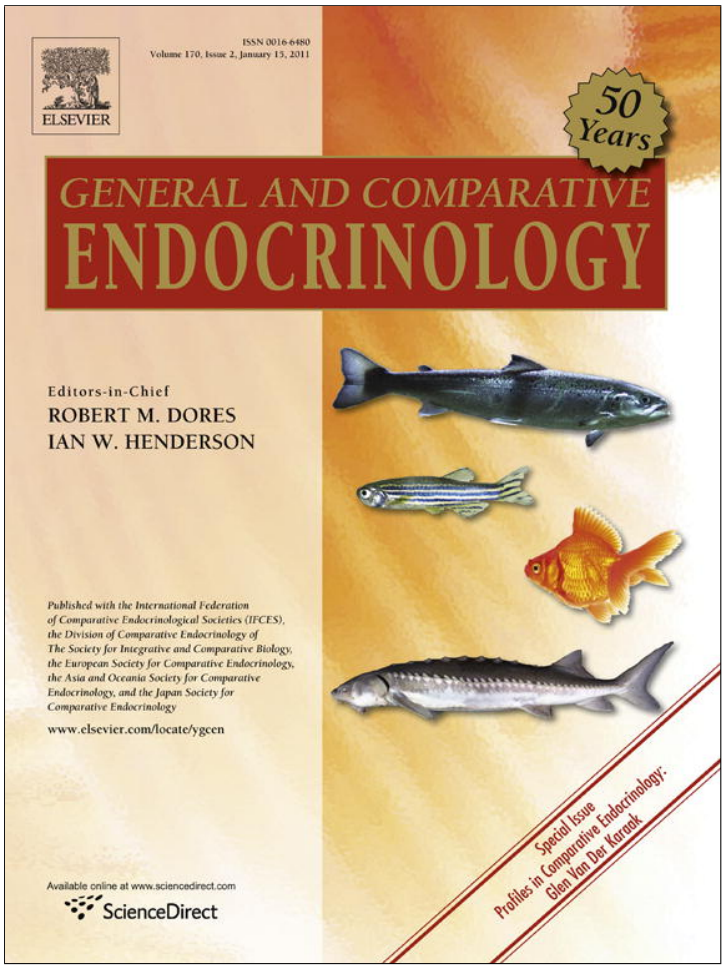

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The glucocorticoid stress response is attenuated but unrelated to reproductive investment during parental care in a teleost fish

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ABSTRACT

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

1. Introduction

The premise of life-history theory is that individuals should make optimal trade-offs among competing functions in order to maximize their fitness [8,34,35,48,49]. Investment in current versus future reproduction is one such trade-off because energy allocated towards current reproductive opportunities sequesters resources from growth and survival, thereby decreasing future reproductive potential [49]. Recently, the endocrine system, and in particular the glucocorticoid hormones, have attracted interest as potential mediators of these trade-offs [13,37,54]. The glucocorticoid hormones, are best known for their rapid elevation in response to a challenge, initiating a suite of physiological changes collectively termed a stress response [29,41]. In the short-term, this response is considered adaptive because it initiates a suite of system-level and whole-organism responses that promote survival of the individual through a challenge [19,41,52]. However, these physiological changes also interrupt other important functions including reproduction (e.g., [18,43,44,51]) and parental care behavior (e.g., [9,44]). The underlying mechanisms are varied, and include actions of glucocorticoids at various locations within the hypothalamus–pituitary–gonadal (HPG) axis responsible for the synthesis and secretion of reproductive hormones [16,18,30], as well as actions that are independent of the HPG-axis (e.g., behavioral effects mediated through brain receptors; [41]). Baseline levels of glucocorticoids also serve important biological functions, and may play a role in preparing an organism to appropriately respond to a future challenge [40,41]. Whereas elevation of glucocorticoids in response to stress inhibits reproduction, increases in baseline glucocorticoid levels may benefit reproductive activity by mobilizing energy reserves for the challenges associated with mating or parental care [30,40]. Therefore, both baseline

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glucocorticoid levels and the extent of glucocorticoid elevation during a challenge are thought to mediate the trade-off between current and future reproduction, and may function in a complementary fashion [40]. Whereas increases in baseline levels are associated with increased investment in current reproduction [3,4,30], augmented glucocorticoid levels during a stress response are thought to shift resources away from current reproductive investment and towards survival and future reproduction [5,50].

