Parental care behaviour in response to perceived paternity is not mediated by 11-ketotestosterone in bluegill sunfish

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ARTICLE INFO

ABSTRACT

Parental care is critical for the survival of many young animals, but parental care can be costly to the individual providing care. To balance this cost, parents can allocate their care to offspring based on their value, which can be dependent on the offspring's relatedness to the parent. Bluegill (*Lepomis macrochirus*) is a fish characterized by uniparental male care and high levels of cuckoldry. While parental males of this species have been shown to adaptively adjust their care in response to paternity, the mechanisms for this adjustment are not well understood. Androgens are steroid hormones that are associated with parental care behaviours in many species including bluegill. Here, we test the hypothesis that circulating androgen concentrations mediate the adjustment in care provided by bluegill parental males by manipulating perceived paternity and then measuring circulating 11-keto-testosterone concentration and parental care behaviour. We show that males with higher perceived paternity provide higher levels of nurturing and nest defense behaviour, but contrary to expectations, we found that these males had lower concentrations of 11-ketotestosterone. Furthermore, we found positive correlations between individual circulating plasma 11-ketotestosterone concentrations and nurturing behaviour, but not with the aggressive behaviours that differed between paternity treatments. While bluegill make behavioural changes in response to perceived paternity, these changes do not appear to be modulated by 11-ketotestosterone.

1. Introduction

Parental care is essential to the survival of many young animals. Quality parental care can increase the rate of survival to hatch/birth, growth rate, and even future reproduction later in life (Clutton-Brock, 1991; Klug & Bonsall, 2014). While the benefits of parental care are well-documented, parental care can be costly. Providing care often requires parents to forego potential breeding opportunities, reduce their foraging rate, and can make them more susceptible to predation and parasitism (reviewed by Alonso-Alvarez and Velando, 2012). Parental investment theory indicates that these competing factors should result in parents assessing offspring value and then investing more care in high-quality or otherwise more valuable offspring (Trivers, 1972).

Parental investment theory predicts that parents should alter their level of care to reflect the reproductive value of their brood (Trivers, 1972). In an evolutionary context, this response to value is adaptive provided that parents invest more in offspring that are most likely to contribute to the parents' fitness (Westneat & Sherman, 1993). Reproductive value may be based on the number and size of the offspring as well as the relatedness of the offspring to the parent (Westneat & Sherman, 1993). Offspring number and size typically can be assessed fairly easily with large broods valued over small broods and large or otherwise healthy offspring valued over small or sickly offspring (Montgomerie & Weatherhead, 1988). To determine the relatedness of offspring to a parent, however, requires some form of kin recognition and discrimination (Mateo 2004).

Kin recognition mechanisms can allow parents to allocate parental care based on paternity. This allocation of care in response to relatedness, or genetic value, is widespread across taxa, including for example the rhacophorid frog (*Kurixalus eiffingeri*, Chen et al., 2011), scissortail sergeant (*Abudefduf sexfaciatus*, Manica, 2004), sarasins ährenfisch (*Telematherina sarasinorum*, Gray et al., 2008), bluegill sunfish (*Lepomis macrochirus*, Neff & Gross 2001; Neff 2003), and at least 52 species of birds (reviewed in Moller and Birkhead, 1993). Animals can recognize kin directly or indirectly. Indirect kin recognition involves using context-based cues such as location or the number of intrusions by brood parasites to determine the likelihood that individuals encountered are related (Mateo, 2004). For example, in cliff-nesting birds, finding a

nestling in one's nest is a strong indication of relatedness because nestlings are unable to move between nests (Cullen, 1957). Bluegill males use the number of sneakers around their nest on the day of spawning to make inferences about their paternity within the nest (Neff, 2003). Direct kin recognition occurs when animals use methods such as familiarity, or phenotype matching to interpret direct cues of relatedness (Holmes & Sherman, 1982). Familiarity is based on prior association, in the sense that individuals remember the phenotypes of individuals they have interacted with in situations normally associated with kinship and later, recognize and treat those individuals as kin (Mateo 2004). Phenotype matching is most common in species with large broods, including fish (Hain, 2015). This form of recognition occurs when individuals form a 'template' of what related individuals look, smell or sound like based on the phenotypes of family members encountered during development (Holmes & Sherman, 1982). These individuals later compare this template to phenotypes of individuals they encounter to determine if they are related (Holmes and Sherman, 1982). In promiscuous species with mixed broods, individuals may use self-referent phenotype matching whereby the kin template is formed using their own phenotype (Hain and Neff, 2006). Specifically, individuals born into broods with mixed relatedness should be more likely to form a self-referent template to determine relatedness of other individuals.

