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Oxidative stress associated with paternal care in smallmouth bass (*Micropterus dolomieu*)

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ABSTRACT

In species that provide parental care, care for offspring is often accompanied by an increase in locomotor activity and a decrease in feeding opportunities which can negatively impact endogenous energy reserves. Depletion of parental energy stores and declines in nutritional condition can cause physiological disturbances, such as an imbalance between free radical production and available antioxidants, known as oxidative stress. Using the teleost smallmouth bass (*Micropterus dolomieu*) as a model, we tested if the energetic challenge associated with sole paternal care was associated with oxidative stress. Blood samples from parental males were collected throughout parental care, during egg, embryo, and larval stages of offspring development, and assayed for both antioxidant capacity and oxidative damage. A reduction in oxygen radical absorbance capacity was observed during the parental care period, indicating a decrease in resistance to oxidative stress. Although no change was observed in the reduced:total thiol ratio, a significant increase in the concentration of both oxidized and total thiols occurred during the parental care period. No increase in the oxidative stress markers 8-hydroxy-2-deoxyguanosine, protein carbonyls and lipid peroxides was observed. We concluded that oxidative stress did not occur as a result of parental care in the male smallmouth bass. This study provides evidence that participation in energetically taxing activities, such as parental care, can result in a decrease in antioxidant resources, but may not always result in oxidative stress.
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1. Introduction

Parental care can be broadly defined as any action which aims to increase an individual's fitness by increasing offspring survival rates (Williams, 1966; Trivers, 1972; Elmen and Oring, 1977; Reynolds, 1996). In general, provision of parental care requires expenditure of endogenous energy reserves (Fitzgerald et al., 1989; Reznick, 1992; Gillooly and Baylis, 1999; Mackereth et al., 1999), resulting in decreased growth (Cox et al., 2010), immune response (Bertrand et al., 2006; Costantini and Møller, 2009), and longevity (Rose and Charlesworth, 1980; Stearns, 1992). In addition to the physiological toll of parenting, caring for offspring also limits future mating opportunities (Coleman et al., 1985; Magrath and Komdeur, 2003). Consequently, individual fitness is optimized by balancing costs such as energy expenditure, and loss of future reproductive opportunities, with current offspring survival (Williams, 1966). Though many costs of parental care are well defined, many proximate costs such as oxidative stress remain largely unknown (Harshman and Zera, 2006).

Few studies have examined the link(s) between oxidative stress and reproduction (see Salmon et al., 2001; Alonso-Alvarez et al., 2004, 2010; Garratt et al., 2011), and even fewer have examined these using a wild model organism. Salmon et al. (2001) demonstrated that induction of fertility in female *Drosophila melanogaster* resulted in both an increase in egg production and increase in susceptibility to oxidative stress, providing the first evidence that oxidative stress may represent a cost of reproduction. Oxidative stress was attenuated in female house mice providing parental care (Garratt et al., 2011). Alonso-Alvarez et al. (2004) reported that an increase in oxidative stress resulted from biparental care in zebra finch (*Taeniopygia guttata*) which correlated with parental investment and brood size. More recently Alonso-Alvarez et al. (2010) demonstrated that older zebra finches produced smaller broods and had higher oxidative stress levels than younger breeding pairs. They also demonstrated that breeding effort promotes oxidative stress, as female zebra finches endured higher oxidative stress levels than males. Thus, oxidative stress may represent an additional and previously uncharacterized consequence of parental care (Alonso-Alvarez et al., 2004; Monaghan et al., 2009).

Oxidative stress is the imbalance between endogenous free radical production through normal metabolism and the ability to absorb or repair oxidative damage (antioxidant capacity) and represents an important cause of cellular damage (Das and White, 2002; Valko et al., 2007). Without the buffering power of antioxidants, free radicals attack biological macromolecules leading to inactivated proteins, damaged membranes, and most importantly, cause mutations in DNA (Finkel and Holbrook,

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2000; Valko et al., 2007). These point mutations have been suggested to be an underlying cause of many disease pathologies, as well as the aging process (Harman, 1956). The evolutionary theory of aging has suggested that point mutations in DNA, resulting from free radical attack, accumulate throughout the lifespan of an organism and result in aging and eventually death (Beckman and Ames, 1998; Finkel and Holbrook, 2000; Barja, 2007; Buffenstein et al., 2008). As the effects of oxidative stress appear to be both costly and diverse, oxidative stress may represent a physiological trade-off of parental care (Costantini, 2008, 2010). For example, older individuals of iteroparous species (i.e., who reproduce more than once) that have fewer future reproductive opportunities may weigh the cost of oxidative stress differently than younger individuals which may be more likely to abandon their current brood to avoid oxidative stress (Alonso-Alvarez et al., 2006; Cohen et al., 2009; Alonso-Alvarez et al., 2010; Metcalfe and Alonso-Alvarez, 2010). Thus, oxidative stress may be an important, and largely unexplored physiological trade-off involved in parental care as it pertains to life-history theory.

