

## RESEARCH ARTICLE

# Oxidative ecology of paternal care in wild smallmouth bass, *Micropterus dolomieu*

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## ABSTRACT

Physiologically, oxidative stress is considered a homeostatic imbalance between reactive oxygen species production and absorption. From an ecological perspective, oxidative stress may serve as an important constraint to life-history traits, such as lifespan, reproduction and the immune system, and is gaining interest as a potential mechanism underlying life-history trade-offs. Of late, there has been much interest in understanding the role of oxidative stress in the ecology of wild animals, particularly during challenging periods such as reproduction. Here, we used a long-term study population of a fish with sole-male parental care, the smallmouth bass, *Micropterus dolomieu*, to examine the associations among oxidative stress indicators and life-history variables in nest-guarding males. In addition, we investigated the potential role of oxidative stress as a physiological mediator of the life-history trade-off decision of paternal smallmouth bass to stay with or abandon their brood. We found that oxidative stress was significantly related to the life history of paternal smallmouth bass, such that older, larger fish with greater reproductive experience and larger broods nesting in cooler water temperatures had lower levels of oxidative stress. However, we found no significant correlation between oxidative stress and nesting success, suggesting that oxidative stress may not be involved in the decision of male smallmouth bass to abandon their brood. Wild fish have been relatively understudied in the emerging field of oxidative ecology, and this study makes noteworthy contributions by revealing interesting connections between the life histories of paternal smallmouth bass and their oxidative status.

**KEY WORDS:** Oxidative stress, Smallmouth bass, Life history, 8-OHdG, Antioxidant

## INTRODUCTION

Although oxygen is essential for all aerobic life on the planet, the dependence of vertebrates upon oxygen for most physiological processes comes at a cost. A small portion of the oxygen used for

metabolic processes is converted into harmful reactive oxygen species (ROS) (Apel and Hirt, 2004). ROS are continuously produced in all tissues of vertebrates during normal metabolism and are capable of travelling in both the circulatory and lymphatic systems (Valko et al., 2007). Although subcellular components, such as DNA, proteins and lipids, are susceptible to attack by ROS, cells have developed effective mechanisms to prevent this damage, repair this damage or both. Antioxidants play an essential role in maintaining ROS at low levels within the cell (Sies, 1997). Oxidative stress can arise as a result of a homeostatic imbalance between the production of ROS and their detoxification by specific degradative enzymes, antioxidant vitamins and other radical scavengers (Lesser, 2006; Sies, 2000). It is important to note, however, that the antioxidant capacity of an organism may not be an accurate indicator of oxidative stress; an organism with a high antioxidant capacity may still exhibit oxidative stress if they also face high ROS production (Costantini and Verhulst, 2009). Furthermore, oxidative stress can be tissue specific with levels of oxidative damage, ROS and antioxidant defences varying across different organs of multicellular organisms (Kammer et al., 2011; Oruc et al., 2004).

The oxidative status of an organism can be affected by various environmental factors. For example, exogenous antioxidants, some of which are obtained exclusively from diet, have been shown to have positive impacts on lifespan in numerous model organisms, such as *Mus musculus*, *Drosophila melanogaster* and *Caenorhabditis elegans*, as reviewed by Sadowska-Bartosz and Bartosz (2014). Studies have found increases in the biomarkers of oxidative damage as a result of higher predation threat in the Catarina scallop (*Argopecten ventricosus*; Guerra et al., 2013) and damselfly larvae (*Enallagma cyathigerum*; Guerra et al., 2013; Janssens and Stoks, 2013). Levels of oxidative stress have also been shown to increase in fish living outside of their thermal optima (Vinagre et al., 2012). The contraction of skeletal muscles during exercise naturally generates ROS that are a product of that contraction, and in rats prolonged exercise and excess ROS cause oxidative stress to that muscle tissue (Powers and Jackson, 2008). Hypoxic stress also results in the release of ROS from mitochondria in multiple species (Murphy, 2009). Finally, a variety of different anthropogenic factors, including pesticides (Slaninova et al., 2009) and metals (Sevcikova et al., 2011), can also influence the oxidative status of fish. In summary, it is clear that oxidative stress plays a role in the ecology of wild animals and, as a result, there has been increasing interest in understanding this 'oxidative ecology' (Beaulieu et al., 2013), particularly during challenging life-history periods such as reproduction.

For any organism, reproduction is a major life-history event that also comes at a cost (Bell, 1980; Reznick, 1985), thereby providing a classic example of a life-history trade-off, i.e. the use of finite resources for current reproductive efforts versus saving those

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resources for investment in future reproductive opportunities (Williams, 1966). This trade-off is particularly relevant for animals that provide parental care, given their potential requirement for high investments of energy and resources during reproduction (Trivers, 1972). Recently, the association of oxidative stress with reproduction has been investigated in a number of organisms, including the house mouse (*Mus musculus domesticus*; Garratt et al., 2011), the zebra finch (*Taeniopygia guttata*; Alonso-Alvarez et al., 2010) and the smallmouth bass (*Micropterus dolomieu*; Wilson et al., 2012). The relationship between oxidative stress and reproduction is complex and context dependent and, depending on the species and environmental conditions, oxidative stress may have either a stimulatory or inhibitory effect on reproduction. In fact, it may be that oxidative stress is a result of reproduction itself (Metcalf and Alonso-Alvarez, 2010) or that reproduction protects an individual against oxidative stress (Costantini et al., 2014).

