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Effects of maternal cortisol treatment on offspring size, responses to stress, and anxiety-related behavior in wild largemouth bass (*Micropterus salmoides*)

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ABSTRACT

Cortisol, the main glucocorticoid stress hormone in teleost fish, is of interest as a mediator of maternal stress on offspring characteristics because it plays an organizational role during early development. The present study tested the hypothesis that maternal exposure to cortisol treatment prior to spawn affects offspring phenotype using wild largemouth bass (*Micropterus salmoides*). Baseline and stress-induced cortisol concentrations, body size (i.e. length and mass), and behavior (i.e. anxiety, exploration, boldness, and aggression) were assessed at different offspring life-stages and compared between offspring of control and cortisol-treated females. Cortisol administration did not affect spawning success or timing, nor were whole-body cortisol concentrations different between embryos from cortisol-treated and control females. However, maternal cortisol treatment had significant effects on offspring stress responsiveness, mass, and behavior. Compared to offspring of control females, offspring of cortisol-treated females exhibited larger mass right after hatch, and young-of-the-year mounted an attenuated cortisol response to an acute stressor, and exhibited less thigmotaxic anxiety, exploratory behavior, boldness and aggression. Thus, offspring phenotype was affected by elevated maternal cortisol levels despite the absence of a significant increase in embryo cortisol concentrations, suggesting that a mechanism other than the direct deposition of cortisol into eggs mediates effects on offspring. The results of the present raise questions about the mechanisms through which maternal stress influences offspring behavior and physiology, as well as the impacts of such phenotypic changes on offspring fitness.

1. Introduction

Fish may be exposed to a gauntlet of natural and anthropogenic stressors, including but not limited to fisheries interactions, physical habitat loss, exposure to chemical and noise pollution and climate change, many of which are increasing in frequency and intensity (reviewed by [1]). These stressors have been linked to population declines in fish [2], and contribute to the current high rate of biodiversity loss and extinction in freshwater fish [3]. Although a large body of literature explores the effects of exposure to such stressors on adult and even larval fitness-related traits (reviewed in [2]), less effort has been invested in studying intergenerational effects of stressor exposure. Knowledge of intergenerational effects is needed because the propagation of a population relies on the survival and successful reproduction of offspring.

Intergenerational effects of stress prior to spawn largely have been investigated with respect to the female parent because she provisions the developing embryo with an egg yolk into which maternal mRNA, proteins, lipids, and hormones are deposited during vitellogenesis [4]. Pre-spawn maternal stress has been reported to affect offspring survival, morphology, physiology, and behavior. Broadly, the reported effects of maternal stress in teleosts include smaller embryos and larval length, reduced responsiveness to stress, and lower boldness (e.g. [5–7]). Effects on other variables such as survival ([8]; but see [9]), clutch size ([10]; but see [11]), embryo cortisol levels ([12]; but see [9]), and larval anti-predator behavior ([13]; but see [14]) were variable in occurrence. Therefore, effects of maternal stress appear to depend on the type and intensity of the stressor, the duration of exposure, the phase of oogenesis during which the female was exposed to the stressor, and the offspring life-stage assessed. Despite these sources of variation, the literature reports many similar effects on offspring of maternal stress prior to fertilization, independent of the type of stressor. For example, rainbow trout (*Oncorhynchus mykiss*) females subjected to repeated air exposure [15], confinement [16], or chasing, crowding,

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and noise [17] all reared significantly smaller embryos. These observations raise questions about what aspect of the maternal stress response mediates such intergenerational effects.

When a fish is exposed to a stressor, the hypothalamic-pituitary-interrenal (HPI) axis is activated, culminating in the increased production of cortisol [18]. This glucocorticoid (GC) stress hormone is of interest as a mediator of maternal stress effects on offspring because cortisol is among the maternal provisions deposited into developing eggs [19], and it plays an organizational role during early development in teleost fish, affecting embryonic development of the eye and HPI axis, the formation of skeletal and cardiac muscle, and neurogenesis [20,21]. Further, in at least some fish species, egg cortisol concentrations are positively correlated with circulating maternal plasma cortisol concentrations ([12,22]; but see [23]).

To investigate the role of cortisol in mediating effects of maternal stress, previous studies in teleosts have treated fertilized eggs with exogenous cortisol and have observed effects on offspring, including malformations, a reduced or absent cortisol response to an acute stressor, and less aggression and boldness [21,24–26]. Although treating eggs with exogenous GCs is a logistically simple approach, it does not take into account other possible maternal effects following stressor exposure, such as ovarian follicle buffering against egg hypercortisolism [23,27] or epigenetic effects [28]. As such, experimental elevation of maternal cortisol may be a more appropriate proxy for maternal stress. Few studies have adopted this approach, let alone in a field setting [6,22,29–31].

Wild, adult female largemouth bass exposed to exogenous cortisol via a cortisol-infused cocoa butter implant prior to spawn exhibited reduced energetic stores and elevated circulating plasma cortisol levels relative to untreated control females [32]. Importantly, O'Connor et al. [32] also observed ovarian cortisol concentrations in treated females that were higher than those in untreated control fish even 9–13 days post-injection (DPI). Thus, the purpose of the present study was to test the hypothesis that experimentally-elevated maternal cortisol affects offspring phenotype in wild largemouth bass. Male bass create a nest and then court females, with each female selecting which male(s) will receive her eggs. After fertilization, the female departs and the male provides extended parental care (up to ~ 5 weeks) until the developing offspring acquire antipredator tactics. Male abandonment of the nest during this period results in loss of the brood, often to predation. The effects of maternal cortisol treatment were assessed in the present study by measuring offspring growth, the cortisol response of offspring to an acute stressor, and behavior at different developmental stages. The behaviors examined included aggression, fear and boldness in offspring experiencing visual exposure to a predator, and anxiety and exploratory behavior in novel environments. Based on previous research, the offspring of cortisol-treated bass would be predicted to be smaller than those of control females, with an attenuated cortisol response to an acute air stressor, greater anxiety, and reduced boldness, fear, aggression and exploratory behavior.

2. Materials and methods

2.1. Experimental design

To test the hypothesis that experimentally-elevated maternal cortisol affects offspring phenotype in wild largemouth bass, two treatment groups were generated; adult female bass that were treated with cortisol, and control animals. Control and cortisol-treated female bass were then allocated to (separate) ponds populated with (untreated) adult male bass, and allowed to reproduce. Ponds were monitored for the appearance of nests and development of broods, allowing offspring to be collected at the embryo, egg-sac fry (ESF), free-swimming fry (FSF), and young-of-the-year (YOY) stages (Table 1). Offspring characteristics that were assessed included length and mass, baseline whole-body cortisol concentrations and cortisol concentrations following exposure to a stressor, and behaviors indicative of anxiety, exploration, boldness and aggression.

