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Regulation of hypothalamic–pituitary–interrenal axis function in male smallmouth bass (*Micropterus dolomieu*) during parental care

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ABSTRACT

Male smallmouth bass (*Micropterus dolomieu*) provide sole parental care until offspring reach independence, a period of several weeks. During the early parental care period when males are guarding fresh eggs (MG-FE), cortisol responsiveness is attenuated; the response is re-established when males reach the end of the parental care period and are guarding free-swimming fry (MG-FSF). It was hypothesized that attenuation of the cortisol response in male smallmouth bass during early parental care reflected modulation of hypothalamic–pituitary–interrenal (HPI) axis function. Male smallmouth bass were sampled at the beginning (MG-FE) and end of the parental care period (MG-FSF), before and/or 25 min after exposure to a standardized stressor consisting of 3 min of air exposure. Repeated sampling of stressed fish for analysis of plasma cortisol and adrenocorticotropic hormone (ACTH) levels was carried out. Males significantly elevated both plasma cortisol and ACTH levels when guarding free-swimming fry but not during early parental care. Control and stressed fish were terminally sampled for tissue mRNA abundance of preoptic area (POA) and hypothalamic corticotropin-releasing factor (CRF) as well as head kidney melanocortin 2 receptor (MC2R), steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage enzyme (P450scc). No significant differences in either hypothalamus CRF or head kidney P450scc mRNA abundance were found across parental care stages or in response to stress. However, POA CRF mRNA abundance and interrenal cell MC2R and StAR mRNA abundances failed to increase in response to stress in MG-FE. Thus, the attenuated cortisol response in males guarding fresh eggs may be explained by hypoactive HPI axis function in response to stress. The present is one of few studies, and the first teleost study, to address the mechanisms underlying resistance to stress during the reproductive/parental care period.

1. Introduction

All centrarchid fish provide sole male parental (i.e., paternal) care although the level of parental investment varies markedly among species (Warren, 2009). Male smallmouth bass (*Micropterus dolomieu*) invest extensively in parental care because males provide care to offspring over a period of 4–6 weeks in the late spring and early summer. During this period, males exert considerable energy providing nest defence, either directly by aerating the eggs and defending the nest from predators, or indirectly by limiting their foraging opportunities (Cooke et al., 2006, 2002; Ridgway, 1988; Ridgway et al., 1991). Indeed, although they rarely move more than 3 m away from the nest, telemetry revealed that bass swim the equivalent of over 40 km/day (Cooke et al., 2002). The energy exerted during this period can negatively impact adult growth as well as survival probability over the following winter (Ridgway et al., 1991). Owing to these energy limitations, a life-history trade-off exists between investing in defence of a current brood (current reproductive outcome) and investing in growth and survival (future reproductive outcome) (Williams, 1966).

Abbreviations: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; cAMP, 3′-5′-cyclic adenosine monophosphate; cDNA, complementary deoxyribonucleic acid; CRF, corticotropin-releasing factor; P450scc, cytochrome P450 side chain cleavage enzyme; DEPC, diethylpyrocarbonate; EDTA, ethylenediaminetetraacetic acid; HPA, hypothalamic–pituitary–adrenal; HPI, hypothalamic–pituitary–interrenal; MG-FE, males guarding fresh eggs; MG-FSF, males guarding free-swimming fry; MC2R, melanocortin 2 receptor; mRNA, messenger ribonucleic acid; POA, preoptic area; RM ANOVA, repeated measures ANOVA; RNA, ribonucleic acid; RT-PCR, reverse transcription-polymerase chain reaction; SEM, standard error of the mean; StAR, steroidogenic acute regulatory protein.

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Indeed, reproductive holidays (i.e., when fish skip reproduction in one or more years despite being sexually mature) are common for smallmouth bass, which is presumably a reflection of this trade-off (Barthel et al., 2008; Gravel et al., 2010).

A rise in glucocorticoid levels in response to a stressor initiates a sequence of physiological effects that are important to the survival of an organism (Wendelaar Bonga, 1997). Cortisol is the main glucocorticoid in teleost fish, and acts to mobilize energy resources to cope with increased energy demand during stress and to restore homeostasis (Barton, 2002; Mommsen et al., 1999; Wendelaar Bonga, 1997). Stress-induced levels of cortisol are adaptive, but when stressors are severe or prolonged, cortisol can negatively impact reproductive function (reviewed by Fuzzen et al., 2011; Schreck, 2010; Schreck et al., 2001). For example, nest abandonment in male smallmouth bass increased when cortisol levels were raised exogenously (Dey et al., 2010; O'Connor et al., 2009). Owing to the negative impacts of high cortisol levels on current reproductive outcome, 'resistance to stress' (reviewed by Wingfield and Sapolsky, 2003) may allow for successful reproduction in fish invested in defending a current brood. O'Connor et al. (2011) found that male smallmouth bass attenuated their cortisol response to a standardized stressor at the early stages of parental care, with the stress response being re-established toward the end of the parental care period. The aim of the present study was to investigate the mechanisms underlying attenuation of the cortisol response during early parental care in smallmouth bass.