In teleost fish, parental care is often undertaken by males (Blumer, 1979; Breder, 1936; Smith and Wootton, 1995). The smallmouth bass is a freshwater fish species in which only the males exhibit nest- and brood-guarding behaviour. This paternal care requires the significant investment of energy and resources from the male for up to four weeks, until the offspring are able to feed and evade predators (Brown, 1984; Cooke et al., 2002; Ridgway, 1988). Caring for the brood entails nest maintenance, oxygenation of eggs and recently hatched larvae, and protection from potential brood predators, which limits feeding by the male while at the same time requiring the male to be significantly more active (Cooke et al., 2002; Hanson et al., 2009). Due to a variety of circumstances, the male may choose to abandon its brood before the offspring reach independence, resulting in the predation of the entire brood and hence an unsuccessful reproductive attempt (Steinhart et al., 2008). This post-spawning life-history decision for male smallmouth bass (i.e. whether to continue guarding its brood or to abandon it) rests upon the male's assessment of the value of that current brood compared with the expected value of its future broods (Williams, 1966). For smallmouth bass, mating success (i.e. the number of eggs deposited in the nest) is a good indicator of current brood value and, as expected, parental males invest more energy in defending larger broods (Ridgway, 1988; Suski et al., 2003). The self-assessment of an individual male's future reproductive opportunities, however, is more complex and likely to be related to the age, size and physiological status of the parental male, which in turn is affected by the individual's age at maturation and previous reproductive history. Smallmouth bass can mature at various ages both within and among populations, from as young as 2–3 years to as old as 6–7 years (Barthel et al., 2008), which can result in an array of different growth trajectories and longevity expectations. In general, if age at maturation is similar among males within a population, then younger (and smaller) males usually have less valuable current broods (i.e. they receive fewer eggs) when compared with older (and larger) males. At the same time, however, these younger, smaller males would expect to have greater opportunities for future reproduction, simply because they presumably would have a longer period until senescence. All other considerations being equal, the younger, smaller males would, therefore, be expected to abandon their nests more readily than larger, older ones. In addition, because of allometric differences in their energy stores (Mackereth et al., 1999), smaller males also acquire relatively more physiological costs during parental care than larger males (Hanson and Cooke, 2009), creating a difference in their survival probability to the next year (Suski and

Ridgway, 2007). These smaller, younger males increase their survival likelihood both by spawning later in the season, when water temperatures are higher (and developmental rates of offspring are faster) and by curtailing their parental care activities somewhat sooner than larger, older males. Both of these strategies, however, reduce the relative survival of their brood (Parkos et al., 2011). As a result, smaller, younger males that spawn and provide enough parental care to raise their broods to independence, do so even though, when compared with their larger and older counterparts in that same population, the probability of their survival to spawn again and the value of their current brood size are both lower than for older, larger males.

Here, we explore the role that oxidative stress might play in the complex reproductive ecology of smallmouth bass, *M. dolomieu* Lacepède 1802. We propose that oxidative stress may be correlated with life-history traits, such as male age and reproductive history. As oxidative stress results in a lowered physiological condition for parental care-providing smallmouth bass (i.e. due to the deleterious effects of ROS damage at both the cellular and organismal levels), oxidative stress may, therefore, provide the physiological mechanism driving life-history trade-offs (Birnie-Gauvin et al., 2017) such as the decision of whether or not to spawn in the spring, as well as the decision of each male that did spawn whether to continue to guard or to abandon its brood throughout the parental care period. In this study, we considered the following alternative hypotheses for oxidative stress relative to life history at the beginning of the parental care period in smallmouth bass, outlined below.

The null hypothesis is that oxidative stress plays no role in the life-history decision of male smallmouth bass. It may also be possible that these males require a certain threshold level of oxidative stress to serve as a signal for them to initiate spawning. In either case, in a given smallmouth bass population, the null hypothesis would predict that at the beginning of the reproductive season, oxidative stress levels would be similar among all of the males that choose to spawn, regardless of size or age. However, if oxidative stress does play a role in the reproductive ecology of smallmouth bass, we would expect that oxidative stress would be correlated to male age and reproductive experience, and would be predictive of whether parental males ultimately abandon or succeed in their reproductive attempts. We therefore propose two alternative hypotheses that take this theory into consideration.

Our first alternative hypothesis is that the ability to reduce oxidative stress by lowering ROS increases with age, prior reproductive experience or both, and provides different starting points for individual males of different ages and experience levels as they begin spawning activities. The relationship between oxidative stress and age is complex and not fully understood (Lapointe and Hekimi, 2010), and oxidative stress has been shown to decrease in older individuals in other species such as the rhesus macaque (Georgiev et al., 2015) and the zebra finch (Alonso-Alvarez et al., 2010). Under this hypothesis, we theorise that oxidative stress levels in nest-guarding fish accumulate over the reproductive period and ultimately act as a trigger to terminate parental care when they reach a certain level. For example, older, larger male smallmouth bass may come out of the overwintering period with lower oxidative stress than younger, smaller males. Older males may therefore initiate spawning with lower levels of oxidative stress than smaller, younger ones and would therefore be capable of accumulating more oxidative stress during parental care before they reach some physiological threshold that would trigger a decision to abandon

the brood. This hypothesis predicts that those males with the lowest levels of oxidative stress at the initiation of spawning activities would be older fish that, because of these low initial oxidative stress levels, would be able to invest in their broods for longer and would be less likely to abandon their broods prematurely.

Our second alternative hypothesis is that oxidative stress might accumulate with age, prior reproductive experience or both, providing different starting points for individual males of different ages and prior experience as they begin spawning activities [i.e. an idea that stems from both the free radical theory of ageing (Harman, 1956) and the mitochondrial theory of ageing (Wei et al., 2001; but also see review by Metcalfe and Alonso-Alvarez, 2010)], and it is this variation that influences the life-history decision to stay or leave the brood (i.e. the levels of initial oxidative stress inform the individual of its 'relative age' and hence its likelihood for survival to the next year). This hypothesis predicts that those males with the highest levels of oxidative stress at the initiation of spawning activities would be older, more experienced fish. These fish, however, still choose to invest in their broods longer, because those high levels inform them of their lowered probability for survival and future reproductive efforts.

## MATERIALS AND METHODS

### Study site, study animals and sampling procedure

The fish used in the current study were parental care-providing males captured in Miller's Lake, part of the Mississippi River system in eastern Ontario, Canada (44°56'26.5"N, 76°41'55.0"W). This population has been the subject of a long-term (>20 years) study on the reproductive ecology of smallmouth bass, and the reproductive history of all adult males reproducing in the system is known for the study period (Barthel et al., 2008). For a full description of the study site, see Barthel et al. (2008). Briefly, the study site is composed of distinct upstream riverine (Mississippi River) and downstream lacustrine (Miller's Lake) habitats, separated by a 1 m waterfall that is not a complete barrier to smallmouth bass movement. The study site is bordered upstream by a 12 m waterfall and downstream by an extensive series of rapids and waterfalls, both of which serve as barriers to at least upstream movement. Radio telemetry studies have not detected fish movement out of the study site in either direction (Barthel et al., 2008). The study site is relatively isolated and receives negligible fishing pressure.

