

# Stress and Parental Care in a Wild Teleost Fish: Insights from Exogenous Supraphysiological Cortisol Implants

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## ABSTRACT

Male largemouth bass (*Micropterus salmoides*) provide sole parental care over a 4–6-wk period to a single brood, fanning the eggs to keep them oxygenated and free of silt and defending the brood until the offspring develop antipredator tactics. During this period, fish are highly active and have few opportunities for feeding, so this activity is energetically costly. To understand some of the consequences of stress during this challenging period, we injected fish with cortisol suspended in coconut oil to experimentally raise circulating cortisol in parental males for the first week of the parental care period. We compared parental care behavior between cortisol-treated, sham-treated (injected only with coconut oil), and control parental males. We further compared physiological parameters associated with metabolism and reproductive function between cortisol-treated and control males. The cortisol injections resulted in supraphysiological levels of circulating plasma cortisol, giving us insight into po-

tential maximal effects of stress during parental care. At these supraphysiological levels, the cortisol-treated fish displayed higher concentrations of circulating glucose and cholesterol and lower concentrations of circulating triglycerides when compared with control fish, with no change in plasma concentrations of total protein. Plasma concentrations of androgen were similarly unaffected by cortisol treatment. In the short term (initial 1–2 wk), parental care of eggs and egg-sac fry was maintained by all groups, with no differences observed in behavior (e.g., tending, vigilance, defense) among the groups. However, the cortisol-treated fish abandoned their offspring at a higher rate than in the control or sham groups. The fish treated with cortisol also tended to develop external Saprolegnial infections, indicative of compromised immune function. These data demonstrate that exogenous cortisol elevation during parental care results in changes in energy use and a decrease in immune function. Interestingly, the data also suggest resistance to stress during parental care in largemouth bass, with no changes in parental care behavior before abandonment.

## Introduction

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

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is surprising given that parental care is relatively common, appearing in approximately 20% of fish families (Gross 2005), and an extensive background exists on the physiology of the stress response in fish (see reviews in Schreck 1981; Barton and Iwama 1991; Wendelaar-Bonga 1997; Mommsen et al. 1999), making them an excellent comparative model for understanding the physiology of parental care behavior.

Resource limitation is an often-cited basis for trade-offs in reproductive function (Trivers 1972; Calow 1985; Webb et al. 2002). A stress response is energetically costly (Davis and Schreck 1997) and may reduce the scope available for other functions, including the reproductive effort before spawning (Priede 1985). In fish, this is particularly interesting because growth is indeterminate and fecundity is correlated with body size (Heino and Kaitala 1999). Stress during reproduction should carry the same energetic costs as stress during nonreproductive periods, but how this will affect the parental care behavior of a fish, and the ultimate reproductive success of a fish, has never been examined in wild fish.

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

This study investigated the effect of exogenous cortisol elevation during parental care in the largemouth bass (*Micropterus salmoides*) in freshwater lakes in eastern Ontario, Canada. Largemouth bass are a long-lived (up to ~20 yr) top predator in many temperate lentic systems. The adults of the species have few natural piscine predators, but the young are heavily preyed on by both conspecifics and other fish and, as a result, require diligent parental care during early development (Coble 1975). Otherwise, brood predators will decimate an undefended nest within minutes (Philipp et al. 1997). The male finds and defends a territory, where he scrapes a nest by digging in the substrate until a shallow depression is formed and then courts a female, who chooses among competing males (Brown 1984). The spawning event lasts for up to several hours, and once complete, the female departs and the male then provides sole parental care for 4–6 wk (Cooke et al. 2006). During this time, the offspring develop through a series of predictable stages. We use the same terminology as Cooke et al. (2002) to describe these stages: during the “egg” stage, the developing embryo is entirely contained within the yolk sac; at the “egg-sac fry” stage, the developing embryo has hatched but remains attached to the egg sac; the “swim-up fry” stage commences when the larvae

are free of the egg sac but are not yet fully motile; finally, during the “free-swimming fry” stage, the larvae are fully motile in the water column. Parental care entails fanning the eggs to provide oxygen and remove silt, removing debris, and defending the offspring against potential brood predators (Coble 1975). This parental care is energetically costly (Cooke et al. 2002, 2006) and, combined with reduced feeding opportunities, can result in males losing significant mass during the parental care period (Cooke et al. 2002).

Here we focus on the behavior (brood tending, vigilance, and defense) and nest success of largemouth bass in which circulating cortisol concentrations were elevated by means of an intraperitoneal injection of cortisol in coconut oil, compared with sham controls, which were administered to the vehicle without cortisol, and untreated controls. Such exogenous manipulations of cortisol titers are extremely common in the study of fish comparative physiology (see review by Gamperl et al. [1994]). To date, however, these manipulations have been largely restricted to the laboratory environment (see review by Mommsen et al. [1999]). We further investigated potential physiological mechanisms of behavioral change by comparing indicators of energy use (glucose, cholesterol, triglycerides, and total protein) and androgen between cortisol-treated largemouth bass and reference control parental males with offspring at the same developmental stage.

