Does nutritional status mediate the consequences of elevated cortisol on wild fish? Field manipulations using wild smallmouth bass.

By

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Abstract

Parental care is an essential life-history component of reproduction for many animal species, which entails a suite of behavioural and physiological investments to enhance offspring survival. These investments can incur costs to the parent, reducing their energetic and physiological condition, future reproductive capabilities, and survival. In fishes, relatively few studies have focused on how these physiological costs are mediated. Male smallmouth bass provide parental care for developing offspring until the brood reaches independence. During this energetically demanding life stage, males cease active foraging as they vigorously defend their offspring. Experimental manipulation of cortisol levels (via injection) and food (via supplemental feeding) in parental males was used to investigate the fitness consequences of parental care. Improving the nutritional condition of nest guarding males increased their reproductive success by reducing premature nest abandonment. However, supplemental feeding and cortisol treatment had no effect on parental care behaviours. Cortisol treatment reduced plasma lymphocyte numbers, but increased neutrophil and monocyte concentrations, indicating a shift in immune function. Supplemental feeding improved the physiological condition of parental fish by reducing the accumulation of oxidative injury. Specifically, supplemental feeding reduced the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) on DNA nucleotides. Increasing the nutritional condition of parental fish can reduce the physiological cost associated with intensive parental activity and improve overall reproductive success, illustrating the importance of nutritional condition as a key modulator of parental fitness.

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Co-Authorship Statement

Stress, nutrition and parental care in a teleost fish: Exploring mechanisms with supplemental feeding and cortisol manipulation

A.J. Zolderdo, D.M. Algera, M.J. Lawrence, K.M. Gilmour, M. Fast, J. Thuswaldner, W. Willmore, and S.J. Cooke.

This manuscript is planned for submission to the Journal of Experimental Biology. This project was conceived by Cooke and Zolderdo. Field work was completed by Zolderdo, Algera, and Cooke. All statistical analyses were conducted by Zolderdo and Cooke. Cortisol analysis were conducted by Lawrence and Gilmour, and 8-hydroxy-2'-deoxyguanosine analysis were conducted by Thuswaldner and Willmore. Fast assisted with white blood cell identification and blood smear preparations. Furthermore, all authors contributed feedback to the manuscript.

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1.0 INTRODUCTION

1.1 Parental Care

Parental care is a common phenomenon typically defined as any behavioural investment made by a parent to promote its offspring's survival and development beyond initial fertilization (Smiseth et al. 2012). There are many forms of parental care involving one (paternal or maternal) or both (biparental) parents (Webb et al., 2002) and ranging in complexity from strategic nest placement to conceal a brood from predators, to lengthy gestation periods followed by numerous years of rearing and teaching offspring (Royle et al. 2012). Parental care can be found among many invertebrate and vertebrate taxa (Trivers, 1972; Clutton-Brock 1991; Cockburn, 2006; Royle et al., 2012), including fish (Blumer, 1979; Baylis, 1981; Gross and Sargent, 1985), but unlike in other taxa, sole paternal care is the predominant form of parental care among fish (Blumer 1979; Gittleman, 1981; Blumer, 1982). Providing parental care is energetically costly, because resources are diverted away from physiological maintenance of the individual, potentially resulting in reduced growth, immunocompetence, and longevity of the parent (Alonso-Alvarez and Velando, 2012). This increased physiological burden also may increase the potential for oxidative stress damage, which increasingly is becoming recognized as a physiological cost associated with parental care and overall fitness (Bell 1980; Metcalfe and Monaghan, 2013). Indeed, parental care reflects a fitness trade-off between the current energetic and physiological costs of reproduction, and the maintenance of parental energetic and physiological condition for future survival and reproduction (Alonso-Alvarez and Velando 2012).

1.2 The Stress Response

Wild fish are frequently exposed to natural and anthropogenic stressors ranging temporally from seconds (e.g., predator avoidance) to spanning entire life-history stages (e.g., habitat alteration) (Barton, 2002). 'Stressors' and 'stress' act in a cause-and-effect relationship, where stress is a response of the body to a demand made upon it (the stressor) that threatens the body's homeostatic state (Mommsen *et al.,* 1999; Barton, 2002; Schreck *et al.,* 2010). The stress response elicits a suite of physiological and behavioural responses that mitigate the negative consequences of a stressor(s) in order to maintain homeostasis. However, the homeostatic set points that the physiological and behavioural responses maintain can change temporally (e.g., adjustments to seasonal temperature changes) and with life history stage (e.g., spawning and reproduction)

(McEwen and Wingfield, 2003; Romero *et al*., 2009). To account for this variability in homeostatic set points, Romero *et al.* (2009) developed the Reactive Scope Model (RSM), which provides a framework that outlines how homeostasis is maintained temporally across various life-history stages. The RSM can be broken down into four categorical ranges that model the concentrations/levels of various physiological and behavioural mediators involved in the stress response. The first range is Predictive Homeostasis, which consists of normal circadian and seasonal variations in mediator responses to predictable challenges (e.g., daily changes in photoperiod and temperature). The second range is Reactive Homeostasis, which is the range of mediator(s) needed to respond to unpredictable or threatening challenges. This is typically referred to as the stress response in an organism, and is considered an adaptive response. Together, the Predictive and Reactive ranges comprise the normal reactive scope of the physiological and behavioural mediators needed to maintain homeostasis in a dynamic environment (Romero *et al*., 2009).

However if a stressor(s) becomes chronic or particularly severe, the concentrations of physiological mediators are forced above the Reactive Homeostasis range, which can lead to Homeostatic Overload (Romero *et al*., 2009). When an organism enters into a state of Homeostatic Overload the physiological mediators themselves become pathological, and cannot be maintained at overload concentrations without causing damage to the organism (e.g., high blood pressure for extended time periods can lead to cardiovascular disease). Homeostatic Failure is the fourth range, and occurs when an organism cannot maintain the necessary concentrations of physiological mediators at the minimum level required for normal homeostasis (e.g., an organism's inability to maintain blood pressure). In both the Homeostatic Overload and Failure states, severe damage or death can occur rapidly if left unchecked (Romero *et al*., 2009). The RSM outlines an adaptive framework for coping with predictable and unpredictable stressors faced by organisms throughout their life cycles, and provides a clear and concise structure for categorizing the stress response at the individual level (Romero *et al*., 2009).

In fish, the initial reaction to a stressor elicits a neuroendocrine response cascade, involving the release of catacholamines from the chromaffin tissue, and the activation of the hypothalamic-pituitary-interrenal (HPI) axis (Schreck *et al*., 2001; Barton, 2002). Activation of the HPI axis stimulates the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary region.

ACTH is a tropic hormone responsible for triggering the release of corticosteroids (GCs) (e.g., cortisol) into circulation (Mommsen *et al.,* 1999; Barton, 2002). GCs elicit the secondary physiological responses which are changes at the blood and tissue level that can disrupt ion balance as well as increase catabolism of carbohydrates, proteins, and lipids (Barton, 2002). These secondary responses can directly impact aspects of animal performance by altering their metabolic activity, respiration, ion balance, blood pH, and immune function (Mazeaud et al. 1977) (Fig. 1). For example, increased concentrations of GCs can facilitate the increased energy requirements for certain parental care behaviours (Wilson and Wingfield, 1992; Nelson, 2000; Comendant et al., 2003; Jessop et al., 2002; Romero, 2002; Magee et al., 2006). However, high concentrations of GCs over a sustained period of time can cause reproductive failure (Moore and Jessop, 2002; Love et al., 2004; Magee et al., 2006; O'Connor et al., 2009; Dey et al., 2010). GCs also mediate aspects of immunity. Acute increases in GCs can boost immune function, by increasing energy availability (Dhabhar, 2000; Dhabhar and McEwen, 1997), whereas chronic increases in GCs suppress immune function. Specifically, chronically elevated GC concentrations redistribute leukocytes throughout the body, and alter the GC receptors on lymphatic and leukocyte cells (Cidlowski et al., 1996; Leonard and Song, 2002; French et al., 2007). Elevated concentrations of GCs also exacerbate the impacts of oxidative stress damage, via increased respiration and metabolic turnover (Behl et al., 1997; reviewed in Srivastava and Kumar, 2015). Oxidative stress occurs when production of reactive oxygen species (ROS) exceeds an organism's ability to neutralize and repair the oxidative damage caused by ROS accumulation (DiGuilo et al. 1989). ROS are a normal by-product of metabolism, and cause significant damage to biomolecules (i.e., proteins, lipids, and DNA) unless neutralized by antioxidant processes (Beckman and Ames, 1998; Finkel and Holbrook, 2000; Fletcher et al., 2013). Elevated metabolic activity during a stress response increases the potential for ROS production, which can further increase the animals energetic and physiological requirements associated with repairing the ROS damage.

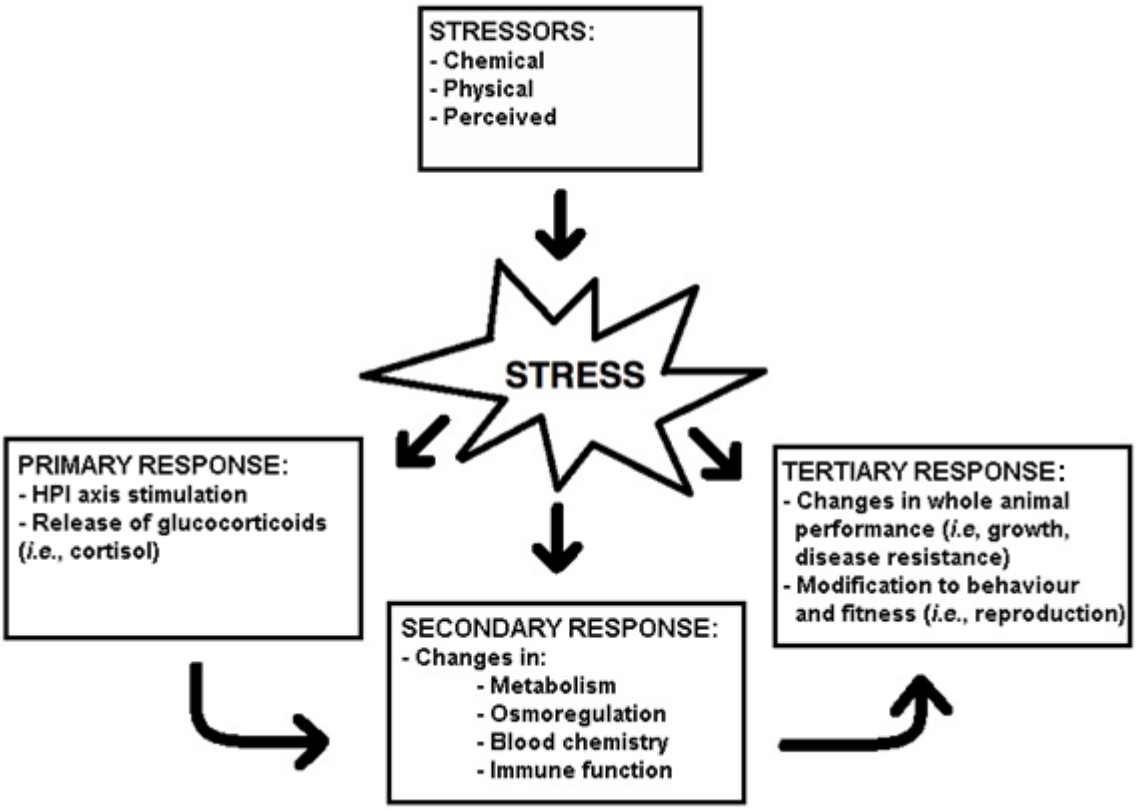

Figure 1: An overview of what occurs when the stress response is initiated via stressors. Adapted from Barton (2002).

1.3 Nutrition and energetics

Although GCs in high concentrations can directly impact aspects of animal performance (e.g., reproduction and immunity), the extent of these negative fitness costs may be influenced by the nutritional condition of the animal. In fish, all biological processes are powered through the metabolism of fats, carbohydrates, and proteins, which are obtained through natural forage (e.g., macrophytes, invertebrates, fish) (Barton and Schreck, 1987; Cargnelli and Gross, 1997; Blair *et al.*, 2012). The total amount of food energy ingested by a fish is allocated towards growth (somatic and reproduction), metabolism and cellular maintenance (standard, and tissue repair), physical activity (foraging for food), and excretion (nutrient metabolism and digestion) (Parazo, 1990; Redpath *et al*., 2009; Blair *et al.,* 2012). In optimal situations, the metabolic demands placed on

the energy budget are maintained in balance with growth requirements (Parazo, 1990; Redpath *et al*., 2009). However, in situations where food sources are reduced below optimal levels (e.g., overwinter or during reproduction), the energy budget for growth is reallocated towards essential systems such as metabolism and cellular maintenance. Trade-offs in energy partitioning between various physiological systems can be influenced by food availability. Animals that are able to maintain an optimal nutritional status can sufficiently allocate energy resources towards mediating the impacts of additional challenges (McEwen and Wingfield, 2003; French *et al.*, 2006; French and Moore, 2008; McConnachie et al., 2012). Although much research attention has been focused on how additional challenges (e.g., stress) influence the fitness trade-offs of a parent (e.g., Tuomi and Haukioja, 1983; Reznick et al., 2000; French et al., 2006, 2007, 2011), comparatively little is known about how the nutritional condition of a care-giving parent can mediate the impacts of additional challenges during the parental care period. Food intake often is lowered for parental-care providing animals owing to reductions in both opportunity and interest in feeding (Hanson et al., 2009). Supplemental feeding has been employed as an experimental technique to investigate the physiological costs, behavioural changes, and reproductive success of parental animals, primarily in terrestrial species (e.g., French et al., 2006, 2007, 2011; French and Moore, 2007; but see Ridgway and Shuter, 1994).

