress response?

As well as raising mechanistic questions, this thesis raises further ecological questions. The findings of this dissertation indicate that fish respond differentially to

stressors based on their life history, and also that responding to stressors can have long-term consequences at the individual and population levels. This link between individual physiology and population dynamics is a first step towards understanding the evolutionary consequences of stress (Calow and Forbes 1998). However, the next step is to understand the dynamics of the interaction between life history and endocrine regulation. For example, if allostatic load increases, will life-history trade-offs change? As individuals need to invest a greater proportion of their energy budget into responding to stressors, will there be population-level shifts in life-history variation? Taking this one step further, some evidence suggests that evolution is possible over very short timescales (Ashley et al. 2003), and it has also been established that stress coping style (i.e., the extent of the cortisol stress response following a challenge) is a heritable trait (Pottinger and Carrick 1999; Øverli et al. 2002), and therefore subject to selection. As external challenges continue to increase, will we see a corresponding shift in how populations and species deal with the additional stressors? Such questions are particularly relevant as anthropogenic changes continue to impact natural ecosystems. In addressing these and similar questions, it will be necessary to continue using an integrative framework. The combination of controlled mechanistic studies with broad-scale ecological studies is required to understand the ecology of stress, and to predict the population-level consequences of stress in wild animals.

APPENDIX I

METHODOLOGY FOR ACHIEVING PHYSIOLOGICAL LEVELS OF

CIRCULATING CORTISOL USING EXOGENOUS CORTISOL IMPLANTS IN

BLACK BASS

i.i. Introduction

Chapters 4, 5, and 6 of this thesis included experimental manipulation of circulating

cortisol concentrations. In these experiments, the methodology used resulted in

supraphysiological concentrations (i.e., concentrations that were higher than those that

naturally occur during an endogenous stress response). Owing to the field-based nature

of Chapters 4, 5, and 6 experiments could not be repeated, and the supraphysiological

dose remains a study limitation for this thesis.

For future studies where a physiological cortisol dose is desired, I present here a

methodology that achieves physiological concentrations of circulating cortisol in black

bass.

i.ii. Materials and methods

i.ii.i. Study animals and cortisol dosage. To verify the dosage, n=10 largemouth bass

were caught by electrofishing from reservoirs in the area surrounding the Sam Parr

Biological Station near Kinmundy, Illinois (38°46'N, 88°50'W) on March 24, 2007. Fish

were held in a common 400 L tank with flow-through reservoir water for 24 hrs to allow

them to recover from electrofishing. Following the recovery period, a subset of

largemouth bass (n=6) was measured (TL; 398 ± 8 mm [mean $\pm$ SEM]), weighed, and

injected with an intraperitoneal injection of cocoa butter (5 mL kg^{-1} body weight)

impregnated with 10 mg mL^{-1} hydrocortisone 21-hemisuccinate (Sigma H4881; Sigma-

Aldrich Inc., St. Louis, MO). The remaining fish (n=4) were weighed and measured (395

$\pm$ 12 mm), but not injected. Fish were held singly in covered raceways with flow-through

reservoir water for 24 hrs. Fish were quickly netted out of raceways and non-lethally

sampled for blood as described previously (Chapter 2, Section 2.3.2). Sampling for each

fish took <90 s. Blood samples were handled and stored as described previously

(Chapter 2, Section 2.3.2).

i.ii.ii. Hormone analysis. Cortisol concentrations were determined as described

previously (Chapter 2, Section 2.3.4). All samples for each species were run in a single

assay. Intra-assay variability was 6.2 %.

i.ii.iii. Statistical analysis. Basic descriptive statistics (mean, range, SEM) were

generated for circulating cortisol concentrations in each group of fish. Analyses were

performed in the statistical packages JMP, version 7.0.1 (SAS Institute Inc., Cary, NC).

i.iii. Results and discussion

The methodology described above resulted in an elevated, but physiological, level of

circulating cortisol in largemouth bass (Table i.i). The same methodology successfully

achieved similar physiological levels in smallmouth bass (Dey et al. 2010). The

circulating cortisol concentrations achieved with the implants were variable, but

parameters fell within the endogenous cortisol concentrations observed following

exhaustive exercise in largemouth bass (Chapter 4, Section 4.4.1). Further studies

examining the time course of this injection are necessary.

i.iv. Tables

	[cortisol] (ng mL^{-1})			
	Mean	Minimum	Maximum	SEM
Control	6	2	8	1
Cortisol-treated	125	50	222	32

Table i.i. Circulating cortisol concentrations for control and cortisol-treated largemouth bass using a revised dosage.