## Material and Methods

### *Animals, Treatments, and Collection of Blood Samples*

All fish were sampled under an Ontario Ministry of Natural Resources Scientific Collection Permit (to S.J.C.) and handled in accordance with the guidelines of the Canadian Council on Animal Care as administered by Carleton University (B07-02) and Queen's University. From May 10 to 28, 2007, 52 largemouth bass guarding nests with fresh eggs (0–1 d old) were identified by snorkeling in Long Lake, a private research lake managed by the Queen's University Biological Station (QUBS) in eastern Ontario (44°31'N, 76°20'W). No angling or other disturbance was permitted during the study period, so there was no possibility that fish were captured or harvested during this period. Mean daily water temperatures (~1 m below the surface) increased from 15° to 21°C across parental care. The nests were individually marked with a numbered tile, and the fish were randomly assigned to one of four treatment groups: (1) control ( $n = 15$ ), (2) sham treatment ( $n = 8$ ), (3) low-dose cortisol treatment ( $n = 14$ ), and (4) high-dose cortisol treatment ( $n = 15$ ). The control animals were neither captured nor handled in any way. Treatment fish were captured by rod-and-reel angling, landed with a rubber-mesh net within 10 s, and placed in a foam-lined trough filled with fresh lake water. Fish were then uniquely identified by intraperitoneal implantation of a passive integrated transponder (PIT) tag (2 mm × 9 mm). The fish were briefly air-exposed (~10 s) while mass was determined using a portable electronic balance and

Table 1: Physiological parameters (mean  $\pm$  SE) for parental male largemouth bass

	Androgen (ng mL <sup>-1</sup> )	Total Protein (mmol L <sup>-1</sup> )	Cholesterol (mmol L <sup>-1</sup> )	Triglycerides (mmol L <sup>-1</sup> )	Glucose (mmol L <sup>-1</sup> )
Reference controls:					
2006 ( $n = 18$ ) <sup>a</sup>	.178 $\pm$ .059	38.7 $\pm$ .93	14.1 $\pm$ .81 <sup>AB</sup>	.725 $\pm$ .06 <sup>A</sup>	2.32 $\pm$ .09 <sup>A</sup>
2007 ( $n = 6$ )	.140 $\pm$ .031	40.5 $\pm$ 1.65	14.7 $\pm$ .74 <sup>AB</sup>	.650 $\pm$ .21 <sup>AB</sup>	2.86 $\pm$ .29 <sup>A</sup>
2008 ( $n = 9$ )	.141 $\pm$ .022	37.0 $\pm$ 1.07	12.9 $\pm$ 1.01 <sup>A</sup>	.660 $\pm$ .14 <sup>AB</sup>	2.24 $\pm$ .15 <sup>A</sup>
Cortisol ( $n = 16$ )	.168 $\pm$ .038	39.5 $\pm$ 1.01	16.8 $\pm$ .81 <sup>B</sup>	.471 $\pm$ .06 <sup>B</sup>	7.34 $\pm$ .58 <sup>B</sup>

Note. Plasma from cortisol-treated parental males was taken at the egg-sac fry stage 5–6 d after cortisol injection. Plasma from reference control males was taken at the equivalent egg-sac fry stage from untreated control fish in nearby lakes over 3 yr. Different letters indicate statistical difference ( $\alpha = 0.05$ ).

<sup>a</sup>  $n = 7$  for androgen only.

returned to the water-filled trough for administration of an intraperitoneal injection of coconut oil (*Cocos nucifera*; Sigma C1758, Sigma-Aldrich, St. Louis, MO) containing cortisol (hydrocortisone; Sigma H4001, Sigma-Aldrich). This method is an established means of raising plasma cortisol in fish and creates a slow-release cortisol implant that will elevate plasma cortisol for approximately 5–6 d (see Gamperl et al. 1994). All fish were injected with 0.005 mL of coconut oil per gram of fish body weight. Sham treatment fish (total length = 358  $\pm$  12 mm [mean  $\pm$  SE]) were injected with pure coconut oil. Low-dose cortisol treatment fish (total length = 365  $\pm$  7 mm) were injected with 10 mg mL<sup>-1</sup> cortisol in coconut oil, while high-dose cortisol treatment fish (total length = 377  $\pm$  10 mm) were injected with 40 mg mL<sup>-1</sup> cortisol in coconut oil. These doses were chosen on the basis of literature values used to induce exogenous stress responses in teleost fish (see Gamperl et al. 1994). During treatment, a snorkeler was deployed to protect the eggs from nest predators and remained with the nest until the parental male returned following release. All techniques were performed without anesthesia (see Cooke et al. 2005 for rationale), and total treatment time was typically <90 s.