Table 1
Stages at which offspring were assessed, together with sample size and age at which offspring were collected or tested.

	Embryo	Egg-sac fry (ESF)	Free-swimming fry (FSF)	Young-of-the-year (YOY)
Description	0–2 DPF spawn to hatch	2–4 DPF hatch to swim-up	5 – 30 DPF swim-up to paternal abandonment/ brood dispersal	> 30 DPF paternal abandonment/ brood dispersal to 1 year
Mass and length	10 per nest	10 per nest	10 per nest ≤ 10 DPF	10 per pond ~ 45 DPF
Whole-body [cortisol]	> 24 per nest	> 24 per nest	> 24 per nest ≤ 8 DPF	20 per pond 35–50 DPF
Behavior			> 15 per nest 8–12 DPF	> 60 per pond 35–50 DPF

DPF, days-post-fertilization.

2.2. Study site and animals

The study was conducted at Sam Parr Biological Station (38°43′N, 88°45′W; Illinois Natural History Survey, Kinmundy, Illinois, USA) using wild adult largemouth bass collected from nearby lakes, as in O'Connor et al. [32]. Experimental protocols complied with the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching, and were approved by institutional animal care committees at the University of Ottawa (protocol BL-2118) and Carleton University (protocol B10-09). Adult largemouth bass (> 30 cm in length; n = 34 males and 25 females; mean mass ± standard error, SEM = 500 ± 43 g) were collected on April 1st and 2nd, 2014, from Forbes Lake (38°71'N, 88°75'W) and Lake Shelbyville (39°47'N, 88°71'W) by electrofishing, and transported in 500 L insulated tanks to the Sam Parr Biological Station, where they were held under natural photoperiod in 400 L tanks supplied with aerated fresh water from the adjacent Forbes Lake for 2–3 days to recover.

On April 4th, 2014, fish were individually netted from the holding tanks, weighed, and sexed as per Benz and Jacobs [33]. Male bass were distributed equally by number and weight into six experimental ponds (4–7 males per pond). Female bass were alternately assigned to either control or cortisol treatment groups (three ponds per maternal treatment group, 4–5 females per pond distributed equally by weight). The six 0.4 ha research ponds were man-made, contained natural earthen sediment, and were exposed to weather and predators, thus allowing the experiment to be conducted under semi-natural conditions.

Females assigned to the cortisol treatment group were placed in a water-filled trough with their ventral side exposed and given a 5 mL kg^{-1} intraperitoneal injection of 10 mg mL^{-1} hydrocortisone 21-hemisuccinate (H4881 Sigma-Aldrich, St. Louis, MO, USA) emulsified in melted cocoa butter (NOW Health Group Inc., Bloomingdale, IL, USA). O'Connor et al. [32] reported that circulating plasma cortisol concentrations following this treatment were comparable to endogenous levels following ecologically-relevant stressors such as catch-and-release angling, confinement, and exhaustive exercise, and that ovarian cortisol concentrations were elevated at 9–13 DPI. Female bass assigned to the control treatment were untreated. In previous studies, sham-treated bass (i.e. vehicle injection only) have exhibited circulating cortisol concentrations intermediate in value between those of control and cortisol-treated fish, probably owing to stress associated with handling and/or implant administration [34]. Because the treatment objective was to elevate cortisol levels, this endogenous stress response constrains the usefulness of sham-treated animals. For this reason, for consistency with O'Connor et al. [32], and to ensure

sufficient sample sizes, a sham-treated group was not included in the experimental design.

2.3. Sampling and fish husbandry

Nest locations were identified by observing patrolling and guarding behavior of male bass, and offspring were collected at four stages (Table 1); embryos, ESF, FSF and YOY. Embryos were collected using a turkey baster, ESF and FSF were collected using a fine hand-held dip net, and YOY were collected via seine net. Offspring were euthanized for measurement of mass and length, and whole-body cortisol concentrations (see Table 1 for sample sizes and age at collection). A third subset of FSF and YOY (see Table 1 for sample sizes and ages at test) was brought into the field station and held until fish were subjected to behavioral tests (one test per individual). The FSF collected for behavioral tests were held at ambient temperature for at least two days in mason jars (~ 20 fry per jar per nest) containing artificial pond water made by mixing tap water with a commercial product that removes iron from tap water and inhibits fungal and bacterial blooms using potassium permanganate (Jungle Labs "Pond Water Clear", Spectrum Brands Inc., Atlanta, GA, USA). Water was changed daily and FSF were fed by adding zooplankton-rich pond water daily. The YOY were held for approximately two weeks in 10 L tanks (10 fish per tank per pond, n > 60 per pond) containing aerated pond water changed every 4–5 days and were fed frozen bloodworms daily.

2.4. Assessment of offspring growth

Embryo diameter and fry length were quantified using ImageJ (https://imagej.nih.gov/ij/) from photographs taken under a dissecting microscope (10 ×) using a micrometer scale slide. The fork length of YOY was measured against a 30 cm ruler.

2.5. Assessment of offspring cortisol responses

Offspring collected from their resident pond were either immediately euthanized and flash frozen with liquid nitrogen (baseline cortisol levels), or were exposed to a standardized stressor (1 min air exposure) and euthanized after 5 min of recovery in a holding container of fresh pond water (stress-induced cortisol levels). Stress-induced cortisol concentrations were not measured for embryos because the species tested to date have not responded to external stressors prior to hatch (e.g. sea bass, *Dicentrarchus labrax*, [72]; zebrafish, *Danio rerio*, 2). Samples were stored at − 80 °C for later analysis of cortisol concentrations.

Cortisol was extracted from embryos (yolk included) and fry as per Jeffrey and Gilmour [5] and quantified by enzyme-linked immunosorbent assay (EIA). In short, samples thawed on ice were homogenized on ice in 200 μL of 5 × diluted extraction buffer from a commercial cortisol EIA kit (Cortisol EIA assay kit, #402710 Neogen, Lexington, KY, USA) using a battery-operated pestle grinder (Kimble Chase Kontes, Rockwood, TN, USA). Homogenates were extracted thrice with 1 mL ether (anhydrous diethyl ether, #AC615080010 Fisher Scientific, Ottawa, ON) each time. After each addition of ether, samples were vortexed, incubated for 15 min (30 min the first time), centrifuged for 5 min at 3000 *g* at 4 °C, and flash frozen at − 80 °C. The ether extraction supernatants were combined and dried in a fume hood under forced air at room temperature. The cortisol residue was reconstituted in 10 μL extraction buffer (Neogen) per embryo/fry in the original sample. To aid reconstitution, samples were heated for 5 min at 65 °C and vortexed at least twice or until cortisol was completed dissolved.