Fish used in the current study represent a subset of the fish used by O'Connor et al. (2012) to determine associations between life history and plasma physiology. As these fish are part of a long-term study population, it was necessary for all sampling to be non-lethal, and so only plasma samples were obtained. If we had used a lethal sampling approach to obtain tissues such as heart, brain or liver, we would have removed the majority of nesting bass for a given year, which would have serious ecological consequences. Previous studies have used plasma for the assessment of oxidative protection and damage (reviewed in Birnie-Gauvin et al., 2017), including research on nesting bass (see Wilson et al., 2012). In addition, other studies on teleost fish have compared different tissues and revealed that plasma concentrations are related to those in other tissues (Taylor et al., 2015).

For full details of the study and the sampling procedures, see O'Connor et al. (2012). Briefly, in May and June 2009, snorkellers identified parental males on nests. Only those fish guarding fresh eggs (<24 h old) were selected for sampling; this timing was required to account for decreases in androgen concentration over the course of parental care (O'Connor et al., 2011). The snorkeller assigned an egg score to each nest; the egg score is between 1 and 5,

and indicates relative egg number, with 1 indicating few eggs and 5 indicating a large number of eggs (Stein and Philipp, 2015). Nest-guarding males were then caught using targeted rod and reel angling, and blood plasma samples were obtained using caudal puncture and lithium-heparinised 3 ml Vacutainer-style syringes (Becton, Dickinson, Franklin Lakes, NJ, USA). Separated plasma samples (initially frozen in liquid nitrogen in the field and then transferred to a -80°C freezer) were used to determine circulating concentrations of androgens and cortisol for a previous study (O'Connor et al., 2012), and antioxidant capacity and oxidative damage in the current study. Scales were collected to allow determination of age, and water temperature was recorded before the fish was released.

Smallmouth bass reproduce in both the river and lake habitats and tend to show high nest-site fidelity (Barthel et al., 2008). Parental males in the two habitats differ in physiology (O'Connor et al., 2012) and life history (Barthel et al., 2008; O'Connor et al., 2012). For simplicity, and to eliminate any discrepancies caused by nesting location, only the fish from Miller's Lake were used in the current study, resulting in a sample size of  $N=57$  individuals. See Table 1 for measured characteristics from these fish.

### Antioxidant capacity

Antioxidant capacity was determined using the oxygen radical absorbance capacity (ORAC) assay as described by Gliwa et al. (2011). Reactions were carried out in triplicate in 96-well Costar microplates to develop fluorescence decay curves using a fluorometric microplate reader (fX800 Multi-Detection Microplate Reader; Biotek, Winooski, VT, USA), measuring excitation and emission peaks at 485 nm and 528 nm, respectively, using Gen5 software (Biotek). Potassium phosphate buffer (pH 7.4) was used to prepare other necessary solutions and as a diluting agent. Owing to the high antioxidant capacity of the plasma samples, dilutions of 1:200 were necessary. Aliquots (20  $\mu$ l) of diluted samples were added in triplicate to the wells. A standard curve was prepared by adding 20  $\mu$ l of a synthetic antioxidant, Trolox (prepared to concentrations of 100  $\mu$ mol l<sup>-1</sup>, 50  $\mu$ mol l<sup>-1</sup>, 25  $\mu$ mol l<sup>-1</sup>, 12.5  $\mu$ mol l<sup>-1</sup> and 6  $\mu$ mol l<sup>-1</sup>), in triplicate to the wells. Potassium phosphate buffer (20  $\mu$ l) served as a blank and rutin (200  $\mu$ mol l<sup>-1</sup>, 20  $\mu$ mol l<sup>-1</sup> and 10  $\mu$ mol l<sup>-1</sup>) functioned as a positive control. Working fluorescein solution (120  $\mu$ l), prepared to a final concentration of 0.129  $\mu$ mol l<sup>-1</sup> from a concentrated stock solution, was added to each well using a multichannel pipette. The plate was then warmed to 37°C by the microplate reader and incubated at this temperature for 20 min. Following this incubation period, 60  $\mu$ l of 0.16  $\mu$ mol l<sup>-1</sup> 2,2'-azobis (2-amidinopropane) dihydrochloride was rapidly added to each well and the plate was placed back in the microplate reader. The plate was shaken vigorously for 15 s before fluorescence was read every 60 s for 50 min. Only the inner 60 wells were used to prevent temperature from having an effect on the outer wells. Protein concentration of

**Table 1. Summary of life history and physiological data collected from parental care-providing male smallmouth bass from Miller's Lake, ON, Canada (N=57)**

Variable	Mean	Median	s.e.m.	Range
Age (years)	5.2	4.0	0.3	3.0–12.0
Total length (mm)	285	268	8	196–482
Number of previous nests	0.9	0.0	0.2	0.0–7.0
Egg score	2.5	2.0	0.1	1.0–5.0
Water temperature (°C)	15.4	15.5	0.1	14.0–16.5

each plasma sample (1:200 dilution) was subsequently determined using the BCA assay (Smith et al., 1985).