A subset of the low-dose cortisol ( $n = 7$ ; total length = 363  $\pm$  10 mm) and high-dose cortisol ( $n = 9$ ; total length = 363  $\pm$  16 mm) fish were recaptured between May 19 and May 28, 2007, 5–6 d after the initial cortisol treatment, at the egg-sac fry offspring development stage. After capture, these fish were placed in a water-filled, foam-lined trough, nonlethally sampled for ~1.5 mL of blood by caudal puncture, using lithium heparinized 3-mL vacutainers (B.D. Vacutainer, Franklin Lakes, NJ) and 21-gauge, 1.5-inch needles, and scanned with a PIT-tag reader to confirm identity. All fish were then released. As with the cortisol treatment, a snorkeler remained with the nest until the parental male returned, and all techniques were performed without anesthesia, with a total handling time <90 s. Mean daily water temperatures (~1 m below the surface) increased from 16° to 21°C during this period. The physiological parameters (plasma concentrations of androgen, triglycerides, cholesterol, total protein, and glucose) of these animals were compared with reference values obtained using the same sampling method from parental fish over 3 yr from two nearby lakes. Long Lake, our focal study lake, is a relatively small lake

(~20 ha), and the fish used for the behavioral component of the study comprised the entire population of nesting males in the lake. We chose to capture size-matched nesting males from nearby water bodies over multiple years to obtain reference values for the physiological component of this study, rather than reduce the sample size of untreated controls for the behavioral data. Because none of the reference groups (i.e., across years or lakes) were different from one another in any of the parameters examined (see Table 1), we are confident that the differences we observed in the cortisol-treated group were a result of the exogenous cortisol implants, rather than a year or lake effect. Largemouth bass with offspring at the egg-sac fry stage ( $n = 18$ ; total length = 365  $\pm$  19 mm) were obtained May 15 to 16, 2006 (water temperature ~18°C), from Lake Opinicon, a nearby public lake that is part of the Rideau River system in eastern Ontario (44°30'N, 76°20'W). Largemouth bass with offspring at the egg-sac fry offspring development stage were also sampled on June 2, 2007 (water temperature ~20°C;  $n = 6$ ; total length = 376  $\pm$  18 mm), and June 4–11, 2008 (water temperature ~20°C;  $n = 9$ ; total length = 368  $\pm$  13 mm), from Charleston Lake, another nearby lake that is part of the Gananoque River system in eastern Ontario (44°32'N, 75°59'W).

To validate the cortisol dose for this species and to verify the time course of cortisol elevation, 36 size-matched largemouth bass were captured, using the same rod-and-reel methods as described for Lake Opinicon, between May 9 and May 13, 2007, and transported in aerated coolers to fish-holding facilities at QUBS. These fish were assigned to a high-dose cortisol treatment ( $n = 18$ ), low-dose cortisol treatment ( $n = 6$ ), sham treatment ( $n = 6$ ), or control treatment ( $n = 6$ ) group and treated as described earlier. After injection, the fish were placed in individual opaque experimental chambers (~12 L) supplied with a constant flow of freshwater from Lake Opinicon until blood sampling. To avoid biasing the data, five blood samples (three high-dose cortisol, one low-dose cortisol, and one control fish) that took more than 3 min between opening the experimental chamber and completing the blood sample were discarded. To verify the cortisol doses, a subsample of the high-dose cortisol treatment fish ( $n = 5$ ; total length = 369  $\pm$  12 mm) and all fish from the low-dose cortisol treatment ( $n = 5$ ; total length = 357  $\pm$  14 mm), sham treat-

ment ( $n = 6$ ; total length =  $368 \pm 13$  mm), and control treatment groups ( $n = 5$ ; total length =  $370 \pm 23$  mm) were removed after 3 d from the experimental chambers and sampled for blood as described earlier. To verify the time course for cortisol elevation, a second subsample of the high-dose cortisol treatment fish ( $n = 6$ ; total length =  $381 \pm 12$  mm) was sampled for blood after 6 d, and the remaining group ( $n = 4$ ; total length =  $348 \pm 22$  mm) was sampled after 9 d.

For a comparison of the exogenous cortisol elevation to endogenous cortisol elevation caused by stress in this species, an additional group of size-matched fish ( $n = 6$ ; total length =  $354 \pm 17$  mm) was captured from Lake Opinicon between September 24 and 27, 2007 (water temperature  $\sim 18^\circ\text{C}$ ), transported back to QUBS as described earlier, and individually placed in a 100-L tank where they were chased to exhaustion (loss of equilibrium) by tail pinching (3 min). They were then held in aerated coolers for 30 min, and blood was sampled as described previously.

Blood samples were held in water-ice slurries for no more than 1 h until they were centrifuged at 10,000 g for 5 min (Compact II Centrifuge, Clay Adams, Parsippany, NJ). Plasma samples were flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until analysis.