Cortisol was extracted from individual YOY using a protocol adapted for the larger mass of body tissue from the protocols of Sopinka et al. [9] and Jeffrey and Gilmour [5]. The YOY were powdered in liquid nitrogen with a mortar and pestle prior to homogenization with a handheld homogenizer (PowerGen 125, Fisher Scientific). Reagent volumes were scaled up from those used in the fry protocol based on the proportional increase in body tissue being processed. Extraction efficiencies determined by spiking homogenates with a known amount of tritiated cortisol (^{3}H-hydrocortisone, 250 μCi, #NET396250UC Perkin Elmer, Waltham, MA, USA) were 61.7% and 65.3% for embryos/fry and YOY, respectively. Extracts were stored at − 80 °C until quantification using a commercial EIA kit (Neogen). Samples were assayed in duplicate, and inter- and intra-assay coefficients of variation were 5.8% and 2.5%, respectively.

2.6. Assessment of offspring behavior

Individuals were subjected to a single behavior test to avoid habituation or stress associated with repeated handling. Between trials, the water in the test tank was changed. Fry were subjected to an open-field test or a novel tank diving test. These tests also were conducted on YOY, as well as an emergence test, a predator recovery test, or a mirror aggression test. For each test, the behavior of five fry from each nest (five individual trials) and ten YOY per pond was recorded (Vixia HF-R400 camcorder, 30 fps; Canon, Brampton, ON). All test chambers had opaque walls to minimize disturbance from movement outside the tank. Fish were allowed to acclimate for 2 min prior to recording in a 'starting chamber' (a piece of PVC pipe) for the open-field and mirror tests [35]; and for 5 min on one side of an opaque removable divider for the emergence and predator exposure tests [36]. The order in which individuals from each maternal treatment group (as well as from each nest or pond containing fry and YOY, respectively) were tested was randomized. Behavioral endpoints were extracted from videos of open-field test trials using automatic tracking (see below), and behavioral endpoints were extracted from all other test recordings by individuals who were blind to the treatment group to which the offspring belonged.

2.6.1. Open-field test (both FSF and YOY)

This commonly-used behavior test assessed exploration and anxiety [35,36]. In the present study, the open-field test was conducted using an all-white behavioral arena (10 cm diameter × 3 cm high for fry and 14 cm diameter × 5 cm high for YOY), which maximized visual contrast, allowing automated tracking of video recordings using the tracking program id Tracker [37]. Behavioral endpoints were then extracted from the coordinate data using Microsoft Excel and MATLAB scripts. After a 2 min acclimation period, the starting chamber was removed and the test subject was recorded for 5 min. Behavioral endpoints included the total distance traveled and percentage of time spent in the outer third of the test arena, which is indicative of an anxiety-related behavior termed thigmotaxis, the tendency of an anxious animal to avoid open areas, preferentially situating itself in corners or along a wall [38,39].

2.6.2. Novel tank diving test (both FSF and YOY)

This behavior test, first conducted by Levin et al. [40], assessed an anxiety-related behavior called geotaxis, the tendency of a fish to seek protection in a novel environment by diving down until it overcomes its geotaxic anxiety and explores the top of the exposed water column [40–42]. The novel tank diving test is conceptually similar to the open-field test but assesses vertical rather than horizontal anxiety-related and exploratory behavior. Offspring behavior was recorded for 5 min upon introduction of the individual to the chamber (18 × 10 cm, 10 cm deep), with the lack of acclimation period ensuring that behavior was assessed in a novel environment. The percentage of time spent in the bottom 'safe' area was determined together with the latency to enter the upper water column, a novel area. The latter provided a measure of exploratory behavior.

2.6.3. Emergence test (YOY)

Similar to the open-field test, horizontal exploratory behavior was assessed during an emergence test by quantifying the time taken by an

individual fish to emerge through an opening and enter a novel environment as per Burns [36] and Wilson et al. [43,44]. A single YOY was placed in a refuge separated from the main portion of the experimental chamber (a 250 L glass aquarium) by a white, opaque, removable divider, and allowed to acclimate for 5 min. The divider was then lifted 5 cm. The latency of a subject to emerge from the refuge was recorded to a maximum of 15 min, after which time the fish was recorded as having failed to emerge.

2.6.4. Predator recovery test (YOY)

In a test chamber similar to that used for the emergence test, the subject was again acclimated in the refuge for 5 min. The opaque removable divider was then lifted to completely reveal the other side of the tank, which contained a live predator separated from the test subject by a static glass divider. The predator was an adult largemouth bass (a common nest predator; 54, [71]) angled from Forbes Lake, Illinois. Adult bass were housed in a 60 L tank filled with aerated pond water, and were not fed on the day of recording to maximize aggression towards the YOY on the other side of the glass divider. All YOY froze in response to the threat of a predator. The act of freezing represented fear-related behavior because the threat (i.e. predator) was imminent, as opposed to the open-field test, which assessed anxiety-related behavior and exploration because of the absence of any imminent threat [42]. After 2 min of visual predator exposure, the opaque divider was lowered and the latency of the YOY to behaviorally recover from the acute stressor and begin moving was measured to a maximum of 10 min. Longer latency to move after freezing was indicative of greater fear, and a failure to begin moving at all within 10 min was defined as a failure to recover (or reduced boldness).

2.6.5. Mirror test of aggression (YOY)

Using a 4.5 L tank with a mirror (8 cm × 12 cm) attached to the inner side of one wall, the test subject was acclimated to the experimental chamber for 2 min with an opaque removable cover in front of the mirror. The cover was then removed, and behavior was recorded for 25 min. Fish respond to their own reflection as if it were a conspecific, often exhibiting aggression. Aggressive behavior was quantified as latency to approach the mirror, percentage of time an individual spent in tail whipping behavior, and percentage of individuals that initiated tail whipping during the 25 min trial. The protocol was adapted from studies on other teleost species (e.g. daffodil cichlid, *Neolamprologus pulcher*, 4, [70]); mozambique tilapia, *Oreochromis mossambicus*, [45]).

