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RESEARCH ARTICLE

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

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## ABSTRACT

Parental care is an essential life-history component of reproduction for many animal species, and it 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 implantation) 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.

**KEY WORDS: Smallmouth bass, Immune function, Oxidative stress, Nutrition**

## INTRODUCTION

Parental care is a common phenomenon typically defined as any behavioural investment made by a parent to promote the survival and development of its offspring 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, paternal care is relatively common among fish (Blumer, 1979, 1982; Gittleman, 1981). Providing parental care is energetically costly, as resources are diverted away from physiological maintenance of the individual, potentially resulting in reduced growth, immunocompetence and longevity of the parent (Alonso-Álvarez 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-Álvarez and Velando, 2012).

The endocrine system is recognized as an important mediator of these fitness trade-offs (Zera and Harshman, 2001; Adkins-Reagan, 2005), with a particular focus on the effects of stress or resistance to stress during reproduction (Wingfield, 1994; Wingfield and Sapolsky, 2003). In fish, as in other vertebrates, exposure to a stressor elicits a neuroendocrine response cascade involving the release of catecholamines from chromaffin tissue, and activation of the hypothalamic-pituitary-interrenal (HPI; HP-adrenal in tetrapods) axis (Schreck et al., 2001; Barton, 2002). Activation of the HPI axis results in an increased production and circulation of glucocorticoids (GCs; cortisol in teleost fish) (Mommsen et al., 1999; Barton, 2002). GCs are widely accepted as energy-mobilizing hormones that promote the catabolism and redistribution of endogenous energy resources away from physiological processes such as immunity and reproduction, and redistributes these energy resources towards essential life-support functions to restore homeostasis (Mommsen et al., 1999); this use of energy reserves makes the physiological response to a stressor energetically expensive (Barton, 2002).

Stress plays an important role in regulating parental investment in vertebrates (Wilson and Wingfield, 1992; Romero, 2002; Pereyra and Wingfield, 2003; Love et al., 2004). 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

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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 other 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 (Di Giuilo et al., 1989). ROS are a normal byproduct 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., 2012). Elevated metabolic activity during a stress response increases the potential for ROS production.

Although GCs in high concentrations can impair reproduction and immunity, the extent of these negative fitness costs may be influenced by the nutritional condition of the animal. The physiological costs of chronic stress can be reduced when animals have access to abundant food, reducing the demand on energy reserves (Tuomi et al., 1983; Reznick et al., 2000; French et al., 2006, 2007; French and Moore, 2008; Fletcher et al., 2012). Parental care is an energetically demanding life-history period. Although much research has focused on how additional challenges (i.e. stress) influence the fitness trade-offs of a parent (e.g. Tuomi et al., 1983; Reznick et al., 2000; French et al., 2006, 2007), 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 animals that provide parental care, 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 organisms (e.g. French et al., 2006, 2007; French and Moore, 2008; but see Ridgway and Shuter, 1994).

Smallmouth bass (*Micropterus dolomieu* Lacépède 1802) 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 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 (Pflieger, 1966). 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; 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 removing 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 during the care period experienced decreased reproductive success (e.g. premature nest abandonment) and compromised immune capacity (Dey et al., 2010). Smallmouth bass feed opportunistically during the care period (Ridgway and Shuter, 1994; Hanson et al., 2009), 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.

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 paternal 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.

## MATERIALS AND METHODS

### Experimental fish and treatments

From 17 to 22 May 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 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 in the ventral midsection between the pectoral fins and the cloaca of cocoa butter (5 ml $kg^{-1}$ body mass) containing emulsified hydrocortisone 21-hemisuccinate (Sigma H4881; Sigma-Aldrich, St Louis, MO, USA) (10 mg $ml^{-1}$ of cocoa butter), using a 16-gauge needle and 10 ml syringe. This method has been used in previous studies to elevate circulating plasma cortisol levels for up to 6 days (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 studies that have shown that a sham injection of cocoa butter does not induce the semi-chronic cortisol elevation that is characteristic of cortisol injections (O'Connor et al., 2009). Moreover, the control fish serve to some extent as a sham because they have to be captured for initial assessment.

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### 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 litres) 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 (Fitzgerald and Caza, 1993; O'Connor et al., 2009).