APPENDIX II

CIRCULATING ANDROGENS ARE INFLUENCED BY PARENTAL

NEST DEFENCE

ii.i. Abstract

While social interactions influence vertebrate endocrine regulation, the dynamics of regulation in relation to specific behaviours have not been clearly elucidated. In the current study, we investigated whether androgens or glucocorticoids (cortisol) play a functional role in aggressive offspring defence behaviour in wild smallmouth bass, a teleost fish with sole paternal care. We measured circulating androgens and cortisol concentrations in plasma samples taken from parental males following a simulated nest intrusion by a common nest predator, the bluegill sunfish. To understand whether endocrine regulation changes across the parental care period, we looked both at males guarding fresh eggs and at males guarding hatched embryos. Plasma androgen levels increased in males subjected to a simulated nest intrusion when compared to sham controls. Androgen concentrations in males guarding egg-sac fry were lower than in males guarding fresh eggs, but circulating androgens was positively correlated with the level of aggression towards the nest predator at both offspring development stages. However, there was no increase in cortisol levels following a simulated nest intrusion, and no relationship between cortisol and any measured parameter. These results suggest that androgens play an important role in promoting aggressive nest defence behaviour in teleost fish.

ii.ii. Introduction

The challenge hypothesis, proposed in a classic paper by Wingfield et al. (1990),

provides a framework for understanding patterns of androgen regulation across the

vertebrates. As originally outlined, the challenge hypothesis was based on the premise

that elevated androgens are important in male vertebrates for mate attraction, territory

defence, and reproduction, but are generally incompatible with paternal care (Hegner and

Wingfield 1987; Silverin 1986). Thus, the endocrine response of a male to a territorial

challenge during paternal care will depend on several factors, notably the frequency of

territorial challenges, the mating system, and the level of paternal care. For example, an

individual with few territorial challenges, a monogamous mating system, and high

paternal care is expected to maintain androgens at relatively low levels, and respond to a

territorial challenge by rapidly and transiently increasing circulating androgens.

Alternately, an individual with frequent territorial challenges, a polygamous mating

system, and little to no paternal care is expected to maintain continually high androgen

concentrations, and a further increase in response to a territorial challenge may not be

possible (Wingfield et al. 1990). This general pattern has been observed in a range of

vertebrates (see reviews for birds, Wingfield et al. 1990; fish, Hirschenhauser et al. 2004;

across taxa, Hirschenhauser and Oliveira 2006).

One postulate of the challenge hypothesis is that androgens are involved only in

conspecific aggression (e.g., competition over mates or territories), while other types of

aggression (e.g., anti-predator aggression) are regulated by different proximate

mechanisms (Wingfield et al. 1990). An interesting exception may be aggressive

offspring defence by parental care-providing teleost fish. In fish, parental care is thought

to have arisen from ancestral territory defence (Gross and Sargent 1985), and unlike in

other taxa, parental care often continues despite high androgen levels (e.g., Desjardins et

al. 2005; Dey et al. 2010).

Cortisol, the primary glucocorticoid in teleosts, also may play an important role in

mediating aggressive nest defence behaviour in teleost fish. Glucocorticoids have been

implicated in mobilizing the energy reserves required for energetically costly behaviours

such as nest defence (Nelson 2000). However, glucocorticoids also play an important

role in mediating stress (Mommsen et al. 1999; Sapolsky et al. 2000), and high levels of

glucocorticoids are often associated with reproductive suppression (Schreck et al. 2001;

Fuzzen et al. 2011), reduced annual reproductive activity (e.g., Magee et al. 2006), and

nest abandonment (e.g., Chapter 4, Section 4.4.3).

The current study investigated the potential roles of circulating androgens and

cortisol in aggressive nest defence behaviour in a teleost fish, the smallmouth bass.