In an effort to increase offspring survival, some fish such as male smallmouth bass (*Micropterus dolomieu*), provide protection for their offspring for an extended duration at significant energetic cost to the parent. Male smallmouth bass care for their offspring for a period of 4 weeks or longer until offspring develop into juveniles and gain independence (Ridgway, 1988). This care is intensive, since offspring survival is highly dependent on the diligence of the male parent (Philipp et al., 1997; Knotek and Orth, 1998). The male must be constantly vigilant, as in many environments there are a great variety of aquatic predators attempting to consume offspring (Kieffer et al., 1995; Steinhart et al., 2005; Gravel and Cooke, 2009). As a result of these activities, nesting males are much more active than non-nesting males, spending 5 to 20% of their time swimming at greater than 80% of critical swimming speed (Cooke et al., 2002). During the remaining time, the male maintains its nest by fanning eggs to provide oxygen and clearing the nest of silt and debris. Due to these energetically demanding activities, males have limited opportunities for feeding and are considered to be voluntarily anorexic (Hinch and Collins, 1991; Hanson et al., 2009a). Both voluntary anorexia and vastly increased activity levels result in depletion of energy reserves throughout the parental care period (Mackereth et al., 1999; Cooke et al., 2002; Cooke et al., 2006).

In this study, we tested whether oxidative stress could be an additional, uncharacterized, cost of parental care using parental male smallmouth bass as a model. Pike et al. (2007) demonstrated that antioxidant availability increases the ability of males to provide parental care under stressful conditions (i.e. hypoxia) in three-spined stickleback (*Gasterosteus aculeatus*). Fish must possess sufficient antioxidant defenses that depend, in part, on a dietary supply of essential antioxidants, such as vitamin E (Martínez-Álvarez et al., 2005). Starvation has been linked to an increase in pro-oxidants, a decrease in exogenous antioxidants and an increase in endogenous antioxidants (Morales et al., 2004). Since smallmouth bass exhibit voluntary anorexia throughout parental care (Mackereth et al., 1999; Hanson et al., 2009a), we predicted that antioxidant capacity would decrease during this period. We also predicted that the increase in activity levels throughout parental care would lead to an increase in free radical production which, in the absence of antioxidants, would result in an increase in oxidative damage and ultimately lead to oxidative stress. To determine if oxidative stress results from parental care, we measured the levels of three markers of oxidative stress: 8-hydroxy-2-deoxyguanosine (8-OHdG), lipid peroxides, and protein carbonyl groups. Our objectives were two-fold: first, to determine if a decrease in antioxidant capacity occurs over parental care, and second, to determine if that decrease in antioxidant capacity results in oxidative damage.

2. Materials and methods

All research was conducted in accordance with guidelines of the Canadian Council of Animal Care as administered by Carleton University and Queen's University (B09-06) and with a scientific collection permit from the Ontario Ministry of Natural Resources. Blood samples from wild adult male smallmouth bass were collected between May 11th and June 5th, 2009, on Indian Lake (44°35′N, 76°19′W) at three time points, determined by the developmental stage of their offspring: fresh eggs, embryonic and larval fry. The fresh egg stage was considered to be the cleavage phase of embryo development (Balon, 1975) and was characterized by near-transparent eggs with no obvious organogenesis or fungal deposition. The embryonic stage was considered to be the transition from the last embryo stage, (eleuthro-embryonic) to first larval stage (protopterygiolarval) and was characterized by loss of egg-sack and development of swimming ability. Finally the larval stage was considered to be the late protopterygiolarval stage (Balon, 1975) and was characterized by black coloration, and development of schooling behaviors. Characterization of each stage was completed by visual observation of physical traits and behaviors of offspring in the nest. Snorkeling surveys were completed whereby snorkelers located and marked smallmouth bass nests, which had eggs that were determined to be in the fresh egg stage of development, with numbered polyvinylchloride (PVC) tags. Nest location was recorded and the number of eggs was categorized between a low value of 1 to a high value of 5 (Suski and Philipp, 2004). For the purpose of this study we focused on fish with intermediate egg scores (i.e., 3 or 4) to control for variation in brood size which is known to be positively correlated with parental vigilance and nest aggression (e.g., Suski and Philipp, 2004; Hanson et al., 2009b). The majority (>90%) of nests for smallmouth bass have an egg score of either 3 or 4. In total 29 parental males were sampled during this study. Nests were all between 1 and 3 m in depth on a cobble-gravel substrate. Marked nests were returned to at each of the three aforementioned stages of offspring development. At each stage different males were captured and sampled. Parental male smallmouth bass were caught by rod and reel from either the boat, or by the snorkeler and landed within 20 s of capture. Fish were placed in a foam lined trough filled with fresh lake water and blood was sampled via caudal venipuncture using a vacutainer tube (3 mL, lithium–heparin anticoagulant, Becton-Dickson; 21 G, 11/2′ long syringe; BD, NJ, USA). All fish were sampled within 2 min from time of initial hooking. Approximately 2 mL of blood was obtained, placed on ice water slurry for <1 min, and immediately centrifuged for 5 min at 1500 rpm (Clay Adams Compact II Centrifuge, Becton-Dickson; Sparks, MD, USA). Erythrocytes and plasma were separated and flash frozen in liquid nitrogen and transferred to an ultra-cold freezer where they were stored at −80 °C until analysis. Fork length of fish was then measured and fish was immediately released.