Directly after completion of both behavioural assessments, each fish was captured using rod and reel by an experienced angler either from the boat, or via snorkeling from the water depending on nest accessibility. When nests were located in areas inaccessible by angling from the boat (i.e. underneath a tree), the boat was anchored 5–10 m away from the nest and a snorkeler was employed to capture the nest-guarding male using the same rod and reel from a more appropriate angling location. Heavy action angling gear was used to minimize fight duration (<20 s to land each fish after hookset irrespective of whether hooked from the boat or hooked by the snorkeler), 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 injury. 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 avoid the use of anaesthesia, which is required to accurately weigh fish, mass ($M$, in g) was estimated from total length ($L_T$, in mm) using the equation $M$=6.581$L_T$–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 (22–27 May 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 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; 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, USA). The filled vacutainer was then 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).

### Feeding protocol

In total, 26 parental males (13 control fish and 13 cortisol-treated fish) were fed supplemental diets. Feeding commenced directly after the initial sampling event (17–22 May), and fish were fed every 2–3 days for a total of five 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 mass for both crayfish and worms was calculated using a subset of individuals ($n$=30 for crayfish, $n$=18 for worms). 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 versus fasting).

### 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 (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 100× 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 ***g*** for 5 min. The plasma was decanted, separated into two aliquots and flash-frozen in liquid nitrogen. The red blood cell pellets were also flash-frozen in liquid nitrogen. Plasma and red blood cell aliquots were stored at −80°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 (*Oncorhynchus* spp.), these 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 radioimmunoassay kit (ImmuChem Cortisol Coated Tube RIA Kit, MP Biomedicals, Solon, OH, USA) previously validated for use in smallmouth bass (O'Connor et al., 2009; Dey et al., 2010). Inter-assay variability for the cortisol assay was 15.9%.

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### DNA extraction and digestion

DNA was extracted from blood cell pellets (red and white blood cells) using a whole cell lysis buffer [10 mmol $l^{-1}$ Tris-HCl (pH 8.0), 5 mmol $l^{-1}$ $MgCl_2$, 0.32 mol $l^{-1}$ sucrose, 1% (w/v) Triton X-100, 0.1 mmol $l^{-1}$ desferrioxamine] according to Wilson et al. (2012). A volume of 1 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 pH 7.4. The tube was vortexed on high speed for 2–10 min (time varies according to the density of the sample) and was left on ice for 5 min, followed by centrifugation at 1150 ***g*** for 15 min at 4°C. The supernatant was removed and discarded, and 1 ml of nuclear lysis buffer [20 mmol $l^{-1}$ Tris-HCl (pH 8.0), 4 mmol $l^{-1}$ EDTA, 100 mmol $l^{-1}$ NaCl] and 500 µl of 10% SDS were added to the pellet. The pellet was vortexed vigorously for 2 min, and then 10 µl of proteinase K solution (20 mg $ml^{-1}$) was added to each pellet. Samples were left to incubate in a 55°C water bath for 2 h. After the incubation period, samples were cooled on ice for 5 min, and then 4 ml of 5.3 mol $l^{-1}$ NaCl solution was added to each tube. Samples were then vortexed on a medium speed for 30 s, followed by centrifugation at 1900 ***g*** for 20 min at 4°C. 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 37°C for 20 min to dry. DNA was resuspended in 300 µl of Tris-HCl at pH 8.5 and was left to redissolve overnight at room temperature. Total DNA concentrations were determined using a Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) at an absorbance of 260 nm. All 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentrations were standardized against total DNA concentration (ng $ml^{-1}$).