#### Biochemical and Endocrine Analysis

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

Plasma cortisol was determined using a commercial kit (ImmunoChem Cortisol  $^{125}\text{I}$  RIA kit, MP Biomedicals, Orangeburg, NY) and a Cobra Auto-Gamma (Hewlett-Packard, Palo Alto, CA) following the methods outlined by Gamperl et al. (1994). A subset of samples (5%–20%) was run in duplicate within and between each assay. Intra-assay variability (% CV) was 11.1%, while interassay variability (% CV) was 12.9%. Steroids were extracted from aliquots (100  $\mu\text{L}$  of plasma and 900  $\mu\text{L}$  of distilled water) using  $3 \times 5\text{-mL}$  ethyl acetate washes and resuspended in phosphate-buffered saline (pH 7.6) containing 0.3% gelatin, and androgen was measured in duplicate by  $^3\text{H}$ -radioimmunoassay using a testosterone antibody, following the methods outlined by McMaster et al. (1992). Cross-reactivity of the testosterone antibody with 11-ketotestosterone was 7.5%. Intra-assay variability (% CV) was 3.3%, and inter-assay variability (% CV), measured using aliquots of an internal pooled standard, was 15.2%.

#### Behavioral Data Collection

All nesting bass identified in Long Lake were included in the behavioral analysis (control  $n = 15$ , sham treatment  $n = 8$ , low-dose cortisol treatment  $n = 14$ , and high-dose cortisol treatment  $n = 15$ ), representing the entire male reproductive effort in the lake for that year. The day after treatment (the day after nest identification for the control animals) all fish were observed according to a behavioral protocol by one of three trained snorkelers. These observations were repeated in the same order every other day, for up to 2 wk, to collect data at the egg stage and the egg-sac fry and swim-up fry offspring developmental stages. Behavioral metrics entailed the following:

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

2. Turning score. Fish were observed at the same 3-m distance for three separate 1-min periods. The fish was required to be directly above the nest for the entirety of each 1-min period. During each 1-min period, the number of one-quarter turns made above the nest was recorded. The numbers of one-quarter turns from each of the three separate 1-min periods were averaged to calculate the turning score. Turns above the nest are a measure of vigilance, as the parental male monitors the surrounding area for nest predators; more vigilant parents will receive higher turning scores (Hinch and Collins 1991).

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

Monitoring of the nest by snorkeling continued every other

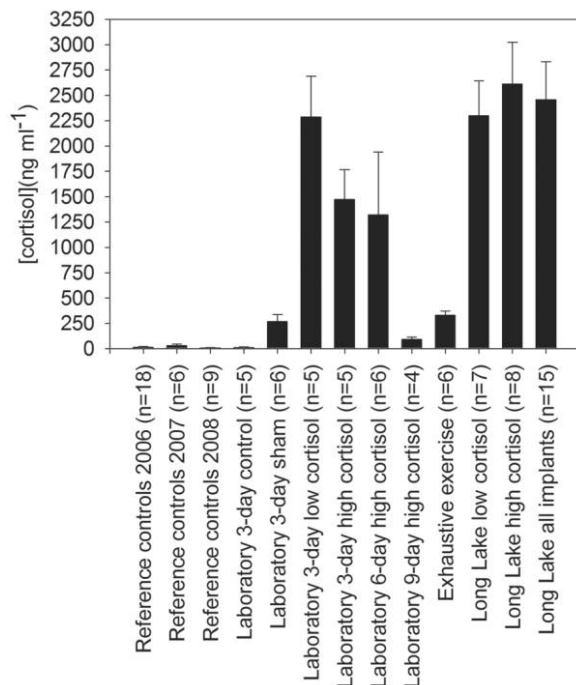


Figure 1. Plasma cortisol concentrations (mean  $\pm$  SE) achieved by the exogenous cortisol treatment and sham treatment and the approximate time course of cortisol elevation. This can be compared with the endogenous cortisol elevation as a result of exhaustive exercise and reference levels of plasma cortisol in control largemouth bass.

day for up to 2 wk to record presence of the parental fish and developmental stage of the offspring. Permanent departure of the parental fish or total predation of the brood before the swim-up fry stage was considered a failed reproductive attempt, while reaching the swim-up fry stage was considered a proxy of nest success (Philipp et al. 1997). Any evidence of Saprolegnial infection was also noted during this monitoring, with a record of where the infection occurred (head, body, fins), and the approximate percentage of the body surface area affected.