1.4 Experimental Biology to Understand Parental Care

Supplemental feeding during the parental care period involves the addition of food resources (natural or artificial) to the care-giving parent(s) to improve their nutritional condition (Boutin, 1990). Supplemental feeding can be a valuable experimental tool that can be used to investigate life-history trade-offs (e.g., reproduction and parental care), especially in care-providing fish species (Ridgway and Shuter, 1994; Pike et al., 2007; Hanson et al., 2009). However to date, relatively few studies have utilized this technique to explore such trade-offs in fish populations, despite its common use in avian ecology research (reviewed in Boutin, 1990). Among the limited research using fish, it has been observed that increasing food resources during the care period can alter parental behaviours, physiological condition, and reproductive outcome (Ridgway and Shuter, 1994; Hanson et al., 2009; Pike et al., 2007).

Manipulating GC concentrations is another valuable experimental technique commonly used to investigate life-history trade- offs (Sopinka et al. 2015; Crossin et al. In Press). While there are

several methods that are readily used to increase plasma GC levels naturally (e.g., capture and handling of study species, manipulating environmental temperature and diet, and isolating/restricting animal movements), as well experimentally (e.g., impregnating food or water sources with GCs, and implanting GCs via injection), there are important benefits and drawbacks associated with each technique. Naturally elevating plasma GC levels often requires repeat manipulation and interaction with the study species, however these techniques generally elicit a natural stress response involving the animal perceiving the stressor and reacting as it would under natural conditions (Glasper and DeVries, 2005; French et al., 2006; Martin et al., 2006; O'Connor et al., 2011). These natural techniques often initiate acute stress responses, thus requiring consistent repetition of the stressor to maintain chronically elevated concentrations of GCs. Whereas, experimental GC manipulations involving the implantation of GCs within food/water sources or by injection, can provide a method to chronically elevate plasma GC levels while minimizing researcher interaction with the study species (reviewed in Gamperl et al., 1994). It is important to note that these experimental techniques do not entirely mimic the stress response in that they fail to include the full cascade of changes that are initiated by sensing or perceiving a stressor. In fish, only a handful of studies have investigated the role of endogenous GC concentrations throughout the parental care period, and of those studies, only a select few have manipulated GC levels in wild fish during the care period (O'Connor et al*.,* 2009, 2011; Hanson et al., 2009; Dey et al, 2010). From these select studies, the results suggest that elevating cortisol levels in wild fish species produces physiological changes consistent with changes in energy metabolism and carbohydrate catabolism. Elevating plasma cortisol levels has also been shown to decrease body condition and increase nest abandonment rates in parental male centrarchids (O'Connor et al., 2009; Dey et al., 2010).

1.5 Smallmouth bass (*Micropterus dolemieu*)

Smallmouth bass (*Micropterus dolomieu*) are one of the many teleost species that utilize paternal care, the most common form of parental care among Teleostei. Smallmouth bass spawn in the early spring, typically when water temperatures reach approximately 14°C. Males move into the shallow littoral regions of lake and river systems to establish a breeding territory and construct nests. Nests are small saucer shaped depressions excavated in the bottom substrate that serve as sites for courtship, fertilization, and brood rearing (Fig. 2). Females spawn with desirable males and depart the nest site after egg deposition. The male remains in the nest site (i.e., a 10 m^2 nesting

area) and provides sole parental care to the developing offspring until they reach independence, which can take approximately 1 month (Turner and MacCrimmon, 1970; O'Connor et al., 2009; Hanson et al., 2009). Males are active in performing parental duties, including aggressively defending their brood from nest predators (e.g., *Lepomis spp.*), and may swim upwards of 40 km a day, engaging nest predators, without actively leaving the nest site (Cooke et al., 2002). Other paternal duties include fanning the brood to increase oxygenation and remove debris from the nest. Parental males curtail active foraging to provide parental care (Hinch and Collins, 1991) and experience reductions in appetite hormones (Hanson et al., 2009). Consequently, parental males rely on endogenous energy stores to power the metabolic demands of parental care and homeostasis.

Physiological and nutritional manipulation of male smallmouth bass during the care period can influence their reproductive outcome. Ridgway and Shuter (1994) demonstrated that supplemental feeding of parental males improved fitness either by increasing parental survival, or by increasing care duration and overall year-class reproductive success. Conversely, parental males subjected to an additional stressor (i.e., elevated cortisol via injection) during the care period experienced decreased reproductive success (i.e., premature nest abandonment) and compromised immune capacity (Dey et al., 2010). Smallmouth bass feed opportunistically during the care period (Hanson et al., 2009; Ridgway and Shuter, 1994), making the smallmouth bass a useful species to investigate how resource availability combined with additional challenges impact reproductive success and the physiological status of parental nest guarding males.

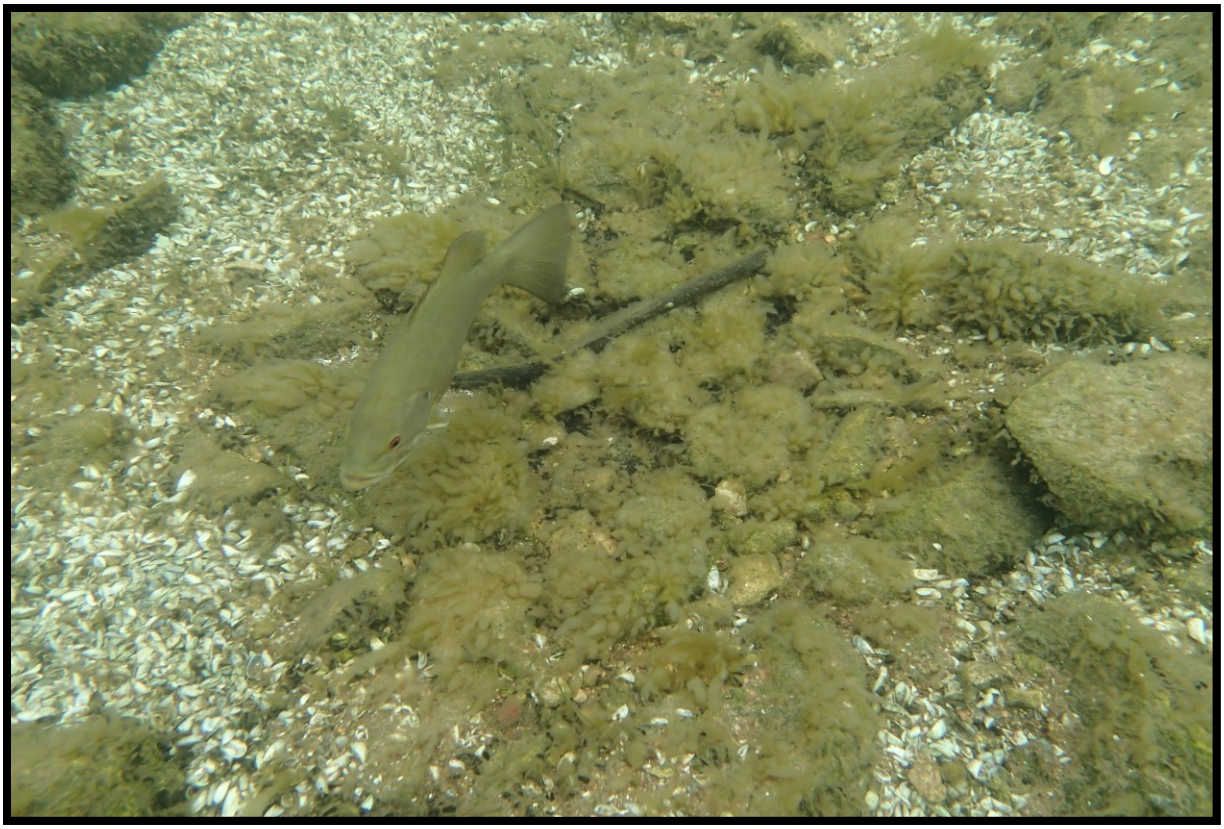

Figure 2: A parental male smallmouth bass guarding his nest from potential brood predators. This photo was taken when the brood were at the initial egg-stage of development (approximately 2-5 days old).

1.6 Research Objectives

Here we use male smallmouth bass to explore the nexus of stress, nutrition and parental care to identify mechanisms that influence parental care success and parental fitness. We tested the hypothesis that nutritional condition modulates the physiological and fitness costs associated with parental care and additional stressors faced during the care period. Using control and cortisol-treated nesting smallmouth bass, this study examined whether supplemental feeding influenced parental care behaviours, reproductive success, or fish condition (including immune status and oxidative stress) relative to unfed fish. Based on previous research addressing aspects of nutritional condition and stress in parental smallmouth bass, we predicted that increasing the nutritional status

of cortisol-treated males through supplemental feeding would positively influence parental care duration, reproductive success, immunity, and physiological condition.

2.0 METHODOLOGY

2.1 Experimental fish and treatments

From May 17 to 22, 2014, 55 nest-guarding male smallmouth bass were identified by snorkeling surveys in the littoral regions of interconnected lakes in the Rideau watershed in eastern Ontario, Canada. During this period smallmouth bass are actively spawning and Ontario Provincial Law strictly prohibits recreational angling for this species. The study sites were rigorously monitored for angler activity, and sampled fish that bore evidence of angling or capture (i.e., visible hook wound in the mouth) were excluded from the study. Upon identification of a nest with an actively guarding male, a brood survey was conducted. The snorkeler visually assessed and recorded brood age and density; density was scored from 1 (low) to 5 (high) based on the method of Suski and Philipp (2004). Each nest was individually marked with a numbered polyvinyl chloride (PVC) tile, and the guarding males were randomly assigned to one of four treatment groups: control (n=14), control plus food (n=13), cortisol (n=15), and cortisol plus food (n=13).

Cortisol-treated males were given an intraperitoneal injection of cocoa butter (5 ml kg^{-1} body weight) containing emulsified hydrocortisone 21-hemisuccinate (Sigma H4881; Sigma-Aldrich Inc., St. Louis, MO) (10 mg ml^{-1} of cocoa butter). This method has been used in previous studies to elevate circulating plasma cortisol levels for up to 6 d (Gamperl et al., 1994; Dey et al., 2010). Control fish were handled in the same way as cortisol-treated males but did not receive an intraperitoneal injection of any kind. A sham treatment group was not included due to previous validation on the effectiveness of the cocoa-butter cortisol injection process in elevating plasma cortisol levels in centrarchid species (e.g., O'Connor et al., 2009; McConnachie et al., 2012; but see Dey et al., 2010). Moreover, the control fish serve to some extent as a sham because they have to be captured for initial assessment.

2.2 Behavioural assessments and sampling protocol

Upon completion of the initial brood survey, the snorkeler conducted two behavioural assessments on the guarding male. First, brood nurturing behaviour was calculated based on the proximity of the guarding male to its nest over time (i.e., tending vigilance).With the snorkeler positioned 3 –

5 m from the nest for 3 min, nest tending was scored every 20 s. Fish greater than 2 m from the nest were given a score of 0, and a score of 1 was given to fish positioned within 2 m of the nest. The total sum of the tending scores over 3 min (out of 9) provided an index for brood-nurturing behaviour. More vigilant parental males spent more time within the nest site and received a higher brood-nurturing score.

An aggression test was performed directly after the brood-nurturing evaluation. The snorkeler placed a clear glass jar (volume = 3.78 liters) containing a nest predator (a pumpkinseed sunfish, *Lepomis gibbosus*, approximately 100 – 140 mm in total length) on the edge of the nest for 60 s and counted the number of attacks made on the jar by the parental male. A score of 1 was given each time the fish made contact with the glass container. The sum of attack scores over 60 s provided an index of parental aggression. This method is commonly used to measure parental aggression in nesting fish (Fitgerald and Caza, 1993; O'Connor et al., 2009; Dey et al., 2010).