### DNA digestion

Samples for the digestion period were chosen based on DNA concentration. All samples fell within a range of 4500–5500 ng $µl^{-1}$ (28 in total). A total of 100 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), and then 1 µl of 0.1 mmol $l^{-1}$ desferrioxamine solution was added to each tube, followed by 100 µl of nuclease P1 buffer [40 mmol $l^{-1}$ sodium acetate (pH 5.3), 0.2 mmol $l^{-1}$ ZnCl2, 0.1 mmol $l^{-1}$ desferrioxamine]. Each sample was heated on a 97°C hot block for 3 min, followed by a cooling period of 3 min on ice. Then 16 µl of nuclease P1 buffer [in 20 mmol $l^{-1}$ sodium acetate (pH 5.3), 5 mmol $l^{-1}$ $ZnCl_2$, 50 mmol $l^{-1}$ NaCl, 50% glycerol] was added to each sample, followed by an incubation period at 37°C for 30 min. A volume of 20 µl of alkaline phosphatase buffer [500 mmol $l^{-1}$ Tris-HCl (pH 8.0), 1 mmol $l^{-1}$ EDTA, 2 mmol $l^{-1}$ $ZnCl_2$, 50 mmol $l^{-1}$ $MgCl_2$] was added to each sample, followed by a 1 h incubation period at 37°C. Afterwards, 18.5 µl of 0.1 HCl was added to each sample to neutralize the reaction. Each sample was centrifuged at 13,000 ***g*** for 5 min. The supernatant was transferred to a new tube and digested sample was run on a 1% agarose gel next to its respective undigested form to ensure full digestion of DNA. The supernatant was stored at −20°C for 2 weeks, and was thawed on ice after this period, when the experiment was resumed to conduct the DNA damage assay.

### 8-OHdG enzyme immunoassay

Oxidative stress was quantified by measuring the production of 8-OHdG using the 8-OHdG EIA Kit (Cayman Chemicals, Ann Arbor, MI, USA) 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 (4500–5000 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, Winooski, VT, USA) and values from each sample were calculated based on the absorbance of standard 8-OHdG concentrations (10.3, 23.1, 52.0, 117.1, 263.4, 592.6, 1333 and 3000 pg $ml^{-1}$).

### Statistical analyses

All statistical analyses were conducted using JMP 9.0.1 with significance levels ($\alpha$) 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). A natural log transformation was applied to datasets that had skewness values >1 relative to a normal distribution of 0. However, if skewness values were <−1 relative to a normal distribution of 0, the data were transformed by squaring all values. Two-way ANOVA followed by either a Student's *t*-test or a 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 (Zar, 1999). 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. All values are presented as means±1 s.e.m.

## RESULTS

### Cortisol manipulations

As expected, the cortisol implants elevated plasma cortisol levels in treatment fish, regardless of supplemental feeding (two-way ANOVA, $F_{1,28}$=86.2474, $P$<0.001). *Post hoc* analysis revealed that plasma cortisol levels were highest in both cortisol treatment groups compared with the control groups (Fig. 1, Tables 1, 2). A complete list

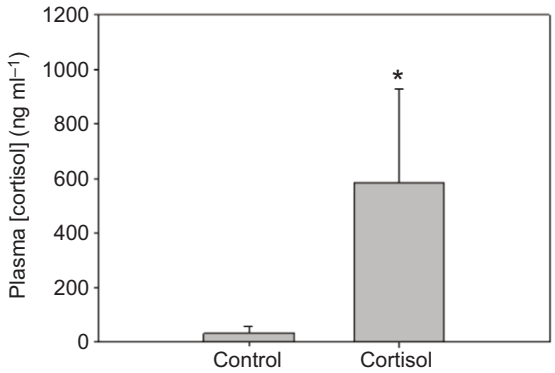


**Fig. 1. The effects of cortisol manipulation on circulating plasma cortisol concentrations in parental smallmouth bass.** Data are presented as means±s.e.m. for control fish ($n$=17) and cortisol-treated fish ($n$=12). Asterisk represents a significant difference at a level of $\alpha$=0.05 between control and cortisol treatment groups. See Results for details.



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of *P*-values for all two-way ANOVAs can be found in Tables 1, 3 and 4. Only *P*-values that were significant are reported in the text.

### 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; $F_{1,2}$=1.842, *P*=0.190 for worms). 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).

### 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 either the initial or post-treatment assessments (Fig. 2, Table 3). Parental care duration also appeared to be independent of cortisol and food treatments (Fig. 2, 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 with the non-fed treatment groups [chi-square test, $\chi^2(1)$=4.115, *P*=0.035, *n*=53; Fig. 2].