2.7. Statistical analyses

Offspring traits were compared between control and cortisol-treated females. All data were expressed as mean values ± 1 SEM. All data analysis in the present study was conducted using R (version R 3.1.1; R Core Team, 2013). The assumptions of normally distributed residuals and homoscedasticity were first tested for each measured trait in R using the Shapiro-Wilk test and Bartlett's test, respectively. Where significant deviation from normal distribution of residuals was detected, the data were transformed using a Box-Cox power transformation (MASS R package), with the value of lambda (λ) indicating the appropriate power, e.g. $\lambda = 0.5$ indicates a square root transformation. The fixed effect of maternal cortisol treatment was assessed using one-way analysis of variance (ANOVA; aov R package) except for cortisol concentrations, where the effects of both maternal treatment and air stressor exposure were evaluated in a two-way ANOVA. To take into account additional variables that could influence a particular dependent variable (e.g. nest and/or pond source, maternal ID), the Akaike information criteria (AIC) of at least two models were compared, a general linear model testing only the fixed effect of maternal treatment on the response variable and at least one linear mixed effects model (nlme R package) that incorporated any possible categorical variable (such as nest/maternal ID or pond) as random effect(s). For more than one random effect, multiple mixed effects models were created in stepwise order. That is, variations of the base model plus each individual random variable separately were created, and additional models combining the random effects were also included in the AIC model comparison. Candidate models were compared using the Restricted Maximum Likelihood estimation to find the optimal random structure. The model with the lowest AIC value was deemed the best fit and was used to conduct the ANOVA. For response variables with binomial distribution (e.g. success versus failure to emerge into a novel environment), a chi-square test of independence was conducted. The size of the effect of maternal cortisol treatment on each offspring trait was estimated using either the eta-squared (η^2; etaSqured in the lsr R package; https://cran.r-project.org/web/packages/lsr/lsr.pdf) or the phi coefficient (ϕ; assocstats in the vcd R package; https://cran.r-project.org/web/packages/vcd/vcd.pdf) for ANOVA and chi-square tests, respectively. Table S1 summarizes all statistical tests performed. A fiducial limit of significance of 0.05 was used for all statistical tests.

3. Results

Ponds containing control (17.1 ± 1.2 DPI) and cortisol-treated (19.0 ± 1.4 DPI) females did not differ significantly in spawning date (one-tailed ANOVA, pond included as a random effect; $P = 0.158$), with all spawning events occurring between 13 and 28 days post-treatment. There was also no effect of female cortisol treatment on the spawning success within ponds (74.6 ± 13.0% and 79.4 ± 10.4% of males with eggs in their nests for the ponds containing control and cortisol-treated females, respectively; ANOVA; n = 3 ponds per maternal treatment group; $P = 0.789$).

Maternal cortisol treatment significantly influenced ESF mass right after hatch but was otherwise without effect on offspring length or mass (Fig. 1; ANOVA at each life-stage except YOY mass, where an ANOVA on log transformed data with pond included as a random effect was conducted; $P > 0.05$ at each life-stage except ESF-1, n = 4–10 nests at each life-stage except YOY where n = 30 individuals; see Table S1 for full statistical results at each life-stage). That is, ESF of cortisol-treated females had significantly higher mass right after hatch compared to those of control females (Fig. 1B, Student's *t*-test; $P = 0.016$).

Embryos of control and cortisol-treated females did not differ significantly in whole-body cortisol concentrations (Fig. 2A; one-tailed ANOVA on reciprocal transformed data; $P = 0.417$). No significant effect of maternal cortisol treatment (MT) or air exposure was detected on the whole-body cortisol concentrations of ESF (Fig. 2B; two-way ANOVA; $P = 0.836$ for MT, $P = 0.419$ for air, $P = 0.497$ for MT × air) or FSF (Fig. 2C; two-way ANOVA on $\lambda = 0.250$ transformed data; $P = 0.588$ for MT, $P = 0.122$ for air, $P = 0.934$ for MT × air). However, YOY of control females exhibited significantly higher cortisol concentrations when exposed to the stressor, whereas YOY of cortisol-treated females failed to respond significantly to the air stressor; YOY of control and cortisol-treated females did not differ in baseline cortisol levels (Fig. 2D; two-way ANOVA on $\lambda = 0.500$ transformed data; $P = 0.037$ for MT × air).

The behavioral traits of thigmotaxic anxiety (i.e. tendency to hug the wall), exploration, fear, boldness, and aggression in YOY were significantly affected by maternal treatment. During the open-field test, YOY of cortisol-treated females spent significantly less time in the outer ring of the arena compared to YOY of control females, and thus exhibited significantly less thigmotaxic anxiety (Fig. 3B; ANOVA on $\lambda = -0.17$ transformed data including pond as a random effect; $P = 0.031$); for FSF, the difference was not significant (Fig. 3A; ANOVA; $P = 0.157$). Exploratory behavior in the open-field test did not otherwise differ significantly between offspring of cortisol-treated and control females (Tables 2 and 3). The novel tank diving test did not reveal effects of maternal cortisol treatment on offspring geotaxis (indicative of anxiety) or exploratory behavior (Tables 2 and 3).

Additional assessments of behavior were carried out only in YOY.