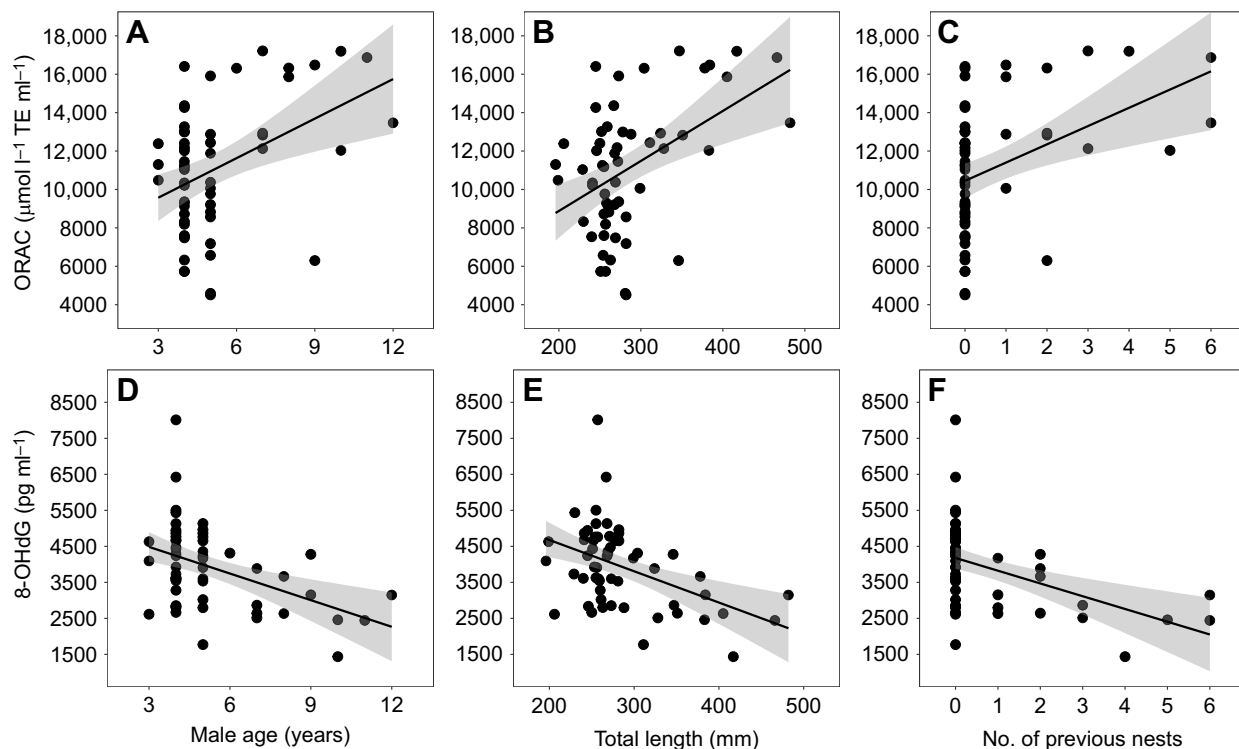
### DNA oxidative damage

Oxidative damage to DNA was quantified by measuring the amount of three oxidised guanine species in plasma: 8-hydroxy-2'-deoxyguanosine (8-OHdG; DNA), 8-hydroxyguanosine (RNA) and 8-hydroxyguanine (DNA/RNA). The concentrations of these species were determined using a DNA/RNA oxidative damage enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA, catalogue number: 589320). No pre-assay preparation of the sample was required but the plasma was diluted 1:20 using reconstituted EIA buffer to compensate for the high concentration of 8-OHdG present. Diluted samples (50  $\mu\text{l}$ ) were then added, in triplicate, to the goat anti-mouse IgG-coated plate. EIA standards were prepared at decreasing concentrations (3000, 1333, 592.6, 263.4, 117.1, 52.0, 23.1 and 10.3  $\text{pg ml}^{-1}$ ) by serial dilution with the EIA buffer; 50  $\mu\text{l}$  of these standards were also run on the plate in triplicate. Two blank wells and one well for determination of total activity were included on the plate. Two non-specific binding wells, containing 100  $\mu\text{l}$  EIA buffer, were included as negative controls. Maximum binding wells that contained 50  $\mu\text{l}$  of EIA buffer were run in duplicate. Acetylcholinesterase (AChE) tracer solution (50  $\mu\text{l}$ ) was added to each well except those used for measurement of total activity and the blank well. Monoclonal antibody solution (50  $\mu\text{l}$ ) was then added to each well with the exception of the blank, wells for total activity and wells for non-specific binding. The plates were then covered for an 18 h incubation period at 4°C. The wells were emptied and rinsed five times with the wash buffer. Reconstituted Ellman's Reagent (200  $\mu\text{l}$ ) was then added to each well and 5  $\mu\text{l}$  of the AChE

tracer solution was added to the total activity well only. The plate was then shielded from the light and placed on an orbital shaker for 90 min to allow optimum development of colour. Finally, absorbance of the wells was measured at 412 nm on an Epoch microplate reader (Biotek) using Gen5 Data Analysis Software (2.00.18, Biotek).

### Protein carbonyl content (PCC) analysis

Protein carbonyl groups were analysed using a Biocell protein carbonyl enzyme ELISA assay kit (Northwest Life Science Specialities, Vancouver, WA, USA, catalogue number: BPCK01) as a measure of protein oxidative damage. Protein concentration of the plasma samples was predetermined using the BCA assay (Smith et al., 1985); 5  $\mu\text{l}$  of the sample was then appropriately diluted in 200  $\mu\text{l}$  dinitrophenylhydrazine (DNP) solution. Diluted plasma samples (200  $\mu\text{l}$ ) were added to each well. A standard curve was also prepared using six oxidised protein standards. Each sample and standard was run in triplicate on the plate. The plate was then sealed and incubated at 4°C for 18 h. EIA buffer (300  $\mu\text{l}$ ) was used to wash the wells five times. Diluted blocking solution (250  $\mu\text{l}$ ) was added to each well followed by a 30 min incubation period at room temperature. The wells were then washed with EIA buffer as before. Anti-DNP-biotin-antibody (200  $\mu\text{l}$ ) was added to each well and the plate was incubated at 37°C for 1 h; this was again followed by the wash procedure described above. Finally, 200  $\mu\text{l}$  of streptavidin-horseradish peroxidase was added to each well, incubated for 1 h at room temperature, then subsequently washed using the previously described method. Chromatin reagent (200  $\mu\text{l}$ ) was added to each well to allow for colour development. The reaction was followed at 650 nm on the Epoch microplate reader (Biotek) until the highest concentration standard reached an absorbance of 0.5. At this point



**Fig. 1. The relationships between observed variables used to derive 'life history' and 'oxidative stress'.** Correlations between antioxidant capacity (A–C) and DNA oxidative damage (D–F) and each of male age, total length and number of previous nests are shown ( $N=57$ ). Oxidative stress parameter values represent the mean from experiments performed in triplicate. Spearman rank correlations were applied to test for a significant correlation between variables ( $P$ -values are presented in Table 2). Relationships between these life-history variables and protein oxidative damage are not included as no significant correlations were detected in our univariate analysis. ORAC, oxygen radical absorbance capacity; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

the reaction was inhibited using 100  $\mu$ l of the stopping reagent, resulting in a colour and absorbance change that was measured at 450 nm on the microplate reader.

### Statistical analysis

Previous research in this system has shown that there are correlations among many of the life-history variables, e.g. older males are also larger, have more reproductive experience, have nests with higher egg scores and tend to nest earlier in the year at cooler water temperatures than smaller males (Barthel et al., 2008; O'Connor et al., 2012). To assess our alternative hypotheses, while also taking into account the known correlations among measured variables, we used two statistical approaches. First, we used univariate Spearman rank correlations to assess the pairwise correlations between observed variables (male age, number of previous nests, total length, egg score and water temperature) and our measured oxidative stress parameters (antioxidant capacity, protein oxidative damage and DNA oxidative damage). Additionally, we used a logistic regression approach to test for the effect each of our three oxidative stress parameters had on the success of the nesting period for each individual fish.