Cortisol elevation reflects activation of the hypothalamic–pituitary–interrenal (HPI) axis in fish (HP-adrenal in other vertebrates). When the HPI axis is activated, corticotropin-releasing factor (CRF) is released from the preoptic area (POA) of the brain to the pituitary corticotropes (reviewed by Bernier et al., 2009; Flik et al., 2006; Lederis et al., 1994). Stimulation of corticotropes by CRF causes the release of adrenocorticotropic hormone (ACTH), the main secretagogue of cortisol (reviewed by Lederis et al., 1994; Wendelaar Bonga, 1997). Circulating ACTH binds to melanocortin 2 receptors (MC2R), G protein-coupled receptors on interrenal cells in the head kidney (Aluru and Vijayan, 2008), a structure homologous to the mammalian adrenal gland. Binding of ACTH to MC2R activates a cAMP-signaling cascade that facilitates the movement of cholesterol to the inner mitochondrial membrane, via steroidogenic acute regulatory protein (StAR), where it is cleaved to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc) in the first and rate-limiting step of cortisol synthesis (Aluru and Vijayan, 2008; Hagen et al., 2006; Mommsen et al., 1999). Because key mediators of cortisol synthesis are expected to increase in response to a stressor (e.g., Aluru and Vijayan, 2006, 2008; Jeffrey et al., 2014) it was hypothesized that attenuation of the cortisol response in male smallmouth bass during early parental care was a result of modulation of HPI axis activity.

To test the above hypothesis, male smallmouth bass guarding nests were sampled during early and late parental care, when the cortisol response is, respectively, attenuated and re-established (O'Connor et al., 2011). Circulating cortisol and ACTH levels were assessed together with mRNA abundance of POA and hypothalamus CRF and head kidney MC2R, StAR and P450scc in males pre- and post- exposure to a stressor.

2. Materials and methods

2.1. Experimental animals

In the spring of 2012, male smallmouth bass [$N = 27$, total length = 36.8 ± 0.9 cm (mean ± SEM); size range = 29.5–46.0 cm] guarding nests were identified by snorkelling on Charleston Lake in eastern Ontario. Males were sampled on two occasions representing the beginning (May 17, 2012; water temperature 16 °C) and end (May 29, 2012; water temperature 20 °C) of the parental care period when males were guarding fresh eggs (MG-FE; $N = 13$) and free-swimming fry (MG-FSF; $N = 14$), respectively. Only males with an egg or fry score of 3–4 (where the maximum score of 5 represents a nest of >4000 eggs/fry and the minimum score of 1 represents a nest of <500 egg/fry) were sampled (O'Connor et al., 2011).

Male smallmouth bass were either sampled as unstressed controls ($N = 14$) or were exposed to a standardized stressor ($N = 13$). Males were angled using a standard rod-and-reel with barbless hooks and a rubber mesh landing net and immediately were placed in a foam-lined trough filled with fresh lake water (O'Connor et al., 2011). Once a fish was hooked, it was landed and placed in the trough within 20 s and total length was measured. A baseline blood sample (1.5 ml) was collected immediately from all fish by caudal puncture using EDTA-coated 3 ml vacutainers (21G needle; BD Vacutainer). Subsequently, fish were either euthanized as controls or subjected to 3 min of air exposure in a damp, foam-lined plastic tub. Stressed fish were allowed to recover in individual tubs filled with fresh lake water until a second blood sample (O'Connor et al., 2011) was collected 25 min post-stress, and again until 2 h post-stress, when fish were euthanized. O'Connor et al. (2011) validated this approach and reported that the peak GC responsiveness occurred 25 min post-stressor. Fish were euthanized via cerebral percussion in accordance with approved standard operating procedures approved by institutional animal care committees (protocol # B10-09, Carleton University) and in accordance with the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching. Hypothalamus, POA and head kidney tissue were collected immediately after phlebotomy (after blood withdrawal for control fish and 2 h post-stressor for stressed fish). Blood samples were centrifuged at 10,000*g* for 5 min in the field. Plasma and tissue samples were flash frozen in liquid nitrogen and stored at −80 °C for later analysis of plasma cortisol and ACTH levels or mRNA abundance of HPI axis-related genes.

2.2. Hormone analysis

Circulating levels of cortisol and ACTH were determined by radioimmunoassay using commercially available kits (MP Biomedicals). All samples were analyzed together in a single assay where intra-assay variability (% CV) was 6.79% for ACTH. In our hands, the intra-assay variability for cortisol is typically 7.3% (Jeffrey et al., 2014). These kits have been validated for use on teleost samples (Doyon et al., 2006; Gamperl et al., 1994; Lim et al., 2013; O'Connor et al., 2011).