Despite a variety of attempts, no control males (i.e., non-reproductive males) of reproductive age, in similar water temperatures were captured. Although males could be caught and sampled pre-spawn, the sex of smallmouth bass cannot be determined externally. Therefore both males and females may have been captured and would have to be sacrificed to determine sex which was not desirable for these relatively long-lived and recreationally important fish. Similarly, holding wild bass in captivity at a variety of temperatures to obtain control values would have induced stress from captivity. Without controls, conclusions about oxidative stress may only be compared among the stages of parental care. Nonetheless, we regard these data as highly relevant because temperature variation contributes to the stress encountered during parental care in wild fish. Relative differences among stages of parental care can provide meaningful information on changes across periods that are associated with significant alterations in nutritional condition (e.g., Cooke et al., 2002; Hanson and Cooke, 2009).

2.1. Thiol determination

Oxidized, reduced and total thiol levels were determined using a method similar to that employed by Winters et al. (1995). Varian (Agilent Technologies, Santa Clara, CA) Pro-Star high pressure liquid chromatography (HPLC) with ProStar 210 Pumps, ProStar 410

Autosampler, ProStar Prime Purge Box, and ProStar 363 Fluorescence Detector was operated at an excitation wavelength of 330 nm and an emission wavelength of 376 nm. A Varian Polaris column (250×4.6 mm and packed with 5 μm particle size C_{18} packing material) was used for separation purposes. Isocratic elution was performed with solvent A (65% v/v acetonitrile, 35% v/v water, 0.1% v/v 85% phosphoric acid, and 0.1% v/v 18 M glacial acetic acid) at a flow rate of 0.5 mL/min. HPLC chromatograms were analyzed using Varian Galaxy software (version 1.9.302.530). Thiol samples were prepared by *N*-(1-pyrenyl)maleimide (NPM) derivatization. Briefly, total thiol levels were determined by first mixing a 2:1 v/v ratio of serine borate buffer (SBB) (100 mM Tris HCl, 10 mM borate, 5 mM serine, 1 mM diethylenetriaminepentaacetic acid (DTPA), pH 8.3) to plasma then adding an equal volume of a 2 mM dithiothreitol (DTT) solution. Samples were incubated at room temperature for 30 min, until all thiols were reduced. NPM (3 mM in acetonitrile) was then added to the samples for a final ratio of 3:1 v/v NPM resulting in a final volume of 1 mL. Samples were incubated for 5 min at room temperature before the reaction was halted with 10 μL of 10 N HCl. Reduced thiol levels were determined using the same protocol with the exception that the 2 nM DTT solution was replaced with water. Oxidized thiols were calculated by subtracting reduced thiols from total thiols. Solutions were stored at room temperature and protected from light until assayed. It was determined that NPM-SH bond remained stable at room temperature for up to 24 h. All thiol concentrations were determined using area-under-the-curve and extrapolated from a standard curve generated from reduced glutathione (0 nM–7500 nM), and are therefore reported as glutathione equivalents.

2.2. Antioxidant capacity

Resistance to free radical attack was determined using the ORAC assay as outlined in Lucas-Abellan et al. (2008). The ORAC analyses were completed using a Fluostar Optima microplate reader (BMG Labtech; Offenburg, Germany) and black 96-Well Costar microplates. Fluorescence was measured with an excitation wavelength of 485 nm and emission of 520 nm. Fluorescence data were analyzed using Optima software (9.15.31, Optima Technology Corporation, Rio Rico, Arizona). The reaction was carried out in 75 mM sodium phosphate buffer (pH 7.4). A stock solution of fluorescein (1:200 v/v in 75 mM sodium phosphate buffer (pH 7.4); 5.85 μM) was prepared from concentrated stock solution of fluorescein (11:25 w/v in 75 mM sodium phosphate buffer (pH 7.4); 1170 μM). Stock fluorescein solution (150 μL; 3.51 μM final concentration) and sample (20 μL) were added to each well in triplicate, and pre-incubated at 37 °C for 30 min before rapidly adding 306 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH; 73.44 mM final concentration) using multichannel pipette. The microplate was immediately placed in the microplate reader and fluorescence was measured every 35 s for 60 min. A blank was prepared with sodium phosphate buffer instead of the sample, while Rutin (16 μM final concentration) was substituted for sample as a positive control. A standard curve was prepared using 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (0, 50, 100, 200 and 400 μM) as an antioxidant and run with each assay. All reaction mixtures were prepared in triplicate. In order to avoid a temperature effect, only the inner 60 wells were used for experimental purposes, while the outer wells were filled with 300 μL of distilled water. The area under the fluorescence decay curve (AUC) was determined and related to Trolox AUC to determine Trolox equivalents. Total protein of samples was determined using the Bradford assay (Bradford, 1976) and final values were reported in Trolox equivalents/mg total protein.