If interactions between endocrine agents mediate individual decisions regarding optimal investment in current versus future reproduction [37], then life-history theory predicts that circulating hormone levels should reflect current reproductive investment. Individuals with high investment in current reproduction should display higher baseline glucocorticoid levels, while attenuating the glucocorticoid response and/or maintaining hormones important for reproduction when faced with a challenge [50]. Conversely, individuals with lower current reproductive investment should display low baseline glucocorticoid levels and a robust glucocorticoid response to a challenge, at the expense of maintaining reproductive hormones. In the current study, we investigated the influence of reproductive investment on the glucocorticoid (cortisol) and reproductive hormone (androgen) concentrations of smallmouth bass (*Micropterus dolomieu*), a teleost fish with annual sole-male parental care. In teleost fish, cortisol is the primary glucocorticoid [29], and androgens are implicated in male parental care (see review by Oliveira et al. [32]). We used a standardized stress protocol that involved capture and 3 min of air exposure, and we collected blood samples prior to and 25 min following the stressor (a period of time that corresponds to the peak of cortisol elevation following a stressor for this species; [11]). We first tested whether baseline and post-stress cortisol and androgen levels varied in parental care-providing males compared to non-parental fish. We then investigated the possibility that baseline and post-stress cortisol and androgen levels varied with offspring age or brood size during parental care. Life-history theory predicts that parental investment should increase as offspring develop and the probability of offspring survival increases, but then decrease again as offspring achieve independence [42]. Supporting this prediction, activity levels in smallmouth bass (indicative of nest defence and fanning, and thus of reproductive investment) peak when the offspring are approximately 2 weeks old [11]. Thus, we evaluated baseline and post-stress circulating cortisol and androgen concentrations at three offspring development stages. For nest-guarding fish, larger broods result in higher reproductive success per brood [45]. Therefore, male smallmouth bass should have higher investment in larger broods. Indeed, males with larger broods expend more energy defending the nest than do males with smaller broods [39,47]. Thus, for an experimental measure of reproductive investment, we manipulated brood size (reduced, enlarged, sham-treated, or unmanipulated) 24 h prior to evaluating baseline and post-stress circulating cortisol and androgen concentrations among treatment groups.

We predicted that parental care-providing smallmouth bass would display higher baseline cortisol levels and attenuate the cortisol response in comparison to non-parental fish. Within parental care-providing fish, we predicted that males would exhibit the highest baseline cortisol levels and the greatest attenuation of the cortisol stress response in the middle of parental care, when energetic demands are highest [11]. We predicted that androgen levels would be high during early parental care, following nest establishment [20,26]. We further predicted that males guarding enlarged brood sizes would display the highest baseline cortisol levels, the greatest attenuation of the cortisol response, and the highest androgen levels when compared with males guarding control, sham, or reduced broods.

2. Methods

2.1. Series 1: Brood age

During May and June 2008, male smallmouth bass guarding nests at various stages of development were identified by snorkeling on Charleston Lake, a public lake in eastern Ontario that is part of the Gananoque River system (44°32′N, 75°59′W). For all males, an egg or fry score was assigned. An egg score is a standard and highly repeatable measure of the relative number of eggs within a nest, and ranges from 1 (low, <500) to 5 (high, >4000; [33,47]). Fry scores are based on a similar relative scale. For standardization, only males with an egg or fry score of 3 or 4 were included. For the purposes of this study, offspring development stages were as outlined in [11]. The 'egg' stage denotes freshly laid and fertilized eggs. 'Egg-sac fry' appear approximately 1 week post-fertilization, when the yolk sac has been partially absorbed, and the larvae have developed tails and are able to wriggle within the nest. At this stage, the larvae are still feeding endogenously, and are unable to swim. The 'free-swimming fry' stage occurs approximately 3 weeks post-fertilization, when the offspring have almost entirely absorbed the yolk sac, and have gained the ability to swim. Larvae at this stage subsist on a combination of endogenous and exogenous feeding, and can be found in the water column above the nest. The parental male is still required to defend the brood, since free-swimming fry have not yet fully developed predator avoidance tactics [6].