#### Statistical Analyses

Before subsequent ANOVA tests, plasma cortisol concentrations between the high- and low-dose cortisol treatment groups were compared. Normal distribution for all parameters was tested using the goodness-of-fit test and for homogeneity of variance using Levene's test. Meeting these assumptions, plasma cortisol levels between the low- and high-cortisol treatment groups were compared using a Student's *t*-test. No difference in circulating plasma cortisol levels between the groups was detected (Student's *t*-test,  $t_{14} = 0.59$ ,  $P = 0.56$ ; Fig. 1), and so these groups were combined into a single cortisol treatment group for all subsequent analysis ( $n = 16$ ; total length =  $371 \pm 6$  mm).

Plasma androgen, triglyceride, cholesterol, plasma protein, and glucose concentrations were compared between the cortisol and control treatment groups. Data for triglyceride concentra-

tion did not meet the assumptions of normality and were log<sub>10</sub>-transformed before further analysis (nontransformed data are presented in Fig. 2). Data for glucose concentration did not meet the assumptions of equality of variances, and so a Welch's ANOVA was performed (Zar 1999). For all other variables, concentrations of plasma constituents were compared among the groups using ANOVAs. Tukey's post hoc multiple comparisons tests were performed to identify the source of differences where significant differences were detected by ANOVA.

Behavioral metrics were compared among the cortisol, sham, and control treatment groups and between offspring development stages. However, high abandonment rates in some of the treatment groups compromised sample size for repeated measures when data from parents with swim-up fry were included. This offspring development stage was dropped from the analysis, and only individuals with data at both the egg stage and the egg-sac fry stage were retained. This approach resulted in a final sample size of  $n = 13$  for the control group,

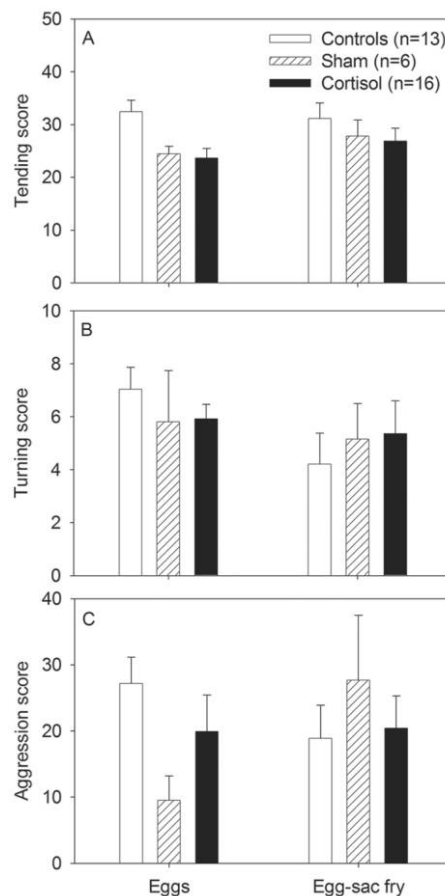


Figure 2. Measurements of behavior in control, sham-treated, and cortisol-treated parental males at the two earliest offspring development stages: the egg stage and the egg-sac fry stage. Tending score (A) is a measure of brood care, turning score (B) is a measure of vigilance, and aggression score (C) is a measure of brood defense. Two-way ANOVAs were performed for all differences in behavioral measures for both treatment differences and offspring stage differences. None of the behavioral measures were significant ( $\alpha = 0.05$ ).

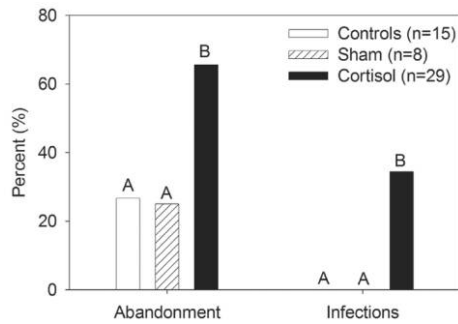


Figure 3. Proportion of control, sham-treated, and cortisol-treated males who permanently left the nest area before the swim-up fry stage, resulting in a reproductive failure for the nesting attempt and displayed evidence of minor (<30% of either the head or fins affected) Saprolegnial infections. Different letters among treatment groups indicate statistical difference ( $\alpha = 0.05$ ).

$n = 6$  for the sham treatment group, and  $n = 16$  for the combined cortisol treatment group. When data for a single individual included multiple behavioral observations at a single offspring development stage, as occurred for six fish at the egg stage and two fish at the egg-sac fry stage, the mean of the observations from the individual was taken for that stage. Data for the turning score and the aggression score did not meet the assumptions of normality and were log<sub>10</sub>-transformed before further analysis (nontransformed data are presented in Fig. 3). Relationships among the treatment groups and across egg stages were tested using two-way repeated-measures ANOVAs using treatment group and offspring development stage as independent variables. Behavioral metrics at the egg stage were also compared between fish that ultimately abandoned their offspring and fish that were successful in their reproductive effort using Student's *t*-tests. Data for the turning score and the aggression score did not meet the assumptions of normality, and were log<sub>10</sub>-transformed before further analysis (nontransformed data are presented in Fig. 4).