Directly after completion of both behavioural assessments, each fish was captured using rod and reel by an experienced angler on the boat, or by a snorkeler in the water. Heavy action angling gear was used to minimize fight duration (< 20 s to land the fish after hookset), so that stress and anaerobic exercise associated with capture would be minimized. A net was used to hoist fish into the boat to reduce potential mouth and jaw damage. Fish were immediately placed in a foam-lined trough filled with fresh lake water. Total length and body condition were measured and recorded, and fish were assigned to one of the four treatment groups (see above). Only fish of lengths 330 – 450 mm were used in the study to minimize any potential effects of size on parental effort and reproductive outcome.

To avoid the use of anesthesia, which is required to accurately weigh fish, weight (in g) was estimated from total length (in mm) using the equation, weight = 6.581(total length) – 1629.1, following the methods of Dey et al. (2010). Capture and processing took no longer than 3 min. During this period, a snorkeler remained on the nest to defend it until the parental male returned (< 5 min after release), so that there would be no loss of brood during the male's absence.

Five or six days after initial capture and treatment (May 22 – 27, 2014), a second round of sampling was undertaken. A snorkeling survey was conducted to assess nest abandonment rates (presence or absence of the guarding male and brood). This round of sampling corresponded with the egg hatching stage of brood development, termed 'egg-sac fry'. At this stage, yolk sacs are clearly

visible, and fry begin to develop distinguishable anatomical features, most notably a defined head and tail. Egg-sac fry are highly vulnerable to predation, and remain concentrated in the nest. Parental care activity remains elevated at this stage, enabling parental care behaviours to be quantified (Hanson et al., 2009; O'Connor et al., 2009; Dey et al., 2010).

Parental behaviours were reassessed for all remaining fish (n = 44), followed by immediate capture, as described above. A blood sample (approximately 1 ml) was withdrawn from the caudal vasculature using a 21- gauge needle and a 3 ml vacutainer syringe containing lithium heparin (B.D. Vacutainer, Franklin Lakes, NJ), and the vacutainer was placed in an ice-water slurry for temporary storage and transport (i.e., <120 min) prior to analysis (see below). All blood samples were collected within 90 s of capture. As before, a snorkeler remained above the nest to defend it until the parental male returned (<5 min after release).

2.3 Feeding Protocol

In total, 26 parental males (13 control fish and 13 cortisol-treated fish) were fed supplemental diets during the care period. Feeding commenced directly after the initial sampling event (May 17 – 22), and fish were fed every 2-3 d for a total of 5 times. The fifth feeding coincided with the beginning of the 'black fry' stage of brood development, where the brood becomes fully mobile, ranging outside of the nest site during the day. It is during this period that guarding males also become more mobile, and begin to actively forage (Hanson et al., 2009). This provided a logical time to cease supplemental feeding. Supplemental food consisted of locally collected crayfish (*Orconectes spp*.) from Lake Opinicon, and earthworms (*Lumbricus terrestris*) purchased from local marinas. The average weight (g) for both crayfish and worms were calculated using a subset of individuals (n = 30 for crayfish, and n = 18 for worms, respectively). During the feeding intervals a snorkeler alternately dropped a crayfish or worm into the nest until fish stopped ingesting the food items. Food items that were not ingested were removed from the nest site. The total number of crayfish and worms consumed were tallied to provide an index of nutritional condition (i.e., fed vs. fasting).

2.4 Analysis of Blood Samples

Blood glucose levels were measured using a handheld blood glucose meter (Accucheck Compact Plus, Roche, Basel, Switzerland), a technique that has been validated for measurement of blood glucose levels in fish (Cooke et al., 2008; Stoot et al. 2014). A single drop of blood was pipetted onto a microscope slide, and smeared across the slide using the edge of another clean glass

microscope slide. The blood smear was then fully submerged in Buffered Differential Wright-Giemsa stain (Camco Quick Stain II). After 10 s, the slide was removed and allowed to air dry. Duplicate blood smears were prepared for each sample. Blood smears were examined under oil immersion using a Leica DME light microscope, and leukocytes were identified and counted. For each smear, 200 cells were counted at 1,000 X magnification. Cell types were identified based on Pickering et al. (1982) and Yasutake and Wales (1983).

The remaining blood was then centrifuged at 10,000 x g for 5 min. The plasma was separated into two aliquots and flash frozen in liquid nitrogen. The red blood pellets were also flash frozen in liquid nitrogen. Plasma and red blood cell aliquots were stored at – 80 ^{o}C for later analysis.

Plasma samples were analyzed for biochemical indicators of nutrition and physiological status, including cholesterol, total protein, triglycerides, and calcium, sodium, potassium, chloride, and magnesium ions. In previous work on smallmouth bass and Pacific salmonids (Oncrohynchus spp.) theses biochemical indicators have reflected various states of nutritional and physiological condition, allowing fasting and feeding conditions to be distinguished (Wagner and Congleton, 2004; Congleton and Wagner, 2006; Dey et al., 2010). These analyses were conducted by IDEXX Laboratories, Markham, Ontario. Plasma cortisol concentrations were determined in a single assay using a commercial radioimminoassay kit (MP Biomedicals, Orange-burg, NY) previously validated for use in smallmouth bass (O'Connor et al., 2009; Dey et al., 2010). Intra-assay variability for the cortisol assay was 15.9%.

2.5 DNA Extraction

DNA was extracted from blood cell pellets (red and white blood cells) using a whole Cell Lysis Buffer (10 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 0.32 M sucrose, 1 % (w/v) Triton X-100, 0.1 mM desferrioxamine) according to Wilson et al (2012). One mL of Whole Cell Lysis Buffer was added to each 1 mL sample of blood, along with 2 mL of cold, sterile Milli-Q water at a pH of 7.4. The tube was vortexed on high speed for 2-10 minutes (time varies according to the density of the sample) and was left on ice for five minutes, followed by centrifugation at 3,500 rpm for 15 minutes at 4ºC. The supernatant was removed and discarded. One mL of Nuclear Lysis Buffer (20 mM Tris-HCl (pH 8.0), 4 mM EDTA, 100 mM NaCl) and 500 µL of 10% SDS were added to the pellet. The pellet was vortexed vigourously for two minutes. Ten µL of proteinase K solution (20 mg/mL) was added to each pellet. Samples were left to incubate in a 55ºC water bath for two hours.

After incubation period, samples cooled on ice for five minutes. Four mL of 5.3M NaCl solution was added to each tube. Samples were then vortexed on medium speed for 30 seconds, followed by centrifugation at 4,500 rpm for 20 minutes at 4ºC. The supernatant was then poured into a new tube, to which 5 mL of cold isopropanol was added. The tube was inverted ~10 times to precipitate DNA. DNA was then removed with a wide bore tip and was transferred to an Eppendorf tube. DNA was rinsed with 70% ethanol and was incubated at 37ºC for 20 minutes to dry. DNA was resuspended in 300 µL of Tris-HCl at pH 8.5 and was let to redissolve overnight at room temperature. Total DNA concentrations were determined using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA) at an absorbance of 260 nm. All 8-OHdG concentrations were standardized against total DNA concentration (ng/mL).

2.6 DNA Digestion

Samples for the digestion period were chosen based on DNA concentration. All samples fell within a range of 4,500-5,500 ng/µL (28 in total). One hundred µg of dissolved DNA was used for each sample. The appropriate volume was added to an Eppendorf tube for each sample (between 18 and 21 µL). One µL of 0.1mM desferrioxamine solution was added to each tube, followed by 100 µL of Nuclease P1 Buffer (40 mM sodium acetate (pH 5.3), 0.2 mM ZnCl2, 0.1 mM desferrioxamine). Each sample was heated on a 97ºC hot block for 3 minutes, followed by a cooling period of three minutes on ice. Sixteen µL of Nuclease P1 (in 20 mM sodium acetate (pH 5.3), 5 mM ZnCl2, 50 mM NaCl, 50 % glycerol) was added to each sample, followed by an incubation period at 37ºC for 30 minutes. Twenty µL of Alkaline Phosphatase Buffer (500 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2 mM ZnCl2, 50 mM MgCl2) was added to each sample, followed by a one-hour incubation period at 37ºC. Afterwards, 18.5 µL of 0.1 HCl was added to each sample to neutralize the reaction. Each sample was centrifuged at 13,000 X g for five minutes. The supernatant was transferred to a new tube and digested sample was run on a 1% agarose gel next its respective undigested form to ensure full digestion of DNA. The supernatant was stored at -20ºC for two weeks, and was thawed on ice after this period when the experiment was resumed to conduct the DNA damage assay.

2.7 8-hydroxy-2'-deoxyguanosine (8-OHdG) EIA

Oxidative stress was quantified by measuring the production of 8-hydroxy-2'-deoxyguanosine (8-OHdG) using the EIA kit (8-OHdG EIA Kit, Cayman Chemicals, Ann Arbor, MI) according to

the manufacturer's instructions. Twenty-eight red blood cell samples, seven from each treatment group, were used for 8-OHdG quantification based on their high DNA concentrations (4,500-5,000 ng ml^{-1}), enabling optimal 8-OHdG detection. Samples outside of this concentration were not used for DNA damage analysis. Digested DNA samples were diluted 1:500 in Whole Cell Lysis Buffer to fall within the standard curve, and 50 µl of each diluted sample was used for the determination of 8-OHdG. Absorbance was measured at 412 nm using an Epoch Microplate Spectrophotometer (Biotek Instruments, Inc., Winooski, VT) and values from each sample were calculated based on the absorbances of standard 8-OH-dG concentrations (10.3, 23.1, 52.0, 117.1, 263.4, 592.6, 1,333, 3,000 pg/mL).

2.8 Statistical analyses

All statistical analyses were conducted using JMP 9.0.1 with significance levels (α) set at 0.05. Prior to analyses, datasets were tested for normality and heterogeneity of variance using Shapiro-Wilk's test and Levene's test, respectively. If non-normality and/or unequal variance occurred within a dataset, it was transformed prior to analysis (Zar, 1999; Mutulsky, 2010). A natural log transformation was applied to datasets that had skewness values > 1 relative to a normal distribution of 0. However, if skewness values < -1 relative to a normal distribution of 0, the data were transformed by squaring all values (Mutulsky, 2010). Two-way analysis of variance (ANOVA) followed by either a Student's t-test or Tukey's test for post-hoc analysis was used to evaluate the effects of cortisol treatment and food supplementation on male physiological condition and immune function, parental care behaviours, and duration of parental care. Nest abandonment rates between cortisol and food treatment groups were analyzed using Pearson's chi-square test (Motulsky, 2010). Weight of total food consumed and food item preference were analyzed using Student's t-test and one-way ANOVA, respectively. Pearson's correlation was used to analyze the relationship between total number of food items consumed and parental care behaviours.

3.0 RESULTS

3.1 Cortisol Concentrations

As expected the cortisol implants were sufficient in elevating plasma cortisol levels in treatment fish, regardless of supplemental feeding (two-way ANOVA, $F_{3, 25} = 86.2474$, $P < 0.001$). A

student's t-test post-hoc analysis revealed that plasma cortisol levels were highest in both cortisol treatment groups compared to the control groups (Fig. 3, tables 1, 2). A complete list of *P* values for all two-way ANOVAs can be found below on tables 1, 2 and 3. Only *P* values that were significant are reported in text.

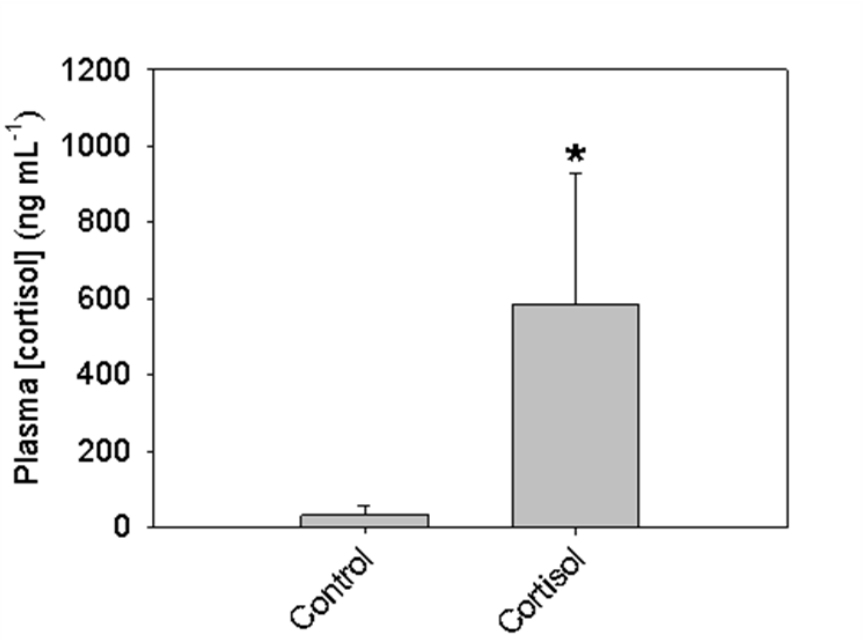

Figure 3: The effects of cortisol manipulations on circulating plasma cortisol concentrations in smallmouth bass across 2 treatment groups. Data presented as mean values (± SE) for control fish (n = 18), and cortisol-fish (n = 12). A single star represents a statistical difference at a level of $\propto$ = 0.05 between control and cortisol treatment groups for plasma cortisol concentrations. See text for details.