2.3. RNA and first strand cDNA synthesis

RNA extraction and cDNA synthesis were performed as in Jeffrey et al. (2012). Briefly, total RNA was extracted from tissues (10–100 mg) using TRIzol reagent (Invitrogen) according to the manufacturer's protocol, and quantified using a NanoDrop® ND-1000 UV–Vis Spectrophotometer. Tissues were homogenized by repeatedly passing the solution of TRIzol and tissue though syringes with 18G and 23G needles. Prior to cDNA synthesis, 1 μg of POA and hypothalamus, and 2 μg head kidney RNA were treated with deoxyribonuclease I (amplification grade, DNase; Invitrogen) according to the manufacturer's protocol. The cDNA was synthesized by reverse transcription using 100 U of RevertAid H Minus M-MuLV Reverse Transcriptase (Fermentas) and 0.2 μg of random hexamer primer (IDT ReadyMade Primer) according to the manufacturer's protocol.

2.4. Gene sequences

Partial nucleotide sequences for CRF, MC2R, StAR and P450scc were generated from cDNA synthesized from POA (for CRF) and head kidney (for MC2R, StAR and P450scc) using PCR with gene-specific primers (Table 1) that were designed using Primer3 (SimGene.com) based on conserved sequences for several teleost fish species. For StAR and P450scc, a second set of primers was used (forward and reverse 2) to extend the sequence, where forward 2 was a nested primer within the sequence produced from forward and reverse 1 primer sets. Primers for CRF were based on conserved regions in *Carassius auratus* (AF098629), *Cyprinus carpio* (AJ317955), *Danio rerio* (NM001007379), *Dicentrarchus labrax* (JF274994), *Gasterosteus aculeatus* (ENSGACT00000003899), *Haplochromis burtoni* (EF363131), *Oncorhynchus mykiss* (AF296672), *Oryzias latipes* (NM001128518), *Platichthys flesus* (AJ555623), *Salmo salar* (NM001141590), *Takifugu rubripes* (ENSTRUT00000022109), and *Tetraodon nigroviridis* (ENSTNIT00000000684). Primers for MC2R were based on conserved regions in *C. carpio* (AJ605725), *D. rerio* (AY161848), *G. aculeatus* (ENSGACT00000004920), *Gadus morhua* (ENSGMOT00000000108), *O. latipes* (ENSORLT00000005412), *O. mykiss* (NM001124680), *T. rubripes* (AY227793), and *T. nigroviridis* (AY332239). Steroidogenic acute regulatory protein primers were based on the *Micropterus salmoides* sequence for StAR (DQ166820). Primers for P450scc were based on conserved regions in *D. rerio* (BC154309), *G. aculeatus* (ENSGACT00000006237), *G. morhua* (ENSGMOT00000009165), *Ictalurus punctatus* (AF063836), *Odontesthes bonariensis* (GQ381266), *O. latipes* (ENSORLT00000009022), *O. mykiss* (S57305), *Tautogolabrus adspersus* (GU596480), *T. nigroviridis* (ENSTNIT00000011435), and *T. rubripes* (ENSTRUT00000035401).

In each case, 25 μl reaction compositions were as follows; 2 μl cDNA, 0.25 μl Choice Taq DNA polymerase (Denville), 0.2 mM dNTPs (Invitrogen), and 0.2 μM primer. All PCR reactions were performed using a Bio-Rad S1000 Thermal Cycler (Bio-Rad) with the following cycling conditions; annealing temperature 55 °C (30 s) and elongation temperature 72 °C (30 s) for 38 cycles. Amplicons were run on 1.5% agarose (Life Technologies) gels with ethidium bromide (Fisher Scientific), extracted using QIAquick gel extraction kit (QIAGEN), and cloned using QIAGEN PCR cloning kit and Subcloning Efficiency DH5α Competent Cells (Invitrogen) following the manufacturers' protocols. Plasmids were extracted using QIAprep Spin Miniprep Kit (QIAGEN) and were sequenced by GenScript USA Inc. Gene cloning resulted in partial sequences; 386 bp for CRF, 397 bp for MC2R, 403 bp for StAR, and 861 bp for P450scc. These sequences were sufficient to generate gene-specific primers for real-time RT-PCR.

Table 1
Oligonucleotide primer sets used for gene cloning.

Gene	Primers (5′–3′)
CRF	Forward – CGC TAT GAA TGT AGG GCT ATT G Reverse – TCT GTT GCT TTG CGC TTG CTG
MC2R	Forward – CAC TCG CCC ATG TAC TGC TT Reverse – CGT GCC AGC AGG AAC ATG TA
StAR	Forward 1 – TGG AGC AAA TGG GGG AGT GG Reverse 1 – GAT TGT CTT TGG GAT CCA GC Forward 2 – ATG TCC ACT CAG CAC CCG AA Reverse 2 – TCA GCA GGC GTG AGC CAT CT
P450scc	Forward 1 – GAG ATT CCT GGA CTG TGG AAG Reverse 1 – GGA GGT GGT CTT GAA CAT GAG Forward 2 – ATC CTG TTC AAA GCG GAG GG Reverse 2 – TCC TGG AGG TTG GGA TGT CT

CRF, corticotropin-releasing factor; MC2R, melanocortin 2 receptor; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage enzyme.