Abandonment and Saprolegnial infection rates were compared among the cortisol, sham, and control treatment groups using likelihood ratio tests. All analyses were performed in the statistical packages JMP, version 7.0.1 (SAS Institute, Cary, NC). The level of significance for all tests ( $\alpha$ ) was assessed at 0.05. All results are stated mean  $\pm$  SE.

## Results

### Cortisol Implant Effectiveness

The high and low doses selected for this study were combined into a single cortisol treatment group for all subsequent analyses, with a supraphysiological plasma cortisol concentration of  $2,457 \pm 375$  ng mL<sup>-1</sup> 5–6 d after initial cortisol injection (Fig. 1). In the parallel laboratory study, sham treatment also resulted in an elevation of plasma cortisol, with plasma cortisol concentrations of  $269 \pm 66$  ng mL<sup>-1</sup> 3 d after initial coconut oil injection, comparable to the levels seen during the apparent

endogenous cortisol stress response 30 min following exhaustive exercise (Student's *t*-test,  $t[10] = 0.78$ ,  $P = 0.45$ ; Fig. 1).

### Physiological Parameters

Of the nutritional parameters examined, only total protein concentration was insensitive to supraphysiological cortisol treatment (ANOVA,  $F_{3,45} = 1.12$ ,  $P = 0.35$ ; Table 1). Cholesterol was significantly higher in the cortisol-treated group than in control fish (ANOVA,  $F_{3,45} = 3.86$ ,  $P = 0.02$ ; Table 1), with a similar pattern for glucose (Welch's ANOVA,  $F_{3,45} = 43.85$ ,  $P < 0.01$ ; Table 1). Conversely, triglycerides were significantly lower in the cortisol-treated group (ANOVA,  $F_{3,45} = 2.89$ ,  $P < 0.05$ ; Table 1). There was insufficient plasma to run androgen analyses on 11 samples from the reference controls in 2006, leaving a sample size of  $n = 7$ ; other reference control group sample sizes and the cortisol treatment group sample size remained the same for androgen as for the nutritional

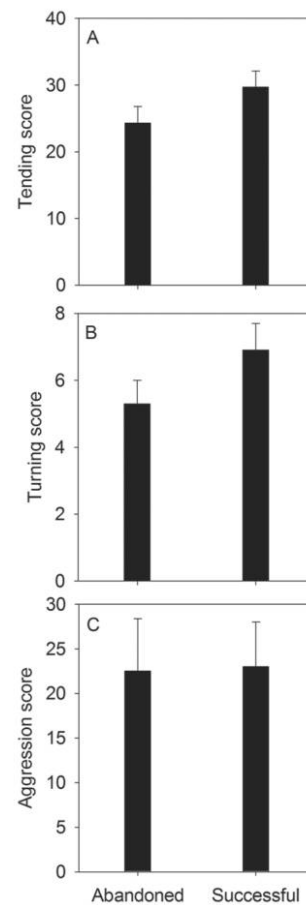


Figure 4. Measurements of behavior in cortisol-treated parental males at the egg stage, comparing fish that ultimately abandoned their broods with fish that were ultimately successful in their reproductive effort. Tending score (A) is a measure of brood care, turning score (B) is a measure of vigilance, and aggression score (C) is a measure of brood defense. Student's *t*-tests were performed between the fish that abandoned and fish that were successful, and none of the behavioral measures were significant ( $\alpha = 0.05$ ).

indices. Androgen levels were unaffected by cortisol treatment (ANOVA,  $F_{3,34} = 0.20$ ,  $P = 0.90$ ; Table 1).

#### Whole-Animal Parameters

Parental care behavior was not affected by supraphysiological cortisol treatment in this study. There were no differences in the tending score (two-way ANOVA with repeated measures,  $F_{3,34} = 0.81$ ,  $P = 0.45$ ), turning score (two-way ANOVA with repeated measures,  $F_{3,34} = 0.69$ ,  $P = 0.51$ ), or aggression score (two-way ANOVA,  $F_{3,34} = 2.89$ ,  $P = 0.07$ ) at any offspring development stage among the three treatment groups (Fig. 2). There was, however, a difference in the rates of early abandonment among the groups (likelihood ratio test,  $\chi^2_{2,N=52} = 8.25$ ,  $P = 0.02$ ), and in the rates of Saprolegnial infections among the groups (likelihood ratio test,  $\chi^2_{2,N=52} = 9.82$ ,  $P = 0.01$ ; Fig. 3). These Saprolegnial infections were mild, isolated to the head or fins, and covered <30% of the affected region. Abandonment was low for the control and sham-treated groups, and behavioral data at the egg stage for fish that abandoned were available for only a small number of control ( $n = 3$ ) and sham-treated fish ( $n = 1$ ). As a result, behavior between fish that abandoned and fish that were successful were analyzed using only cortisol-treated fish. There were no differences in tending score (Student's  $t$ -test,  $t[24] = 0.21$ ,  $P = 0.83$ ), turning score (Student's  $t$ -test,  $t[24] = 1.33$ ,  $P = 0.19$ ), or aggression score (Student's  $t$ -test,  $t[24] = 1.58$ ,  $P = 0.13$ ) between fish that abandoned their offspring and fish that were ultimately successful (Fig. 4).