Table 1: Statistical comparison of indicators of nutritional status in parental male smallmouth bass among the four treatment groups sampled from Lake Opinicon and Sand Lake, Ontario. Ion, glucose, total protein, cholesterol and triglyceride concentrations were measured in plasma, whereas 8-OHdG concentration was assessed using red blood cells. Italicized and boldfaced values indicate a statistically significant difference at $\propto = 0.05$.

	Cortisol			Food Supplement			Interaction		
Physiological variable	*df*	*F-ratio*	*P-value*	*df*	*F-ratio*	*P-value*	*df*	*F-ratio*	*P-value*
Calcium (mmol L^{-1})	1	0.259	0.613	1	2.528	0.119	1	1.734	0.195
Chloride (mmol L^{-1})	1	8.939	***<0.005***	1	1.640	0.207	1	4.581	***<0.05***
Magnesium (mmol L^{-1})	1	0.559	0.459	1	0.257	0.614	1	9.068	<0.01
Potassium (mmol L^{-1})	1	4.611	<0.05	1	0.428	0.516	1	0.359	0.552
Sodium (mmol L^{-1})	1	0.090	0.765	1	1.789	0.188	1	0.218	0.642
Cholesterol (mmol L^{-1})	1	8.323	***<0.001***	1	1.119	0.296	1	2.840	0.099
Cortisol (mmol L^{-1})	1	86.247	***<0.001***	1	0.940	0.342	1	0.008	0.930
Glucose (mmol L^{-1})	1	176.499	***<0.001***	1	1.439	0.237	1	4.645	***<0.05***
Total Protein (g L^{-1})	1	71.284	0.124	1	4.338	***<0.05***	1	0.612	0.438
Triglycerides (mmol L^{-1})	1	2.012	0.163	1	9.624	***<0.005***	1	0.899	0.348
8-OHdG (ng mL^{-1})	1	40.892	***<0.001***	1	64.233	***<0.001***	1	6.692	***0.016***

Table 2: Indicators of nutritional condition in parental smallmouth bass, among the four treatment groups, which were sampled from Lake Opinicon and Sand Lake, Ontario. Ion, glucose, total protein, cholesterol and triglyceride concentrations were measured in plasma, whereas 8-OHdG concentration was assessed using red blood cells.

Physiological Variable	Control	Cortisol	Control + Food	Cortisol + Food
Calcium (mmol L^{-1})	2.48 ± 0.16 (2.20-2.80) n = 11	2.43 ± 0.18 (2.30-2.90) n = 10	2.24 ± 0.36 (1.20-2.70) n = 11	2.42 ± 0.24 (2.00-2.90) n = 11
Chloride (mmol L^{-1})	106.6 ± 3.5 (99-110) n = 10	104.4 ± 10.1 (80-110) n = 10	113.1 ± 4.2 (111-120) n = 11	102.8 ± 7.26 (92-115) n = 11
Magnesium (mmol L^{-1})	1.03 ± 0.062 (0.95-1.06) n = 11	0.92 ± 0.058 (0.79-0.99) n = 10	0.93 ± 0.14 (0.83-1.02) n = 11	0.99 ± 0.11 (0.88-1.16) n = 11
Potassium (mmol L^{-1})	3.52 ± 0.63 (2.80-4.30) n = 10	2.84 ± 0.65 (1.80-2.90) n = 10	3.26 ± 0.63 (2.40-3.90) n = 11	2.96 ± 1.19 (1.20-6.20) n = 11
Sodium (mmol L^{-1})	153 ± 5 (144-164) n = 10	155 ± 6 (144-165) n = 10	151 ± 5 (137-158) n = 11	150 ± 8 (130-159) n = 11
Cholesterol (mmol L^{-1})	11.4 ± 1.4 (9.50-13.9) n = 11	12.0 ± 1.3 (9.70-13.7) n = 10	10.4 ± 1.4 (8.20-12.7) n = 11	12.4 ± 1.7 (9.20-16.2) n = 11
Cortisol (ng mL^{-1})	34.7 ± 20.3 (10.2-69.2) n = 10	619.2 ± 292.8 (197.9-943.2) n = 6	27.3 ± 27.0 (10.8-85.2) n = 8	554.6 ± 353.8 (87.0-1003.9) n = 6
Glucose (mmol L^{-1})	3.0 ± 0.6 (2.10-4.10) n = 11	5.8 ± 1.1 (4.30-7.90) n = 10	2.5 ± 0.2 (2.20-2.90) n = 11	6.2 ± 1.5 (4.30-9.40) n = 12
Triglycerides (mmol L^{-1})	1.60 ± 0.78 (0.82-3.47) n = 11	1.41 ± 0.33 (0.85-1.98) n = 10	2.62 ± 1.03 (1.02-4.44) n = 11	1.91 ± 0.64 (0.66-2.77) n = 11

Total Protein (g L^{-1})	45 ± 4 (39-53) n = 11	46 ± 4 (39-53) n = 10	40 ± 7 (18-46) n = 11	44 ± 3 (39-48) n = 11
8-OHdG (ng mL^{-1})	255.8 ± 94.8 (144.9-390.2) n = 7	471.8 ± 40.3 (408.4-521.9) n = 7	125.3 ± 48.6 (50.9-193.7) n = 7	216.8 ± 30.0 (184.4-232.7) n = 7

Values are presented as mean ± (SD) with minimum and maximum values contained within parentheses.

3.2 Food Consumption

Total number of food items consumed did not differ between control and cortisol treatment groups (one-way ANOVA, $F_{1, 22} = 0.626$, $P = 0.437$), and there was no effect of treatment type on the proportion of each food type consumed (one-way ANOVA, $F_{1, 22} = 0.891$, $P = 0.355$, for crayfish; $F_{1, 22} = 1.842$, $P = 0.190$ for worms, respectively). However, overall worms were preferred by parental smallmouth bass regardless of treatment type (76.5 ± 39.9 g worms consumed per fish, 18.1 ± 9.0 g crayfish consumed per fish; Student's t-test, d.f. = 35.51, t = 3.80, $P < 0.001$).

3.3 Parental Care Behaviour

Parental care behaviours were initially assessed during the early egg development stage for all 55 parental males. No differences in brood size were found between cortisol treatment groups (chi-square test, $\chi 2$ (4) = 6.653, $P = 0.194$, n = 55) or food treatment groups (chi-square test, $\chi 2$ (4) = 4.217, $P = 0.334$, n = 55). This is an important consideration because brood size can influence parental care behaviour in nest-guarding smallmouth bass (Ridgway, 1988; Hanson et al., 2009). Neither parental aggression nor brood-tending behaviour were significantly affected by cortisol treatment or food supplementation, for both the initial and post treatment assessments (Fig. 4; Table 3). Parental care duration also appeared to be independent of cortisol and food treatments (Fig. 4; Table 3). Nest abandonment rates were found to be highest within the cortisol treatment group, although this trend was not statistically significant (chi-square test, $\chi 2$ (1) = 0.895, $P = 0.344$, n = 53). However, there was a significant effect of food treatment in reducing nest abandonment rates compared to the non-fed treatment groups (chi-square test, $\chi 2$ (1) = 4.115, $P = 0.035$, n = 53) (Fig. 4).

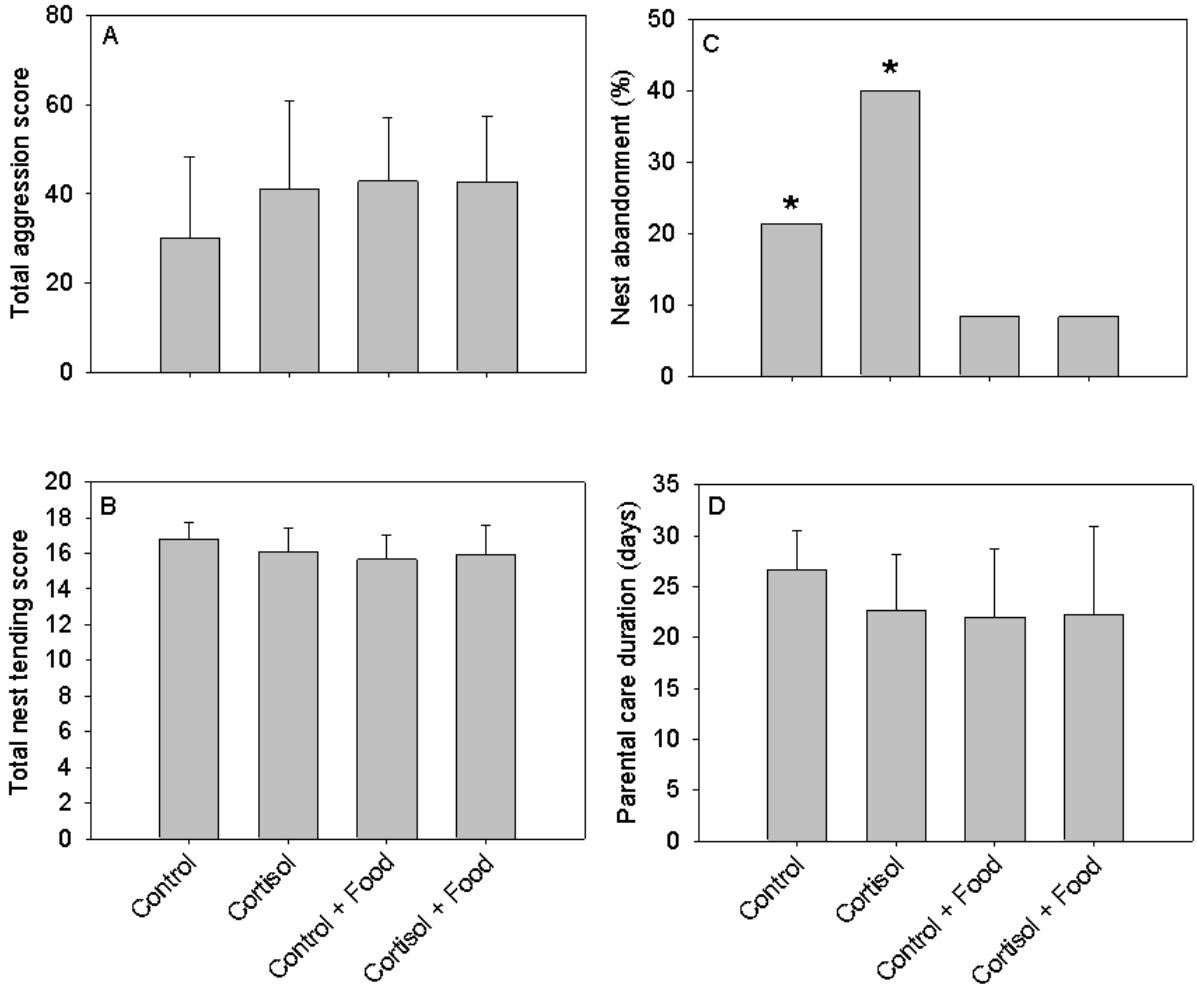

Figure 4: Comparison of parental care behaviours in parental smallmouth bass sampled from Lake Opinicon and Sand Lake, Ontario. Data for total aggression (**A**), nest tending (**B**), and parental care duration (**D**) are presented as mean values (± SE) for control fish (n = 11), cortisol-fish (n = 10), control plus food fish (n = 12), and cortisol plus food fish (n = 12). Data for nest abandonment (**C**) are presented as mean values (± SE) for control fish (n = 14), cortisol-fish (n = 15), control plus food fish (n = 12), and cortisol plus food fish (n = 12).Total aggression score represents the mean value of both the first and second aggression tests. Similarly, the total tending score represents the mean value of both the first and second nest tending measurements. Data for the total aggression scores and total nest tending scores were only used for individual fish that were subjected to both rounds of behavioural assessments. Fish that abandoned their nests prior to the second round of behavioural assessments were not included. Data for nest abandonment includes all fish that were sampled during the 2014 field season. Parental care duration was measured as the total number of days a parental male smallmouth bass was actively guarding his brood. A single star represents a statistical difference at a level of $\propto = 0.05$ between control and cortisol treatment groups for plasma cholesterol concentrations. See text for details.

Table 3: Comparison of behavioural metrics for parental male smallmouth bass among the four treatment groups that were sampled from Lake Opinicon and Sand Lake, Ontario. The total aggression score is the summed values of both the first and second aggression tests. The total tending score is the summed values of both the first and second nest tending measurements. Data for the total aggression scores and total nest tending scores were only used for individual fish that were subject to both rounds of behavioural assessments. Duration is a measurement of total parental care investment over time, and is measured in the total days the parental males remained in the nest site and actively engaged in parental care duties.