2.5. Semi-quantitative real-time RT-PCR

Semi-quantitative real-time RT-PCR was used to assess the mRNA abundance of target genes. Primers for CRF, MC2R, StAR and P450scc (Table 2) were generated using Primer3 (SimGene.com). The ribosomal 18S subunit was used as a reference gene, and primers were retrieved from Robertson et al. (2009). Primer specificity was verified by sequencing of amplicons for each gene-specific primer set. In addition, standard curves were generated for each primer set to optimize the reaction composition based on the efficiency of the reaction (Table 2). Real-time RT-PCR reactions were carried out using SYBR green III mastermix kit (Stratagene) and Bio-Rad CFX96 Real-Time PCR System (Bio-Rad). For all reactions, the manufacturer's instructions were followed with the exception that reaction volumes were scaled to 10 μl rather than 20 μl. Primer concentrations were 0.07 μM for MC2R, 0.1 μM for CRF and P450scc, and 0.12 μM for 18S and StAR; in addition, cDNA was diluted 25-fold for CRF, 10-fold for MC2R, StAR and P450scc, and 1000-fold for 18S. In each case, the cycling conditions were 95 °C (10 s) and 60 °C (6 s) for 39 cycles. Dissociation curves were generated for each product to confirm primer specificity and no template controls (cDNA replaced with water for real-time RT-PCR) as well as no reverse transcriptase controls (cDNA synthesis reaction carried out without reverse transcriptase) were included to eliminate the possibility of genomic DNA contamination. The mRNA abundance of each gene was calculated relative to the control MG-FE using the modified delta-delta Ct method (Pfaffl, 2001) with 18S as the normalizing gene, as in Jeffrey et al. (2012).

2.6. Statistical analyses

All data are expressed as mean values ± 1 standard error of the mean (SEM). For plasma ACTH and cortisol concentrations, only repeated samples from stressed fish were used, and these data were analyzed by two-way repeated measures analysis of variance (RM ANOVA). For mRNA abundance, data were analyzed by two-way ANOVA. Data were transformed using a square root transformation where they did not pass the normality test (for

Table 2
Oligonucleotide primer sets used for real-time RT-PCR.

Gene	Primers (5′–3′)	Product size (bp)	Accession Nos.
18S	Forward – GCA AAG CTG AAA CTT AAA GGA ATT G Reverse – TCC CGT GTT GAG TCA AAT TAA GC	80	EU502753
CRF	Forward – CAT AAG CAG CTT CGG AGA CC Reverse – GAC ATC TCC ATC ATC TCC CG	117	KJ534544
MC2R	Forward – TCA CCA AAA CCT GGG AGA AC Reverse – AGC AGG GAG TCC ATC ACA TC	106	KJ534545
StAR	Forward – TGT TGT CAG AGC GGA GAA TG Reverse – AAA GTC CAC CTG CGT CTG AG	160	KJ534546
P450scc	Forward – ATC TTC AAC CAA GCG GAC CG Reverse – TTG CCA GGA CTC CTG GGT AT	94	KJ534547

CRF, corticotropin-releasing factor; MC2R, melanocortin 2 receptor; StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side chain cleavage enzyme.

StAR). Where trends were present in the data but no overall (i.e., by parental care stage or stress treatment for the entire ANOVA model) statistical differences were present, within-stage differences were further analyzed by one-tailed Student's *t*-test (plasma ACTH, POA CRF and head kidney StAR) for the purpose of discussion. Our overall statistical power was low given conservation concerns with sampling wild fish engaged in reproduction so we felt that this approach would not lead to spurious type I errors given the overall conservatism associated with low sample size. All analyses were carried out using Sigma Plot V.11. The level of significance (α) was 0.05.

3. Results

Plasma cortisol levels were significantly elevated by a standardized stressor in MG-FSF but not in MG-FE, confirming that the cortisol response during the early period of parental care was attenuated relative to that later in the parental care period (Fig. 1A; two-way RM ANOVA $P = 0.067$ for parental care stage, $P < 0.001$ for sampling time and $P = 0.024$ for stage × time). The post-stress cortisol levels of MG-FSF were significantly higher than those of MG-FE. Plasma ACTH levels appeared to be elevated in response to stress but this trend was not significant with analysis by two-way RM ANOVA (Fig. 1B; $P = 0.747$ for stage, $P = 0.066$ for sampling time and $P = 0.444$ for stage × time). However, when males at different parental stages were analyzed separately, ACTH levels were significantly elevated in MG-FSF (one-tailed Student's *t*-test, $P = 0.0175$) but not in MG-FE (one-tailed Student's *t*-test, $P = 0.250$).