On May 22, 25, and 26, 2008 (water temperature 14–16 °C), 25 parental male smallmouth bass (350–466 mm) guarding nests with fresh eggs (0–1 day old) were identified. On June 2 and 5, 2008 (water temperature 17–18 °C), a different group of 23 males (339–493 mm) guarding nests that contained egg-sac fry was identified. Finally, on June 12 and 13, 2008 (water temperature 20–21 °C), a final group of 23 males (273–465 mm) guarding free-swimming fry was identified. Individual fish were sampled at only one stage. Once a nest was identified, the parental male was captured using standard rod-and-reel angling and a rubber mesh landing net, and immediately placed in a foam-lined trough filled with fresh lake water, a procedure that took less than 30 s from hooking the fish. A blood sample (~1 mL) was withdrawn by caudal puncture, using lithium-heparinized 3 mL vacutainers (B.D. Vacutainer, Franklin Lakes, NJ) and 21 gauge, 38 mm needles. Fish were then subjected to a standardized stressor consisting of 3 min of air exposure (a period of time that is sufficient to elevate circulating cortisol; [11]). During air exposure, fish were placed on a damp foam mat within a 35 × 60 cm plastic tub covered by a loosely-fitted lid to shield the fish from direct sun. After the stressor, fish were placed in individual 50 L coolers filled with fresh lake water for a 25 min recovery period, before a second blood sample was withdrawn as described above. Total length (TL) was measured and the fish was released.

To provide a comparison group of non-nesting control fish, 53 mature fish of reproductive size (273–502 mm) were captured in May and June 2009, from several lakes in eastern Ontario across a range of water temperatures that corresponded to the water temperatures during parental care (see Table 1). Identifying the sex of smallmouth bass that are non-reproductive is not reliable without dissecting the animals, so these non-reproductive fish were likely a mixture of males and females. Upon capture, all fish were subjected to the sampling regime and standardized stressor described above.

In all groups, sampling took place throughout the day (between 9:00 and 19:00). Blood samples were held in water–ice slurries for <1 h, and then centrifuged at 10,000*g* for 5 min (Compact II Centrifuge, Clay Adams, NJ). Plasma samples were flash frozen in liquid nitrogen and stored at −80 °C until analysis. Blood samples were excluded when more than 90 s elapsed between the start and

Table 1
Water temperatures and capture locations for non-reproductive smallmouth bass (*Micropterus dolomieu*) used as a comparison group for parental care-providing fish.

Water temperature (°C)	Date	Number of captured fish	Lake	Coordinates
13.5–14.0	May 5, 2009	4	Sand	44°32′N, 76°14′′W
14.5–15.0	May 11, 2009	8	Sand	44°32′N, 76°14′′W
15.5–16.0	May 16, 2009	7	Sand	44°32′N, 76°14′′W
17.0–17.5	June 11, 2009	10	Sand	44°32′N, 76°14′′W
18.0–18.5	June 16, 2009	12	Big Rideau	44°45′N, 76°14′′W
21.0–22.0	June 24, 2009	12	Big Rideau	44°45′N, 76°14′′W

end of the blood sampling procedure. This resulted in the exclusion of three males guarding free-swimming fry and one non-reproductive fish.

2.2. Series 2: Brood size

Between May 12 and 14, 2008 (water temperature 14 °C) male smallmouth bass guarding nests with fresh eggs (0–1 days old) were identified by snorkelling on Sand Lake, a public lake that is part of the Rideau River system in eastern Ontario (44°30′N, 76°20′W). All nests were individually marked with a numbered tile, and the brood size of each nest was classified according to egg score. Again for standardization, only males guarding nests with egg scores of 3 and 4 were included in this study. In total, 57 parental fish were randomly assigned to one of four treatment groups: controls ($n = 14$), shams ($n = 14$), enlarged nests ($n = 14$), and reduced nests ($n = 15$). Eggs were removed from reduced nests to reduce the egg score to 1 (<500 eggs). Fresh eggs from other nests were added to enlarged nests to double the brood size, resulting in an egg score of 5 (>4000 eggs). Eggs were removed from and then returned to sham nests, with no net gain or loss of eggs, while control nests were not manipulated. Turkey basters and mason jars filled with fresh lake water were used for all egg transfers [39]. Between 18 and 30 h following brood size manipulation, all male parental fish were captured, subjected to the standardized stress protocol and sampled for blood as described above. Sampling for all groups took place throughout the day.