### 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 days, 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, $F_{1,41}$=4.645, *P*=0.037; Tables 1, 2). *Post hoc* analyses revealed plasma glucose levels to be highest within cortisol-treated fish regardless of food supplementation (Fig. 3). Plasma cholesterol levels were found to be significantly affected by cortisol treatment alone (two-way ANOVA, $F_{1,40}$=8.323, *P*=0.006; Tables 1, 2). *Post hoc* analysis revealed that cortisol-treated fish had higher concentrations of plasma cholesterol than control fish (Fig. 3). Plasma total protein and triglyceride concentrations were found to be significantly affected only by food supplementation (two-way ANOVA, $F_{1,39}$=4.338, *P*=0.043 for total protein and $F_{1,39}$=9.624, *P*=0.003 for triglycerides; Tables 1, 3). *Post hoc* analyses revealed that plasma total protein levels were higher in non-fed treatment groups compared with food-supplemented groups (Fig. 4). For triglyceride levels, fed fish had higher triglyceride concentrations than non-fed treatment groups (Fig. 4).

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, $F_{1,39}$=4.581, *P*=0.038; Tables 1, 2). *Post hoc* analyses indicated that control plus food treatment fish had the highest circulating plasma chloride concentrations compared with all other treatments groups (Fig. 5). Analysis of the log-transformed plasma potassium concentrations indicated a significant effect of cortisol treatment on circulating potassium concentrations (two-way ANOVA, $F_{1,40}$=4.611, *P*=0.037; Tables 1, 2). *Post hoc* analysis revealed higher plasma potassium concentrations in control fish compared with cortisol-treated fish (Fig. 5). 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, $F_{1,40}$=9.068, *P*=0.004; Tables 1, 2) was detected. *Post hoc* analysis indicated that plasma magnesium concentrations were highest in the control and cortisol plus food treatment groups, and lowest in the cortisol and control plus food treatment groups (Fig. 5).

### Oxidative stress indicators

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 their interaction

**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**

| | Cortisol | | | Food supplement | | | Interaction | | |
|---|---|---|---|---|---|---|---|---|---|
| Physiological variable | d.f. | *F*-ratio | *P*-value | d.f. | *F*-ratio | *P*-value | d.f. | *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 | **<0.05** |

Ion, glucose, total protein, cholesterol and triglyceride concentrations were measured in plasma, whereas 8-OHdG concentration was assessed using red blood cells. Bold values indicate a significant difference from the control at $\alpha$=0.05.

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**Table 2. Indicators of nutritional condition in parental smallmouth bass, among the four treatment groups, sampled from Lake Opinicon and Sand Lake, Ontario**

| Physiological variable | Control | Cortisol | Control+Food | Cortisol+Food |
|---|---|---|---|---|
| Calcium (mmol $l^{-1}$) | 2.48±0.16 | 2.43±0.18 | 2.24±0.36 | 2.42±0.24 |
| | (2.20–2.80) | (2.30–2.90) | (1.20–2.70) | (2.00–2.90) |
| | *n*=11 | *n*=10 | *n*=11 | *n*=11 |
| Chloride (mmol $l^{-1}$) | 106.6±3.5 | 104.4±10.1 | 113.1±4.2 | 102.8±7.26 |
| | (99–110) | (80–110) | (111–120) | (92–115) |
| | *n*=10 | *n*=10 | *n*=11 | *n*=11 |
| Magnesium (mmol $l^{-1}$) | 1.03±0.062 | 0.92±0.058 | 0.93±0.14 | 0.99±0.11 |
| | (0.95–1.06) | (0.79–0.99) | (0.83–1.02) | (0.88–1.16) |
| | *n*=11 | *n*=10 | *n*=11 | *n*=11 |
| Potassium (mmol $l^{-1}$) | 3.52±0.63 | 2.84±0.65 | 3.26±0.63 | 2.96±1.19 |
| | (2.80–4.30) | (1.80–2.90) | (2.40–3.90) | (1.20–6.20) |
| | *n*=10 | *n*=10 | *n*=11 | *n*=11 |
| Sodium (mmol $l^{-1}$) | 153±5 | 155±6 | 151±5 | 150±8 |
| | (144–164) | (144–165) | (137–158) | (130–159) |
| | *n*=10 | *n*=10 | *n*=11 | *n*=11 |
| Cholesterol (mmol $l^{-1}$) | 11.4±1.4 | 12.0±1.3 | 10.4±1.4 | 12.4±1.7 |
| | (9.50–13.9) | (9.70–13.7) | (8.20–12.7) | (9.20–16.2) |
| | *n*=11 | *n*=10 | *n*=11 | *n*=11 |
| Cortisol (ng $ml^{-1}$) | 34.7±20.3 | 619.2±292.8 | 27.3±27.0 | 554.6±353.8 |
| | (10.2–69.2) | (197.9–943.2) | (10.8–85.2) | (87.0–1003.9) |
| | *n*=10 | *n*=6 | *n*=8 | *n*=6 |
| Glucose (mmol $l^{-1}$) | 3.0±0.6 | 5.8±1.1 | 2.5±0.2 | 6.2±1.5 |
| | (2.10–4.10) | (4.30–7.90) | (2.20–2.90) | (4.30–9.40) |
| | *n*=11 | *n*=10 | *n*=11 | *n*=12 |
| Triglycerides (mmol $l^{-1}$) | 1.60±0.78 | 1.41±0.33 | 2.62±1.03 | 1.91±0.64 |
| | (0.82–3.47) | (0.85–1.98) | (1.02–4.44) | (0.66–2.77) |
| | *n*=11 | *n*=10 | *n*=11 | *n*=11 |
| Total protein (g $l^{-1}$) | 45±4 | 46±4 | 40±7 | 44±3 |
| | (39–53) | (39–53) | (18–46) | (39–48) |
| | *n*=11 | *n*=10 | *n*=11 | *n*=11 |
| 8-OHdG (ng $ml^{-1}$) | 255.8±94.8 | 471.8±40.3 | 125.3±48.6 | 216.8±30.0 |
| | (144.9–390.2) | (408.4–521.9) | (50.9–193.7) | (184.4–232.7) |
| | *n*=7 | *n*=7 | *n*=7 | *n*=7 |