#### Discussion

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

#### Limitations of Intraperitoneal Cortisol Injections

The use of cortisol in coconut oil to raise plasma cortisol created some confounding factors associated with elevated cortisol as a result of the capture and handling stress of administering injections (see Gamperl et al. 1994). The use of a sham treatment group does not entirely control for these factors because the sham treatment can be a stressor that can raise plasma cortisol, perhaps chronically. Indeed, the sham treatment fish in the laboratory verification study showed plasma cortisol levels approaching those of the exhaustively exercised fish (Fig. 1). We do not believe that this methodological limitation reduces the validity of the study, but it does require acknowledgment. Our cortisol-treated fish were subjected to handling stress and received an additional treatment of a cortisol-impregnated vehicle, while the sham treatment fish were subjected to handling stress and injection with the vehicle alone. The control and reference control groups were untreated and unhandled in any way. Our results represent the comprehensive effects of handling stress and extended cortisol elevation during parental care, rather than an isolated effect of elevated plasma cortisol.

The field setting for this study precluded a pilot study on the effectiveness of the cortisol dose, with water temperatures dictating a simultaneous laboratory study. As a result, the doses selected based on literature from rainbow trout (*Oncorhynchus mykiss*; Gamperl et al. 1994) resulted in plasma cortisol concentrations an order of magnitude higher than desired in largemouth bass (Fig. 1). This could be a result of interspecific differences, or it could be a result of warmer water temperatures and therefore a softer coconut oil implant and faster release of cortisol; studies in rainbow trout are generally conducted at water temperatures of  $\sim 12^\circ\text{C}$ , while water temperatures during parental care in largemouth bass can exceed  $21^\circ\text{C}$ . Despite the pharmacological dose, we observed a typical glucose response to elevated plasma cortisol (see reviews in Schreck et al. 1992; Mommsen et al. 1999), and the fish were able to maintain parental care behavior at control levels for  $\sim 1$ – $2$  wk. Therefore, we believe that our results are still interesting and valid in their direction and overall message. Although limited by methodology, this is the first study to have examined elevated plasma cortisol during parental care in a fish and provides unique insight into the physiological mechanisms that drive whole-animal consequences of stress during parental care and a framework for further studies on this topic.

#### Changes in Energy Mobilization

The results of this study showed a significant effect of supraphysiological cortisol treatment on three of the four nutritional parameters examined: glucose, cholesterol, and triglycerides (Fig. 2). The plasma hyperglycemic effects of cortisol in fish have been well documented (see reviews in Schreck et al. 1992; Mommsen et al. 1999), and despite the supraphysiological dose of cortisol in this study, the increase in plasma glucose is consistent with previous work, indicating that elevations of plasma

cortisol increase carbohydrate catabolism (Axelrod and Reisine 1984). The relationships between cortisol elevation and protein and lipid metabolism are less clear-cut, and studies to date have yielded varied and often conflicting results (see reviews in Van der Boon et al. 1991; Mommsen et al. 1999). In this particular study, feeding may also be a confounding effect. In largemouth bass, foraging is reduced during parental care and only occurs opportunistically (Cooke et al. 2006). Cortisol treatment in juvenile rainbow trout causes anorexia and loss of condition even when animals are supplied with ample food (Gregory and Wood 1999). It is possible that cortisol treatment of parental male largemouth bass caused a similar rejection of opportunistic meals. Particularly, for total plasma protein, any potential increases in plasma protein as a result of an increase in protein catabolism (e.g., Mommsen et al. 1999) might be countered by decreases in plasma protein as a result of fasting (Congleton and Wagner 2006). Similarly, potential feeding differences complicate the interpretation of results for plasma lipids. Increases in cholesterol seen in the cortisol-treated fish could indicate increased catabolism of stored lipid reserves, a widely accepted effect of cortisol (Mommsen et al. 1999), but in this case may be confounded by a decrease in lipid availability as a result of fasting or by the use of coconut oil as a vehicle for the cortisol. Although extensive interspecific variation requires caution when applying studies from rainbow trout to largemouth bass, studies in juvenile rainbow trout have also shown that triglycerides decrease during periods of starvation (Congleton and Wagner 2006). Taken together, the increase in cholesterol and decrease in triglycerides suggests a change in lipid metabolism that could be a direct result of cortisol effects on lipid catabolism, an indirect effect as a result of changes in feeding behavior, or a combination of these effects. The increase observed in plasma cholesterol in cortisol-treated bass could also indicate a decrease in steroidogenesis consistent with a decrease in endogenous cortisol production as a result of negative feedback (Wenderlaar-Bonga 1997).