Behavioural endocrinology is a rapidly expanding field with hormones being proposed as a proximate mechanism to mediate parental care behaviour (Numan & Insel, 2003; Smiley et al; 2019). In particular, androgens have been shown to be critical to the establishment and then maintenance of territories and courtship by modulating behaviour. However, increases in androgens can suppress nurturing parental care and immune response, which requires parents to regulate their androgens during the parental care period (Wingfield et al. 1990). There is a large body of research demonstrating that testosterone in birds mediates aggressive behaviour during the breeding and parental care season. Typically, increases in testosterone increase behaviours like singing, posturing, and attacking (Nelson, 1995; Wingfield et al., 2000). The challenge hypothesis was proposed by Wingfield et al. (1990) as a way for parents to balance the trade-off of aggressive behaviour and parental care, by increasing androgen synthesis only in response to challenges to avoid suppressing other forms of parental care and immune response. The challenge hypothesis has since been supported in several taxa including fish, mammals, and reptiles (reviewed in Moore et al., 2020). While the challenge hypothesis broadly explains androgen regulation, much of the research is focused on mammals and birds where testosterone is the primary androgen. When tested in fish, T elicits a response consistent with other taxa, but at a lower response than 11-ketotestosterone (Moore et al., 2020). Unaromatizable 11-ketotestosterone is the active metabolite of testosterone in fishes (Borg, 1994). While fish synthesize 11-ketotestosterone, testosterone, and 11ß-hydroxytestosterone, 11-ketotestosterone is found at the highest levels in the breeding season and has been found to be more effective than testosterone in stimulating secondary sexual characteristics including reproductive behaviour and parental care in many fishes (reviewed in Borg, 1994). Thus in fishes, 11-ketotestosterone is likely to be the primary androgen, underscoring the nuanced nature of behavioural endocrinology across species.

Bluegill are endemic to North America and have been extensively studied for their alternative reproductive tactics. In bluegill, parental care is performed by males called "parentals" (Gross, 1982). Parental males establish territories within colonies, build nests, court and spawn with females, and then provide sole care for the offspring by oxygenating eggs, cleaning the nest, and defending the brood from nest predation (Gross, 1982). Parental males sometimes nest and spawn multiple times during the breeding season (Gross, 1982). The bluegill mating system is highly promiscuous, with about 25% of the broods being sired by precocious males called cuckolders (Neff 2001; Neff & Clare 2008; Garner & Neff 2013). In Lake Opinicon, cuckolder males mature at age 2 years and use a sneaking tactic where they hide in vegetation around the

nests and dart into nests to fertilize eggs when the parental male is spawning with a female (Gross & Charnov, 1980). At about 4 years of age, cuckolders switch tactic and instead use female mimicry (Gross, 1982). These mimics orbit around the nests like satellites and enter the nest while the parental male is spawning with a female. "Satellite" males then fertilize the female's eggs while acting as if they are also spawning with the parental male.