Specifically, we determined whether a simulated intrusion by a potential nest predator

would elicit an androgen or a cortisol response in parental care-providing fish. To

understand whether the androgen or cortisol response is maintained across parental care,

we studied smallmouth bass guarding fresh eggs versus embryos. Smallmouth bass

spawn annually, with males establishing territories and clearing an area of substrate for

the nest, and then providing sole paternal care to a single brood of offspring for

approximately 6 weeks (Coble 1975). With this social system, the challenge hypothesis

predicts high androgen levels during territory establishment, and decreasing levels across

parental care, a pattern that has been documented in the smallmouth bass (Chapter 3, Section 3.4.2). The challenge hypothesis further predicts that a conspecific territorial challenge during parental care will elicit a transient and rapid androgen response. If androgens perform a functional role in aggressive nest defence during parental care, then we predict that a simulated nest intrusion by a nest predator (i.e., a small fish that is not a threat to the territory or male) should elicit an increase in circulating androgen concentrations. Further, if cortisol liberates stored energy reserves to fuel aggression, then an increase in circulating cortisol levels following a simulated nest intrusion should also occur.

ii.iii. Materials and methods

ii.iii.i. Study animals and field data collection. On May 25 and 26, 2008 (water temperature 15 - 16ºC), male smallmouth bass guarding nests with fresh eggs (0 - 1 day after laying and fertilization) were identified on Charleston Lake, a public lake that is part of the Gananoque River system in eastern Ontario (44°32'N, 75°59"W). All nests were individually marked with a numbered tile, and the brood size of each nest was classified according to egg score. The egg score is a standard and highly repeatable measure of the relative number of eggs within a nest, and ranges from 1 (low, <500) to 5 (high, >4000; Philipp et al. 1997; Suski et al. 2003). For standardization, only males guarding nests with intermediate egg scores of 3 - 4 were included in this study.

A subset of parental fish was sampled immediately. Fish were first subjected to a simulated nest predator intrusion. Bluegill sunfish are common nest predators of

smallmouth bass, and simulated nest intrusions of bluegill sunfish elicit aggressive nest

defence behaviours (e.g., Hanson et al. 2009). A bluegill sunfish (TL 150-200 mm) in a

3.8 L glass jar was placed in the nest by a snorkeler who then retreated to a distance of

approximately 2 m for 3 min of behavioural observations. Aggressive behaviours were

defined in Chapter 4. Briefly, a "yawn" is when the parent opens and closes its mouth in

the direction of the glass jar, a "charge" is when the parent moves rapidly towards the jar

but without making physical contact, and a "hit" refers to the smallmouth bass making

physical contact with the jar. Snorkelers recorded the sum of all three aggressive

behaviours for each individual during the 3 min period. After 3 min, the snorkeler

removed the jar. After 25 min, a standard time to allow circulating cortisol

concentrations to rise following a stressor (e.g., Chapters 2 and 3) and also sufficient for

androgens to increase following a simulated territorial intrusion in cichlid fish (e.g.,

Desjardins et al. 2005), fish were quickly captured by rod-and-reel angling and sampled

for 1 mL of blood as described previously (Chapter 2, Section 2.3.2). Only fish that

could be captured and blood sampled within 5 min of the first angling attempt were

included in the study. Fish were measured (TL) and released. In total, blood samples

were obtained from 24 males (TL=399.1 ± 8.7 mm [mean ± SEM]) guarding fresh eggs.

Blood samples were also obtained from 6 males (TL=413.8 ± 17.4 mm) selected for a

"sham" treatment, in which an empty jar was placed in the nest. On June 5 2008 (water

temperature 17.5 ºC), smallmouth bass guarding egg-sac fry (TL=399.2 ± 9.8 mm, n=19)

were subjected to a simulated nest intrusion and sampled for blood as described above.

Blood samples were handled and stored as described previously (Chapter 2, Section

2.3.2).

ii.iii.ii. Hormone analysis. Cortisol concentrations were determined as described previously (Chapter 2, Section 2.3.4). All samples were run in a single assay. Intra-assay variability was 7.3 %. Androgen concentrations were also determined as described previously (Chapter 3, Section 3.3.3). All samples were run in a single assay, and intra-assay variability was 5.2 %. There was insufficient plasma to run an androgen assay for 2 males guarding fresh eggs (resulting in a sample size of 22) and for 1 male guarding egg-sac fry (resulting in a sample size of 18).

ii.iii.iii. Statistical analysis. No difference in male TL was found among the groups ($F_{2,46}$=0.314, p=0.73). Therefore, this parameter was not included as a covariate, and Student's *t*-tests were used to compare aggression scores, circulating cortisol levels, and circulating androgen levels between fish guarding fresh eggs subjected to the simulated nest intrusion, and the sham group. To investigate whether the intensity of aggression was related to hormone levels, ANCOVA models were run with androgen or cortisol as the independent variable, offspring development stage (eggs or egg-sac fry) as the dependent variable, and aggression (aggressive behaviours in 3 min) as the covariate. The interaction between group and aggression was included in the model.