To determine whether brood manipulation itself was affecting the endocrine response of parental males to the standardized stressor, we conducted a follow-up study that used sham brood size manipulation (i.e., nest disturbance) of varying duration. On May 26, 2009 (water temperature 16 °C) 32 male smallmouth bass guarding nests with fresh eggs with egg scores of 3 or 4 were identified by snorkeling on Charleston Lake. Nests were individually identified with numbered tiles, and fish were randomly assigned to one of three treatment groups: control ($n = 11$), short nest disturbance ($n = 8$), and long nest disturbance ($n = 9$). Nest disturbance involved the snorkeler using a turkey baster to mimic egg transfer, but without any direct contact between the turkey baster and the eggs, for 5 min (long) or 1 min (short). Control nests were not disturbed. All fish were captured 18–30 h following nest disturbance, subjected to a stress protocol and sampled using the methods described above. Sampling for all groups took place throughout the day. As before, fish were excluded if more than 90 s elapsed between the start and end of the blood sampling procedure. This resulted in the exclusion of one short disturbance fish.

2.3. Hormone analysis

Cortisol was determined on non-extracted plasma samples using a commercial kit (ImmunoChem Cortisol ^{125}I RIA kit; MP Biomedicals, Orangeburg, NY) previously validated for teleost fish [17]. Intra-assay variability (% CV) was 7.3%. Inter-assay variability was 6.9%. For the measurement of androgens, plasma samples were extracted three times using 5 ml ethyl acetate, and the dried extract was resuspended in phosphate-buffered saline (pH 7.6) containing 0.3% gelatin and measured following the methods outlined in McMaster et al. [28]. Androgen content in the extract was measured in duplicate by ^{3}H-radioimmunoassay using a testosterone antibody provided by Medicorp (AS0116, Medicorp Inc., Montréal, QC). Cross-reactivity of the testosterone antibody with 11-ketotestosterone was 7.5%, and thus we describe our measurement as ''androgens'' rather than specifically ''testosterone''. All samples for each experiment were run in single assays. Intra-assay variability was 3.7–14.3%.

2.4. Statistical analysis

Because male and female non-reproductive fish were used as a comparison group for parental care-providing fish, the distribution of hormone levels for non-reproductive fish was examined for evidence of bimodality. Baseline, post-stress, and change in cortisol concentrations were compared among parental care-providing and non-reproductive fish using analysis of covariance (ANCOVA) tests. Parental care status (egg stage, egg-sac fry stage, free-swimming fry stage, or non-reproductive) was the independent variable, with water temperature and sampling time of day as covariates. Androgen concentrations were compared among parental fish using similar ANCOVA tests; non-reproductive fish were excluded from these analyses. Finally, we compared baseline to post-stress hormone levels using paired Student's *t*-tests.

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

In all cases, residuals were tested for normal distribution using Shaprio–Wilks goodness-of-fit tests, and for homogeneity of variance by visual inspection [53]. All analyses were performed using the statistical package JMP, version 7.0.1 (SAS Institute Inc., Cary, NC). The level of significance for all tests (α) was 0.05. All results are presented as mean ± 1 standard error of the mean (SEM).

3. Results

3.1. Series I: Brood age

Neither baseline nor post-stress cortisol concentrations for non-reproductive fish deviated from a normal distribution (Shaprio–Wilks

goodness-of-fit tests, $p > 0.05$); there was no evidence of bimodality. This observation suggests that non-parental male and female fish did not differ with respect to cortisol stress responses, and validates our use of these fish as a comparison group for the parental males.

There were no differences in baseline cortisol concentrations with reproductive status or stage, nor was there an effect of temperature or sampling time (baseline ANCOVA whole model $F_{5,110} = 2.064$, $p = 0.075$; Figs. 1A and 2A). However, cortisol levels increased significantly following the standardized stressor ($t_{109} = 15.521$, $p < 0.001$), and both parental care status (post-stress status effect $F_{3,3} = 5.086$, $p = 0.002$; Figs. 1B and 2B) and water temperature (post-stress temperature effect $F_{1,1} = 17.451$, $p < 0.001$; Fig. 1B) significantly affected post-stress cortisol concentrations (post-stress ANCOVA whole model $F_{5,108} = 11.256$, $p < 0.001$; Figs. 1B and 2B). There was no effect of sampling time (post-stress timing effect $F_{1,1} = 1.557$, $p = 0.215$). Post-stress cortisol concentrations increased with water temperature (Fig. 1B) and were lowest in fish guarding fresh eggs, returning to non-reproductive levels in fish guarding free-swimming fry (Figs. 1B and 2B). Similarly, the change in cortisol levels also increased with water temperature and was lower in the fish providing care (change ANCOVA whole model $F_{5,104} = 10.169$, $p < 0.001$; status effect $F_{3,3} = 4.861$, $p = 0.003$; temperature effect $F_{1,1} = 12.785$, $p < 0.001$; timing effect $F_{1,1} = 1.329$, $p = 0.252$).