Ion, glucose, total protein, cholesterol and triglyceride concentrations were measured in plasma, whereas 8-OHdG concentration was assessed using red blood cells.
Values are presented as means±s.d. with minimum and maximum values contained within parentheses.

(two-way ANOVA, $F_{1,27}$=6.691, $P$=0.016; Table 1, Fig. 6). *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. 6).

### Immune function

Five categories of leukocytes were identified in the blood smear preparations: 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, $F_{1,43}$=12.401, $P$=0.001; Table 4). *Post hoc* analysis revealed that lymphocyte numbers were lower in cortisol-treated fish than control fish (Fig. 7). Moreover, cortisol treatment had a significant impact on plasma neutrophil and monocyte proportions (two-way ANOVA, $F_{1,43}$=4.264, $P$=0.045 for neutrophils and

**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**

| | Cortisol | | | Food supplement | | | Interaction | | |
|---|---|---|---|---|---|---|---|---|---|
| Behavioural variable | d.f. | *F*-ratio | *P*-value | d.f. | *F*-ratio | *P*-value | d.f. | *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 |

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.



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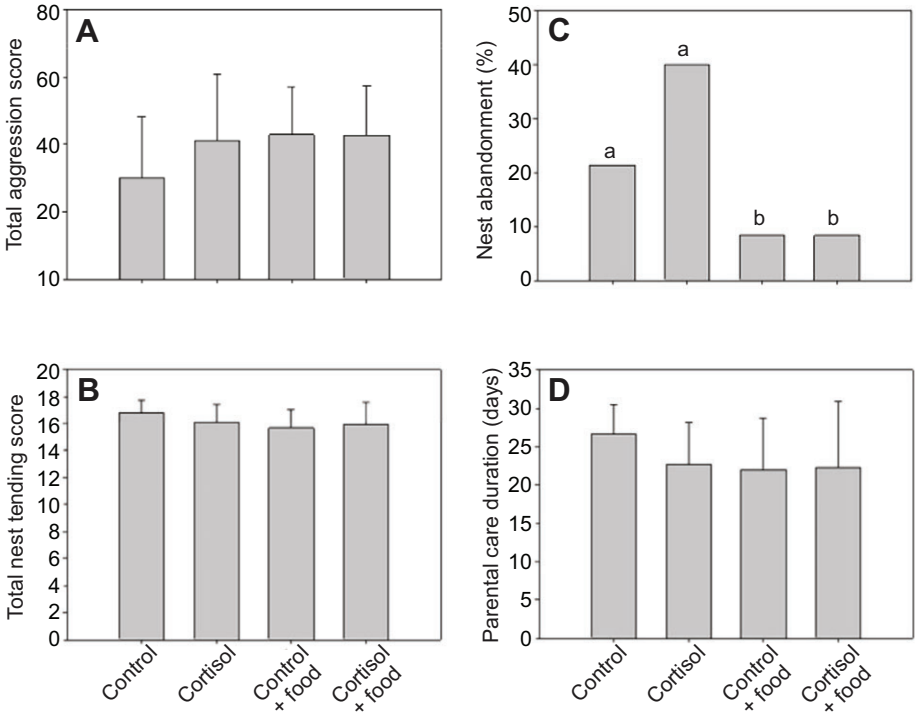