	Cortisol			Food Supplement			Interaction		
Behavioural variable	*df*	*F-ratio*	*P-value*	*df*	*F-ratio*	*P-value*	*df*	*F-ratio*	*P-value*
Aggression Score #1	1	0.032	0.857	1	1.117	0.296	1	0.889	0.350
Aggression Score #2	1	2.296	0.137	1	1.479	0.230	1	0.696	0.409
Total Aggression Score	1	1.152	0.289	1	2.015	0.163	1	1.225	0.274
Tending Score #1	1	1.425	0.239	1	0.181	0.672	1	0.041	0.840
Tending Score #2	1	0.028	0.886	1	2.562	0.117	1	2.269	0.139
Total Tending Score	1	0.348	0.558	1	2.832	0.100	1	1.490	0.229
Duration	1	0.899	0.348	1	1.711	0.198	1	1.596	0.287

3.4 Nutritional Status

A key focus of this study was to examine the effects of cortisol elevation and supplemental feeding on biochemical correlates of nutritional status and immune function in smallmouth bass during the parental care period. The blood sampling period coincided with the egg hatching stage of brood development. At this stage, cortisol-treated fish had been exposed to elevated cortisol levels for approximately 5 - 6 d, and all fish in the food treatment groups had been fed to satiation in two independent feeding sessions. Analysis of log transformed plasma glucose concentrations revealed significant effects of cortisol treatment and the interaction between cortisol and food treatments (two-way ANOVA, $F_{3, 41} = 4.645$, $P = 0.037$; Tables 1, 2). A Tukey's post-hoc analysis revealed plasma glucose levels to be higher within cortisol-treated fish regardless of food supplementation (Fig. 5). Plasma cholesterol levels were found to be significantly affected by cortisol treatment alone (two- way ANOVA, $F_{3, 40} = 8.323$, $P = 0.006$; Tables 1, 2). A Student's t-test post-hoc analysis revealed that cortisol treated fish had higher concentrations of plasma cholesterol than control fish (Fig. 5). Plasma total protein and triglyceride concentrations were found to be significantly affected only by food supplementation (two-way ANOVA, $F_{3, 39} = 4.338$, $P = 0.043$ for total protein and $F_{3, 39} = 9.624$, $P = 0.003$, for triglycerides; Tables 1, 2). A Student's t-test post-hoc analyses revealed that plasma total protein levels were higher in non-fed treatment groups compared to food-supplemented groups (Fig. 6). For triglyceride levels, fed fish had higher triglyceride concentrations than non-fed treatment groups (Fig. 6).

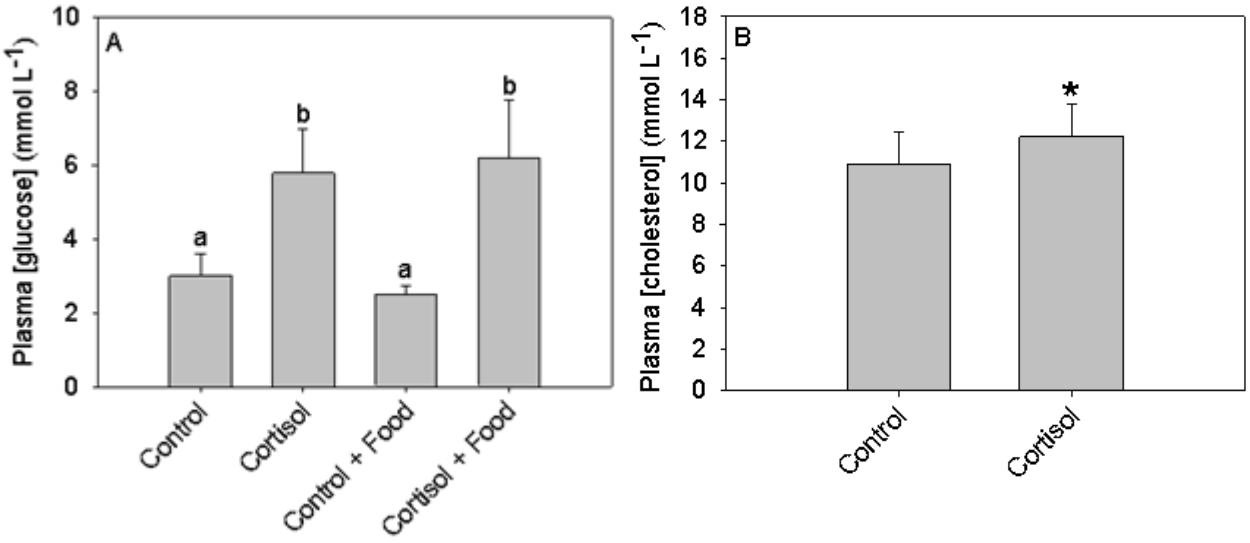

Figure 5: Plasma glucose (**A**) and cholesterol (**B**) concentrations among four treatment groups of parental smallmouth bass. Figure 3B represents the main statistical effect of cortisol treatment, as the interaction term between supplemental feeding and cortisol was not significant. Plasma glucose data are presented as mean values (± SE) for control fish (n = 11), cortisol-fish (n = 10), control plus food fish (n = 11), and cortisol plus food fish (n = 12). Plasma cholesterol data are presented as mean values (± SE) for control fish (n = 22), and cortisol-fish (n = 21). Letter assignments of "a", and "b" denote a significant difference at a level of $\propto = 0.05$ among treatment groups for plasma glucose concentration. A single star represents a statistical difference between control and cortisol treatment groups for plasma cholesterol concentrations. See text for details.

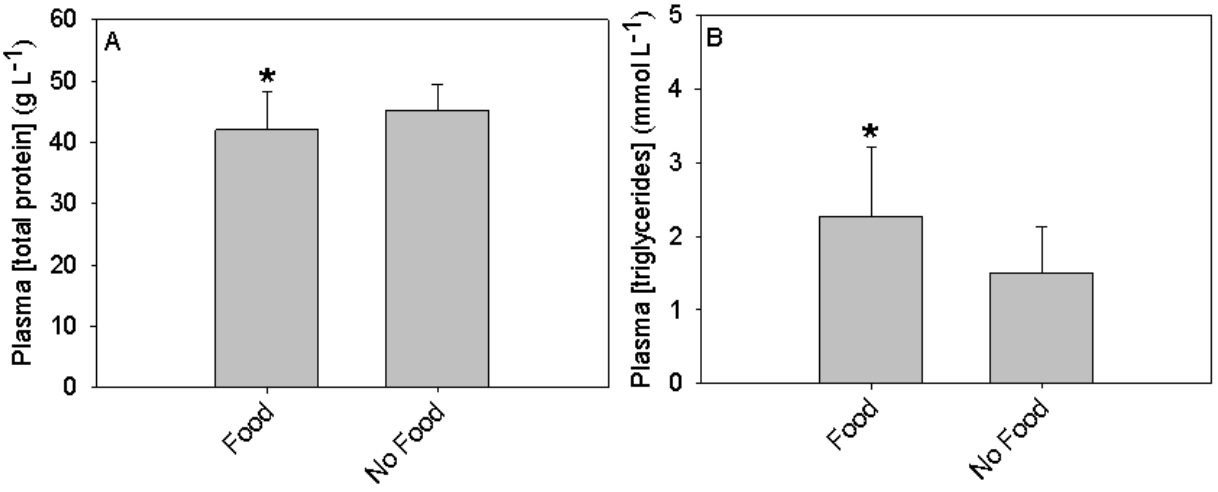

Figure 6: Plasma total protein (**A**) and triglyceride (**B**) concentrations between fed and non-fed treatment groups of parental male smallmouth bass. Data are presented as mean values (± SE) for food treatment fish (n = 22), and non-fed treatment fish (n = 21). The figures for both plasma total protein and plasma triglycerides, represent the main statistical effect of supplemental feeding, as the interaction term between supplemental feeding and cortisol was not significant. A single star represents a statistical difference between fed and non-fed treatment groups for plasma total protein and plasma triglyceride levels. See text for details.

Plasma chloride concentrations were transformed by squaring the data values to correct normality prior to analysis. Circulating plasma chloride levels were significantly affected by cortisol treatment and the interaction between cortisol treatment and food supplementation (two-way ANOVA, $F_{3, 39} = 4.581$, $P = 0.038$; Tables 1, 2). A Tukey's post-hoc analysis indicated that control plus food treatment fish had the highest circulating plasma chloride concentrations compared to all other treatments groups (Fig. 7). Analysis of the log transformed plasma potassium concentrations indicated a significant effect of cortisol treatment on circulating potassium concentrations (two-way ANOVA, $F_{3, 40} = 4.611$, $P = 0.037$; Tables 2, 3). A Student's t-test post-hoc analysis revealed higher plasma potassium concentrations in control fish, compared to cortisol-treated fish (Fig. 7). Plasma magnesium concentrations were transformed by squaring the values to fix normality prior to analysis. A significant interaction of cortisol treatment and food supplementation on plasma magnesium levels (two-way ANOVA, $F_{3, 40} = 9.068$, $P = 0.004$; Tables 2, 3) was detected. However, a Tukey's post-hoc analysis indicated that plasma magnesium concentrations did not significantly differ across any of the 4 treatment groups (Fig. 7). 8-OHdG data were log transformed prior to analysis to correct for normality. 8-OHdG levels were significantly affected by supplemental feeding, cortisol treatment, and the interaction between food supplementation and cortisol treatment (two-way ANOVA, $F_{3, 24} = 6.691$, $P = 0.016$; Tables 1, 2; Fig. 8). A Student's t-test post-hoc analysis indicated that 8-OHdG levels were lowest in the control plus food group and highest in the cortisol treatment group. However, 8-OHdG levels did not differ statistically between the control group and the cortisol plus food treatment group (Tables 1, 2; Fig. 8).

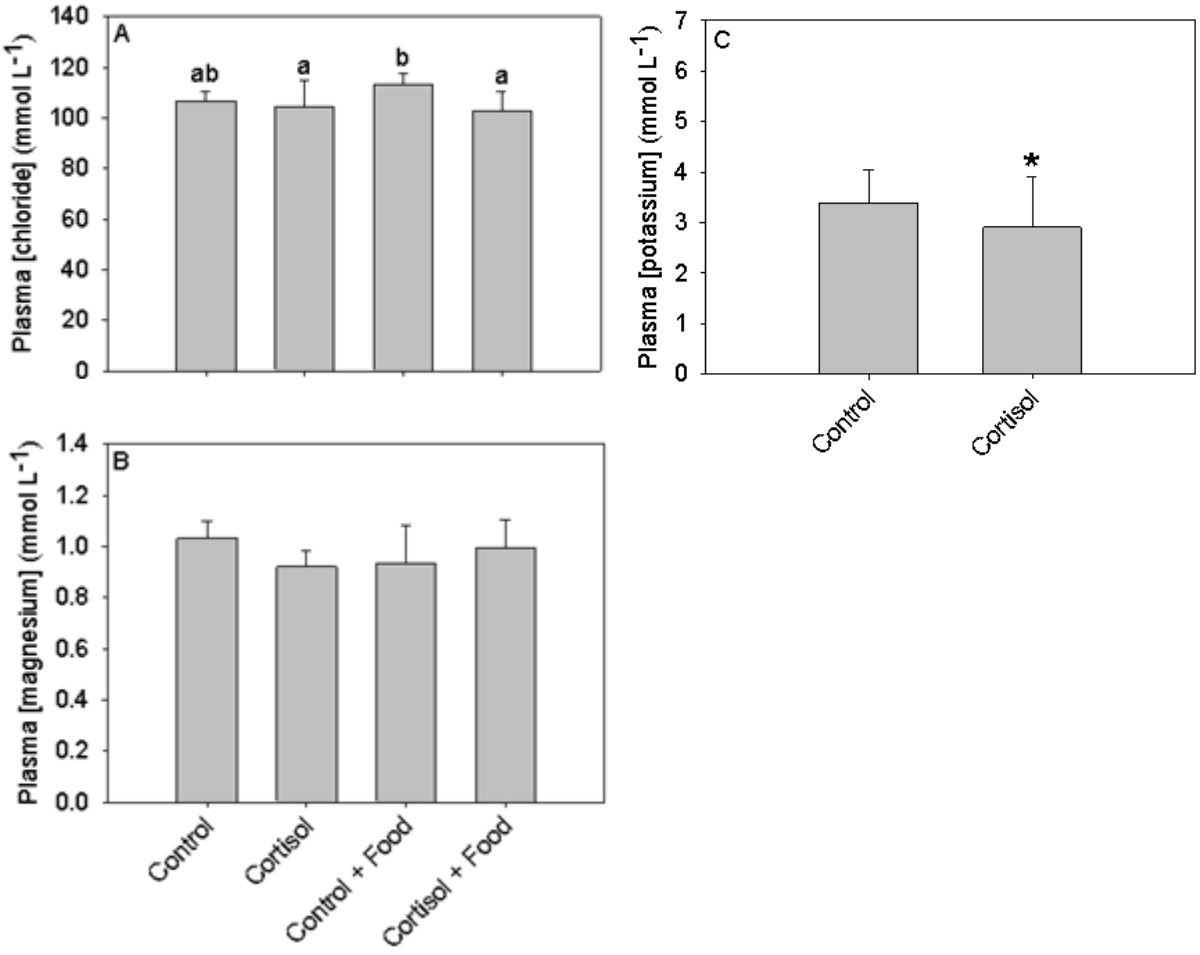

Figure 7: Comparison of plasma chloride (**A**) and plasma magnesium (**B**) concentrations among the four treatment groups of parental smallmouth bass; and differences in plasma potassium (**C**) levels between control and cortisol-treated parental smallmouth bass. Plasma chloride and plasma magnesium data are presented as mean values (± SE) for control fish (n = 10), cortisol-fish (n = 10), control plus food fish (n = 11), and cortisol plus food fish (n = 11). Plasma potassium data are presented as mean values (± SE) for control fish (n = 10), cortisol-fish (n = 10), control plus food fish (n = 11), and cortisol plus food fish (n = 11). Figure 5C represents the main statistical effect of cortisol treatment, as the interaction term between supplemental feeding and cortisol was not significant. Letter assignments of "a" and "b" denote a significant difference at a level of $\propto = 0.05$ between treatment groups. Stars represent statistical differences between treatment groups at a level of $\propto = 0.05$.

