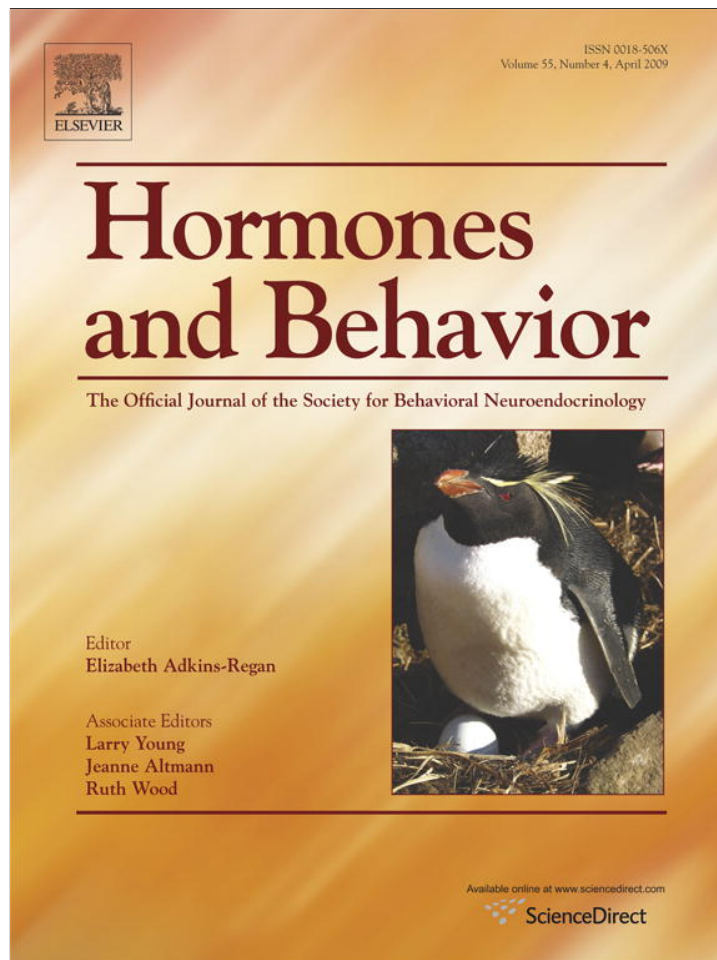


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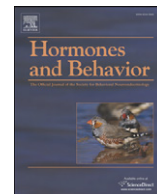
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Paternal aggression towards a brood predator during parental care in wild smallmouth bass is not correlated with circulating testosterone and cortisol concentrations

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ABSTRACT

Male smallmouth bass (*Micropterus dolomieu*) provide sole parental care including frequent aggressive actions towards conspecifics and potential brood predators. Failure to defend the brood through continual vigilance results in predation reducing the number of offspring and promoting abandonment by the nesting male. However, little is known about how biochemical and endocrine factors and brood size collectively influence paternal aggression. Behavioral assays were conducted during the egg stage of offspring development by placing a brood predator in a jar on the nest to quantify aggression (number of attacks on the potential brood predator in a minute). To determine the correlates of parental aggression, we temporarily removed fish from their nests and measured circulating levels of testosterone and indicators of the primary (plasma cortisol) and secondary stress response (plasma glucose, Cl^- , Na^+ , K^+) from non-lethal blood samples. While the male was removed from the nest, a snorkeler quantified the size of the brood. Brood size was positively correlated with male aggression. The only biochemical correlate of parental aggression was plasma glucose, which also had a positive relationship with brood size. When the effect of brood size was removed, no biochemical or endocrine factors were predictive of male aggression. Hence, brood value appeared to influence parental aggression independent of biochemical or endocrine status. While several-fold individual differences in aggression towards brood predators were noted, the role of androgens and glucocorticoids in mediating these behaviors is currently not well understood.

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Introduction

Uniparental male care for offspring has been theorized to have developed as a result of territory defense by male teleost fishes (Gross and Sargent, 1985). The most prevalent form of male parental care amongst teleost fishes is nest guarding which often includes aggressive actions towards conspecifics as well as potential brood predators (Gross and Sargent, 1985). A large body of literature suggests that androgens and stress hormones may play a role in mediating aggression between individuals during mating and territory establishment when inter-individual aggressive actions between potential rivals are frequent (Wingfield et al., 1990; Oliveira et al., 2002). In general, it has been noted that circulating levels of androgens generally decline during parental care in fish (Kindler et al., 1989; Knapp et al., 1999; Pankhurst et al., 1999; Oliveira et al., 2002; Páll et al., 2002a) as the likelihood of these aggressive encounters decrease. Additionally, chronically high levels of circulating androgens are generally thought to be incompatible with parental behavior (Wingfield et al., 1990). However, within the context of extended

parental care after mating, little work has evaluated the potential role of circulating levels of androgens on aggressive actions towards potential brood predators.