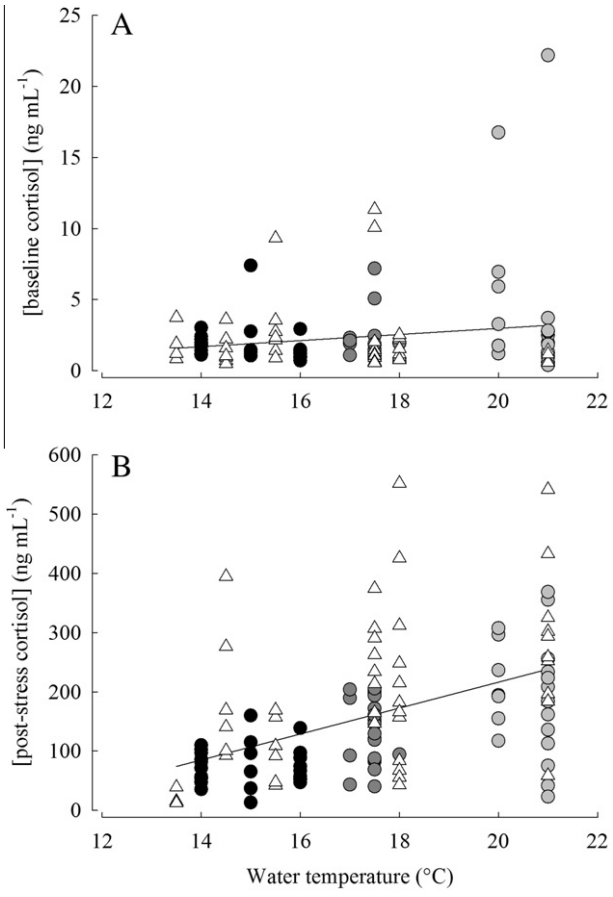

Fig. 1. The relationship between (A) baseline and (B) post-stress circulating cortisol concentrations and water temperature among smallmouth bass (*Micropterus dolomieu*) guarding offspring at various development stages, and non-parental fish. Fish providing care to eggs are represented by closed black circles (●), fish providing care to egg-sac fry are represented by closed dark grey circles (●), fish providing care to free-swimming fry are represented by closed light grey circles (●), and non-reproductive fish are represented by open triangles (△). Neither offspring development stage nor water temperature affected (A) baseline cortisol concentrations, but both factors had a significant effect on (B) post-stress circulating cortisol concentrations ($p < 0.05$; see Section 2.4).