2.3. Lipid analysis

Lipid peroxide concentration was determined using thiobarbituric acid (TBA) reactive substances (TBARS) assay as outlined in Uchiyama and Mihara (1978) and Willmore and Storey (1997). Briefly, plasma samples were thawed on ice and plasma was homogenized in 1:1 ratio with 0.2% v/v phosphoric acid. An equal volume of 2% v/v phosphoric acid was added for a final concentration of 1.1% v/v phosphoric acid in a ratio of 1:2 plasma to phosphoric acid. An aliquot of the sample solution (400 μL) was added to TBA solution in a 1:1 ratio. TBA solution was prepared with 1% w/v TBA in 10 mL of 50 mM NaOH containing 100 μL of 10 mM BHT. A 200 μL volume of 7% v/v phosphoric acid was added to make the final volume 1 mL. Individual blanks were prepared by replacing the TBA solution with 3 M HCl. Sample pH was adjusted to 1.6 with 10 M HCl as needed. Sample and blank solutions were heated to 100 °C for 15 min, and cooled to room temperature before 1.5 mL of butanol was added. Each sample was vortexed for 40 s and centrifuged for 10 min at 1600 rpm using an IEC multi RF benchtop centrifuge. The organic phase of each solution was scanned from 400 nm to 650 nm using Varian (Agilent Technologies, Santa Clara, CA, USA) Cary 100 Bio UV Vis spectrophotometer and quartz cuvettes. Final absorption values were obtained using the formula $(A_{533}-A_{600})$ samples $-(A_{533}-A_{600})$ blanks. Final TBARS concentration was determined using a TBA-malondialdehyde (MDA) standard curve (0–10 μM). The Bradford assay (Bradford, 1976) was used to determine total concentration of protein in samples. Final concentrations are reported as MDA equivalents/mg total protein.

2.4. Protein carbonyl group analysis

Oxidative protein damage was assessed through Western blot analysis using the OxyBlot™ Protein Oxidation Detection Kit (Chemicon International, Inc; Billerica, MA, USA). Briefly, 15 μg of total protein was reacted with 2,4-dinitrophenylhydrazine (DNPH) for 15 min, followed by neutralization with a neutralization solution (32% v/v glycerol, 17.5% w/v trometamol). Individual controls were run without DNPH derivatization. Sample and controls were run side-by-side and resolved in 12% SDS-PAGE, transferred to a polyvinyl difluoride (PVDF) membrane, blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline with Tween-20 (PBST) and then incubated with a rabbit anti-DNPH antibody as the primary antibody (1:150 dilution) for 1 h at room temperature. After washing, the membrane was incubated with the secondary antibody goat anti-rabbit IgG (1:300 dilution) conjugated to horseradish peroxidase and detected by Renaissance Western Blot Chemiluminescence Reagent (NEN Life Science Products; Boston, MA, USA). Reactive bands were visualized by 15 s exposure to autoradiography film. Film was scanned and analyzed using AlphaEaseFC software (Alpha Innotech/Cell Biosciences; Santa Clara, CA, USA). Oxidized proteins were standardized against total protein in lanes through staining with Ponceau Red dye. Membranes were scanned and band densitometry was performed using AlphaEase FC software.

2.5. 8-OHdG determination

Erythrocytes were homogenized in 1:5 v/v lysis solution (1% w/v Triton X-100, 0.32 M sucrose, 5 mM $MgCl_2$, 10 mM Tris–HCl, and 0.1 mM desferrioxamine). Samples were centrifuged at 1600 rpm for 10 min at 4 °C using microcentrifuge (Heraeus Biofuge 13; Biodirect; Taunton, MA, USA), pellets were collected and process repeated three additional times. Samples were incubated for 10 min at 37 °C after 200 μL enzyme reaction solution (1% w/v SDS, 5 mM EDTA-Na_2, 10 mM Triton X-100 (pH 8.0), 0.1 mM desferrioxamine) containing 70 units RNase A (from bovine pancreas; Sigma-Aldrich) was added to final pellet. After 10 min incubation with RNase A, 5 units of proteinase K (from *Tritirachium album*; Sigma-Aldrich) were added in 10 μL enzyme reaction solution and samples were incubated for an additional 1 h. DNA was extracted using 1:1 ratio of NaI solution (7.6 M NaI, 20 mM EDTA-Na_2, 40 mM Tris–HCl, 0.1 mM desferrioxamine) with gentle mixing followed by a 1:1 ratio of 100% isopropanol. Samples were centrifuged at 16,000 g for 5 min at 4 °C. The DNA pellet was washed with 1 mL of 40% v/v isopropanol and centrifuged at 16,000 g