Parental males are able to discriminate between larvae they have sired and larvae sired by other males. Such kin discrimination happens using both indirect cues of paternity (nest intrusion by sneakers during spawning) and direct phenotype matching of olfactory cues released by larvae after egg hatching (Neff & Gross, 2001; Neff 2003; Neff & Sherman 2003). This ability to discriminate based on paternity leads to differences in parental care: parental males with high paternity provide more aggressive parental care against brood predators than males with low paternity (Neff & Gross 2001; Neff 2003). Prior work has also been able to elicit aggressive behaviour by exposing parental males to exogenous 11-ketotestosterone via subcutaneous implant (Cunha et al., 2019; Rodgers et al., 2012). In these studies, exposure to high concentrations of 11KT resulted in increased aggressive nest defensive behaviour (Cunha et al., 2019; Rodgers et al., 2012). Taken together, 11-ketotestosterone may regulate aggressive behaviour in bluegill during the breeding season, and this aggressive behaviour should vary based on paternity. This, in the current study, our objective is to elucidate the role of 11-ketotestosterone in adaptive adjustments of parental care behaviour in bluegill. We hypothesize the manipulation of perceived paternity will lead to changes in nurturing and defensive behaviour, and the underlying mechanism of these behavioural adjustments is 11-ketotestosterone. We predict males with experimentally reduced perceived paternity will reduce the quality of their parental care and frequency of care behaviours. Furthermore, if 11-ketotestosterone mediates these behaviour changes, we expect males with lower perceived paternity to have lower circulating 11-ketotestosterone concentrations. To test this, we subjected parental males to either a direct paternity manipulation where paternity in the nest was altered, or to a visual manipulation where males perceived the visual cue of nest intrusion by sneakers on the day of spawning. We then measured changes in the circulating concentration of 11-ketotestosterone and parental care behaviour.

2. Methods

2.1. Species and study site

We studied a population of bluegill in Lake Opinicon (44°34′N, 76°19′W), Ontario, Canada. This 890-hectare lake has been a study site for this species since the mid-1970s (Gross, 1982). In Lake Opinicon, bluegill breed from late May to July. During this time, parental males enter the littoral zone and build nests in colonies of up to 300 males. Parental care lasts between 7 and 10 days, with the eggs hatching around day 3. From 2018 to 2021 swimmers equipped with snorkelling gear monitored bluegill reproductive behaviour along a 2 km stretch of the littoral zone of the lake. When a colony formed, we tagged each nest with an individually-numbered ceramic tile. A single swimmer mapped the colony to record the position of each nest after spawning and assigned each nest an egg score from 1 to 5 as a proxy of the number of eggs in the nest (Claussen, 1991; Cargnelli and Gross, 1996). This score is based on the percentage of the nest covered in eggs, and is highly correlated with the number of eggs and larvae in the nest (Claussen, 1991).

2.2. Direct manipulation of paternity

Our objective in this study was to replicate the experimental paternity manipulation of Neff (2003), while adding blood sampling to assess the effect of paternity manipulation on circulating 11-ketotestosterone (11KT) levels. Parental males were paired based on their assigned egg

scores the morning after spawning was observed at each colony (day 1; Fig. 1). The paired males with equal egg scores were then caught one at a time using a dip net and brought to a nearby boat. Nests were covered with a screen to prevent egg predation while the parental male was absent from the nest. We immediately took a 200 μL whole-blood sample from the caudal vein using a 25G needle attached to a 1 mL heparinized syringe. These samples took an average of 85 s to collect from the time of capturing the male (range = 21 to 256 s) and were used to measure baseline circulating concentrations of 11KT. We then measured total body length (mm) and placed the male in a recovery tank while the nest was manipulated. Both males remained on the boat while their nests were manipulated, and each pair of males were returned to their nests after the manipulation was completed. Each male spent<10 min on the boat, and all manipulations took place between 09:00 – 12:00 EST. The Animal Care Committee at Western University (UCC) approved all procedures performed in this study (AUP #2010-214).

Following Neff (2003), we assigned each pair of males to one of two treatments: (1) control; or (2) egg manipulation. For the egg manipulation treatment, we swapped about one-half of each male's eggs between the two nests. These swaps were not performed between neighbouring nests to ensure the foreign eggs introduced were unrelated to the focal male. We performed a sham swap in the nests of males assigned to the control treatment, in which we removed and then returned one-half of the eggs to the original nest. This mimicked the disturbance of the egg swap, but not the reduction in paternity.

On day 2, we recorded each male's parental care behaviours by performing a standardized nest defense test between 14:00–17:00 EST. We presented a natural egg predator (pumpkinseed sunfish, *Lepomis gibbosus*) in a transparent plastic bag on the border of the parental male's nest and recorded the parental male's defensive behaviour for 1 min using a go-pro camera (Hero 5 and 6, San Mateo, California, USA). Later from the videos we quantified three aggressive behaviours (*sensu* Neff, 2003): (1) lateral display; (2) opercular flare; and (3) bite.