In summary, the direct and indirect effects of exogenous cortisol elevation on metabolism are difficult to disentangle; what is clear is that exogenous supraphysiological cortisol elevation alters the mobilization of carbohydrates and lipids from the tissues. Further study is necessary to determine whether stressors during parental care deplete overall energy reserves, which could result in reductions in the lifetime fitness if male largemouth bass are unable to acquire sufficient energetic resources before the next reproductive period such that they forgo reproduction in one or more seasons (e.g., Wiegmann et al. 1992) or could result in overwinter mortality, a common phenomenon associated with low energetic reserves in bass (Hurst 2007).

#### *Maintenance of Plasma Androgen and Parental Care Behavior and a Reduction in Nest Success*

The cortisol-treated group in this study showed no differences in behavior during the egg and egg-sac fry offspring development stages when compared with the sham-treated or control

groups. We also observed no differences in behavior of cortisol-treated fish between fish that ultimately abandoned and fish that were successful, although it is important to note that the low sample size of control and sham-treated fish that ultimately abandoned precluded a comparison using those treatment groups. The lack of a behavioral response was surprising given results from other taxa during parental care and from fish during nonreproductive periods. This result was also surprising given the extent of plasma cortisol elevation found in this study. Previous studies in birds have shown a decrease in aggression and in tending as a result of corticosterone elevation during parental care (Silverin 1986; Wingfield and Silverin 1986). Non-reproductive rainbow trout show decreases in feeding behavior and swimming behavior when treated with cortisol (Gregory and Wood 1999). Similar to our fish model, however, a study in the Lapland longspur bird (*Calcarius lapponicus*) showed that during severe winter storms, the parental birds were able to resist stress for several days before abandoning when the storms persisted (Astheimer et al. 1995). In that case, endogenous corticosterone was elevated when the birds abandoned their broods. In our study, abandonment occurred past the time course for exogenous cortisol elevation in many individuals. This observation, combined with the ability to maintain parental care behavior at controls levels until abandonment, suggests a secondary or tertiary cause of ultimate brood abandonment in fish rather than an immediate or direct response to the supraphysiological cortisol elevation.

We observed no changes in plasma androgen as a result of cortisol treatment. This also differs from the situation in bluegill sunfish, where endogenous cortisol and androgen levels appear to be negatively correlated during the reproductive period (Magee et al. 2006), and sockeye salmon (*Oncorhynchus nerka*), where stress during the reproductive migration causes reproductive hormones, including testosterone, to plummet (Kubokawa et al. 1999; Hinch et al. 2005). It is possible that our androgen assay was not sensitive enough to detect differences among the very low androgen levels seen in our study treatment groups. More research is necessary to fully elucidate the role of androgens in parental care in largemouth bass and the influence of elevated plasma cortisol on androgens. Future studies would likely benefit from the specific measurement of 11-ketotestosterone in this species (Magee et al. 2006).

#### *Synthesis: Resistance to Stress and Life-History Trade-Offs*

The stress response is a plastic trait, and individuals will modulate their stress response depending on their life history and their probability of current and future reproductive success (Stearns and Koella 1986). Resistance to stress during reproduction is generally considered a trade-off in favor of current reproductive opportunity, at the expense of survival probability and potential future reproductive opportunities (Wingfield et al. 1998). Animals with few future reproductive opportunities or valuable current reproductive opportunities should be more resistant to stress during parental care (Wingfield and Sapolsky 2003). This hypothesis has been supported through various



intraspecific studies in birds. For example, as mentioned earlier, older birds are better able to resist stress during reproduction than younger birds (Angelier et al. 2007). Birds at higher latitudes, with fewer annual reproductive opportunities, attenuate their stress response during reproduction when compared with birds in more temperate climates (Silverin et al. 1997). Largemouth bass, with a long life span and limited annual reproductive opportunities, display a life-history strategy that is consistent with the model of stress resistance during parental care (Wingfield and Sapolsky 2003). In this study, the ability of largemouth bass to temporarily maintain parental care (Figs. 2, 4) despite handling stress, suprphysiological increases in plasma cortisol (Fig. 1), and Saprolegnial infections (Fig. 3), suggests that they are resistant to the effects of elevated plasma cortisol at the whole-animal level during the reproductive period. Although it is always possible that other, unstudied variables were involved, the changes in nutritional indices detected in this study suggest that temporary resistance to stress is limited by energetic resources. For fish, the energetic costs to stress may be even more important than in other taxa, since growth in fish is indeterminate and fecundity is related to body size (Heino and Kaitala 1999).

#### Summary and Future Directions

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

yield information on the role of stress in ecological and evolutionary processes.

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