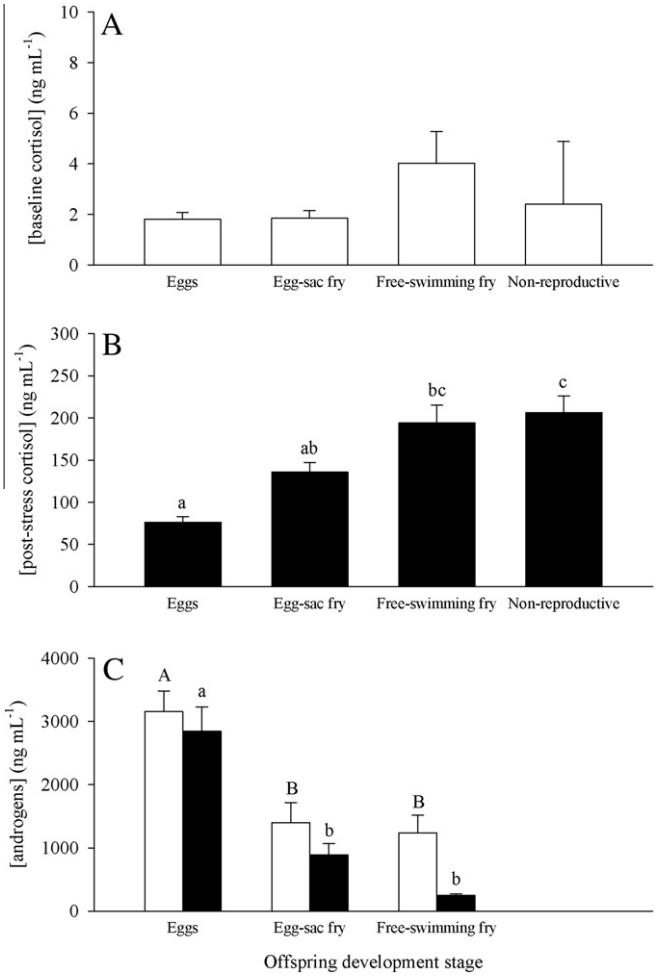

Fig. 2. Circulating (A) baseline and (B) post-stress cortisol and (C) androgen concentrations are depicted across the parental care period in smallmouth bass (*Micropterus dolomieu*). Baseline values are indicated by open bars, while post-stress values are indicated by closed black bars. Both (B) post-stress cortisol and (C) androgen levels varied significantly among offspring development stages ($p < 0.05$; see Section 2.4). Note that androgens were not measured in non-reproductive fish. Values are presented as means ± SEM. Different capital letters indicate statistical differences among baseline groups, while different lower-case letters indicate statistical differences among post-stress groups as indicated by post hoc tests ($p < 0.05$; see Section 2.4).

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

3.2. Series II: Brood size

We found no differences in baseline, post-stress, or change in cortisol concentrations among males in the brood manipulation

experiment (baseline ANCOVA whole model $F_{4,51} = 0.626$, $p = 0.646$; post-stress ANCOVA whole model $F_{4,51} = 1.592$, $p = 0.191$; change ANCOVA whole model $F_{4,51} = 1.649$, $p = 0.177$; Fig. 3 A and B). We again documented an increase in cortisol concentrations following the standard stressor ($t_{54} = 12.065$, $p < 0.001$). There was a marginally non-significant effect of treatment group on baseline androgen concentrations (baseline ANCOVA whole model $F_{4,52} = 2.873$, $p = 0.032$; treatment effect $F_{3,3} = 2.654$, $p = 0.058$; timing effect $F_{1,1} = 2.579$, $p = 0.115$; Fig. 3C). Although we again documented a decrease in androgen levels following the standardized stressor ($t_{54} = -2.225$, $p = 0.030$), there was no difference among treatment groups in the extent of the change in androgen concentrations (change ANCOVA whole model $F_{4,51} = 1.034$, $p = 0.399$), or in post-stress androgen concentrations (post-stress ANCOVA whole model $F_{4,51} = 1.144$ $p = 0.347$; Fig. 3C).

As in the brood manipulation experiment, we found no differences in baseline cortisol concentrations among males in the nest disturbance experiment (baseline ANOVA $F_{2,25} = 2.192$, $p = 0.256$; Fig. 3D). Cortisol levels again increased following the standard stressor ($t_{26} = 10.585$, $p < 0.001$), and in this case both post-stress cortisol concentrations and the change in cortisol concentration were significantly different among treatment groups (post-stress ANOVA $F_{2,25} = 7.161$, $p = 0.004$; change ANOVA $F_{2,25} = 7.161$, $p = 0.004$). The cortisol response to a standard stressor increased with the extent of previous nest disturbance (Fig. 3E). However, we found no differences in baseline, post-stress, or change in androgen concentration among these treatment groups (baseline ANOVA $F_{2,26} = 2.819$, $p = 0.078$; post-stress ANOVA $F_{2,26} = 1.866$, $p = 0.176$; change $F_{2,26} = 0.561$, $p = 0.578$; Fig. 3F), and in this case, androgen levels did not decline following the standardized stress protocol ($t_{54} = -0.503$, $p = 0.619$).