We monitored nests daily to determine the day of hatch, which was expected on day 3. We performed another nest defense test the day after hatch. Immediately after the test, we collected another blood sample from each parental male, as described above, to measure circulating 11KT concentration. Due to a difference in blood sampling methodology post-hatch in 2018, those samples were not analyzed for 11KT, but the behavioural data from those males were used.

2.3. Indirect manipulation of perceived paternity

In a second experiment, we manipulated perceived paternity using an indirect cue – the presence of sneakers during spawning. Following Neff (2003), we placed two transparent plastic tanks (20 × 16 × 10 cm) on opposite sides of parental males' nest on the day of spawning (Fig. 1). We assigned parental males to one of two treatments: (1) control; or (2) sneaker visual cue. We placed two bluegill sneaker males in each tank surrounding the males assigned to the experimental treatment. We left these tanks beside the nest for the duration of the spawning day to present a visual cue of high cuckoldry by sneakers to the parental male. The tanks beside the nests of males assigned to the control treatment remained empty during the day of spawning.

On the day after spawning (day 1), each parental male was caught via dip net and brought to the boat one at a time for initial processing. We measured each male's total body length (mm) and a 200 μL whole-blood sample to measure circulating 11KT. The next morning, we set up GoPro Cameras (Hero 5, 6, or 7) at each parental male's nest. We recorded nurturing behaviour for 30 min between 09:00–12:00 EST. We quantified four nurturing behaviours: (1) rim circling; (2) caudal fan; (3) pectoral fan; and (4) egg consumption (Gross & Macmillan, 1981; Côté and Gross, 1993; Neff 2003). In the afternoon, between 14:00–17:00 EST, we recorded and quantified nest defense parental care behaviour using the same standardized nest defense test from the direct paternity manipulation experiment.

2.4. Hormone analysis

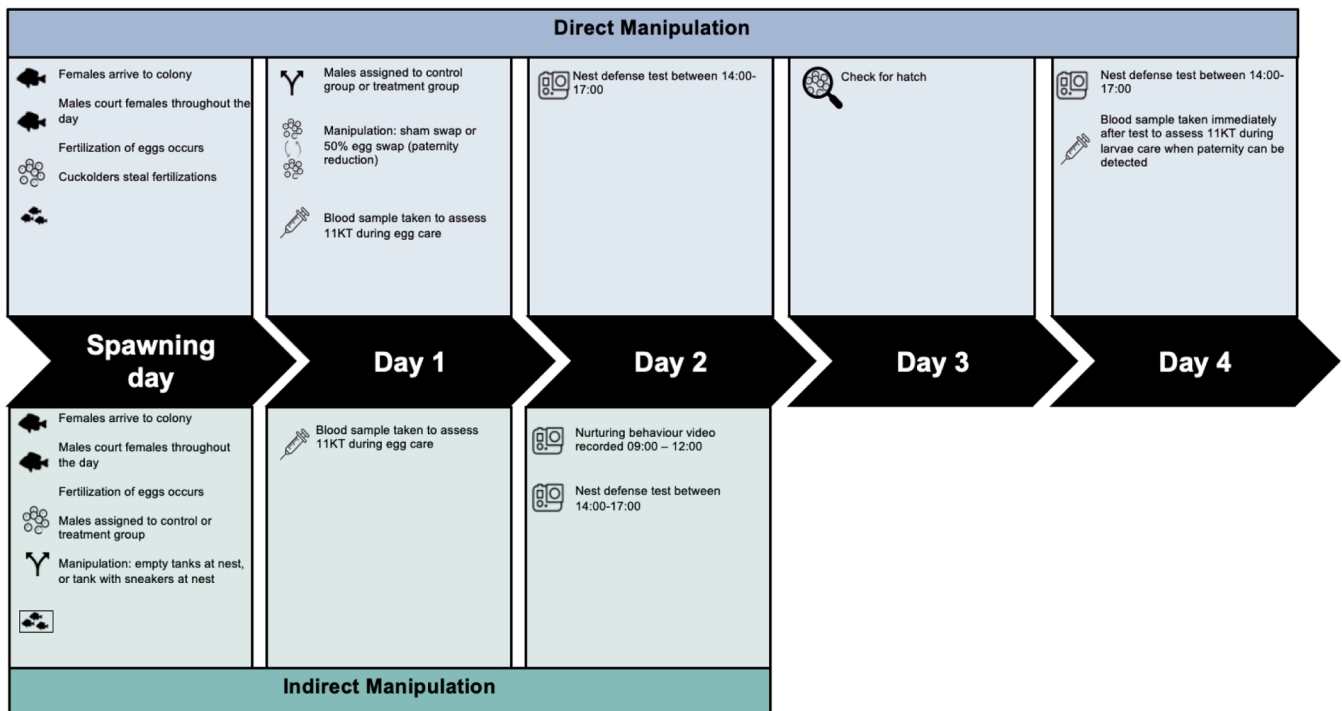We extracted plasma from each blood sample within 8 h of collection