The extended parental care provided by many teleost fish species includes continued aggressive actions towards both conspecifics after the period of territory establishment and brood predators until the cessation of care. Circulating levels of the major androgens in teleost fishes, testosterone and 11-ketotestosterone (11-KT), have been noted to be highest during the courtship and territory defense phases of parental care and then sharply decline as individuals care for eggs (Páll et al., 2002a; Ros et al., 2004; Magee et al., 2006), though there has been some evidence that circulating androgens may not regulate parental behavior (Páll et al., 2002b). However, the correlation between parental aggression and circulating androgens may potentially be confounded by stress during the parental care period. Due to the need to mobilize energy reserves to perform parental care, fish may exhibit increased levels of circulating glucocorticoids (GC) (Nelson, 2000; Magee et al., 2006). Increased levels of GCs have been shown to decrease circulating androgen levels in multiple fish species (reviewed in Barton and Iwama, 1991), though the role of GCs (and the stress response in general) in mediating parental aggression is currently not well understood.

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Male smallmouth bass (*Micropterus dolomieu*) provide extended parental care after spawning. In spring when the water temperatures reach ~15 °C, male bass construct nests in the littoral zone which become the site of courtship and egg deposition (Coble, 1975; Ridgway, 1988). After spawning, females leave the vicinity of the nest and the male assumes the role of sole parental care-giver (Cooke et al., 2006). Parental care typically lasts a month and is highly energetically demanding as males are extremely active and unable to forage normally (Hinch and Collins, 1991; Mackereth et al., 1999; Cooke et al., 2002). Parental care activities include brood maintenance (consisting of fanning the brood to provide aeration and prevent silt deposition; Coble, 1975; Ridgway, 1988) and aggressive nest defense against potential brood predators as well as conspecifics (Coble, 1975; Ridgway, 1988). For adult bass, the potential brood predators tend to be small fishes (primarily *Lepomis* spp.) and thus pose no potential direct predation risk to the parent. Aggressive nest defense increases offspring survival by deterring predation on vulnerable eggs or young (Sargent and Gross, 1986) and previous work has noted that the level of aggressive nest defense varies among individuals and is positively correlated with brood size and male body length (Ridgway, 1988; Suski and Philipp, 2004). However, the proximate mechanism through which this effect is exerted has yet to be tested.

The objective of this study was to determine the biochemical and endocrine correlates of parental aggression towards brood predators in wild nest guarding male smallmouth bass. We predicted that parental aggression would be positively correlated with brood size. We predicted that aggression would be positively correlated with plasma testosterone concentrations and negatively correlated with plasma cortisol levels. We also predicted that the negative relationship between plasma cortisol and parental aggression would also result in a negative relationship between measures of the secondary stress response (glucose, Cl^- , Na^+ , K^+) and parental aggression.

Methods

Field techniques

All procedures used in this study were developed with approvals and guidance from the Canadian Council on Animal Care administered by Carleton University and Queen's University. This study was carried out from May 24th to May 26th, 2007 on Charleston Lake, eastern Ontario, Canada (44°32'14"N, 75°59'48"W). Each day, snorkelers surveyed the littoral zone and located smallmouth bass that were actively guarding nests with newly deposited eggs (>1 day old). The snorkeler then placed a numbered polyvinyl chloride (PVC) tile next to the nest and recorded nest location, depth, and egg score (a visual, categorical assessment of the number of eggs ranging from low of 1 to high of 5 based on Suski and Philipp, 2004). The snorkeler placed a jar (volume = 3.78 L) containing a small nest predator (bluegill, *Lepomis macrochirus*, mean total length \pm SEM, 172 \pm 29 mm) on the edge of the nest and counted the number of aggressive acts ('hits' whereby a male made physical contact with the jar containing the brood predator) performed by the parental male during a one minute time period. Fish were subsequently captured (5 to 20 min following behavioral assay) via recreational angling with heavy-action equipment from either the boat or underwater (by the diver) and landed within 20 s of hooking to minimize non-parental care related anaerobic exercise as well as stress response to capture. Upon capture, fish were placed in a foam lined trough filled with fresh lake water and quickly blood sampled (approximately 1.5 mL of blood in a 3 mL vacutainer containing lithium heparin) by the caudal puncture method using a 1.5", 21 gauge vacutainer syringe (Houston, 1990). Finally, total length was measured and presence or absence of injury was noted while the fish was in the trough. The snorkeler remained at the nest site for the entirety of the sampling procedure (191 \pm 4.66 s) and defended the brood until the male returned (typically in under