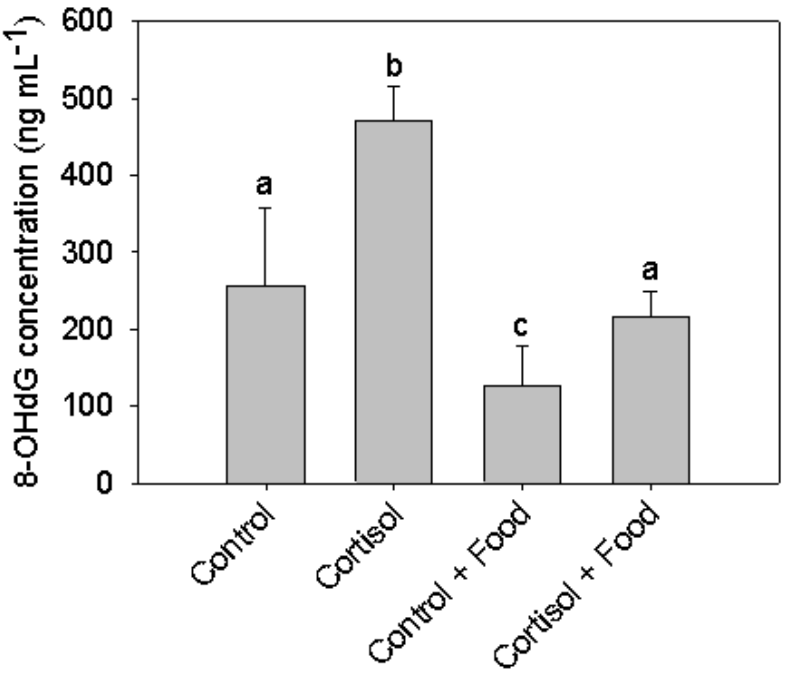

Figure 8: 8-OH-dG concentration (ng/mL) in each treatment group of smallmouth bass after using 8-OH-dG EIA kit Epoch Microplate Spectrophotometer. Data are presented as mean values (± SE) for control fish (n = 7), cortisol-treated fish (n = 7), control + food-treated fish (n = 7), and cortisol-treated + food-supplemented fish (n = 7). Letter assignments of "a", "b", and "c" denote a significant difference at a level of $\propto = 0.05$ among treatment groups. See text for details.

3.5 Immune Function

Five categories of leukocytes were identified in the blood smear preparations including lymphocytes, thrombocytes, granulocytes (eosinophiles, basophils, and mast cells), monocytes, and neutrophils. Cortisol treatment had a significant effect on lymphocyte, neutrophil, and monocyte abundance, whereas leukocyte concentrations appeared to be independent of nutritional status. Specifically, cortisol treatment had a significant effect on the proportion of lymphocytes in the plasma (two-way ANOVA, $F_{3, 40} = 12.401$, $P = 0.001$; Table 4). A Student's t-test post-hoc analysis revealed that lymphocyte numbers were lower in cortisol-treated fish than control fish (Fig. 9). Moreover, cortisol treatment had a significant impact on plasma neutrophil and monocyte proportions (two-way ANOVA, $F_{3, 40} = 4.264$, $P = 0.045$ for neutrophils and $F_{3, 40} = 7.666$, $P = 0.008$, for monocytes; Table 4). A Student's t-test post-hoc analysis indicated that both neutrophil and monocyte concentrations were higher in cortisol-treated fish relative to control fish (Fig. 9).

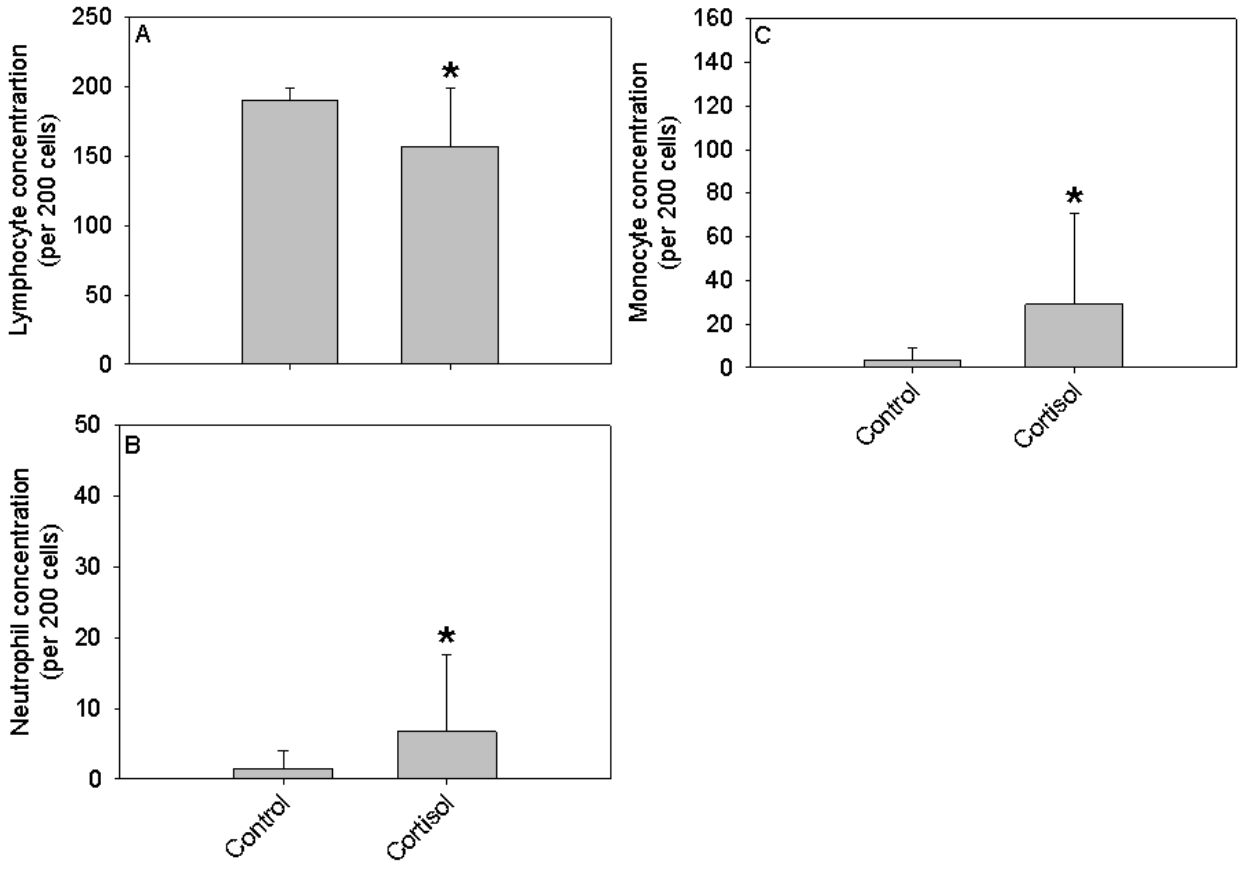

Figure 9: Proportional comparison of lymphocyte (A), neutrophil (B), and monocyte (C) prevalence between control and cortisol-treated fish. Figures represent proportional data based on the mean percentage of specified cell per random 200 cell counts. Data are presented as mean values (± SE) for control fish (n = 10), cortisol-fish (n = 10), control plus food fish (n = 12), and cortisol plus food fish (n = 12). All figures represent the main statistical effect of cortisol treatment, as the interaction term between supplemental feeding and cortisol was not significant. Stars represent statistical differences between treatment groups at a level of $\propto = 0.05$. See text for detail.

Table 4: Effects of cortisol treatment and food supplementation on leukocyte concentrations in parental smallmouth bass sampled from Lake Opinicon and Sand Lake, Ontario. Italicized and boldfaced values indicate a statistically significant difference at $\propto = 0.05$.

	Cortisol			Food Supplement			Interaction		
Leukocyte type	*df*	*F-ratio*	*P-value*	*df*	*F-ratio*	*P-value*	*df*	*F-ratio*	*P-value*
Lymphocyte	1	12.401	***<0.010***	1	0.475	0.494	1	0.634	0.430
Thrombocyte	1	0.036	0.849	1	0.002	0.962	1	0.446	0.508
Granulocyte	1	1.171	0.285	1	2.109	0.154	1	0.372	0.545
Neutrophil	1	4.264	***<0.050***	1	1.721	0.196	1	2.534	0.119
Monocyte	1	7.666	***<0.001***	1	0.000	0.976	1	0.069	0.79

4.0 DISCUSSION

The results of the present study indicate that nutritional condition and elevated plasma cortisol levels had mixed effects on parental behavior, physiology, and reproductive success. Specifically, supplemental feeding had no detectable influence on parental care behaviour or immune capacity. However, nutritional condition was an important mediator of reproductive success, likely by reducing the oxidative stress associated with parental care and reducing the likelihood of nest abandonment. By contrast, elevation of circulating cortisol concentrations to mimic the effects of a chronic stressor suppressed adaptive immunity while enhancing innate immunity, but had no significant effect on parental behaviour or reproductive success. The results of this study underline the complexity of relationships among nutritional condition, stress, and parental care.

4.1 Parental Care Behaviour

Of the three parental care behaviours measured (nest tending vigilance, brood defense aggression, and duration of parental care) none were found to be significantly influenced by plasma cortisol elevation or by supplemental feeding of parental males; i.e., smallmouth bass maintained a similar intensity of parental behaviour regardless of their nutritional condition or exposure to increases in circulating GCs. Behavioural resistance to elevated GCs has been previously documented in both largemouth and smallmouth bass during the parental care period, where both species maintained parental behaviour during chronic elevation of plasma cortisol during the egg and egg-sac fry stage of brood development (O'Connor et al., 2009; Dey et al., 2010). Resistance to stress during periods of parental care also has been widely documented in mammalian and avian taxa, and is thought to be an adaptive response to increase current reproductive fitness (Astheimer et al., 2000; Wingfield and Sapolsky, 2003). The lack of impact of nutritional condition on parental behaviours was unexpected, as supplemental feeding during the reproductive period has been shown to widely influence parental care behaviours in a variety of taxa (Boutin, 1990; Hogstedt, 1981; Schreck et al., 2001; Brown et al., 2010). In avian species, supplemental feeding has been shown to increase parental nest presence, egg incubation time, and increase parental aggression towards nest predators (Dewey and Kennedy, 2001; Lothery et al., 2014; Markman, 2014). Supplemental feeding has also been linked to improving reproductive success. Specifically, magpies (Pica pica) were found to have higher egg hatching success when provided supplemental diets during the breeding season as compared to non-fed individuals (Hogstedt, 1981). In previous research on

parental smallmouth bass, supplemental feeding increased parental care duration (Ridgway and Shuter, 1994) while reducing parental aggression towards simulated brood predators (Hanson et al., 2009). Thus, it was surprising that supplemental feeding of parental smallmouth bass in the present study yielded no differences in care behaviours. The energy requirements for parental care in bass largely are supplied by endogenous energy stores (i.e., lipids), making it essential that parental smallmouth bass build up the required energy reserves needed to sustain parental effort throughout the care period prior to spawning (Smith and Wootton, 1995; Cooke et al., 2006; Hanson et al., 2009; Gravel et al., 2010). Thus the energy gained through food supplements may not have been necessary to improve parental effort during the initial egg and egg-sac fry stages of the brood development period, which was when our behavioural assessments were conducted. It is also possible that the observational methods used to assess nest-tending vigilance and parental aggression towards simulated brood predators were not sufficient to detect subtle changes in care behaviour.