for 5 min at 4 °C. The pellet was then washed with 70% v/v ethanol, centrifuged at 16,000 *g* for 5 min at 4 °C and stored at −20 °C. Isolated DNA was denatured and digested by heating to 100 °C for 3 min, cooling on ice for 2 min, and treating with 40 units of nuclease P1 (from *Penicillium citrinum*; Sigma-Aldrich) in 120 μL nuclease P1 buffer (40 mM sodium acetate, 0.2 mM $ZnCl_2$, 0.1 mM desferrioxamine). The samples were incubated for 30 min at 37 °C before 1 unit of alkaline phosphatase (from bovine intestinal mucosa; Sigma-Aldrich) was added. Samples were incubated for an additional hour before 18.5 μL of 0.1 M HCl was added to neutralize the reaction. Samples were stored at −20 °C.

Samples were diluted 5 fold and 50 μL of the diluted sample was used for the determination of 8-OHdG with a commercial ELISA kit (Highly Sensitive 8-OHdG Check, Japan Institute for the Control of Aging, Fukuroi, Shizuoka, Japan). The sensitivity range of the ELISA was from 0.125 to 10 mg/mL. The monoclonal antibody, N45.1, with an established specificity for 8-OHdG (Toyokuni et al., 1997), was used as a primary antibody. Values from each sample were calculated based on an absorbance at 450 nm of standard 8-OHdG (0.125, 0.25, 0.5, 1, 4, 10 ng/mL). Total DNA concentrations were determined using Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) at an absorbance of 260 nm. All 8-OHdG concentrations were standardized against total DNA concentration (ng/mL).

2.6. Statistical analyses

Statistical analyses were completed using JMP (8.02; SAS Institute; Cary, NC, USA). Data were tested for normality and homoscedasticity. Non-normal data were log10 or sine transformed to achieve normality. The original values are plotted in the figures. ANCOVAs were used to test if the stage of parental care was related to the indicators of oxidative stress using size of parental male as a covariant. The level of significance (α) for all tests was assessed at 0.01 to minimize Type I error associated with multiple statistical tests (Zar, 1999). If significant differences were found, we used the Tukey–Kramer post-hoc test to identify significantly different groups.

3. Results

Size was used as a covariant for all variables. Average fork length of parental males was not statistically different among parental care periods ($F_{2,26} = 0.09$; $p = 0.91$).

3.1. Measures of antioxidant capacity

Total levels of oxidized thiols differed among the stages of parental care ($F_{2,2} = 6.66$, $p = 0.005$; Table 1). Parental males guarding eggs had the lowest levels of antioxidant capacity while parents guarding embryos had the highest values. Although concentrations of oxidized thiols increased between the first two stages of parental care, the amount of reduced thiols did not change significantly throughout parental care ($F_{2,2} = 1.60$; $p = 0.22$; Table 1). Total thiol concentrations were related to the stage of parental care ($F_{2,2} = 5.97$; $p = 0.008$; Table 1), where parents guarding eggs showed the lowest concentrations, parents guarding embryos showed the highest concentrations and parents guarding larvae showed intermediate concentrations. Reduced thiol to total thiol ratios were not related to the stage of parental care ($F_{2,2} = 3.52$; $p = 0.05$; Fig. 1). Total oxygen radical absorbing capacity differed among the stages of parental care ($F_{2,2} = 23.56$; $p < 0.0001$; Fig. 2), where males guarding eggs and embryos had the lowest values and males guarding larvae had significantly higher concentrations.

Table 1
Mean (±S.E.M.) concentrations of oxidized, reduced, and total thiols of plasma from male smallmouth bass guarding fresh eggs, embryos and larvae.

Parental care stage	Sample size	Oxidized thiols (nmol GSH equ)	Reduced thiol (nmol GSH equ)	Total thiol (nmol GSH equ)
Egg	9	1.65 ± 0.12^{a}	0.307 ± 0.029	1.95 ± 0.13^{a}
Embryo	9	2.30 ± 0.11^{b}	0.273 ± 0.029	2.58 ± 0.12^{b}
Larvae	10	2.01 ± 0.12^{ab}	0.227 ± 0.027	2.24 ± 0.12^{ab}

Note — dissimilar letters denote significant differences among the groups (results from ANCOVA with size as covariant $p < 0.01$). All values are reported in glutathione equivalents.