5 min). Blood samples were centrifuged at 10000 \times gravity for 5 min (Clay Adams Compact II Centrifuge, New Jersey), and plasma samples were stored in liquid nitrogen for later analysis.

Lab analyses

Plasma samples were analyzed for concentrations of testosterone and cortisol. Plasma cortisol was determined using a commercial ImmunoChem Cortisol ^{125}I RIA kit (MP Biomedicals, Orangeburg, NY) that has been previously validated for use with fish (e.g., DiBattista et al., 2005). Cortisol intra-assay variability (% CV) was 5.7%. Plasma testosterone was extracted using 3.5 mL ethyl acetate washes, resuspended in phosphate-buffered saline (pH 7.6) containing 0.3% gelatin, and then measured by radioimmunoassay as per the protocol outlined in McMaster et al. (1992). Extraction efficiency was measured by recovery of ^3H -labeled steroid from triplicates of a plasma pool, and assay values were corrected accordingly. All samples were run in duplicate in a single assay. Intra-assay variability (% CV) was 3.3% and cross reactivity with 11-KT was 7.5%.

Additionally, samples were analyzed for concentrations of various plasma-borne biochemical constituents that have been previously identified as indicative of the secondary stress response in fishes (glucose, Cl^- , Na^+ , K^+) (Wedemeyer et al., 1990; Mommensen et al., 1999; Barton, 2002). Biochemical analyses were based upon the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) standard reference model and conducted on a Roche Hitachi 917 analyzer (Basel, Switzerland) following procedural guidelines for standardization and quality assurance established by the Veterinary Laboratory Association Quality Assurance Program, College of American Pathologists, and the Canadian Food Inspection Agency External Proficiency Panel.

Statistical analysis

Normality and heterogeneity of variance of initial physiological data were assessed to determine whether variables needed to be transformed before analysis. Non-normal variables were \log_{10} transformed prior to subsequent analysis. To assess relationships between various biochemical measures and aggression, we utilized a least squares multiple linear regression with total number of aggressive acts by the individual as the independent variable (Zar, 1999). For nominal variables such as egg score, Tukey's HSD *post hoc* tests were used to determine differences between groups (Zar, 1999). Additionally, to remove brood size as a factor, we utilized a least squares multiple linear regression with total number of aggressive acts by the individual as the independent variable within each grouping of brood size (with the exception of the individuals with the lowest number of eggs due to insufficient sample size) (Zar, 1999). All

Table 1

Results of multiple linear regression ($r^2=0.58$) of aggression (number of hits of a model brood predator) by plasma borne indicators of stress and sex hormones as well as brood size in smallmouth bass

Source	Non-transformed mean \pm S.E.	df	F	P-value
Full model		10, 84	10.11	<0.001
\log_{10} glucose (mmol/L)	3.58 \pm 0.17	1, 84	8.19	0.006
\log_{10} K^+ (mmol/L)	2.36 \pm 0.08	1, 84	0.27	0.61
\log_{10} Na^+ (mmol/L)	163.25 \pm 1.18	1, 84	1.02	0.32
\log_{10} Cl^- (mmol/L)	113.18 \pm 1.46	1, 84	0.05	0.83
\log_{10} Testosterone (ng/mL)	1.40 \pm 0.11	1, 84	0.23	0.63
\log_{10} cortisol (ng/mL)	4.19 \pm 0.77	1, 84	0.62	0.44
Egg score		4, 84	23.39	<0.001

Significant statistical results are denoted by bold and italicized text.

Egg score is a visual, categorical assessment of the number of eggs ranging from low of 1 to high of 5.