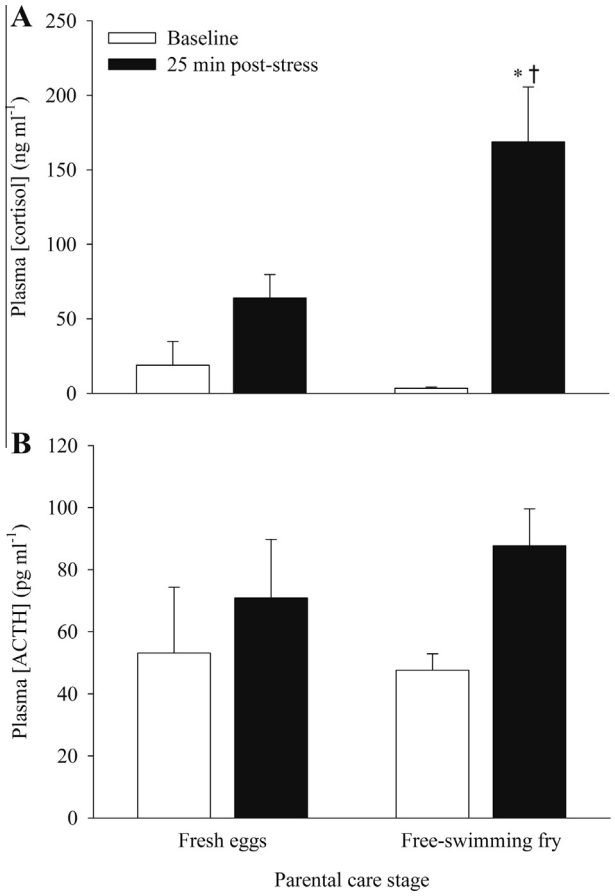

Fig. 1. Plasma (A) cortisol and (B) adrenocorticotripic hormone (ACTH) levels in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE; $N = 6$) or free-swimming fry (MG-FSF; $N = 6$–7). Males were subjected to 3 min of air exposure and blood samples were collected before (baseline) and 25 min after the stressor. Values are means + SEM. An asterisk indicates a significant difference in 25 min post-stress cortisol levels between MG-FE and MG-FSF, and a dagger indicates a significant difference between baseline and 25 min post-stress cortisol levels within a parental care stage (two-way RM ANOVA; see text for details).

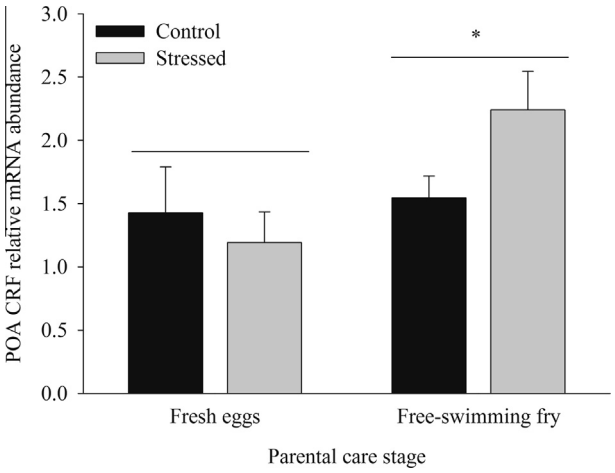

Fig. 2. Relative mRNA abundance of preoptic area (POA) corticotropin-releasing factor (CRF) in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE) or free-swimming fry (MG-FSF). Males ($N = 6$) were euthanized immediately post-capture (control), or 2 h after 3 min of air exposure (stressed). Values are means + SEM. All data were normalized to the mRNA abundance of 18S and expressed relative to the MG-FE control group. An asterisk indicates a significant difference between MG-FE and MG-FSF, with no significant effect of exposure to the stressor (two-way ANOVA; see text for details).

Corticotropin-releasing factor mRNA abundance was assessed in the POA and hypothalamus of control and stressed males at each parental-care stage. No significant differences in hypothalamic CRF mRNA levels were found (data not shown). Preoptic area CRF mRNA levels were significantly higher in MG-FSF compared to MG-FE (Fig. 2; two-way ANOVA, $P = 0.050$ for parental care stage, $P = 0.418$ for treatment group and $P = 0.111$ for stage × treatment). When analyzed individually, CRF mRNA abundance was significantly higher in stress-exposed males in MG-FSF (one-tailed Student's *t*-test, $P = 0.037$) but not in MG-FE (one-tailed Student's *t*-test, $P = 0.302$).

Exposure to a standardized stressor resulted in significantly higher MC2R mRNA abundance in MG-FSF but not MG-FE, and levels of MC2R mRNA in males of the stress-exposed group were significantly higher in MG-FSF compared to MG-FE (Fig. 3; two-way ANOVA, $P = 0.097$ for parental care stage, $P = 0.20$ for treatment group and $P = 0.014$ for stage × treatment). Although no significant differences with parental stage or exposure to a stressor were detected in P450scc mRNA levels (two-way ANOVA, $P = 0.106$ for stage, $P = 0.883$ for treatment group and $P = 0.664$ for stage × treatment), StAR mRNA abundance appeared to be higher in MG-FSF compared to MG-FE (two-way ANOVA, $P = 0.068$ for stage, $P = 0.107$ for treatment group and $P = 0.179$ for stage × treatment). Analysis of these data by parental care stage suggested a trend for StAR mRNA abundance to be higher in males exposed to a stressor than in control males in MG-FSF (one-tailed Student's *t*-test, $P = 0.0585$) but not MG-FE ($P = 0.314$) (see Fig. 4).