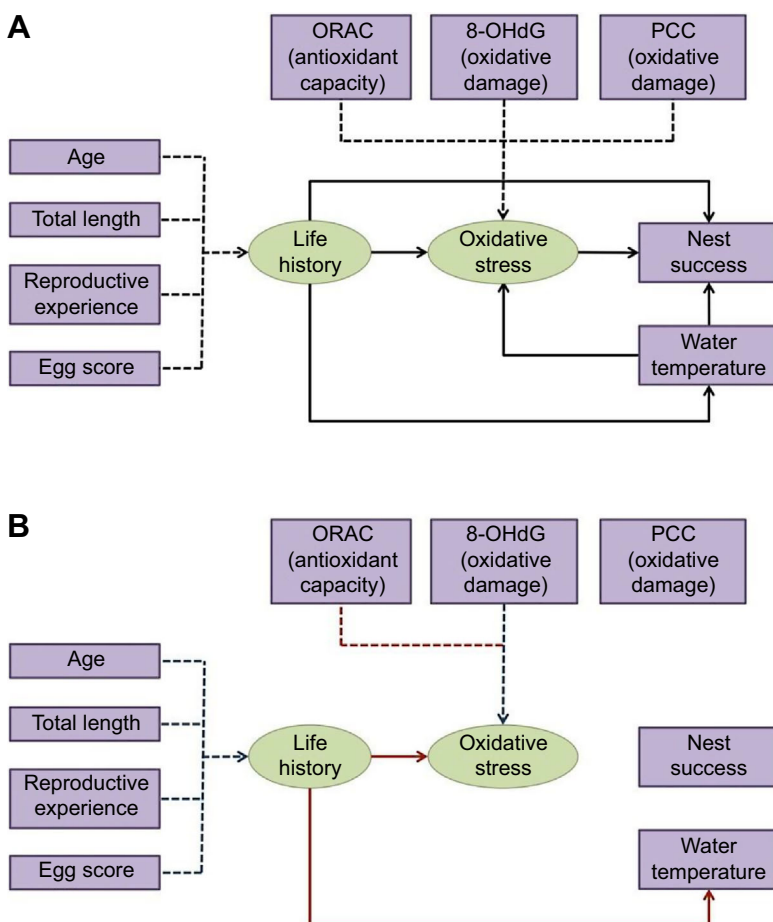
Second, we used a structural equation modelling approach. This approach is based on a multi-step process (Grace et al., 2010; Iriondo et al., 2003). First, the hypotheses are translated into a conceptual model of expected cause-and-effect relationships. In this case, we predicted that life history might influence oxidative stress, and both life history and oxidative stress might influence nest success. We also took into account that in this system, life history influences water temperature at nesting (i.e. older, larger males tend to nest earlier in the year at cooler water temperatures) and, as a

result, we predicted that water temperature might influence oxidative stress, as well as nest success. As the second step in this process, we determined which parameters of the conceptual model could be derived through observed variables. In this case, life history can be derived from male age, male body size (i.e. total length), male reproductive experience (i.e. the number of previous nests) and egg score. Oxidative stress can be derived from antioxidant capacity (i.e. ORAC) and oxidative damage (i.e. 8-OHdG and PCC). Nest success and water temperature can be directly measured. Variables that cannot be directly measured, but are inferred through observed variables (i.e. life history and oxidative stress), are latent variables. See Fig. 2A for the completed conceptual model, with latent and observed variables, as well as the predicted relationships among these variables. Once we had built our conceptual model, we then used the structural equation modelling function in the 'lavaan' package (Rosseel, 2012) to assess the fit of our observed data to the conceptual model. To make effect sizes comparable for all variables, data were scaled and centred prior to analysis. All analyses were conducted using R version 3.2.1 within R Studio (RStudio, Boston, MA, USA).

## RESULTS

### Univariate analysis

Consistent with previous studies (i.e. Barthel et al., 2008; O'Connor et al., 2012), we found strong correlations between many of our life-history and environmental variables (Table 2); older fish were also larger, had a greater number of previous nests, had higher egg scores and were more likely to nest in cooler water temperatures.



**Fig. 2. Conceptual model of the potential relationships between life history, oxidative stress, nest success and water temperature.** Latent variables (i.e. variables that are not directly observed but are inferred from observed variables) are represented as green circles. Observed variables are represented as purple squares. Direct regressions between two variables are represented as solid directional arrows. The inference of latent variables from observed variables is represented as broken lines. (A) In this conceptual model, the latent variable 'life history' is inferred from the observed variables male age, male size (i.e. total length), previous nest experience (i.e. number of previous nests) and egg score, and the latent variable 'oxidative stress' is inferred from antioxidant capacity [i.e. oxygen radical absorbance capacity (ORAC)] and oxidative damage [i.e. 8-hydroxy-2'-deoxyguanosine (8-OHdG) and protein carbonyl content (PCC)]. We predict: that life history will influence oxidative stress and nesting water temperature; that nesting water temperature might influence oxidative stress; and that life history, oxidative stress and nesting water temperature will influence nest success. (B) Model of the significant relationships between life history, oxidative stress, nest success and water temperature identified through the structural equation modelling approach. As above, latent variables are represented as green circles, and observed variables are represented as purple squares. Direct regressions between two variables are represented as solid directional arrows, and the inference of latent variables from observed variables is represented as broken lines. Here, red lines indicate a negative relationship whereas blue lines indicate a positive relationship. Our structural equation modelling approach identified that the latent variable 'life history' is derived from age, total length, reproductive experience and egg score whereas the latent variable 'oxidative stress' is derived from ORAC and 8-OHdG. We found that life history is significantly negatively related to both oxidative stress and water temperature. All other relationships were non-significant ( $P > 0.05$ ; see Tables 1 and 2).