Fig. 1. Timeline of direct paternity manipulation and indirect perceived paternity visual manipulation on bluegill sunfish (*Lepomis macrochirus*). Days in blue represent the timeline of the swapped eggs paternity manipulation protocol, and in green represent the timeline of the visual manipulation protocol.

and stored it at $-20°$ C for transportation back to the University of Western Ontario. We then used enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical, Ann Arbor, MI) to determine the concentration of 11KT in the plasma. We ran each sample in triplicate. Concentrations of 11KT were within the range expected based on previous studies of reproductive hormones in bluegill (Magee et al., 2006; Neff & Knapp, 2009).

2.5. Statistical analyses

We used R Studio (2015) for all statistical analyses. Degrees of freedom were calculated as the adjusted values as calculated in R. For both the direct and indirect manipulation of perceived paternity experiments, we first used t-tests to determine if there was a difference in egg score or body length between treatments. We used a Shapiro-Wilk test to assess the normality of each behaviour and 11KT concentration. We then used t-tests to determine the effect of experimental treatment on each of the nest defense behaviours (lateral displays, opercular flares, bites), and 11KT concentrations. For the indirect paternity manipulation experiment, we also used t-tests to compare the effect of the treatment on the nurturing behaviours (rim circling, caudal fanning, pectoral fanning, and egg consumption).

We used Spearman's rank correlations to determine if there was a relationship between individual fish 11KT concentration and their nest defense and nurturing behaviours. We compared each treatment separately, and further analyzed each treatment per time point (egg care and larvae care).

3. Results

3.1. Direct manipulation of paternity

During the egg stage of care, we collected blood from 145 males ($N_{control} = 71$, $N_{swap} = 74$), and behaviour observations for 74 males ($N_{control} = 32$, $N_{swap} = 42$). The discrepancy between the blood and behaviour sample sizes is largely explained by nest abandonment between the first and second day post-spawning. During the larvae care stage, immediately after the nest defense test, we collected blood from 51 males ($N_{control} = 22$, $N_{swap} = 29$) and behaviour observations from 65 males ($N_{control} = 29$, $N_{swap} = 36$). The discrepancy between blood and behaviour sample sizes during larval care is explained by video quality and water clarity, along with the coagulation of plasma for some blood samples, which prevented use of the ELISA assay. In 2018, 15 control and 9 swap males abandoned their nests after hatch. In 2020, 12 control and 9 swap males abandoned their nests after hatch. In 2021, 13 control and 8 swap males abandoned their nests after hatch.

Body length and egg score were similar between the control and egg swap treatments. At the egg care stage, there was no significant difference in egg score between the control (2.6 ± 1.1; mean $\pm$ SD) and egg swap treatments (2.8 ± 1.0; $t_{120} = -0.84$, $p = 0.40$). At the larval care stage, there was no significant difference in the egg score of males who remained after hatch between the control (2.6 ± 1.0) and egg swap treatments (2.8 ± 1.0; $t_{120} = -0.84$, $p = 0.40$). At the egg care stage, there was no significant difference in body length between the control (195 ± 10 mm) and egg swap treatments (196 ± 12 mm; $t_{140} = -0.37$, $p = 0.71$). At the larval care stage, there was no significant difference in body length between the control (195 ± 10 mm) and egg swap treatments (196 ± 12 mm; $t_{125} = -0.58$, $p = 0.56$).