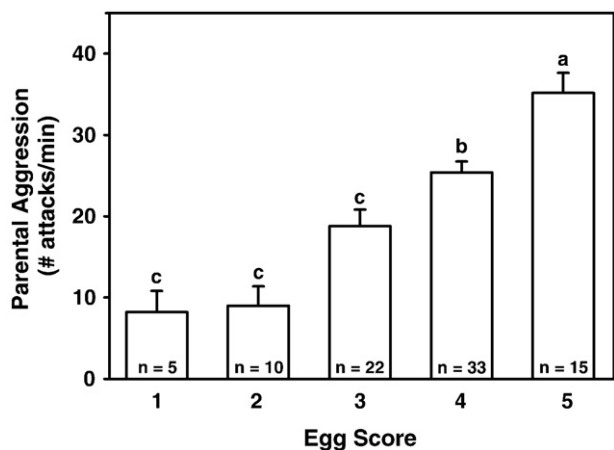


Fig. 1. Relationship between brood size and parental aggression (measured as number of attacks on a potential brood predator) by parental male smallmouth bass. $n_{\text{parental male}} = 85$.

analyses were performed in the statistical package JMP v7.0 (SAS Institute., Cary, North Carolina, U.S.A.) and the level of significance for all tests (α) was assessed at 0.05. All values presented represent means \pm S.E. unless otherwise noted.

Results

Overall, the biochemical and brood size parameters measured in this study described 58% of the variation associated with parental aggression amongst the smallmouth bass sampled (Table 1). Brood size was significantly predictive of parental aggression with brood size being positively correlated to the number of parental aggressive actions (Multiple regression: $df = 84$, $F\text{-value} = 23.39$, $P\text{-value} < 0.001$; Table 1, Fig. 1). Circulating plasma glucose was the only biochemical measure predictive of parental aggression (Multiple regression: $df = 84$, $F\text{-value} = 8.19$, $P\text{-value} = 0.006$; Table 1). Plasma glucose levels were positively related to the number of times a parental male attacked a model brood predator (Fig. 2). To remove the effects of fish with similar brood sizes, we repeated the above analysis within groups of fish with similar brood sizes. The models generated for each brood size group did not explain much of the variation associated with parental aggression, and no biochemical metrics

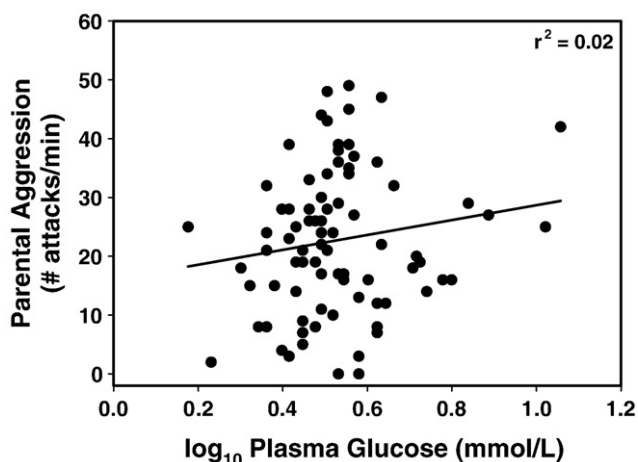


Fig. 2. Positive relationship (multiple regression: $df = 84$, $F\text{-value} = 8.19$, $P\text{-value} = 0.006$) between \log_{10} plasma glucose (mmol/L) and parental aggression (measured as number of attacks on a potential brood predator) by parental male smallmouth bass. $n_{\text{parental male}} = 85$.

Table 2

Results of multiple linear regressions of aggression (number of hits of a model brood predator) by plasma borne indicators of stress and sex hormones within groups of males with similar brood size in smallmouth bass