**Fig. 2. Comparison of parental care behaviours in parental smallmouth bass sampled from Lake Opinicon and Sand Lake, Ontario.** Data for total aggression (A), nest tending (B) and parental care duration (D) are presented as means±s.e.m. for control fish ($n$=11), cortisol-treated fish ($n$=10), control plus food fish ($n$=12) and cortisol plus food fish ($n$=12). Data for nest abandonment (C) are presented as the percentage of males within a group that abandoned their brood prior to brood independence for control fish ($n$=14), cortisol-treated 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 include 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. Dissimilar letters indicate a significant difference at a level of $\alpha$=0.05. See Results for details.

$F_{1,43}$=7.666, $P$=0.008 for monocytes; Table 4). *Post hoc* analysis indicated that both neutrophil and monocyte concentrations were higher in cortisol-treated fish relative to control fish (Fig. 7).

## DISCUSSION

The results of the present study indicate that nutritional condition and elevated plasma cortisol levels had mixed effects on parental behaviour, 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

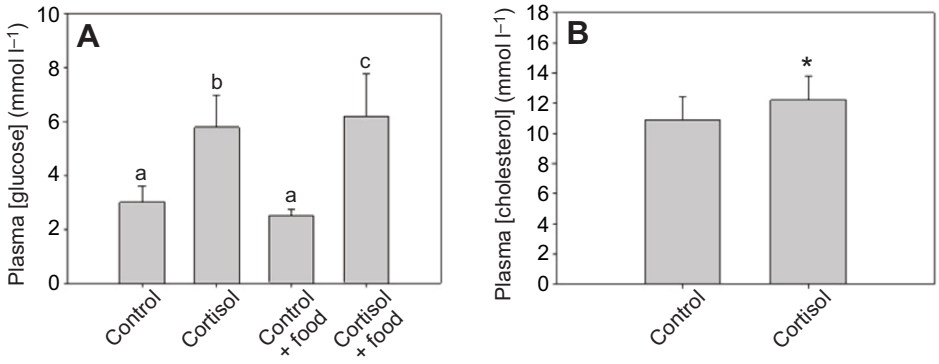


**Fig. 3. Plasma glucose and cholesterol concentrations among four treatment groups of parental smallmouth bass.** (A) Plasma glucose data are presented as means±s.e.m. for control fish ($n$=11), cortisol-treated fish ($n$=10), control plus food fish ($n$=11) and cortisol plus food fish ($n$=12). (B) Plasma cholesterol data are presented as means±s.e.m. for control fish ($n$=22) and cortisol-treated fish ($n$=21), and represent the main statistical effect of cortisol treatment, as the interaction term between supplemental feeding and cortisol was not significant. Different lowercase letters in A denote a significant difference at a level of $\alpha$=0.05 among treatment groups. Asterisk in B represents a significant difference at a level of $\alpha$=0.05 between control and cortisol treatment groups. See Results for details.