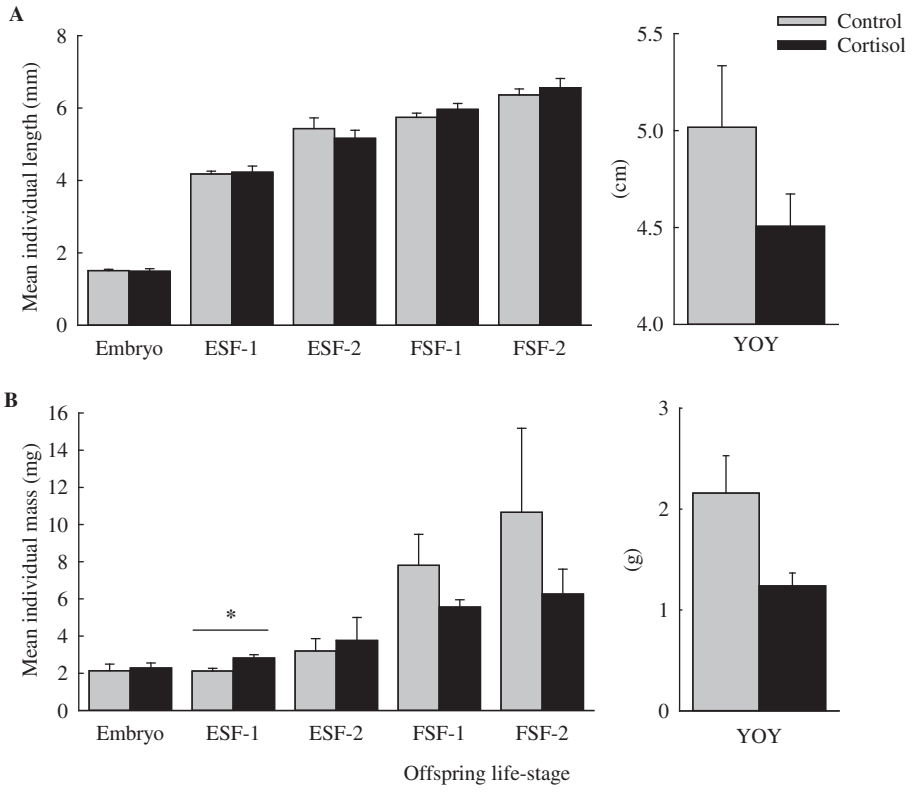

Fig. 1. Length (A) and mass (B) of offspring of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) at early life stages, including embryos (0 DPF; n = 6 and 7–8 nests for offspring of control and cortisol-treated females, respectively), early egg-sac fry (ESF-1; 2 DPF; n = 4–5 and 7–9 nests), late egg-sac fry (ESF-2; 4 DPF; n = 4 and 7 nests), early free-swimming fry (FSF-1; 6 DPF; n = 9 and 9–10 nests), late free-swimming fry (FSF-2; 8–9 DPF; n = 5–7 and 9 nests), and YOY (35–50 DPF; n = 30 and 30 individual YOY). Values are means + SEM. An asterisk indicates a significant difference between maternal treatments within a life-stage (Student's *t*-test; see text for details).

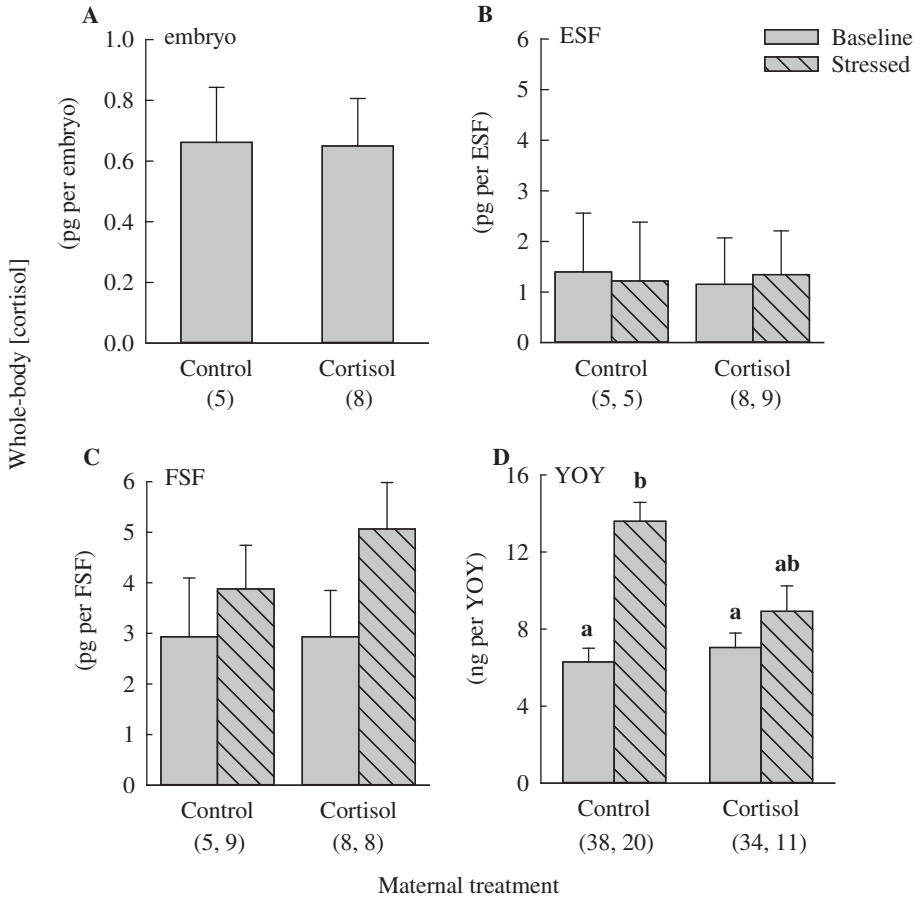

Fig. 2. Whole-body cortisol levels of embryos (A), egg-sac fry (ESF; B), free-swimming fry (FSF; C), and young-of-the-year (YOY; C) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*). Cortisol levels were measured before (baseline) or after (stressed) subjecting the offspring to 1 min of air exposure followed by a 5 min recovery period; only baseline measurements were collected for embryos. From each nest, cortisol content was quantified from groups of 12–30 embryos, ESF and FSF, while YOY from each pond were processed individually. Values are means + SEM, with n values for nests (embryos, ESF, FSF) or individuals (YOY) indicated on the figure in parentheses (baseline, stressed). Treatments that do not share a letter are significantly different from one another (two-way ANOVA; see text for details); where no letters are present, no statistically significant differences were detected.

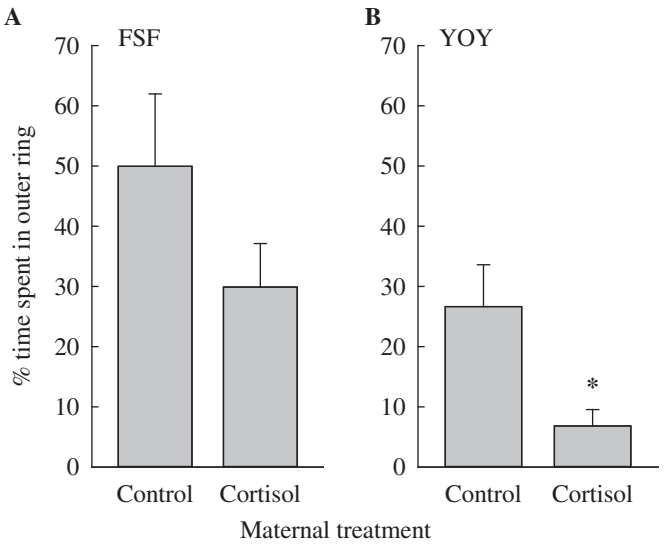

Fig. 3. The percentage of time free-swimming fry (FSF; A) and young-of-the-year (YOY; B) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) spent in the outer ring of the testing arena, thus exhibiting thigmotaxic anxiety, during a 5 min open-field test. Values are means + SEM, n = 26 and 35 FSF, 29 and 24 YOY for control and cortisol-treated females, respectively. An asterisk indicates a significant difference between maternal treatments (ANOVA; see text for details).