**Table 2. Spearman rank correlation coefficients determined between all pairs of observed variables**

	No. of previous nests	Total length (mm)	Temperature (°C)	Egg score	ORAC ( $\mu\text{mol l}^{-1}$ TE $\text{ml}^{-1}$ )	8-OHdG ( $\text{pg ml}^{-1}$ )	PCC ( $\text{nmol mg}^{-1}$ )
Age (years)	<b>0.77****</b>	<b>0.87****</b>	<b>-0.56****</b>	<b>0.37**</b>	<b>0.31*</b>	<b>-0.39**</b>	0.02
Number of previous nests		<b>0.74****</b>	<b>-0.59****</b>	<b>0.37**</b>	<b>0.46***</b>	<b>-0.52***</b>	0.03
Total length (mm)			<b>-0.59****</b>	<b>0.41**</b>	<b>0.341**</b>	<b>-0.40**</b>	0.06
Temperature (°C)				<b>-0.46***</b>	<b>-0.45***</b>	<b>0.33*</b>	0.16
Egg score					0.08	-0.11	-0.08
ORAC ( $\mu\text{mol l}^{-1}$ TE $\text{ml}^{-1}$ )						<b>-0.40**</b>	<b>0.27*</b>
8-OHdG ( $\text{pg ml}^{-1}$ )							-0.21

Significant correlations are shown in bold (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ).

ORAC, oxygen radical absorbance capacity; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PCC, protein carbonyl content.

With respect to oxidative stress, we found a significant, negative correlation between antioxidant capacity (i.e. ORAC) and DNA oxidative damage (i.e. 8-OHdG), such that individuals with a higher plasma antioxidant capacity also exhibited lower levels of DNA oxidative damage (Table 2). Interestingly, we found the reverse relationship between antioxidant capacity and protein oxidative damage (i.e. PCC), such that individuals with a higher antioxidant capacity also had higher protein oxidative damage; DNA oxidative damage and protein oxidative damage were not correlated (Table 2).

Although protein oxidative damage (i.e. PCC) did not significantly correlate with any of our observed life-history variables (Table 2), both antioxidant capacity (i.e. ORAC) and DNA oxidative damage (i.e. 8-OHdG) were correlated with age, number of previous nests, total length and water temperature. Older, larger fish with more reproductive experience nested at cooler temperatures and had both a higher antioxidant capacity and lower DNA oxidative damage (Table 2; Fig. 1).

Results from our logistic regressions indicate that neither antioxidant capacity, DNA oxidative damage nor protein oxidative damage played a significant role in determining the success of the reproductive period of the smallmouth bass (Table 3).

#### Structural equation modelling: latent variables

Our overall structural equation model converged normally after 52 iterations ( $\chi^2=48.89$ , d.f.=23,  $P=0.001$ ). The latent variable 'life history' was derived from male age, total length, reproductive history and egg score (Table 4, Fig. 2B), with all variables being positively correlated (Table 4, Fig. 2B). The latent variable 'oxidative stress' was derived from ORAC (i.e. a measure of antioxidant capacity) and 8-OHdG (i.e. a measure of oxidative stress); these variables were negatively correlated with one another (Table 4, Fig. 2B). Our measure of protein oxidative damage, PCC, did not significantly contribute to our 'oxidative stress' latent variable (Table 4, Fig. 2B).

**Table 3. Logistic regressions investigating the relationship between oxidative stress parameters and the nesting success of paternal smallmouth bass**

Success	Standard error	Z-value	P-value
ORAC ( $\mu\text{mol l}^{-1}$ TE $\text{mg}^{-1}$ )	<0.001	0.04	0.97
8-OHdG ( $\text{pg ml}^{-1}$ )	<0.001	-1.38	0.17
PCC ( $\text{nmol mg}^{-1}$ )	7.45	1.65	0.10

Nesting success is defined as whether the male successfully raised the brood to independence or prematurely decided to abandon its brood.

ORAC, oxygen radical absorbance capacity; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PCC, protein carbonyl content.

#### Structural equation modelling: relationships among variables

We found that life history significantly predicted nesting water temperature. As with previous studies (O'Connor et al., 2012), we found that males with higher life-history values (i.e. older, larger, more experienced males with higher egg scores) nested earlier in the year at cooler water temperatures (Table 5, Fig. 2B). We did not, however, find that water temperature significantly influenced oxidative stress (Table 5, Fig. 2B). In addition, life history directly influenced oxidative stress. Males with 'higher' life-history values (i.e. older, larger, more experienced males with higher egg scores) had lower oxidative stress values (i.e. lower ORAC and higher 8-OHdG) (Table 5, Figs 2B and 3). With this dataset, we did not find that life history, oxidative stress or water temperature directly influenced nest success (Table 5, Fig. 2B).

#### DISCUSSION

Because oxidative stress in an ecological context is an increasingly important field of research, this study aimed to investigate the potential role that oxidative stress may play as a 'physiological messenger' in influencing the life history of male smallmouth bass. We proposed alternative hypotheses that could explain the potential relationships between oxidative stress and life history in parental care-providing smallmouth bass and could shed light on how oxidative stress may influence reproductive decisions, such as the decision to continue care or to prematurely abandon a brood. We found that oxidative stress is associated with the life history of a fish at the beginning of the parental care period,

**Table 4. Parameter estimates for the inference of the latent variable 'life history' from observed variables and the inference of the latent variable 'oxidative stress' from antioxidant capacity (i.e. ORAC) and oxidative damage (i.e. 8-OHdG and PCC)**

Latent variable	Observed variable	Standard		Z-value	P-value
		Estimate	error		
Life history	<b>Age (years)</b>	<b>1.00</b>	<b>0.00</b>	<b>n.a.</b>	<b>n.a.</b>
	<b>Total length (mm)</b>	<b>0.99</b>	<b>0.04</b>	<b>24.48</b>	<b>&lt;0.001</b>
	<b>Number of previous nests</b>	<b>0.92</b>	<b>0.06</b>	<b>14.87</b>	<b>&lt;0.001</b>
	<b>Egg score</b>	<b>0.54</b>	<b>0.12</b>	<b>4.68</b>	<b>&lt;0.001</b>
Oxidative stress	<b>ORAC (<math>\mu\text{mol l}^{-1}</math> TE <math>\text{ml}^{-1}</math>)</b>	<b>-1.00</b>	<b>0.00</b>	<b>n.a.</b>	<b>n.a.</b>
	<b>8-OHdG (<math>\text{pg ml}^{-1}</math>)</b>	<b>0.85</b>	<b>0.27</b>	<b>3.16</b>	<b>0.002</b>
	<b>PCC (<math>\text{nmol mg}^{-1}</math>)</b>	<b>-0.26</b>	<b>0.23</b>	<b>-1.15</b>	<b>0.25</b>

Bold indicates observed variables that significantly contribute to the derivation of the latent variables ( $P < 0.05$ ).