Brood size grouping	Source	Non-transformed mean \pm S.E.	df	F	P-value
Egg score 2	Full model		6, 9	4.92	0.11
	\log_{10} glucose (mmol/L)	3.28 ± 0.36	1, 9	2.02	0.14
	\log_{10} K^+ (mmol/L)	2.37 ± 0.30	1, 9	1.39	0.26
	\log_{10} Na^+ (mmol/L)	166.70 ± 2.23	1, 9	1.27	0.33
	\log_{10} Cl^- (mmol/L)	118.70 ± 2.18	1, 9	1.86	0.16
	\log_{10} Testosterone (ng/mL)	1.31 ± 0.23	1, 9	4.27	0.03
	\log_{10} cortisol (ng/mL)	6.30 ± 2.40	1, 9	0.04	0.97
Egg score 3	Full model		6, 21	2.04	0.13
	\log_{10} glucose (mmol/L)	4.11 ± 0.46	1, 21	2.95	0.01
	\log_{10} K^+ (mmol/L)	2.43 ± 0.15	1, 21	0.001	0.99
	\log_{10} Na^+ (mmol/L)	160.86 ± 2.02	1, 21	1.15	0.27
	\log_{10} Cl^- (mmol/L)	110.27 ± 2.68	1, 21	1.10	0.29
	\log_{10} Testosterone (ng/mL)	2.03 ± 0.29	1, 21	1.26	0.27
	\log_{10} cortisol (ng/mL)	2.75 ± 0.45	1, 21	1.04	0.32
Egg score 4	Full model		6, 32	0.47	0.82
	\log_{10} glucose (mmol/L)	3.41 ± 0.27	1, 32	0.17	0.87
	\log_{10} K^+ (mmol/L)	2.42 ± 0.14	1, 32	0.75	0.46
	\log_{10} Na^+ (mmol/L)	161.91 ± 1.71	1, 32	0.44	0.66
	\log_{10} Cl^- (mmol/L)	111.61 ± 2.82	1, 32	0.89	0.38
	\log_{10} Testosterone (ng/mL)	1.16 ± 0.12	1, 32	0.03	0.76
	\log_{10} cortisol (ng/mL)	4.85 ± 1.70	1, 32	0.26	0.79
Egg score 5	Full model		6, 14	3.00	0.08
	\log_{10} glucose (mmol/L)	3.31 ± 0.16	1, 14	3.14	0.01
	\log_{10} K^+ (mmol/L)	2.22 ± 0.13	1, 14	1.00	0.35
	\log_{10} Na^+ (mmol/L)	168.73 ± 3.93	1, 14	1.81	0.11
	\log_{10} Cl^- (mmol/L)	119.87 ± 2.51	1, 14	1.49	0.18
	\log_{10} Testosterone (ng/mL)	1.16 ± 0.18	1, 14	1.77	0.12
	\log_{10} cortisol (ng/mL)	4.23 ± 1.50	1, 14	0.81	0.44

Egg score is a visual, categorical assessment of the number of eggs ranging from low of 1 to high of 5.

were significantly correlated to the number of aggressive actions performed by an individual (Table 2).

Discussion

In the current study, the most important factor associated with parental aggression in smallmouth bass was the size of the brood (Table 1; Fig. 1). Multiple previous studies have shown a positive relationship between the number of offspring in the nest and parental aggression in smallmouth bass (Ridgway, 1988; Suski and Philipp, 2004) as well as other fish (Pressley, 1981; Coleman et al., 1985) and other taxa (see Montgomerie and Weatherhead, 1988). Large broods represent an opportunity for greater reproductive success, therefore males engage in more aggressive actions to defend the nest and increase offspring survival (Trivers, 1972; Ridgway, 1988; Gross, 2005; Suski and Philipp, 2004). However, aggressive nest defense also comes at a cost of higher energy expenditures as these actions require the utilization of energy to power swimming associated with chasing and attacking brood predators. This occurs at a time when the male is not foraging and is fueling all activity by finite endogenous energy reserves (Mackereth et al., 1999; Cooke et al., 2002). Potentially, if parental male energy levels decrease to a point that could threaten the potential for future reproduction, the individual should abandon the current brood resulting in no reproductive success for that year (Trivers, 1972; Sargent and Gross, 1986). Additionally, as energy expended in parental care needs to be replenished prior to the next reproductive season, overly aggressive individuals may impact future reproductive opportunities (Mackereth et al., 1999). Surprisingly, no biochemical factors measured in this study, with the exception of plasma glucose, were correlated with parental aggression in the

current study (Table 1), though this may be due to an overwhelming effect of brood size.