The emergence test revealed significant effects of maternal cortisol treatment on YOY exploratory behavior. During this test, YOY of cortisol-treated mothers took significantly longer to emerge from the refuge into the novel environment (Fig. 4A; ANOVA on $\lambda = 0.340$ transformed data; $P = 0.012$) and also failed to emerge during the 15 min trial significantly more often than YOY of control females (6.9 ± 4.8% and 27.6 ± 8.4% emergence failure for YOY of control and cortisol-treated females, respectively; chi-square test of independence; n = 29 for each maternal treatment; $P = 0.037$). Similarly, YOY of cortisol-treated females tended to exhibit a longer latency to first move following exposure to a predator (Fig. 4B; ANOVA on $\lambda = 0.160$ transformed data, pond included as a random effect; $P = 0.067$), and were also significantly more likely to remain immobile for the entire 10 min trial than offspring of control females (chi-square test of independence; n = 29 and 28 YOY of control and cortisol-treated females, respectively; $P = 0.008$), indicative of a stronger fear response. That is, 21.4 ± 7.9% of the YOY of cortisol-treated females maintained immobility for the entire observation period whereas all offspring of control females recovered behaviorally from the acute stress of predator exposure. The mirror test of aggressive behavior revealed that YOY of control and cortisol-treated females did not differ in their latency to approach the mirror (Table 3). However, a significantly smaller proportion of YOY of cortisol-treated females initiated aggression during the 25 min trial (Fig. 5A; chi-square test of independence; $P = 0.010$), and those that did spent a smaller percentage of time engaged in aggressive tail whipping behavior compared to YOY of control females (Fig. 5B; ANOVA on $\lambda = 0.005$ transformed data, pond included as a random effect; $P = 0.009$), indicative of lower aggression in the offspring of cortisol-treated females.

4. Discussion

The findings of the present study support the hypothesis that cortisol treatment of wild, female largemouth bass prior to spawn (as a proxy for maternal stress) affects offspring physiology and behavior. Specifically, offspring of cortisol-treated females exhibited an attenuated cortisol response to an acute stressor together with behavior suggestive of less anxiety, as well as less exploratory behavior, boldness and aggression. The offspring effects were detected in the absence of a significant difference in embryo cortisol concentrations, suggesting a role for mechanisms other than increased deposition of maternal cortisol into the eggs. These findings raise questions about the mechanisms through which maternal stress influences offspring behavior and physiology, as well as the impacts of such phenotypic changes on fitness [46].

4.1. Effects of exogenous cortisol administration on female spawning and embryo cortisol concentrations

In some previous studies, stressor exposure in female teleost fish prior to spawn was reported to delay ovulation and spawning ([10,15]; reviewed by [47]) and to reduce spawning success [48]. However, based on counts of male-guarded nests with eggs, cortisol-treated female bass in the present study did not exhibit delayed spawning or lower spawning success. The spawning period was unusually late and short in 2014, following a severe winter and April precipitation that was twice the historical average. The resultant delay between cortisol administration and spawning may have minimized the impact of cortisol treatment on spawning date or breeding success.

Spawning is the culmination of gonadal recrudescence, the timing of which is linked to water temperature and photoperiod cues [49]. Developing oocytes enter the vitellogenic phase of yolk accumulation during the late winter and/or early spring, when plasma vitellogenin concentrations are elevated and gonadosomatic index (GSI) is increasing [49,50]. Plasma concentrations of the sex steroid estradiol increase in female largemouth bass in the lead up to spawning, as oocytes enter the final stage of development and GSI peaks [49,50]. In the research ponds used in the present study, spawning typically takes place mid-April [51] and oocytes are in the vitellogenic phase for approximately a month preceding the onset of spawning [52]. Maternal cortisol deposition into the developing eggs occurs during vitellogenesis [53]. Using the cortisol treatment that was adopted in the present study, O'Connor et al. [32] detected elevated ovarian cortisol concentrations in female bass at 9–13 DPI. A comparable response to

Table 2
Non-significant statistical results for analysis of the behavioral endpoints quantified during the open-field and novel tank diving tests conducted on free-swimming fry (FSF) from control versus cortisol-treated female largemouth bass (*Micropterus salmoides*).

Behavior measured	Control females	Cortisol-treated females	Transformation (λ)	*P*-value
Open-field test	n = 24	n = 33		
Distance traveled (m)	1.46 ± 0.40	1.78 ± 0.37	0.214	0.337
Novel tank diving test	n = 12–23	n = 16–33		
Latency to explore novel upper area (s)	90.8 ± 29.3	134 ± 19.0	0.920	0.321
% time spent in bottom area (i.e. geotaxic anxiety)	93.8 ± 2.3	94.5 ± 1.8	11.55	0.703
% failure to explore	47.8 ± 10.5	51.5 ± 8.8	–	0.786

Values are means ± SEM, for n (indicated in the table) FSF. For the open-field test, data were analyzed by ANOVA with nest included as a random effect in a linear mixed effects model. Where data were transformed to meet the underlying assumptions of parametric tests, the value of lambda (λ) indicates the power to which data were raised. For the novel tank diving test, latency to explore and geotaxic anxiety were analyzed by ANOVA with nest and pond, respectively, included as random effects in a linear mixed effects model, while % failure to explore was evaluated using a chi-square test of independence.

Table 3
Non-significant statistical results for analysis of behavioral endpoints quantified during the open-field, novel tank diving, and mirror aggression tests conducted on young-of-the-year (YOY) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*).

Behavior measured	Control females	Cortisol-treated females	Transformation (ʎ)	*P*-value
Open-field test	n = 28	n = 29		
Distance traveled (m)	1.59 ± 0.28	1.13 ± 0.25	0.250	0.241*
Novel tank diving test	n = 19–28	n = 18–28		
Latency to explore novel upper area (s)	35.5 ± 13.2	92.9 ± 25.3	0.18	0.260*
% time spent in bottom area (i.e. geotaxic anxiety)	98.0 ± 0.6	97.1 ± 0.8	18.33	0.494†
% failure to explore	35.7 ± 9.2	39.3 ± 9.4	–	0.783Δ
Mirror test of aggression	n = 30	n = 30		
Latency to approach mirror (min)	6.93 ± 0.79	8.20 ± 1.12	− 0.800	0.596*

Values are means ± SEM, for n (indicated in the table) YOY. The statistical significance of differences between YOY of control and cortisol-treated females was assessed by ANOVA (*), ANOVA with pond included as a random effect in a linear mixed effects model (†), or chi-square test of independence (Δ). Where data were transformed to meet the underlying assumptions of parametric tests, the value of lambda (ʎ) indicates the power to which data were raised.