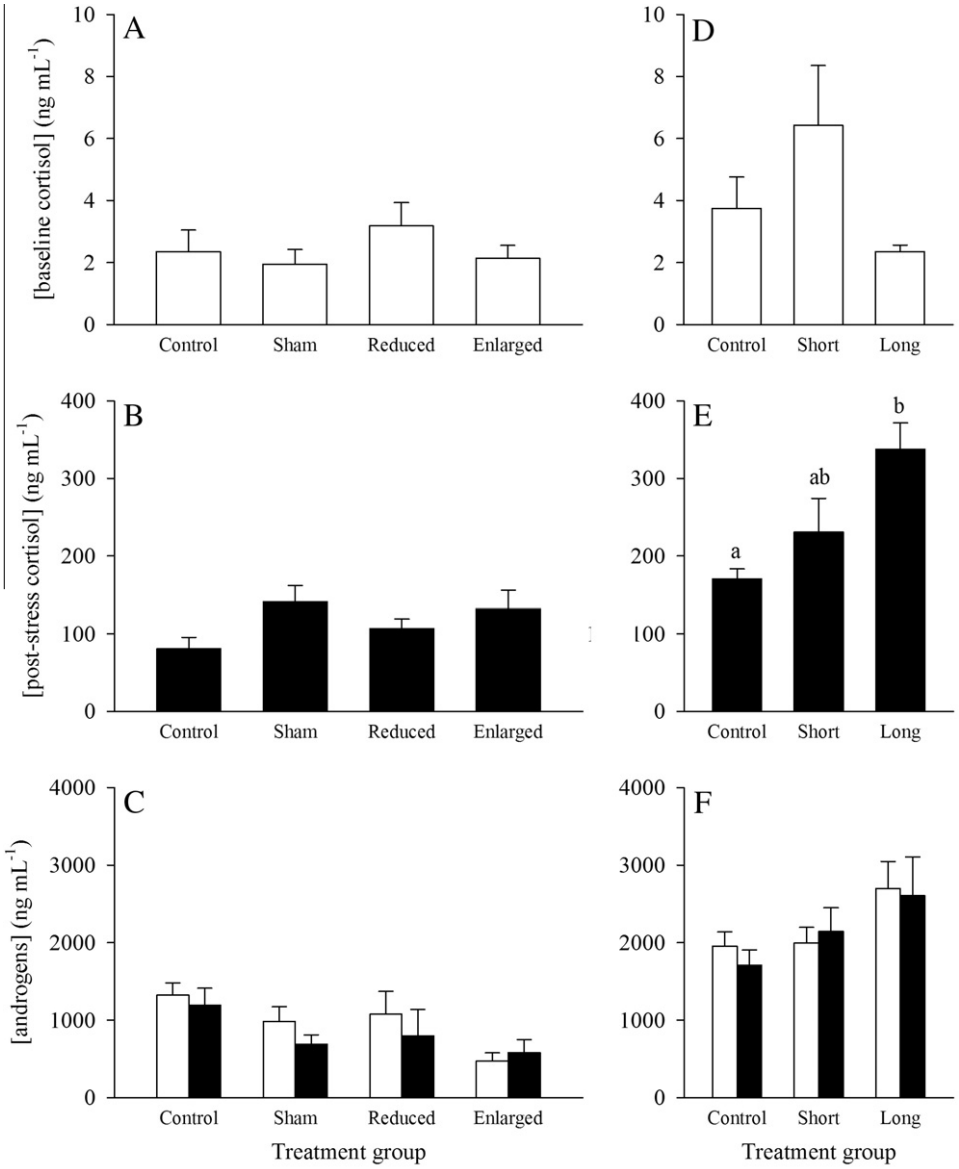

Fig. 3. Circulating (A and D) baseline and (B and E) post-stress cortisol and (C and F) androgen concentrations are depicted among male smallmouth bass (*Micropterus dolomieu*) treatment groups in a brood manipulation study (A–C), and a nest disturbance study (D–F). All brood manipulations and disturbances occurred approximately 24 h prior to capture and sampling of the fish. Post-stress cortisol concentrations varied among fish in the nest disturbance treatment groups. Different lower-case letters indicate statistical differences among post-stress groups as indicated by post hoc tests ($p < 0.05$; see Section 2.4). Differences between the results of the two experiments (i.e., across panels) should be interpreted with caution, as water temperatures varied between the two experiments, and androgen assays for the two experiments were run separately and without an inter-assay control.