4. Discussion

Male smallmouth bass attenuated their cortisol response to a stressor during early parental care, a response that was recovered by the end of the parental care period (see also O'Connor et al., 2011). The results of the present study supported the hypothesis

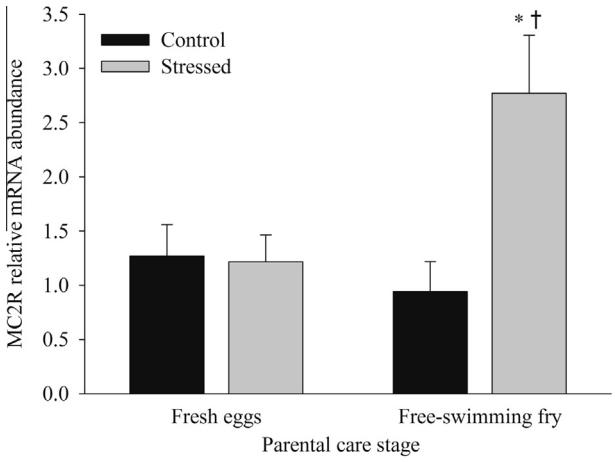

Fig. 3. Relative mRNA abundance of head kidney melanocortin 2 receptor (MC2R) in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE; $N = 6–7$) and free-swimming fry (MG-FSF; $N = 6$). Males were either euthanized immediately post-capture (control), or 2 h after 3 min of air exposure (stressed). Values are means + SEM. All data were normalized to the mRNA abundance of 18S and expressed relative to the MG-FE control group. An asterisk indicates a significant difference between stressed MG-FE and MG-FSF males, and a dagger indicates a significant difference between control and stressed groups within MG-FSF (two-way ANOVA; see text for details).

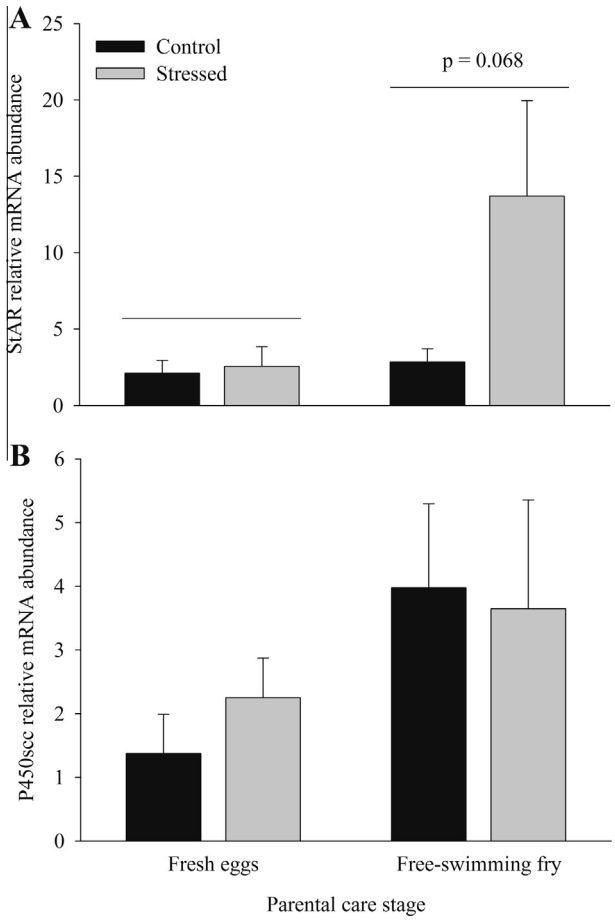

Fig. 4. Relative mRNA abundance of head kidney steroidogenic acute regulatory protein (StAR; (A)) and cytochrome P450 side change cleavage enzyme (P450scc; (B)) in male smallmouth bass (*Micropterus dolomieu*) guarding fresh eggs (MG-FE; $N = 6–7$) or free-swimming fry (MG-FSF; $N = 6$). Males were either euthanized immediately post-capture (control) or 2 h after 3 min of air exposure (stressed). Values are means + SEM. All data were normalized to the mRNA abundance of 18S and expressed relative to the MG-FE control group. Data were analyzed by two-way ANOVA (see text for details).

that this attenuated cortisol response reflected modulation of HPI axis activity. Activity of the HPI axis was modulated at multiple levels, and in directions that would be expected to decrease cortisol synthesis in response to a stressor. With the exception of hypothalamic CRF and head kidney P450scc mRNA abundance, which did not vary significantly with parental care stage, all other variables tested, including POA CRF mRNA abundance, circulating ACTH concentration, and MC2R and StAR mRNA levels (the latter as a trend), increased in response to stress in MG-FSF but not in MG-FE.