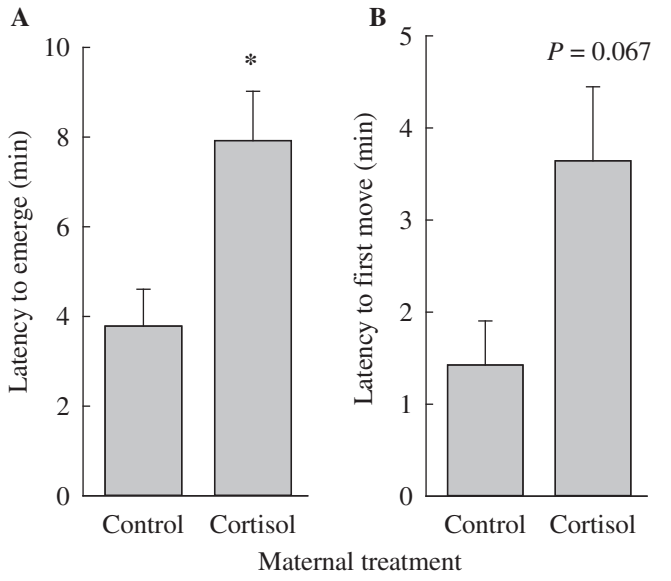

Fig. 4. Latency for young-of-the-year (YOY) of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) to emerge from a refuge into a novel environment (A), and to behaviorally recover and begin moving following visual exposure to a potential predator (B). Values are means + SEM, n = 27–29 individual YOY. An asterisk indicates a significant difference between maternal treatments (ANOVA; see text for details).

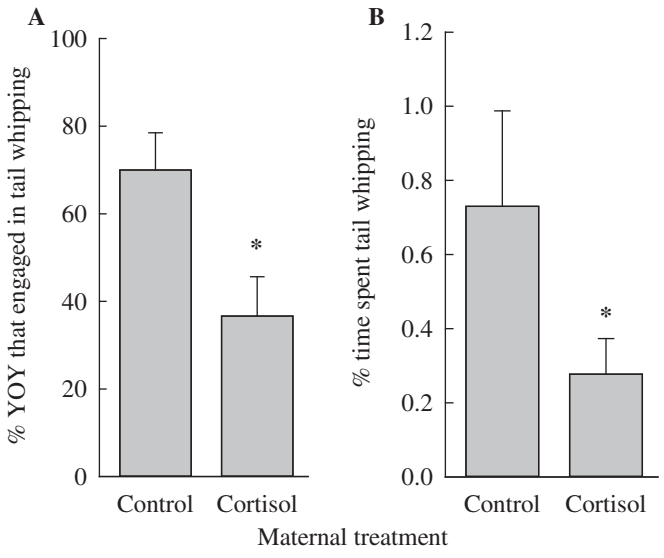

Fig. 5. Percentage of individual young-of-the-year (YOY) offspring of control versus cortisol-treated female largemouth bass (*Micropterus salmoides*) that initiated aggressive tail whipping behavior in response to their reflection in a mirror (A), and the percentage of time that YOY spent engaging in tail whipping behavior during the 25 min mirror trial (B). Values are means + SEM, n = 28–30 individual YOY. An asterisk indicates a significant difference between maternal treatments (chi-square test of independence for % of individuals that initiated aggression and ANOVA for percentage of time spent tail whipping; see text for details).

cortisol treatment would be expected for the female bass of the present study, given that O'Connor et al. [32] studied bass from the same populations as the present study, held in the same research ponds. Spawning in the present study occurred between 13 and 28 DPI, suggesting that maternal ovarian cortisol concentrations would have been elevated only for the latter part of vitellogenesis and the final stage of oocyte maturation. This factor may have contributed to the absence of differences in cortisol content between embryos of control and cortisol-treated females. Alternatively, maternal or embryonic buffering against maternal deposition of cortisol may have occurred [46]. Females may buffer eggs from hypercortisolism via the action of ovarian 11β-hydroxysteroid dehydrogenase type 2 (11βHSD-2), the enzyme that catalyzes the conversion of cortisol to its inactive form cortisone [53]. Zebrafish ovarian tissue treated with cortisol in vitro elevated ovarian follicle 11βHSD-2 transcript abundance seven-fold, potentially accounting for the general lack of effect of maternal cortisol treatment on embryo cortisol content in this species, despite elevated maternal ovarian cortisol levels [23]. Increased efflux of cortisol using ATP-binding cassette (ABC) transporters, as proposed for three-spined stickleback embryos [54], also could have protected bass embryos from effects of maternal cortisol treatment. Clearly, these potential mechanisms require further investigation in largemouth bass. Although studies of embryos reared from cortisol-treated mothers or cortisol-treated eggs typically have reported elevated embryo cortisol levels ([22,25]; but see [23]), the effects of maternal stress on embryo cortisol content have been more variable [5,6,9,11–13]. Importantly, effects of maternal stress on offspring phenotype have been reported in teleosts in the absence of differences in embryo cortisol content [5,9,55], and the results of the present study support these findings. That is, despite the absence of elevated cortisol concentrations in embryos of cortisol-treated females, maternal cortisol treatment significantly affected offspring phenotype, suggesting that mechanisms other than embryo cortisol levels per se may mediate the effects of maternal stress on offspring. The possibility of epigenetic effects is of obvious interest in this regard [28].

4.2. Effects of maternal cortisol treatment on offspring growth

Effects of maternal cortisol treatment were detected only on

offspring mass right after hatch, when early ESF of cortisol-treated female bass were greater in mass than offspring of control females. It is difficult to place this finding in the context of the existing literature for teleosts because previous studies of the effects of maternal stress or cortisol exposure on offspring length and/or mass have yielded variable results. Although several studies reported reduced offspring length and mass [6,10,11,17,22,25,56], others reported increases [13,21,28,57] or no effect [9,12,15,16]. Ostrand et al. [7] reported that pre-spawn exposure of adult largemouth bass (male and female) to multiple, repeated acute stressors resulted in offspring with reduced growth. Species differences and/or differences in the type of maternal stressor or cortisol administration method may account for at least some of this variability but patterns have not emerged to date (see also Ref. [59]).