For all tests, residuals were tested for normal distribution using goodness-of-fit tests, and for homogeneity of variance using Levene's test or by visual inspection (Zar 1999). Assumptions were met in all but one case (see Results and Discussion). All

analyses were performed in the statistical packages JMP, version 7.0.1 (SAS Institute Inc., Cary, NC). The level of significance for all tests (α) was 0.05. All results are stated as mean ± SEM.

ii.iv. Results and discussion

ii.iv.i. Did parental smallmouth bass respond to the bluegill as a nest intrusion?

Parental smallmouth bass exhibited aggressive behaviour towards a bluegill in a jar, and showed no behavioural response to an empty glass jar placed in the nest (Table ii.i). This difference was statistically significant (t_{28}=-6.019, p<0.01), although the uniform zero scores in the sham treatment group violated the test's assumptions.

ii.iv.ii. Is there a role for androgens in aggressive nest defence?

Fish subjected to a simulated brood predator displayed higher circulating androgen levels than those subjected to the empty glass jar (t_{26}=-2.481, p=0.02; Table ii.i). Furthermore, a significant positive correlation was detected between circulating androgen level and the intensity of the aggressive response (full ANCOVA model R^2=0.555, $F_{3,36}$=14.963, p<0.01; aggression term in ANCOVA $F_{1,1}$=4.821, p=0.03; Fig ii.iA). As found in previous studies in this species (Chapter 3, Section 3.4.2) and in the plainfin midshipman fish (*Porichthys notatus*; Knapp et al. 1999), circulating androgen concentrations were higher in fish guarding eggs than in fish guarding egg-sac fry (offspring development stage term in ANCOVA $F_{1,1}$=9.873, p<0.01). There was no significant influence of offspring development stage on the positive correlation between circulating androgen

concentration and aggression (offspring development stage x aggression interaction term in ANCOVA $F_{1,1}$=0.749, p=0.39). Collectively these results demonstrate that circulating androgens are elevated following aggressive nest defence, and this elevation correlates with the intensity of aggressive behaviour.

Hanson et al. (2009) measured circulating androgen levels in smallmouth bass immediately (less than 5 min) following a simulated nest intrusion by a bluegill sunfish and failed to detect a relationship between parental aggression and circulating androgen concentration. The apparent discrepancy in results between Hanson et al. (2009) and the current study is explained by the different time courses of sampling: androgen concentrations in Hanson et al. (2009) likely reflected baseline levels, while androgen concentrations in the current study (collected 25 min after disturbance) reflect post-intrusion levels. Collectively, the results strongly suggest that circulating androgen concentrations transiently increase during parental care in response to a threat to offspring, and play a role in aggressive nest defence. This is supported by a previous study in which parental smallmouth bass treated with an androgen receptor antagonist (cyproterone acetate) displayed reduced aggression towards a simulated nest predator when compared with control paternal fish (Dey et al. 2010). To the best of our knowledge, this is the first example of an androgen increase in a teleost fish in response to what is strictly a threat to the offspring rather than to a conspecific territorial challenge. Overall, however, this finding is consistent with the challenge hypothesis (Wingfield et al. 1990).

ii.iv.iii. Is there a role for cortisol in aggressive nest defence? Cortisol levels of

smallmouth bass subjected to the simulated nest intrusion did not differ from those

subjected to the empty glass jar (t_{28}=0.028, p=0.97; Table ii.i); all parental smallmouth

bass exhibited low plasma cortisol concentrations (Fig ii.iB). Moreover, neither

aggressive behaviour nor offspring development stage affected these uniformly low

plasma cortisol values (full ANCOVA model $F_{3,39}$=0.701, R^2=0.051, p=0.56; Fig ii.iB).

Thus, in the smallmouth bass, cortisol does not appear to play a role in aggressive nest

defence behaviour. This finding is interesting because aggressive nest defence is

energetically costly (Cooke et al. 2002a) and cortisol mobilizes energy reserves during a

stress response (Mommsen et al. 1999). Our results imply that a nest predator does not

invoke a stress response, and that aggression and stress are uncoupled in this system.