We acknowledge that it was not possible to control for water temperature given that it rose steadily during the parental care period for bass (i.e., water temperatures were 16 °C at the FE sampling period and 20 °C at the FSF period), a common problem with studies of parental care in the wild (Wilson et al., 2012). To partially address this issue, in the present study, two additional groups of males were angled, euthanized and sampled outside of the reproductive period (in the fall) at water temperatures (16 and 20 °C) equivalent to those experienced by males engaged in parental care. Only control (non-stressed) fish were used, to decrease the number of fish that had to be euthanized, particularly because distinguishing between males and females is challenging in non-reproductive bass. With the exception of POA CRF mRNA, key mediators of HPI axis function did not vary significantly between males sampled at low or high temperatures, representing water temperatures for MG-FE and MG-FSF, respectively. Preoptic area CRF levels were significantly higher in males sampled at high water temperature compared to those sampled at low water temperature. Although this difference could be related to water temperature, it is also possible that seasonal differences may have been a contributing factor because no significant difference in POA CRF was found between control MG-FE and MG-FSF. Although a potential impact of water temperature cannot be ruled out, the data for non-reproductive fish provide some confidence that temperature effects were of little importance and that parental care stage effects dominated the data set collected.

We also acknowledge that the relatively low sample size in this study made statistical analysis difficult. The most appropriate way to search for differences between parental care stages and treatments was a two-way ANOVA; however, our ability to detect statistically significant differences in the data was constrained by the relatively low N numbers. To this end, Student's t-tests were used to assess differences between treatments within a parental care period where differences were suspected but were not found to be significant by two-way ANOVA. Although increasing the sample size would be preferred, sampling additional males during the parental care period becomes problematic from a conservation perspective and would not be permitted by regulators.

4.1. Modulation of HPI axis function at multiple levels during early parental care

Corticosteroid axis reactivity and reproductive status are tightly linked in that reproductive status can dictate stress responsiveness and corticosteroid levels can affect reproductive success. During the reproductive period and subsequent parental care, robust activation of the stress axis can negatively impact reproduction (e.g., Schreck et al., 2001; Silverin, 1986; Wingfield and Silverin, 1986) as well as parental care behaviors (e.g., Almasi et al., 2008; Kitaysky et al., 2001; Silverin, 1986). The effect of high glucocorticoid levels on reproduction and parental care has been assessed in a number of avian species. Experimental increases in glucocorticoid levels decreased reproductive success in pied flycatchers (*Ficedula hypoleuca*) (Silverin, 1986), decreased territorial behavior in male song sparrows (*Melospiza melodia*) (Wingfield and Silverin, 1986), reduced nestling provisioning in male barn owls (*Tyto alba*)

(Almasi et al., 2008), and increased the time spent away from the nest in black-legged kittiwakes (*Rissa tridactyla*) (Kitaysky et al., 2001). Similarly, nest abandonment increased in male smallmouth bass with exogenously elevated cortisol levels (Dey et al., 2010; O'Connor et al., 2009). Owing to the possible negative impacts of high glucocorticoid levels on reproductive success, it has been hypothesized that 'resistance to stress' (e.g., an attenuated glucocorticoid response to a stressor) is an adaptive response to increase current reproductive success (Wingfield and Sapolsky, 2003). Resistance to stress has been observed in a number of vertebrate species and is a strategy that can vary across species; blockade at the level of the central nervous system, HPA/HPI axis, or HP-gonadal axis is possible (reviewed by Wingfield and Sapolsky, 2003). In the current study and that of O'Connor et al. (2011), an attenuated cortisol response to an acute stressor was found in parental care-providing male smallmouth bass, with the most severe attenuation of the stress response occurring early in the parental care period when males were guarding fresh eggs; the response was re-established by the end of the parental care period when fry were free-swimming. To our knowledge, few studies have assessed stress responsiveness in teleosts providing parental care. However, several studies on avian species reported similar attenuation of the acute stress response during parental care (e.g., Done et al., 2011; Meddle et al., 2003; O'Reilly and Wingfield, 2003; Silverin and Wingfield, 1998; Wingfield et al., 1992). Although the dynamics of reproduction and parental care are different between birds and teleosts (e.g., smallmouth bass), they share similarities in that parental investment can fluctuate over the period of parental care (Sargent and Gross, 1986), which in turn is reflected in the extent of their response to acute stressors. In the Arctic-breeding polygynandrous songbird Smith's longspur (*Calcarius pictus*), for example, males arriving on the breeding ground retained a sensitive stress response, but this response was suppressed once paternity was established during the nestling stage and parental investment was highest (Meddle et al., 2003). In smallmouth bass, parental investment arguably may be higher in the early stages of parental care when the stress response exhibits the greatest degree of attenuation, compared to the late stages of parental care when offspring near independence (Cooke et al., 2002). Although extensive work has documented (presumably) adaptive changes in the stress response during breeding and parental care, few studies have attempted to elucidate the mechanisms through which HPA/HPI axis function is modulated (Meddle et al., 2003; Romero et al., 1998). In the current study, HPI axis function was altered at multiple levels during early but not late parental care, which was in agreement with cortisol responsiveness during these periods.