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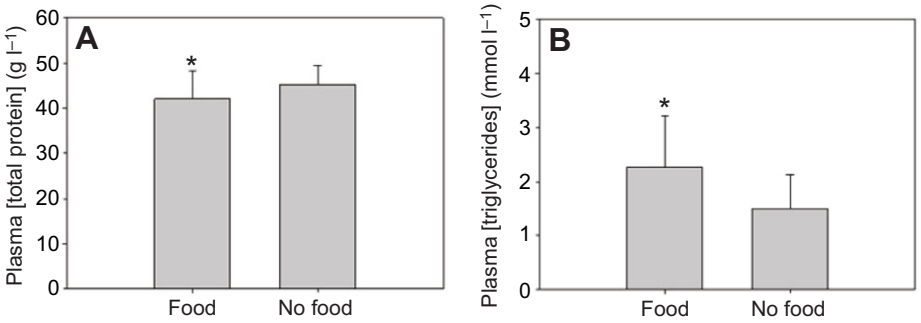


**Fig. 4. Plasma total protein and triglyceride concentrations between fed and non-fed treatment groups of parental male smallmouth bass.** Data are presented as means±s.e.m. for food treatment fish ($n$=22) and non-fed treatment fish ($n$=21). The figures for both (A) plasma total protein and (B) plasma triglycerides represent the main statistical effect of supplemental feeding, as the interaction term between supplemental feeding and cortisol was not significant. Asterisks represent a significant difference at a level of $\alpha$=0.05 between fed and non-fed treatment groups. See Results for details.

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.

Of the three parental care behaviours measured (nest tending vigilance, brood defence 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 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 with 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 males 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.

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 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 with 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

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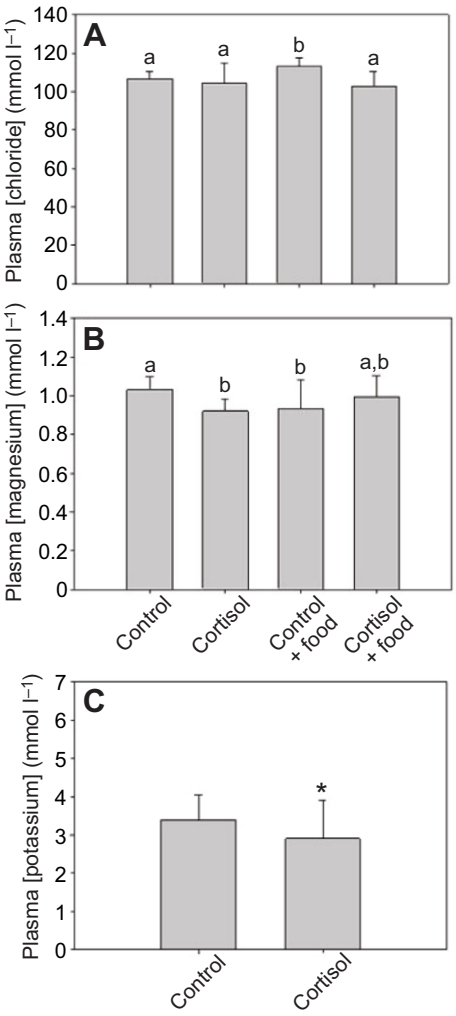


**Fig. 5. Comparison of plasma chloride and magnesium concentrations among the four treatment groups of parental smallmouth bass, and differences in plasma potassium levels between control and cortisol-treated parental smallmouth bass.** (A) Plasma chloride and (B) plasma magnesium data are presented as means±s.e.m. for control fish ($n$=10), cortisol-treated fish ($n$=10), control plus food fish ($n$=11) and cortisol plus food fish ($n$=11). (C) Plasma potassium data are presented as means±s.e.m. for control fish ($n$=10), cortisol-treated fish ($n$=10), control plus food fish ($n$=11) and cortisol plus food fish ($n$=11), and represent the main statistical effect of cortisol treatment, as the interaction term between supplemental feeding and cortisol was not significant. Different lowercase letters denote a significant difference at a level of $\alpha$=0.05 between treatment groups. Asterisk represents a statistical difference at a level of $\alpha$=0.05 between treatment groups.

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). Although the food-supplemented treatment groups had lower levels of plasma protein and higher levels of plasma triglycerides compared with the non-fed treatment groups, the biological significance of these findings is uncertain.

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. Oxidative stress in parental smallmouth bass was assessed through the accumulation of DNA damage, as measured by 8-OHdG formation on DNA nucleotides.