3.2. Measures of oxidative damage

Lipid and protein damage did not differ across the parental care period. The concentrations of MDA in blood plasma of parental males did not vary significantly throughout the observed stages of larval development ($F_{2,2} = 0.34$, $p = 0.71$; Table 2). Similarly, no significant differences were found in protein carbonyl levels throughout parental care period ($F_{2,26} = 0.92$, $p = 0.41$; Table 2).

Interestingly, no clear differences in DNA damage were observed. Contrary to our original hypothesis, the level of DNA damage in the form of 8-OHdG did not differ significantly among parental care stages ($F_{2,17} = 2.34$, $p = 0.13$; Table 2).

4. Discussion

Recently, special focus has been given to the role of antioxidants pertaining to life-history theory and parental care in variety of lab-reared and free-range bird species, especially zebra finches and kestrals (Alonso-Alvarez et al., 2004, 2006, 2007, 2010; Cohen et al., 2009). However, there is a general lack of studies in both non-avian species and natural populations. Additionally, the role of oxidative damage has only recently been included in the study of oxidative stress. It has been suggested that a deficit in antioxidants is not necessarily indicative of oxidative stress (Costantini and Verhulst, 2009; Alonso-Alvarez et al., 2010). This study, therefore seeks to enhance the understanding of the role of oxidative stress in parental care in a non-avian species in a wild setting. Furthermore, this study focuses not solely on antioxidant levels, as have many previous studies, but also on oxidative damage parameters, in an effort to clearly examine the potential for oxidative stress.

Parental care has been suggested to cause oxidative stress, consistent with a decrease in body mass in both male and female parental zebra finches, although this study did not include any measurement of oxidative damage levels (Alonso-Alvarez et al., 2004). Our study used a more integrative approach to examine the role of oxidative stress in a wild population of teleost which provides paternal care. The first objective was to determine if a deficiency in antioxidants existed which would have resulted from parental care. Due to both the increased activity associated with parental care (Cooke et al., 2002) and decreased endogenous energy reserves (Mackereth et al., 1999; Cooke et al., 2006), a reduction in antioxidants, and subsequently a decrease in resistance to oxidative stress, would be consistent with current literature (Alonso-Alvarez et al., 2004; Garratt et al., 2011). Our second objective was to determine if changes in antioxidant status

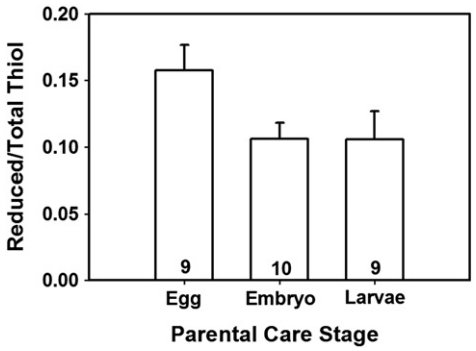

Fig. 1. Plasma reduced:total thiol ratio of parental male smallmouth bass guarding eggs, embryos, and larvae. Significance determined with ANCOVA at $p > 0.01$ with size as a covariant. Sample sizes are shown on bars. Error bars represent standard error of the mean.

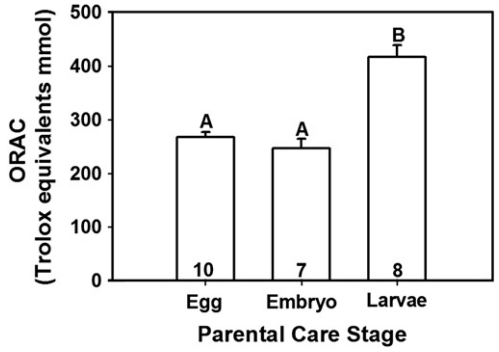

Fig. 2. Plasma ORAC values reported in Trolox equivalents of parental male smallmouth bass guarding eggs, embryos, and larvae. Dissimilar letters (A,B) denote significant differences between groups (Tukey–Kramer *post-hoc* test $p<0.01$). Significance determined with ANCOVA at $p>0.01$ with size as a covariant. Sample sizes are shown on bars. Error bars represent standard error of the mean.

corresponded with increased oxidative damage, which would indicate oxidative stress.

Examination of antioxidant resources, thiols and ORAC values, revealed that overall antioxidant capacity was reduced in males guarding eggs and embryos compared to males guarding larvae, whereas total thiol concentrations increased between males guarding eggs and embryos. Oxidized, reduced and total thiol concentrations were measured in plasma as these are the first biomolecules to be modified during oxidative stress (Halliwell and Gutteridge, 2007). The ratio of reduced to total thiols is commonly used as a measure of oxidative stress. A lower reduced to total thiol ratio represents an imbalance between prooxidants and antioxidants in favor of prooxidants. Our study revealed that the ratio of reduced to total thiols did not change significantly among the egg, embryonic and larval stages of parental care (Fig. 1). There was also no change in reduced thiol levels across parental care. However, we noted significantly higher oxidized thiol and total thiol concentrations in males guarding embryos compared to males guarding eggs. An increase in oxidized thiols suggests that an increase in free radical production was occurring in males providing care for embryos compared to males tending eggs. Although oxidized thiols increased, reduced thiols did not significantly change throughout parental care. This suggested that the increase in the thiol production, associated with the increase in total thiol concentration, was sufficient to combat the increase in oxidative pressure associated with an increase in free radical production.