The observed variables are male age, male size (i.e. total length), previous nest experience (i.e. number of previous nests) and egg score.

ORAC, oxygen radical absorbance capacity; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PCC, protein carbonyl content; n.a., not applicable.

**Table 5. Parameter estimates for the regressions between life history, oxidative stress, nest success and nesting water temperatures**

Dependent variable	Predictor variable	Estimate	Standard error	Z-value	P-value
Water temperature	<b>Life history</b>	<b>-0.73</b>	<b>0.10</b>	<b>-7.59</b>	<b>&lt;0.001</b>
	<b>Life history</b>	<b>-0.30</b>	<b>0.15</b>	<b>-1.97</b>	<b>0.04</b>
Oxidative stress	Water temperature	0.24	0.15	1.62	0.11
	Life history	0.18	0.23	0.79	0.43
Nest success	Oxidative stress	-0.11	0.41	-0.28	0.78
	Water temperature	0.08	0.21	0.38	0.71

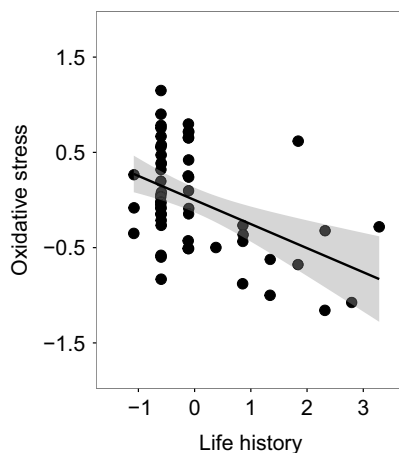
Bold indicates significant relationships between the variables ( $P < 0.05$ ).

such that older, larger males with higher egg scores and greater reproductive experience have lower oxidative stress than younger, smaller males with lower egg scores, which provides support for our first alternative hypothesis. However, we did not find that either oxidative stress or life history significantly influenced the life-history decision of a parental male smallmouth bass to stay with or abandon their brood.

### Individuals with 'higher' life-history values exhibit lower levels of oxidative stress

Our first alternative hypothesis predicted that individual fish would have different initial oxidative levels at the beginning of the reproductive period, with older, larger, more experienced fish having lower initial levels of oxidative stress, because these fish are at a physiological advantage over younger, smaller, less experienced fish. In support of this hypothesis, we found that fish with higher life-history values (i.e. older, larger, more experienced fish with higher egg scores) had lower oxidative stress values (i.e. less DNA oxidative damage and a higher antioxidant capacity) at the beginning of the parental care period.

Accordingly, we found no support for the second alternative hypothesis that proposed that oxidative stress may be higher in older, more experienced fish relative to younger, less experienced



**Fig. 3. The relationship between the latent variables 'life history' and 'oxidative stress' for all individuals ( $N=57$ ) as determined from structural equation modelling ( $P=0.04$ ; Table 5).** The values for life history are derived from male age, male size (i.e. total length), previous nest experience (i.e. number of previous nests) and egg score; high values indicate individuals with relatively high values for all observed variables. The values for oxidative stress are derived from antioxidant capacity [i.e. oxygen radical absorbance capacity (ORAC)] and oxidative damage [i.e. 8-hydroxy-2'-deoxyguanosine (8-OHdG)]; high values indicate individuals with relatively low values for ORAC and relatively high values for 8-OHdG.

fish, as a consequence of ageing, previous reproductive experience or both. Although there is a large amount of evidence supporting the accumulation of oxidative damage to macromolecules with age (Sohal et al., 2002), there is disagreement over the role that oxidative stress may or may not play in ageing, and the correlation is not always so simple (Andziak and Buffenstein, 2006; Lapointe and Hekimi, 2010). Indeed, other studies have found either a negative relationship (Georgiev et al., 2015), one similar to the relationship found in the current study or have found parabolic relationships (Alonso-Alvarez et al., 2010), something that deviates from the commonly held view that oxidative damage increases with age, reproductive experience or both.

There are several potential explanations for why older, larger, more experienced males might enter into the parental care-providing period with lower oxidative stress than younger, smaller, less experienced males. First, smallmouth bass spawn in the spring directly following a challenging overwinter period. Larger males have greater energy stores than smaller males (Mackereth et al., 1999) and, therefore, the lower oxidative stress in the older, larger, more experienced males may reflect the physiological advantage that these larger males have over the smaller males emerging from the winter.

Second, older, larger, more experienced males tend to nest earlier in the year at lower water temperatures. Owing to the strong association between metabolism and water temperature in fish (Clarke and Fraser, 2004; Fry, 1947, 1971), it may be that fish nesting in warmer water, with higher metabolic rates, are in turn faced with increased free radical production and, ultimately, higher oxidative stress. However, our results showed no direct relationship between oxidative stress and nesting water temperature, which suggests that the energetic advantage of large males over small males, rather than water temperature, influenced the pattern that we saw between life history and oxidative stress.

Prior selection may also be an influencing factor on the oxidative status of an individual male smallmouth bass; oxidative stress is considered a disadvantage to an individual, and we would therefore predict that fish with a higher antioxidant capacity and lower accumulated oxidative damage would be selected for (i.e. selection would promote a slower production of ROS, a more efficient oxidative damage repair process or an increase in antioxidant capacity). As a result, those larger, older males with a greater lifetime reproductive output, as compared with smaller, often semelparous, males (Barthel et al., 2008), may be superior from a physiological and oxidative perspective, passing along a strong selection gradient, thereby allowing for iteroparous behaviour and a longer lifespan. Because oxidative stress has deleterious consequences for the general health and physiological status of an organism, individuals with a lower, more beneficial oxidative status would be expected to have a longer lifespan.

### Age of maturation may also influence oxidative stress in reproducing males

Finally, one important consideration that we have until now evaded is the possible effect on life-history decisions of differences in age at first maturation. For many of the fish within our current study sample, this was their first time reproducing. Therefore, for these individuals, differences in age represent differences in age at maturation, and the results we found may be driven at least in part by factors related to age at maturation. We know that, on average, smaller males not only have smaller broods (as shown in the Results section) but also incur relatively greater physiological costs (because they have lower relative energy stores; Hanson and

Cooke, 2009). We may, therefore, expect individuals that mature at a younger age to accumulate greater levels of oxidative damage during courtship, spawning and parental care relative to males that delay maturation.