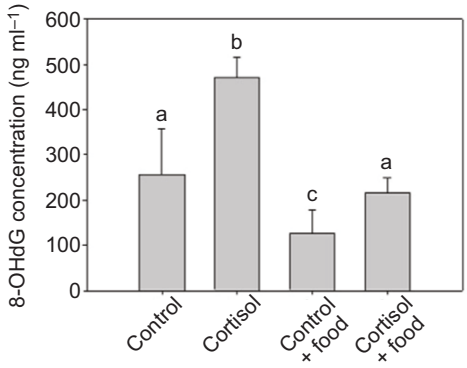


**Fig. 6. 8-OHdG concentration among the four treatment groups of parental smallmouth bass.** Data are presented as means±s.e.m. for control fish ($n$=7), cortisol-treated fish ($n$=7), control plus food treated fish ($n$=7) and cortisol plus food treated fish ($n$=7). Different lowercase letters denote a significant difference at a level of $\alpha$=0.05 among treatment groups. See Results for details.

DNA nucleotides are prominent sites for oxidative damage because of 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 outcompetes 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 fish. Cortisol treatment caused significantly higher concentrations of 8-OHdG when compared with 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 present 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). However, antioxidant capacity was not directly measured in this study, underlining a potential limitation as well as a direction for future research.

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;

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**Table 4. Effects of cortisol treatment and food supplementation on leukocyte concentrations in parental smallmouth bass sampled from Lake Opinicon and Sand Lake, Ontario**

| | Cortisol | | | Food supplement | | | Interaction | | |
|---|---|---|---|---|---|---|---|---|---|
| Leukocyte type | d.f. | *F*-ratio | *P*-value | d.f. | *F*-ratio | *P*-value | d.f. | *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 |

Bold values indicate a significant difference from the control at $\alpha$=0.05.

Kaattari and Tripp, 1987; Barton and Iwama, 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 proportionately higher in cortisol-treated fish compared with 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.

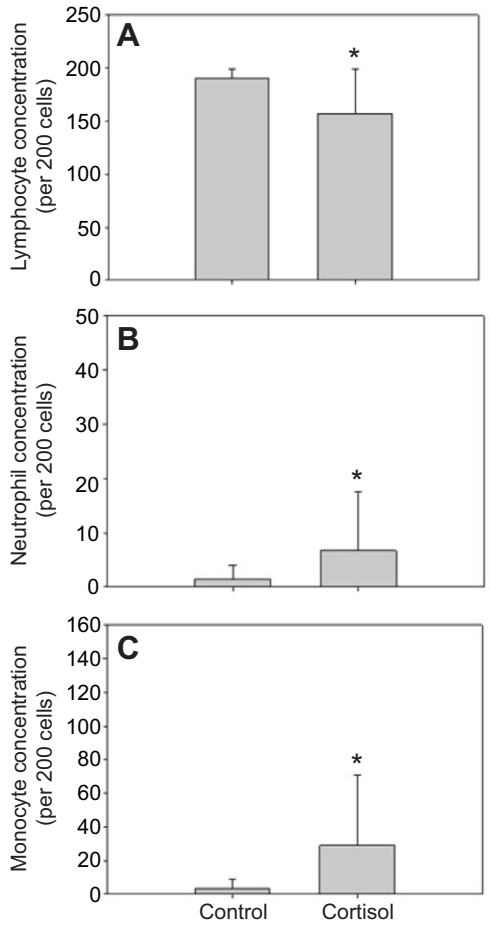


**Fig. 7. Proportional comparison of the prevalence of white blood cell types between control and cortisol-treated fish.** (A) Lymphocyte, (B) neutrophil and (C) monocyte. Figures represent proportional data based on the mean percentage of specified cell per random 200 cell counts. Data are presented as means±s.e.m. for control fish ($n$=10), cortisol-treated 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. Asterisks represent statistical differences between treatment groups at a level of $\alpha$=0.05. See Results for details.

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.

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### Competing interests
The authors declare no competing or financial interests.

### Author contributions
This project was conceived by S.J.C. and A.J.Z. Field work was completed by A.J.Z., D.A.A. and S.J.C. All statistical analyses were conducted by A.J.Z. and S.J.C. Cortisol analysis was conducted by M.J.L. and K.M.G., and 8-hydroxy-2′-deoxyguanosine analysis was conducted by J.T. and W.W. M.D.F. assisted with blood smear preparations and white blood cell identification. All authors provided feedback on the manuscript.

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