4. Discussion

4.1. Did the cortisol stress response vary with reproductive investment?

A key finding of the current study was the attenuation of the cortisol response to a standardized stressor during parental care in smallmouth bass. This finding is consistent with previous work in avian (reviewed by Wingfield and Sapolsky [50]), reptilian and amphibian systems (reviewed by Moore and Jessop [30]), and is the first study to note this phenomenon in a teleost model. We hypothesize that attenuation of the cortisol stress response during parental care may be an adaptive mechanism to avoid elevating cortisol titers beyond a threshold where parental care behaviors can no longer be maintained, in agreement with the "resistance to stress" hypothesis [50]. Previous research demonstrated that parental care behaviors are transiently maintained despite exogenous elevation of circulating cortisol in the smallmouth bass [14] and the congeneric largemouth bass (*Micropterus salmoides*; [31]). However, in both cases fish were ultimately unable to sustain parental care as treatment with cortisol resulted in increased nest abandonment [14,31]. Such results suggest that while parental fish can display transient behavioral resistance to elevations of cortisol, a sustained physiological stress response is ultimately associated with reproductive costs. The attenuation of the cortisol response during parental care documented in this study may be an adaptive mechanism to avoid or at least postpone such negative effects.

Our results provided only weak evidence that post-stress cortisol values in smallmouth bass vary according to reproductive investment within the parental care period. That is, the cortisol stress response of males guarding young at the free-swimming fry stage was no longer significantly different from that of non-reproductive fish, suggesting that the attenuation of the cortisol response disappears as the brood reaches independence. However, with respect to brood size there were no differences among males guarding enlarged, reduced, sham, or control broods. This finding contrasts with that of a previous study showing that in house sparrows (*Passer domesticus*), birds with enlarged broods exhibited a greater attenuation of the glucocorticoid stress response than birds with reduced broods [23]. These results are also in contrast to previous studies in which, at a behavioral level, smallmouth bass defend enlarged broods more vigorously than smaller broods [39,47]. Taken as a whole, these results suggest that the cortisol stress response in bass is coarsely tuned to reproductive state but not finely tuned to investment within the reproductive period.

4.2. Other factors influencing the cortisol stress response

We documented an increase in the cortisol stress response with increasing water temperature in smallmouth bass. This relationship is consistent with findings from previous studies in teleost fish demonstrating a rise in the rate and/or magnitude of cortisol elevation following a stressor with increasing water temperature (e.g., [1,12,21,24,25,36]). The relationship is interesting given that smallmouth bass provide annual parental care that lasts for 4–6 weeks in the early spring, during which water temperatures increase from approximately 13 °C to 20 °C [10,38]. The relationship between water temperature and the cortisol stress response may contribute to the observed decrease in reproductive success in smallmouth bass as water temperature increases [46].

We also demonstrated that recent exposure to stressors can have a significant effect on the cortisol response during a subsequent challenge. Previous studies on smallmouth bass demonstrated that multiple repeated stressors can be cumulative, with fish showing a higher peak cortisol response to the final disturbance than to the initial disturbance [7]. Similar findings have been reported in rainbow trout (*Oncorhynchus mykiss*; [15]), and in chinook salmon (*Oncorhynchus tshawytscha*; [2,27]). It is likely that the parental smallmouth bass in this study perceived nest disturbance as a stressor. Although the fish had recovered from the stress of nest disturbance by the time of capture (based on the low baseline cortisol concentrations measured), the capture-and-standardized-stress likely acted as a repeated stressor, and the cortisol values following air exposure therefore reflected a cumulative effect. Although further research is necessary to understand the nature of the relationship, our results emphasize the importance of considering an individual's recent history when interpreting endocrine data.

4.3. Did androgens vary with reproductive investment?

We documented a decline in mean androgen concentrations across parental care, a result that is consistent with previous work in the congeneric bluegill [26], where mean androgen levels were highest during nest establishment stages, and declined during care stages. These findings suggest that androgens may be more important during nest and territory establishment than during parental care [20,22,26]. In agreement with this pattern, androgen levels exhibited little evidence of varying according to reproductive investment within the parental care period, and did not differ significantly among males guarding manipulated broods.

4.4. Study limitations

Baseline cortisol levels were not affected by any measured parameter, a finding that contrasts with previous studies in the confamilial male bluegill (*Lepomis macrochirus*), where baseline cortisol levels were reduced in males with reduced brood sizes [26]. However, in the current study baseline cortisol levels in all fish were uniformly low, and frequently near the 3 ng ml^{-1} detection limit of the radioimmunoassay kit, making it difficult to detect non-random variation that may have existed.

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

5. Summary

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

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