When the major factor of brood size is removed from the analysis, none of the biochemical factors measured in this study was correlated with parental aggression (Table 2). This is surprising given that previous research has documented the positive correlation between circulating levels of androgens and aggressive acts and territoriality by parental male fishes (Páll et al., 2002a; Ros et al., 2004) and which is consistent with the theory that aggressive brood defense evolved from an ancestral form of territory defense (Gross and Sargent, 1985). Though it has been noted that androgen levels are positively correlated with aggressive territorial actions towards conspecifics during mating, other aggressive actions, such as anti-predator or irritable aggression, are not mediated by circulating androgen levels (Moyer, 1968; Archer, 1988; Wingfield and Marler, 1988; Wingfield et al., 1990). As such, aggression towards brood predators may be a behavior that is not mediated by circulating levels of androgens and may be more aptly described as parental care behavior than an aggressive action. Indeed, in the current study, plasma testosterone levels for all fish were quite low (Table 1) regardless of the number of aggressive actions towards a brood predator. This finding is in concert with the supposition that parental care behaviors are typically incompatible with high levels of androgens in the plasma (Wingfield et al., 1990; Páll et al., 2002a; Magee et al., 2006). In a recent review of modulation of androgen levels by teleost fishes, androgen levels decreased in all fish species surveyed following territory acquisition and spawning (Oliveira et al., 2002), though there are exceptions where androgen levels remain elevated through parental care (Knapp et al., 1999; Rodgers et al., 2006). In species that exhibit extended parental care including aggressive defense of the nest, androgen levels precipitously decrease during the parental care phase of reproduction (Oliveira et al., 2001; Magee et al., 2006). Similarly, a manipulation experiment by Páll et al. (2002b) in which a group of parental male stickleback were subjected to 11-ketoandrostenedione (11KA converts to 11KT in the bloodstream, thereby preventing declines in 11KT across parental care) and a separate group were castrated also provides evidence that parental care behavior is not related to androgen levels. Both treatment of fish with 11KA and castration did not significantly alter courtship or parental behaviors when compared to control fish, indicating that these behaviors are more likely to be modulated through non-gonadal endocrine factors such as prolactin (Páll et al., 2002b).

The majority of work on androgens and GCs in relation to parental care focuses on small bodied fishes defending broods against similar sized conspecifics that are either trying to steal territories (Oliveira et al., 2001; Páll et al., 2002a,b) or are in close proximity due to colonial nesting (Knapp et al., 1999; Magee et al., 2006). In contrast, smallmouth bass territory establishment typically occurs across a short time frame prior to spawning with few subsequent incursions by conspecifics due to the relatively solitary nature of nest sites (Coble, 1975). As such, territorial aggression (and related increases in androgen levels) may be quite muted in smallmouth bass following egg deposition (i.e., termination of spawning and departure of the female) and commencement of parental care. Unlike other model systems commonly studied in the literature, smallmouth bass nest defense is generally characterized by aggressive actions towards brood predators that are much smaller than the parental male and often small enough to be potentially consumed (Coble, 1975). Additionally, in most systems inhabited by smallmouth bass, there are few aquatic organisms that could predate upon an adult bass which suggests that partaking in aggressive parental care poses little to no immediate threat to the welfare of the parental male. While it is currently not known if any of these factors should result in differences in hormonal mediation of parental aggression, it is worth noting for future studies.

Similarly, due to the antagonistic relationship between GC levels and circulating levels of androgens (Pottinger et al., 1996) and due to

the role of GCs in mobilizing energy reserves (Nelson, 2000), male aggression could be mediated through circulating cortisol (Pankhurst and Van Der Kraak, 1997; Pottinger, 1999; Schreck, 1996; Magee et al., 2006). In the current study, there was no relationship between the primary indicator of stress (circulating cortisol), but there was a positive relationship between parental aggression and circulating glucose (a measure of the secondary stress response; Mommsen et al., 1999) (Table 1; Fig. 1). However, this relationship was not present when brood size was removed from the analyses. Participation in a high number of aggressive actions to repel brood intrusions by brood predators may constitute repeated aerobic and anaerobic exercise for these fish (Cooke et al., 2002), reflected by increases in plasma glucose, without an accompanying increase in plasma cortisol. Indeed, we sampled fish 5 to 20 min after their behavior was assayed so an increase in glucose or cortisol that could have resulted from attacking the jar would likely not have fallen by that time. Additionally, increases in plasma glucose concentrations most likely reflect mobilization of endogenous glucose to fuel increases in parental aggression as glucose is a requirement for metabolism in multiple tissues associated with swimming behavior (Mommsen, 1986; Mommsen et al., 1999; Magee et al., 2006).

Parental care consists of a complex interplay of behaviors such as parental aggression and brood maintenance that are modified by potentially conflicting endocrine functions (Wingfield et al., 1990; Pottinger, 1999; Schreck, 1996). The data from the current study suggest that parental aggression in smallmouth bass is primarily dictated by brood size rather than being modulated through hormonal cues such as circulating androgen or GC levels. The current study presents novel information on a teleost fish model that provides extended parental care that includes aggressive actions towards non-threatening brood predators. As such, this study provides new information on the role of the endocrine system in modulating parental care behavior of teleost fishes with differing life histories.

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