4.2 Reproductive Success

Although behaviour was not affected by supplemental feeding during the care period, food supplementation had a positive impact on reproductive success. The parental care period is an inherently challenging and dynamic life-stage requiring a large energetic reserve to maintain parental activities, and sustain homeostatic set-points; and for parental smallmouth bass this inherently challenging period is compounded by a voluntary reduction in feeding, limiting their energetic budget (Ridgway and Shuter, 1994; Cooke et al., 2006; Hanson et al., 2009; Zuckerman and Suski, 2013). Food supplementation throughout the egg and egg-sac fry stage of brood development provided a consistent source of food energy during periods of energy-intensive parental activity, presumably off-setting the high energetic cost of parental care. Similarly, Ridgway and Shuter (1994) observed that supplemental feeding had a density-dependent effect on reproductive success in smallmouth bass. When the spawning population of smallmouth bass was high, supplemental feeding increased current reproductive success compared to non-fed control fish. Conversely, when the spawning population of smallmouth bass was low, the food energy gained through supplemental feeding was reallocated towards future reproduction. Relatively few studies have been conducted using supplemental feeding techniques during the reproductive period, and most have focused on avian and mammalian models (reviewed in Boutin, 1990). Studies employing supplemental feeding techniques in mammals generally have found that

increased access to food resources during reproduction supports larger litter sizes and increased offspring survival (Angerbjorn et al., 1991; reviewed in Boutin, 1990). Similarly, supplemental feeding in avian species has been associated with increased hatching success as well as offspring survival (reviewed in Boutin, 1990; Lothery et al., 2014).

Elevated plasma cortisol levels had no significant impact on the reproductive success of parental smallmouth bass in the present study. However, the nest abandonment rate among the cortisol treatment group was approximately twice as high as the nest abandonment rate in the control group (Fig. 2). Although this finding was not statistically significant, this trend does correspond with previous research examining the impacts of stress during the parental care period of black bass (O'Connor et al., 2009; Dey et al., 2010). O`Connor et al. (2009) attributed the lower reproductive success among cortisol treated fish to the secondary and tertiary effects of cortisol on metabolism and immunity, although these components were not measured in their study. Although we observed no significant difference in reproductive outcomes between control and cortisol-treated fish, cortisol-treated fish had significantly higher levels of plasma glucose and plasma cholesterol, indicating increases in carbohydrate and lipid catabolism, respectively. Hyperglycemia and hypercholesterolemia are physiological states associated with an elevation in metabolic activity, which is a well-documented physiological effect of GCs on vertebrate physiology (Wendelaar Bonga, 1997; Mommsen et al., 1999).

4.3 Oxidative Stress

Oxidative stress can have numerous negative consequences on a variety of biological processes and systems (i.e., inactivate proteins, damage cellular membranes, and degrade DNA), which can impair the physiological condition of an organism. Furthermore, these impairments can incur energetic and physiological costs associated with repairing the cellular damage. Oxidative stress in parental smallmouth bass was assessed through the accumulation of DNA damage, as measured by 8-OHdG formation on DNA nucleotides. DNA nucleotides are prominent sites for oxidative damage due to their high mutagenic and oxidative potential (Cooke et al., 2002; Evans et al., 2004; Shimoi et al., 2002; Wu et al., 2004; Wilson et al., 2012) . Among nucleic base pairs, guanine is most prone to oxidation (Cooke et al., 2002; Wu et al., 2004). When guanine is oxidized by a free hydroxyl group it becomes 8-OHdG, a prominent form of free-radical induced deformation of DNA (Valavanidis et al., 2009; Wu et al., 2004). Under normal metabolic conditions, metabolic

and antioxidant systems isolate and repair oxidative damage. However, when free radical production out-competes antioxidant processes, oxidative stress occurs, and can lead to increased cellular damage over time (Valavanidis et al., 2009). Of the various types of injury inflicted by free radical attack on nucleotides, only 8-OHdG results in point mutations, making it a reliable and accurate measure of oxidative injury (Shimoi et al., 2002; Srivastava and Kumar, 2015). The present study assessed the interplay among nutritional condition, stress, and parental care on oxidative injury in a wild teleost species. Cortisol treatment caused significantly higher concentrations of 8-OHdG when compared to the control group. However, the effect of cortisol on 8-OHdG production was alleviated by supplemental feeding; 8-OHdG levels for the cortisol plus food treatment group were comparable to the levels in the control group (Fig, 6). In addition, supplemental feeding alone appeared to mitigate oxidative stress associated with parental care; 8-OHdG levels were lowest in the control plus food treatment group (Fig, 6). These results are interesting as they identify a potential physiological mechanism that may influence a parental male's decision to 'stay' or 'abandon' its current reproductive effort. In the current study, the buffering effect of feeding on reducing 8-OHdG concentrations could be a direct result of having access to available food energy to support and maintain antioxidant processes; or through the acquisition of antioxidants directly from the supplemental food sources (i.e., essential vitamins). Although antioxidant capacity was not directly measured in this study.

4.4 Immune Function

The immune capacity of parental smallmouth bass was assessed through the proportions of white blood cell types identified from blood smear preparations. Cortisol-treated fish had significantly lower proportions of plasma lymphocyte concentrations than control fish. A stress-induced state of lymphocytopenia has been documented in various taxa, including fish, and is considered a physiological consequence of chronic stress (Pearson et al., 1978; Pickering, 1984; Kattarir and Tripp, 1987; Barton, 1991). Lymphocytes are integral components of both the innate and adaptive immune systems, responsible for long-term adaptive defence against bacterial and viral pathogens (Moretta, 2005). Previous studies have noted that chronic increases in GCs cause apoptotic or necrotic cell death of lymphocytes, leading to reduced phagocytic activity, antibody production, lymphocyte numbers and resistance to pathogens (Chilmonczyk, 1982; Pickering, 1984; Yada and Nakanishi, 2002; Saha et al., 2003; Srivastava and Kumar, 2015). The present study did not determine whether lymphocytopenia in cortisol-treated fish reflected lymphocyte redistribution

within the tissues of the fish or a cytolytic response to elevated cortisol levels. Conversely, plasma monocyte and neutrophil concentrations both were proportionally higher in cortisol-treated fish compared to control fish. Monocytes and neutrophils are key components of innate immunity, and are generally the 'first responders' at a wound/inflammation site (Magnadóttir, 2006). Neutrophils are phagocytic, whereas monocytes play key roles in both phagocytosis and inflammation. Monocytes can either upregulate or downregulate inflammation depending on their activation state (Srivastava and Kumar, 2015). Taken together, these findings indicate that cortisol elevation mimicking chronic stress activates part of the innate immune system through the induction of neutrophils and monocytes in the blood plasma, whereas the adaptive immune system is inhibited owing to the reduction in plasma lymphocyte concentrations.

4.5 Future Research Directions

With respect to future research it is worth considering the disadvantages of solely examining correlates of oxidative stress without measuring antioxidant capabilities. One limitation of this study was that oxidative injury was solely assessed via 8-OHdG production, however antioxidant remediation (e.g., superoxide dismutase or glutathione production) was not considered due to logistical reasons (e.g., insufficient blood samples to run antioxidant assessments). Measuring an animal's antioxidant buffering capacity is valuable in providing a holistic picture of how an animal deals with, or is impacted by, oxidative stress. A study conducted by Wilson et al. (2012) assessed oxidative stress across the parental care period in parental smallmouth bass and noted a strong antioxidant presence to counteract oxidative stress. Future oxidative stress assessments should consider measuring antioxidant concentrations to gain a more thorough understanding of how oxidative stress influences physiology and behaviour.

Another potential limitation of this study was the observational assessments used to measure parental behaviour (e.g., parental aggression and nest tending vigilance). It is possible that the techniques employed in the present study were unable to detect subtle changes in care behaviour between treatment groups. Supplemental feeding has been noted to have a wide range of impacts on parental behaviour across taxa, including smallmouth bass. The fact that changes in parental behaviours were not detected in the present study is peculiar. Perhaps the use of video recording devices (Struthers et al., 2015) or accelerometry biologgers (Brownscombe et al., 2014) could

benefit future behavioural assessments by providing a more thorough means to investigate changes in parental behaviour over time and space.

In the future it would also be of interest to further explore the benefits of nutritional condition on mediating the effects of oxidative stress during challenging life-history periods. To date, the present study is one of the few existing studies to have examined this relationship in the context of life-history trade-offs (via parental care and reproductive outcome), through direct comparisons between fed and fasting treatment groups. However, the dietary constituents of the food sources were not analyzed. Thus, the buffering effect of the food supplements on reducing 8-OHdG concentrations could be a direct result of having access to available food energy to support and maintain antioxidant processes; or through the acquisition of antioxidants directly from the supplemental food sources (i.e., essential vitamins). Future research on nutrition and oxidative stress should be directed towards understanding how specific dietary constituents influence antioxidant capabilities.

4.6 Conclusion

The results of this study underline the complexity of relationships among nutritional condition, stress, and parental care. Cortisol treatment, used as a tool to mimic the effects of chronic stress, altered leukocyte composition in the blood plasma, reducing aspects of adaptive immunity through the reduction of lymphocyte concentrations, and boosting innate immunity by increasing the proportion of neutrophils and monocytes. Cortisol treatment also increased oxidative injury, based on 8-OHdG concentrations. Together, such effects may increase the likelihood of nest abandonment in cortisol-treated, i.e. chronically stressed, males. Supplemental feeding improved the overall reproductive success of parental males, regardless of cortisol elevation, suggesting that the high energetic demands associated with parental care are a key determinant of reproductive success. Moreover, supplemental feeding reduced the generation of 8-OHdG, which is a key indicator of oxidative damage. The accumulation of oxidative damage over time is considered to be an important physiological driver modulating life-history trade-offs between reproduction and survival (Behl et al., 1997; Garratt et al., 2011; Srivastava and Kumar, 2015). The results of the present study indicate that improving parental nutritional status reduces the generation of 8-OHdG, and understanding this link could be crucial in understanding how parental animals allocate energy resources between physiological maintenance and reproductive output. Future research should

focus on measuring not only correlates of oxidative stress, but also, the antioxidant buffering capacity to oxidative stress. This will provide a better understanding of how animals generate and mitigate the physiological damage associated with reproduction and parental care.

References:

Adkins-Regan, E. (2005). Hormones and animal social behavior. Princeton University Press.

Alonso-Álvarez, C. and Velando, A. (2012). Benefits and costs of parental care. In The Evolution of Parental Care (ed. Royle, N. J., Smiseth, P. T. and Kölliker, M.), pp 40-61. Oxford: Oxford University Press.

Barton, C. (2002). Stress in Fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integ. Comp. Biol.* **522**: 517-525.

Barton, B. a. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish Dis.* **1**, 3–26.

Baylis, J. R. (1981). The evolution of parental care in fishes, with reference to Darwin's rule of male sexual selection. *Environ. Biol. Fishes*. **6**(2), 223-251.

Beckman, K. B. and Ames, B. N. (1998). The free radical theory of aging matures. Physiol. Rev. **78**, 547–581.

Behl, C., Lezoualc'h, F., Trapp, T., Widmann, M., Skutella, T. and Holsboer, F. (1997). Glucocorticoids enhance oxidative stress-induced cell death in hippocampal neurons in vitro. *Endocrinology* **138**, 101–106.

Bell, G. (1980). The costs of reproduction and their consequences. *Amer. Nat.* 45-76.

Blumer, L. S. (1979). Male parental care in the bony fishes. Quarterly Review of Biology. 149-161.

Brownscombe, J.W., L.F. Gutowsky, A.J. Danylchuk and S.J. Cooke. (2014). Foraging behaviour and activity of a marine benthivorous fish estimated using tri-axial accelerometer biologgers. *Mar. Ecol. Prog. Ser.* **505**, 241-251.

Cidlowski, J. A., King, K. L., Evans-Storms, R. B., Montague, J. W., Bortner, C. D. and Hughes, F. M. (1996). The biochemistry and molecular biology of glucocorticoid-induced apoptosis in the immune system. *Recent Prog. Horm. Res.* **51**, 457-491.

Clutton-Brock , T. H. (1991). The evolution of parental care. Princeton University Press, New Jersey.

Cockburn, A. (2006). Prevelance of different modes of parental care in birds. *Proc. R. Soc. B.* **272**, 1375-1383.

Comendant, T., Sinervo, B., Svensson, E. I. and Wingfield, J. (2003). Social competition, corticosterone and survival in female lizard morphs. *J. Evol. Biol*. **16**, 948-955.

Cooke, M. S., Lunec, J. and Evans, M. D. (2002). Progress in the analysis of urinary oxidative DNA damage. *Free Radic. Biol. Med*. **33**, 1601–1614.

Cooke, S. J., Philipp, D. P., Wahl, D. H. and Weatherhead, P. J. (2006). Energetics of parental care in six syntopic centrarchid fishes. *Oecologia* **148**, 235–249.

Crossin, G.T., O.P. Love, S.J. Cooke and T.D. Williams. In Press. Glucocorticoid manipulations in free-living animals: considerations of dose delivery, life-history context, and reproductive state. *Funct. Ecol.* **00:000-000**.