Activation of the HPI axis and subsequent cortisol production begins with the release of CRF to the pituitary from neurons whose cell bodies originate in the POA (reviewed by Bernier et al., 2009; Flik et al., 2006; Lederis et al., 1994). Although CRF is produced in other areas of the brain, POA CRF is thought to play the largest role in activating pituitary corticotropes to produce ACTH in teleosts (Bernier et al., 2009). In mammals, CRF is depleted from CRF-containing neurosecretory neurons of the HPI axis in response to stress and elicits an increase in CRF mRNA expression in the paraventricular nucleus, the mammalian equivalent of the POA (e.g., Chen et al., 2004; Imaki et al., 1995). Although this process is less well understood in fish, there is evidence for a similar increase in POA CRF mRNA in response to stress (confinement, Ando et al., 1999; hypoxia, Bernier and Craig, 2005; subordinance, Doyon et al., 2003; repeated chasing, isolation and confinement, Doyon et al., 2005; vortex stress, Fuzzen et al., 2010). In accordance with these findings, POA CRF mRNA levels were elevated in response to stress in MG-FSF but not MG-FE in the present study. Suppression of stress-induced increases in POA CRF mRNA abundance in MG-FE may explain, at least in part, the attenuated cortisol response observed in these fish. Factors such as CRF receptors and CRF-binding protein mediate the downstream actions of CRF (Alderman and Bernier, 2007; Pohl et al., 2001; Seasholtz et al., 2002) and as such, future studies should investigate how these factors may be regulated by parental care stage.

Pituitary corticotropes release ACTH which then binds to MC2R of interrenal cells in the head kidney. Previous studies in teleost fish have shown that ACTH levels increase in response to acute stress (Aluru and Vijayan, 2008; Balm and Pottinger, 1995; Pickering et al., 1986; Sumpter et al., 1986), and that this increase in circulating ACTH levels causes an increase in MC2R mRNA abundance (Aluru and Vijayan, 2008). As expected, plasma ACTH and MC2R mRNA levels increased in response to stress in MG-FSF. Again, however, circulating ACTH and MC2R mRNA levels failed to increase in response to stress in MG-FE. Autoregulation of MC2R mRNA by ACTH is thought to play an important role in maintaining interrenal responsiveness to ACTH (Aluru and Vijayan, 2008). A failure to increase circulating ACTH levels and interrenal MC2R mRNA abundance in response to stress suggests a desensitization of cortisol responsiveness at the level of the interrenal cells which could help to explain the attenuated cortisol response in MG-FE. Stimulation of the interrenal cells with ACTH in males at different stages of parental care could shed further light on whether interrenal insensitivity plays a role in modulation of the cortisol response in these fish. For example, Jeffrey et al. (2014) demonstrated that interrenal cell insensitivity to ACTH was responsible for attenuation of the cortisol response in subordinate rainbow trout.

Cortisol synthesis also is mediated by other factors including StAR and P450scc. Cholesterol is moved to the inner mitochondrial membrane by StAR and cleaved to pregnenolone by P450scc as the first and rate-limiting step in cortisol synthesis (Aluru and Vijayan, 2006, 2008; Hagen et al., 2006). The current study presents some evidence to support an increase in StAR but not P450scc mRNA abundance in response to stress in MG-FSF, a response that was not observed in MG-FE. Like MC2R, both StAR and P450scc mRNA levels have been shown to increase in response to stress in teleosts (Aluru and Vijayan, 2006; Geslin and Auperin, 2004; Jeffrey et al., 2014), and during ACTH-stimulation *in vitro* (Aluru and Vijayan, 2008; Hagen et al., 2006). However, transcriptional regulation of StAR and P450scc remains poorly understood in teleosts since Alderman et al. (2012) and Geslin and Auperin (2004) failed to observe an increase in mRNA levels of these steroidogenic genes following ACTH-stimulation *in vitro* (Alderman and Vijayan, 2012; Geslin and Auperin, 2004) or chasing (Geslin and Auperin, 2004). Geslin and Auperin (2004) suggested that the severity of the stressor and the sampling time may influence transcriptional regulation of and observation of an mRNA increase in both StAR and P450scc. The lack of an increase in StAR in response to stress in males at the early parental care stage provides further evidence for mediation of the cortisol response at the level of the interrenal cells in these smallmouth bass; however, transcriptional regulation of steroidogenic genes in teleosts warrants further investigation.

4.2. Conclusion

The trade-off between current and future reproduction in relation to the glucocorticoid stress response has been documented in avian species as well as other taxa, and evidence for similar trade-offs in teleosts is increasing. However, most of these studies focused on the ecological relevance of the trade-off and did not probe more deeply into the physiological mechanisms underlying changes in the glucocorticoid response during the period of parental care. The present study is one of the first that has attempted to fill this gap. The current study provides evidence to support a multi-level modulation of HPI axis activity during the early

parental care period, with key mediators in the brain (POA CRF), pituitary (circulating levels of ACTH) and interrenal cells (MC2R and StAR) failing to increase in response to stress, accounting for the attenuated cortisol response in male smallmouth bass during early parental care. Further studies into the causes of a hypoactive HPI axis during early parental care are warranted. Of particular interest would be the role of androgens in mediating HPI axis function.

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