4.3. Effects of maternal cortisol treatment on offspring stress-induced cortisol levels

The present study is, to our knowledge, the first to report baseline and stress-induced cortisol concentrations for largemouth bass fry. Maternal cortisol treatment did not affect baseline cortisol concentrations of bass offspring at any of the three developmental stages assessed, in agreement with the majority of previous studies in free-swimming fish fry [5,55,58]. Regardless of maternal treatment group, neither ESF nor FSF mounted a significant cortisol response to acute air exposure, indicating that the capacity of developing bass to perceive external stressors and activate the HPI axis to generate a cortisol response appears during the transition from yolk reabsorption to exogenous feeding, timing that is consistent with that in other teleost species (rainbow trout, *Oncorhynchus mykiss*, [24]; zebrafish, 1,2 [62,69]). By the YOY life-stage, a robust cortisol response was present in offspring of control females. However, the cortisol response to air exposure was attenuated in YOY of cortisol-treated female bass, in agreement with reports of diminished cortisol responses to acute stress in the offspring of stressed or cortisol-treated female salmonids [24,55] and zebrafish [5]. Similarly, cortisol-injected zebrafish eggs yielded offspring with a reduced cortisol response to an acute stressor [21]. Whether an attenuated cortisol response is adaptive, and under what conditions it might be adaptive, remain to be determined. It is conceivable that the physiological costs associated with constant or repeated activation of the HPI axis could be reduced by attenuating the cortisol response.

4.4. Effects of maternal cortisol treatment on offspring behavior

In addition to the effect of maternal cortisol treatment in dampening the physiological (cortisol) response to an acute stressor, YOY behavior was altered by maternal cortisol treatment. Thigmotaxis in the open-field test was reduced in YOY of cortisol-treated females, suggesting reduced anxiety [39]. The open-field test also is used to assess exploratory behavior [59], with the lower thigmotaxis observed in YOY of cortisol-treated females consistent with an increased tendency to explore. However, YOY of cortisol-treated females exhibited a longer latency to leave the refuge and explore the novel environment during the emergence test, and a greater proportion of these offspring failed to emerge during the trial, observations that suggest reduced exploratory behavior [59]. These contrasting results, together with the lack of significant difference in exploratory behavior between treatment groups in the novel tank diving test, suggest that effects of maternal cortisol treatment on YOY behavior are context-specific. In the predator recovery test, YOY of cortisol-treated female bass tended to take longer to begin moving after freezing in response to viewing a predator, and were significantly more likely to fail to recover than YOY of control females. That is, maternal cortisol treatment resulted in offspring with a more persistent fear response. Such latency to resume movement also could be considered a measure of boldness [35,59,60], suggesting that offspring of cortisol-treated female bass exhibited reduced boldness.

The mechanisms through which cortisol treatment of female bass results in offspring that is less anxious, potentially less exploratory, less bold, and with an attenuated cortisol response to a stressor, remain to be determined. In zebrafish larvae that were injected as embryos with cortisol to mimic cortisol transfer by stressed females, the cortisol response to an acute physical stressor was eliminated [21] and thigmotaxis was reduced [61], findings similar to those of the present study. These zebrafish larvae also exhibited increased neurogenesis in the pallium and the preoptic area of the brain, regions that are involved in the control of behavior and control of the HPI axis, respectively [61]. In addition, the mRNA abundances of corticotropin releasing factor (CRF) and proopiomelanocortin (POMC), key players in activation of the HPI axis in the preoptic area and pituitary, respectively, were significantly reduced in cortisol-treated relative to control animals, in accordance with the attenuated cortisol response of these larvae [21]. Collectively, these data suggest that programming of offspring behavior and stress responsiveness by maternal stress may be mediated by actions of cortisol on neurogenesis in specific regions of the developing brain [61]. Even in the absence of differences in embryo cortisol content, as in the present study, changes in factors such as glucocorticoid receptor (GR) abundance could alter cortisol signalling, generating effects in offspring; GRs are maternally deposited into the oocyte together with cortisol [62,63]. Indeed, GR knockdown in zebrafish not only altered development [64,65], but also resulted in changes in the behavioral phenotype of adult zebrafish [66]. Extending these types of mechanistic studies to species such as bass is needed to explore the underlying relationships among maternal stress, cortisol, behavior and HPI axis function.

Maternal cortisol treatment also yielded YOY that were less likely to engage in aggressive behavior, because a significantly smaller proportion of YOY of cortisol-treated female bass initiated tail whipping behavior during the mirror test trial, and those that exhibited this behavior spent less time tail whipping than did YOY of control females. Interestingly, essentially the opposite result was obtained for the offspring of female guppies (*Poecilia reticulata*) exposed to mild stress, where an increased rate of interaction with a mirror image was detected [67]. Opposing effects on aggression were also reported for treatment of brown trout (*Salmo trutta*) eggs with cortisol, with exposure of ova pre-fertilization causing elevated aggression [57], whereas exposure of embryos immediately post-fertilization lowered aggression [25]. Thus, the timing of exposure to cortisol appears to be important in determining effects on aggressive behavior. Whether changes in aggressive behavior are adaptive may depend on the environment experienced by the offspring. For example, salmonid fry compete for feeding territories and the outcome of this competition affects subsequent growth [68], so increased aggression, if it translates into competitive success, could confer greater fitness.

4.5. Conclusions

In summary, the treatment of wild, female largemouth bass with cortisol several weeks prior to spawn had long-lasting effects on offspring phenotype, including increased fear responses, as well as reduced anxiety, boldness, aggression, and potentially exploratory behavior in YOY. Perhaps most striking, however, was the attenuation of the cortisol response to an acute stressor in YOY of cortisol-treated female bass. Further studies are needed to assess the impact of these phenotypic changes on lifetime fitness in largemouth bass, and effects on lifetime fitness likely will be dependent on the type and frequency of stressors present in the offspring environment [46]. It is noteworthy that most effects of maternal cortisol treatment did not manifest in offspring until later in development (up to 60 DPF), emphasizing the importance of intergenerational studies that extend beyond evaluation of embryos or larvae. Whether effects of elevated maternal cortisol on offspring traits would have differed if maternal cortisol manipulations had been conducted earlier or even closer to spawn requires further

investigation, as does the possibility of effects associated with the administration of cortisol but independent of the effects of elevating maternal cortisol per se. Lastly, effects of maternal cortisol treatment were observed in the present study even in the absence of differences in embryo cortisol concentrations, suggesting that maternal cortisol deposition into the egg is not the sole mediator of maternal stress effects on offspring phenotype.

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