Dhabhar, F. S. (2000). Acute stress enhances while chronic stress suppresses skin immunity – the role of stress hormones and leukocyte trafficking. *Neuroimmunomodulation* **917**, 876-893.

Dhabhar, F. S. and McEwen, B. S. (1997). Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. *Brain Behav. Immun.* **11**, 286-306.

Dey, C., O'Connor, C., Gilmour, K. M., Van Der Kraak, G. and S. J. Cooke. (2010). Behavioral and physiological responses of a wild teleost fish to cortisol and androgen manipulation during parental care. *Horm. Behav.* **58**(4), 599-605.

Di Giulio, R. T., Washburn, P. C., Wenning, R. J., Winston, G. W. and Jewell, C. S. (1989). Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environ. Toxicol. Chem*. **8**(12), 1103-1123.

Evans, M. D., Dizdaroglu, M. and Cooke, M. S. (2004). Oxidative DNA damage and disease: induction, repair and significance. *Mutat. Res.* **567**, 1-61.

Farmer, C, G. (2011). Parental Care : The Key to Understanding Endothermy and other convergent features in birds and mammals. *Amer. Nat.* **155**, 326–334.

Fitzgerald, G.J. and Caza, N. (1993). Parental investment in an anadromous population threespine sticklebacks: an experimental study. *Evol. Ecol.* **7**: 279–286.

French, S. S. Matt, K. S. and Moore, M. C. (2006). The effects of stress on wound healing in male tree lizards (Urosaurus ornatus). *Gen*. *Comp*. *Endocrinol*. **145**(61), 128–132.

Gamperl, A., Vijayan, M., and Boutilier, R. (1994). Experimental control of stress hormone levels in fishes: techniques and applications. *Rev. Fish Biol. Fish.* **4**(2), 215-255.

Gittleman, J. L. (1981). The phylogeny of parental care in fishes. *Anim. Behav*. **29**(3), 936-941.

Glasper, E. R. and DeVries, A. C. (2005). Social structure influences effects of pair-housing on wound healing. *Brain. Behav*. *Immun*. **19**, 61–68.

Gross, M. R. and Sargent, R. C. (1985). The evolution of male and female parental care in fishes. *Amer. Zool*. **25**(3), 807-822.

Hanson, K. C., Abizaid, A. and Cooke, S. J. (2009). Causes and consequences of voluntary anorexia during the parental care period of wild male smallmouth bass (Micropterus dolomieu). *Horm. Behav.* **56**(5), 503-509.

Hinch, S. G. and Collins, N. C. (1991). Importance of diurnal and nocturnal nest defense in the energy budget of male small- mouth bass: insights from direct video observations. *Trans Am Fish Soc* **120**:657–663.

Jessop, T. S., Knapp, R., Whittier, J. M. and Limpus, C. J. (2002). Dynamic endocrine responses to stress: evidence for energetic constraints and status dependence in breeding male green turtles. *Gen. Comp. Endocrinol*. **126**, 59- 67.

Kattarir S. A. and Tripp, R. a (1987). Cellular mechanisms of glucocorticoid immunosuppression in salmon. *J. Fish. Biol.* **31**, 129–132.

Leonard, B. E. and Song, C. (2002). Changes in the immune system in rodent models of depression. *Int. J. Neuropsychopharmacol*. **5**, 345-356.

Love, O.P., Breuner, C.W., Vézina, F., Williams, T.D. (2004). Mediation of a corticosterone induced reproductive conflict. *Horm. Behav*. **46**, 59–65.

Magnadóttir, B. (2006). Innate immunity of fish (overview). *Fish Shellfish Immunol.* **20**, 137–151.

Martin, L. B., Glasper, E. R., Nelson, R. J., and DeVries, A. C. (2006). Prolonged separation delays wound healing in monogamous California mice, Peromyscus californicus, but not in polygynous white-footed mice, P. leucopus. *Physiol. Behav*. **87**, 837–841.

Metcalfe, N. B. and Monaghan, P. (2013). Does reproduction cause oxidative stress? An open question. *Trends Ecol. Evol*. **28**(6), 347-350.

Mommsen, T. P., Vijayan, M. M. and T. W. Moon. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol.Fisher.* **9**(3), 211-268.

Monaghan, P., Metcalfe, N. B. AND Torres, R. (2009). Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecol. Lett.* ***12***(1), 75-92.

Moretta, L. (2005). Lymphocyte effector functions. *Curr. Opin. Immunol.* **17**, 303–305.

Nelson, R.J, (2000). An Introduction to Behavioral Endocrinology. Sinauer Associates, Sunderland, Massachusetts.

O'Connor, C.M., Gilmour, K.M., Arlinghaus, R., Van Der Kraak, G. and S.J. Cooke. (2009). Stress and parental care in a wild teleost fish: Insights from exogenous supraphysiological cortisol implants. *Physiol.Biochem. Zool*. **82**,70.

O'Connor, C. M., Yick, C. Y., Gilmour, K. M., Van Der Kraak, G. and Cooke, S. J. (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.

Pearson, C. M., Celements, P. J., and Yu, D. T. Y. (1978). The effects of corticosteroids on lymphocyte functions. *Eur. J. Rheumatol. Injlammation* **1**, 216-225.

Pereyra, M. E. and Wingfield, J. C. (2003). Changes in plasma corticosterone and adrenocortical response to stress during the breeding cycle in high altitude flycatchers. *Gen Comp Endocrinol.* **130**: 222–231.

Peres, H., Santos, S. and Oliva-Teles, A. (2014). Blood chemistry profile as indicator of nutritional status in European seabass (Dicentrarchus labrax). *Fish Physiol. Biochem.* **40**, 1339-1347.

Pflieger, W. L. (1966). Reproduction of the smallmouth bass (Micropterus dolomieui) in a small Ozark stream. *Am. Midl. Nat.* **76**:410-418.

Pickering, A. D. (1984). Cortisol-Induced Lymphocytopenia in Brown Trout , Salmo trutta L . *Gen. Comp. Endocrinol.* **259**, 252–259.

Pike, T. W., Blount, J. D., Lindström, J. and Metcalfe, N. B. (2007). Dietary carotenoid availability influences a male's ability to provide parental care. *Behav. Ecol.* **18**, 1100–1105.

Ray, P. D., Huang, B. W. and Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell. Signal.* **24**(5), 981-990.

Ridgway, M. S. (1988). Developmental stage of offspring and brood defense in smallmouth bass (Micropterus dolomieui). *Can. J. Zool.* **66**, 1722–1728.

Ridgway, M. S. & Friesen, T. G. (1992). Annual variation in parental care in smallmouth bass, Micropterus dolomieu. *Environ. Biol. Fishes* **35**, 243–255.

Ridgway, M. S., and Shuter, B. J. (1994). The effects of supplemental feeding in parental male smallmouth bass. *Environ. Biol. Fishes*. **39**, 201-207.

Reznick, D., L. Nunney, andA. Tessier. (2000). Big houses, big cars, superfleas and the costs of reproduction. *Trends Ecol. Evol.* **15**, 421–425.

Romero, L. M. (2002). Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. *Gen. Comp. Endocrinol.* **128**, 1–24.

Royle, N. J., Smiseth, P. T. and Kölliker, M. (2012). The evolution of parental care. Oxford University Press.

Schreck, C. B., Contreras-Sanchez, W. and Fitzpatrick, M. S. (2001). Effects of stress on fish reproduction, gamete quality, and progeny. *Aquaculture* **197**, 3–24.

Smiseth , P.T., Kölliker, M. and Royle, N. J. (2012). What is parental care? In The evolution of parental care (eds N. J. Royle, P. T. Smiseth, and M. Kölliker), pp. 1–17. Oxford, UK: Oxford University Press.

Smith, C. and Wootton, R. J. (1995). The costs of parental care in teleost fishes. *Rev. Fish Biol. Fish.* **5**, 7–22.

S**truthers, D.P., A.J. Danylchuk, A.D.M. Wilson and S.J. Cooke.** (2015). Action cameras: Bringing aquatic and fisheries research into view. *Fisheries.* **40**(10), 502-512.

Trivers, R.L. (1972). Parental investment and sexual selection. In: Campell B (ed) Sexual selection and the descent of man. Heinemann Press, London, pp 139–179.

Tuomi, J., Hakala, T. and Haukioja, E. (1983). Alternative concepts of reproduc- tive effort, costs of reproduction, and selection in life-history evolution. *Am. Zool.* **23**, 25–34.

Valavanidis, A., Vlachogianni, T. and Fiotakis, C. (2009). 8-hydroxy-2′ -deoxyguanosine (8-OHdG): A Critical Biomarker of Oxidative Stress and Carcinogenesis. *J. Environ. Sci. Heal. Part C* **27**, 120–139.

Wagner, T. and Congleton, J. L. (2004). Blood chemistry correlates of nutritional condition, tissue damage, and stress in migrating juvenile Chinook salmon (Oncorhynchus tshawytscha). *Can. J. Fish. Aquat. Sci.* **61**, 1066–1074.

Webb, J. N., Szekely, T., Houston, A. I., McNamara, J. M. (2002). A theoretical analysis of the energetic costs and consequences of parental care decisions. Philos. *Trans. R. Soc. B-Biol. Sci.* **357**, 331–340.

Wingfield, J. C. (1994). Hormone-behavior interactions and mating systems in male and female birds. The differences between the sexes. Cambridge University Press, Cambridge, 303-330.

Wilson, B. S. and Wingfield, J. C. (1992). Correlation between female reproductive condition and plasma-corticosterone in the lizard Uta stansburiana. *Copeia* **1992**, 691-697.

Wingfield, J. C. and Sapolsky, R. M. (2003). Reproduction and resistance to stress: when and how. *J. Neuroendocrinol.* **15**(8), 711-724.

Yasutake, W. T. and Wales, J. H. (1983). Microscopic anatomy of salmonids: an atlas. U.S. Fish and Wildlife Service, Resource Publication 150,Washington, D.C.

Zar, J. H. (1999). Biostatistical Analysis, 4th ed. Prentice Hall, Upper Saddle River, NJ, USA.

Zera, A. J. and Harshman, L. G. (2001). The physiology of life history trade-offs in animals. *Annu. Rev. Ecol. Syst.* 95-126.

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APPENDIX A - Pictures of Methods: Behavioural assessments, fish capture, fish processing and cortisol implantation:

Figure 10: Methods used to capture and process parental male smallmouth bass. Nest-tending vigilance (A) was conducted by a snorkeler in the water position approximately 3 – 5 m from the nest site to visually observe and record parental behaviour. A clear glass jar (B) housing a bluegill sunfish (a common and abundant nest predator of the smallmouth bass) was placed on the edge of the nest for a 60 s time period, and during this time a snorkeler would observe and record the number of aggressive strikes made by the parental male on the glass jar. All fish were captured via rod and reel by an experienced angler (C). Upon capture fish would be processed according to their treatment. Cortisol treated fish would receive an intraperitoneal injection of cocoa butter-cortisol mixture (D), the amount was determined based on fish size. See above text for detailed expiation.

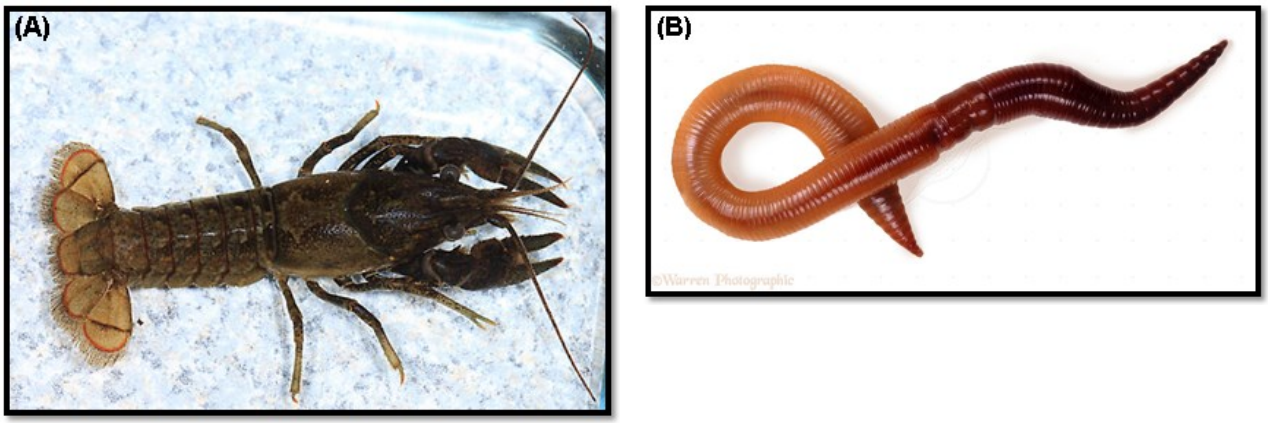

Figure 11: Food sources provided to the food treatment groups including locally captured crayfish (A), and locally purchased earthworms (B). See above text for detailed explanation.