This result is consistent with previous results in parental plainfin midshipman fish,

showing no relationship between offspring development stage and circulating cortisol

(Knapp et al. 1999), but contrasts with aggressive social interactions, where cortisol is

elevated and may play a significant role in mobilizing energy reserves (e.g., Gilmour et

al. 2005). Parental aggression may also be a measure of parental quality (e.g., Tolonen

and Korpimäki 1994).

ii.iv.iv. Summary. The positive correlation between aggression towards a nest predator

and circulating androgen concentration provides support for the hypothesis that

androgens play a role in aggressive nest defence behaviour in teleost fish. The androgen

response to nest predators resembled that proposed by the challenge hypothesis for male-

male conspecific territorial challenges. However, we found no evidence that cortisol

functioned in aggressive nest defence behaviour in this system.

ii.v. Figures
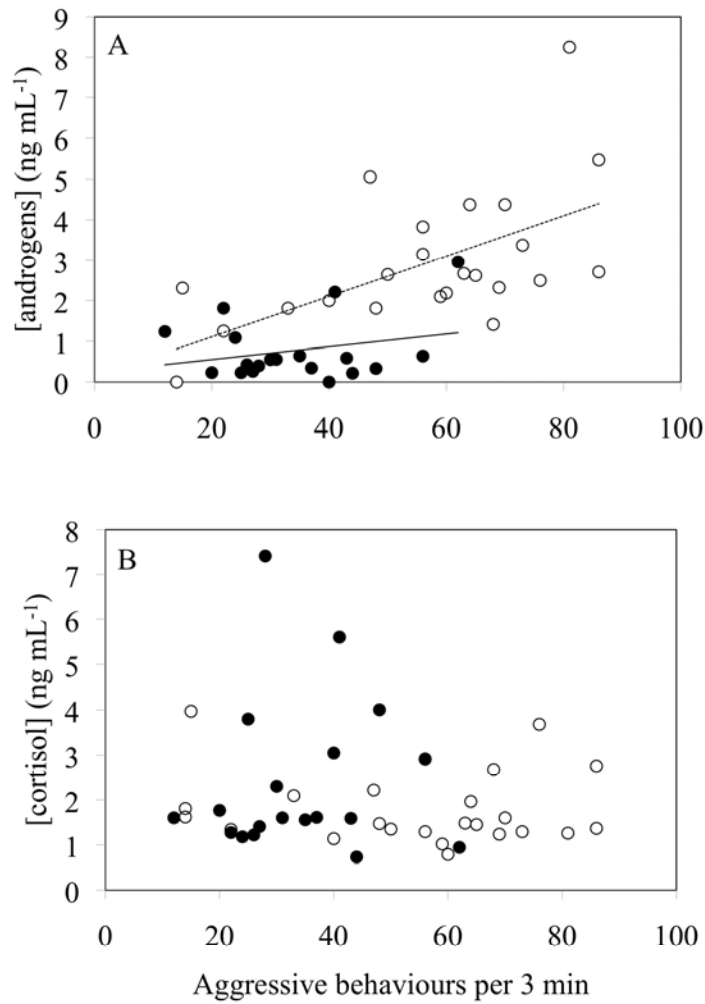
Figure ii.i. Circulating (A) androgen and (B) cortisol concentrations following a simulated nest intrusion for male parental smallmouth bass guarding fresh eggs, represented by open circles (○) or egg-sac fry, represented by closed circles (●). Androgen concentrations were positively correlated to the level of aggression, while

cortisol concentrations were unrelated to aggression ($\alpha=0.05$; see text for statistical

details).

ii.vi. Tables

	Bluegill present	Empty jar	t-statistic	p-value
Aggressive behaviours per 3 min	**54.8 ± 4.5 (24)**	**0.0 ± 0.0 (6)**	**6.019**	**0.01**
[androgens] (ng mL^{-1})	**3.10 ± 0.34 (22)**	**1.39 ± 0.33 (6)**	**2.481**	**0.02**
[cortisol] (ng mL^{-1})	1.8 ± 0.2 (24)	1.8 ± 0.5 (6)	0.028	0.97

Table ii.i. Behavioural responses and circulating steroid (cortisol and androgens) concentrations in male parental smallmouth bass presented with either a simulated (bluegill sunfish in a glass jar placed in the nest) or sham (empty jar placed in the nest) nest intrusion. Mean and SEM are presented, with sample size in brackets. Bold text indicates statistically significant differences between fish presented with the simulated nest intrusion and fish presented with the sham control (α=0.05).

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