The increase in oxidized and total thiols observed in this study occurred concomitant with an increase in water temperature. Smallmouth bass spawn in early spring, typically when water temperatures are 15 °C and provide care for 4 weeks or longer at which time the water temperature would have increased by several °C (Cooke et al., 2006). As such, one of the confounding issues with our study is that water temperature increased across the parental care period. Nonetheless, although somewhat confounding, thermal variability is an inherent component of bass parental care. Controlling temperature across parental care is not possible in the wild and it was equally difficult to obtain control fish across temperatures used here without possibly capturing parental males. Ectothermic animals such as fish have increasing basal metabolic rate with increasing ambient temperature (Fry, 1971), and thus there is a potential for both increased oxygen toxicity at higher water temperatures (Barja, 2007) and an increase in endogenously formed free radicals (Laloutte et al., 2011). Interestingly, although oxidized thiols increased as water temperature increased, all other oxidative stress indicators seemed to be independent of temperature and offspring developmental stage for parental bass in this study.

Another antioxidant parameter measured was ORAC, which measures the ability of the sample to absorb peroxyl radicals generated by AAPH, referred to as resistance to oxidative stress. Our study revealed a significant increase in resistance to oxidative stress between the embryonic and larval fry stages of parental care (Fig. 2). This finding is consistent both with data collected by Cooke et al. (2002) which demonstrated that the parental male is most active between the egg and embryonic stages of development, and with behavioral data, which suggests that opportunistic feeding increases as the parent range increases when offspring become larval (Ridgway, 1988; Hanson et al., 2009a). Therefore, the low levels of antioxidants observed in the egg and embryonic stages may be due to an increase in activity and a decrease in feeding, which may utilize antioxidant reserves. The increase in the larval fry stage of parental care may be due to a return to feeding and an activation of metabolism. Though many antioxidants are endogenously produced, their production may be dependent upon factors obtained from exogenous sources (e.g. glutathione peroxidase is dependent on adequate source of selenium which can only be obtained from diet) (Weiss and Sunde, 1997; Catani et al., 2008). Other antioxidants may only be available through diet (e.g. carotenoids). The increase in dietary antioxidants may account for the increase in antioxidant capacity observed, when males return to feeding, similar to what was seen in Morales et al. (2004) and their study of *Dentex dentex*. Although we demonstrated that a reduction in antioxidants occurred in males guarding eggs and embryos compared to males guarding larvae, a decrease in free radical absorbing capacity does not always indicate that oxidative stress is occurring, as the decrease may be within the ability to withstand oxidative damage (Costantini and Verhulst, 2009). Thus the second objective of this study was to show that an increase in oxidative damage occurred as a result of decreased antioxidant capacity.

Examination of oxidative damage to lipid, proteins and DNA revealed that no oxidative damage occurred as a result of a decrease in antioxidant capacity. Lipid peroxides were used as a marker of oxidative stress as they are formed from free radical attack of lipids, which leads to unstable phospholipids and unsaturated fatty acids. These unstable intermediates cause a cascade of reactions ending in the production of MDA, which contains a highly reactive aldehyde group and can perpetuate damage by forming adducts with a) DNA and cause mutations and b) proteins to form advanced lipoxidation end products (ALEs) (Halliwell and Gutteridge, 2007). Our study revealed that there were no differences in MDA concentrations across the parental care period (Table 2), indicating a lack of lipid peroxidation. This finding is not surprising because there is evidence that an increase in adduct formation between MDA and proteins and MDA and nucleic acids is not necessarily accompanied by an increase in oxidative damage to lipids (Dotan et al., 2004). Furthermore, the TBARS assay has several well established limitations. The nature of lipid peroxidation damage depends on the type of free radical initiator and the membrane and lipid composition (Monaghan et al., 2009). Additionally, the presence of metal ions and other contaminants within the sample that are released during cell lysis can initiate lipid peroxidation during sample preparation. Finally the TBARS assay lacks sensitivity as it measures all aldehydes, only some of which are generated by ROS. These aldehydes may cause a high background

Table 2
Mean (±S.E.M.) concentrations of oxidative stress markers across parental care period.

Parental care stage	Lipid peroxides (MDA equ/mg protein)	Oxidized protein ($IDV_{COOH}/IDV_{Protien}$)	Oxidized DNA (10^{-6} ng 8-OHdG/ng DNA)
Egg	1.56 ± 0.18	4.69 ± 0.76	1.60 ± 0.24
Embryo	1.33 ± 0.19	3.55 ± 0.81	1.70 ± 0.24
Larvae	1.50 ± 0.19	4.48 ± 0.77	0.91 ± 0.27

Note — dissimilar letters denote significant differences among the groups (results from ANCOVA with size as covariant $p<0.01$).

for the assay (Monaghan et al., 2009). Any of these factors may have contributed to the lack of detectable changes in levels of MDA in smallmouth bass plasma during parental care.