Further, we know that the age at first maturation in smallmouth bass can vary not only among populations but also, to at least some extent, within populations as well (Dunlop et al., 2005). Often, those fish with faster growth rates seem to reach maturation at an earlier age (Dunlop et al., 2005; Raffetto et al., 1990). The individual decision of male smallmouth bass to mature or to delay maturation until following years depends, to a great extent, on certain environmental influences. For a population that lives in a habitat that has perennial high survival risks (e.g. river systems with huge winter or spring floods, lentic systems with a high frequency of winter kills), maturing at the earliest possible age could be the best evolutionary strategy (Bertschy and Fox, 1999). For other populations, the driving factor is likely to be a social one (i.e. the ability of a male to compete for mating success with the rest of the population if it were to mature for the upcoming spawning season versus delaying maturation until some subsequent year). In this way, a population of smallmouth bass with a smaller mean total length will promote young and, therefore, small males to mature. Males of the same age and size would be at a disadvantage in a population with a high mean total length and thus would be expected to delay maturation until they reached a larger size. Observations of increased mortality and a tendency towards semelparity in those individual fish that mature at a younger age (Barthel et al., 2008) suggest that delayed maturation may be beneficial to an individual in terms of increasing lifespan and total reproductive output. In zebra finches, increased oxidative damage has been shown to be a consequence of accelerated growth (Alonso-Alvarez et al., 2007), thereby explaining the negative impacts of fast growth and early maturation. Under this hypothesis, specifically for smallmouth bass, if a male reaches maturation at an earlier age, then we would expect that individual to have higher levels of oxidative stress than males that delay maturation. Explicitly investigating age at maturity relative to oxidative stress and comparing first-time nesting males of a given age with males of the same age that choose to delay reproduction is a promising opportunity for future research.

#### **Oxidative stress does not act as a physiological mediator of the decision of a parental male to stay with or abandon its brood**

The life-history trade-off theory associated with reproduction is well established (Williams, 1966) but the ‘currency’ that is used by organisms to mediate this trade-off is still under much debate. Oxidative stress has been suggested as a potential mediator to this trade-off, due to the ability of harmful ROS to damage subcellular components (Monaghan et al., 2009), and recent suggestions that oxidative stress biomarkers can act as indicators of the general health of an organism (Hau et al., 2015). Previous studies have found that older, larger, more experienced males are less likely to prematurely abandon their broods relative to younger, smaller, less experienced males (e.g. O’Connor et al., 2012). We proposed that oxidative stress might mediate this decision. In addition, the pattern of abandonment relative to life history may arise either because older, larger individuals have lower initial levels of oxidative stress (and may, therefore, be further from a theoretical abandonment threshold of oxidative stress) or because older, larger individuals have higher levels of oxidative stress as a result of ageing or reproductive experience. One of these alternatives would then provide the cue that these individuals have fewer future reproductive

opportunities. Although we found that older, larger individuals have lower initial levels of oxidative stress, providing some indirect support for the first hypothesis, we failed to find a direct relationship between oxidative stress and nest success.

Our study has several limitations that need to be taken into consideration in interpreting these results. First, with our dataset, we failed to find a relationship between life history and nest success, which is inconsistent with previous research. The fish investigated in the current study are a subset of the fish used during the study by O’Connor et al. (2012), and they did find a relationship between a life-history trait (i.e. body size) and nest success. Because this relationship has been found previously, our null result suggests that larger sample sizes, or samples with higher variance in life-history traits and nest success, may be necessary to detect relationships, e.g. O’Connor et al. (2012) had a larger sample size of  $N=131$  compared with our subset of  $N=57$ . O’Connor et al. (2012) also used fish from both the riverine and the lacustrine habitats whereas only fish nesting in the lake were used in the current study. Fish from the lake and river differ in several key characteristics, including body size (fish nesting in the river are smaller than fish in the lake) and reproductive success (fish nesting in the river have lower reproductive success than fish in the lake). In this study, we intentionally used only fish from the lake in order to avoid confounds based on habitat type but, in doing so, we also reduced the variance in life-history traits and nest success within our subset of fish. A future study with a larger sample and a wider variance in life-history traits may be necessary to fully elucidate the relationships among life history, oxidative stress and nest success.

Second, our study only looked at initial oxidative parameter measurements (i.e. at the beginning of the parental care period; males found on eggs <24 h old). Because a recent study in smallmouth bass suggested that oxidative stress does not increase throughout the parental care period (Wilson et al., 2012), it could be reasoned that oxidative stress is not an influencing factor on the life-history trade-off decision of male smallmouth bass to either stay with or to abandon their brood. The relationship between oxidative stress and reproduction, however, is complex and often indirect and context dependent (Metcalf and Monaghan, 2013). Further studies that simultaneously assess life history, the accumulation of oxidative stress during parental care and nest success are needed to draw strong conclusions about how life history and the accumulation of oxidative stress might interact to influence nest success.

#### **Conclusion**

Understanding if and how oxidative ecology is associated with different components of parental care and life-history decisions represents a significant knowledge gap, not just for smallmouth bass or teleost fish, but more broadly within parental care-providing vertebrates (Birmie-Gauvin et al., 2017; Costantini, 2016). We found that older, more experienced fish show reduced oxidative stress entering the parental care period. This supports a model in which older parents are more physiologically capable and resistant to oxidative stress, rather than a model where oxidative stress accumulates as a cost of ageing, reproduction or both. This finding is contrary to the commonly held view that oxidative stress accumulates with age (Birmie-Gauvin et al., 2017), and provides insight into physiological mechanisms behind the life-history trade-off of reproductive male smallmouth bass.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: S.J.C., D.P.P., C.M.O. and L.K.E. Methodology: C.M.O. and L.K.E. Formal analysis: C.M.O. and L.K.E. Formal analysis and investigation: C.M.O., B.L.B., D.P.P. and L.K.E. Resources: W.G.W. and C.M.O. Writing – original draft preparation: L.K.E., C.M.O., S.J.C. and D.P.P. Writing – review and editing: L.K.E., C.M.O., S.J.C., D.P.P., B.L.B., W.G.W., K.M.G. and G.V.D.K. Visualisation: L.K.E. and C.M.O. Project administration: L.K.E. Funding acquisition: S.J.C., W.G.W. and G.V.D.K.

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### Data availability

Data are available from Dataverse: <http://doi.org/10.5683/SP/QB7WYT>

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