Protein oxidation was measured by protein carbonyl formation. Oxidation of proteins by ROS can induce protein fragmentation or enzyme inactivation that can disrupt cellular processes and biochemical pathways (Hyslop et al., 1988). It is therefore an important indicator of oxidative stress which is often not measured. As with lipid peroxidation, total protein oxidation did not change significantly over the parental care period. However, results not shown revealed that differential oxidation of proteins may have occurred; albeit at concentrations that do not contribute to statistically significant changes when total protein oxidation is measured as a whole. Future studies should focus on the proteins that are being selectively oxidized in smallmouth bass during the parental care period.

The final measure of macromolecular damage tested was DNA damage, as measured by 8-OHdG formation. There are several types of damage caused by free radical attack on nucleotides, but only 8-OHdG results in point mutations. 8-OHdG was measured with a competitive ELISA, which has been shown to be highly sensitive (Shimoi et al., 2002) compared to the HPLC method. Similar to both the lipid peroxidation and protein carbonyl group analyses, no statistically significant change in the levels of 8-OHdG occurred over the parental care period. Although insignificant, the amount of DNA decreased in males guarding embryos and males guarding larvae (Table 2). This corresponds with the significant increase in antioxidant capacity observed (see Fig. 2).

A degree of variability was observed within each marker of oxidative damage, possibly due to the heterogeneity of samples that cannot be accounted for by the collected measures. For example, damage increases with age and therefore varying ages of fish and/or the variability in the number of reproductive periods, may have resulted in high inter-individual variation (Alonso-Alvarez et al., 2010). Other physiological conditions such as testosterone levels (Costantini et al., 2008a; Negre-Salvayre et al., 2010; Costantini et al., 2011) and nutritional condition (Catani et al., 2008) may influence oxidative stress. Parasite load may also affect oxidative stress, since immune activation increases susceptibility to oxidative damage (Bertrand et al., 2006; Costantini and Møller, 2009). Additionally, variations in behavioral traits (e.g. coping style) among individuals may mediate physiological characteristics such as basal metabolic rate and oxidative stress (Costantini et al., 2008b). Although egg score was recorded for these individuals, most nests were of the same size. Future studies may wish to examine if nest size (i.e. the amount of potential offspring) influences parental investment and oxidative stress. Sample heterogeneity may also have been affected by the high abandonment rate observed in these individuals. As these male smallmouth bass are at the northern extent of their range, the costs of parental care can often outweigh the fitness benefits of a given brood and the male may abandon its nest (Philipp et al., 1997). It is possible that the individuals that maintained parental care through to offspring independence were the best suited to withstand the costs of parental care, and therefore the observations in the final stage of parental care may not be entirely representative of the whole nesting male population. Finally, due to the small number of nesting smallmouth bass at the study sites, sample sizes for each stage were small. Despite the limitations of this study, similar results were reported for female house mice where they have mechanisms to attenuate oxidative stress as a result of the increased energetic demands during parental care (Garratt et al., 2011).

The pro-oxidant buffering capacity in nest-guarding males was sufficient to absorb any ROS and prevent damage from being incurred during parental care. It is also possible that due to restricted caloric intake, fewer ROS were generated (Morales et al., 2004). It has been previously demonstrated that nesting male smallmouth bass have increased oxygen consumption, and thus higher metabolism compared to non-nesting males as a result of both elevated locomotor activity (Cooke et al., 2002) as well as higher levels of cardiac output likely due to high levels of androgens (Cooke et al., 2010). Greater oxygen consumption during periods of starvation may result in fewer ROS being produced, due to increased uncoupling of mitochondria, which increases mitochondrial efficiency (Speakman et al., 2004). Thus the combination of increased activity with restricted caloric uptake may contribute, not to an increase oxidative stress as originally hypothesized, but to the control of oxidative stress throughout parental care. This is supported by the study of Aydin et al. (2007), in which rats were assigned as either restricted caloric intake or fed ad libitum and treated with varying intensities of exercise. They demonstrated that rats on a restricted caloric uptake were protected from exercise-induced oxidative stress compared to rats fed ad libitum. Future studies are required to correlate metabolic rate to ROS production during times of starvation and increased exercise in fish.

5. Conclusions

This study demonstrated that oxidative stress does not occur during parental care in the smallmouth bass, most likely due to an increased antioxidant buffering capacity found during this period. The results of the present study emphasize the importance of examining both the antioxidant capacity as well as oxidative damage parameters when studying oxidative stress, as overall antioxidant capacity was decreased during the first two stages of parental care in the absence of oxidative damage.

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