There was no significant difference in the number of lateral displays performed by males in the control or swap treatments at the egg ($t_{74} = -1.18$, $p = 0.24$) or larval care stages ($t_{65} = -0.62$, $p = 0.54$; Fig. 2a). Similarly, there was no significant difference in the number of opercular flares performed by males in the control or swap treatments at the egg ($t_{74} = 0.27$, $p = 0.79$) or larval care stages ($t_{65} = -0.75$, $p = 0.46$; Fig. 2b). There was no significant difference in the number of bites performed by males in the control or swap treatments at the egg care stage ($t_{74} = -1.19$,
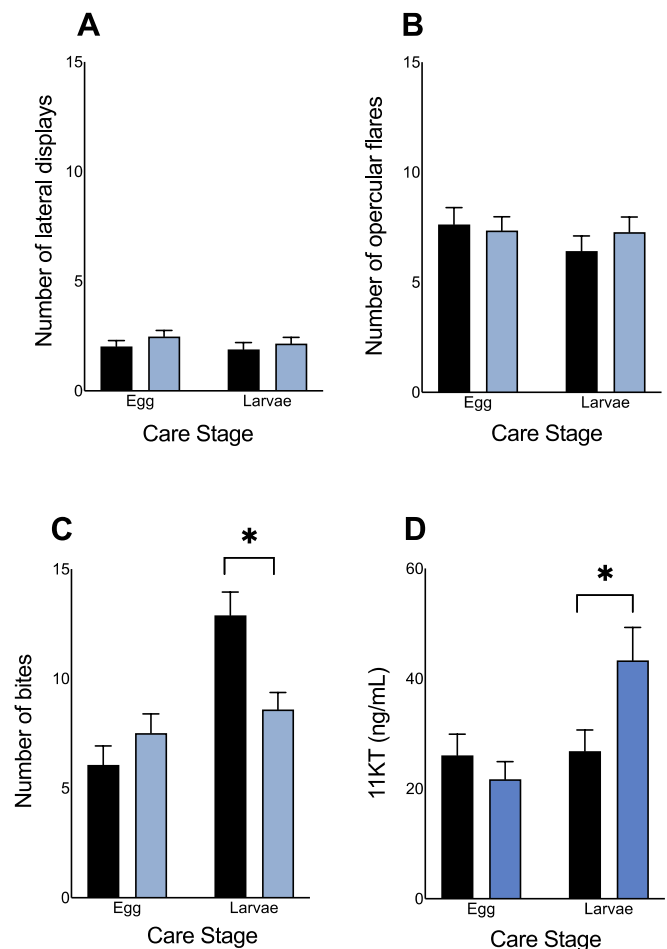

Fig. 2. Nest defense behaviours (A. Lateral Display, B. Opercular Flare, C. Bite) and 11-ketotestoterone concentration (D) by parental male bluegill (*Lepomis macrochirus*) during the egg and larvae stages in response to a natural predator (Mean $\pm$ SEM). Control males (sham swapped eggs) are denoted by black bars while treatment males with experimentally reduced paternity (swapped eggs) are denoted by blue bars.

$p = 0.24$). However, after hatch, during the larval care stage, males in the control treatment performed significantly more bites than males in the experimentally lowered paternity treatment ($t_{65} = 3.38$, $p < 0.01$ Fig. 2c).

Males in the control and swap treatments at the egg care stage had no significant difference in their 11KT concentration ($t_{57} = 0.74$, $p = 0.46$). However, during the larval care stage, males in the experimentally lowered paternity treatment had significantly higher 11KT than males in the control treatment ($t_{51} = -2.44$, $p = 0.02$; Fig. 2d). There was also a significant difference in the change in individual 11KT concentrations with control males increasing on average by 5.14 ng/mL and swap males increasing by 19.8 ng/mL ($t_{57} = -3.83$, $p < 0.01$).

There was no relationship between the number of lateral displays performed by parental males in the control treatment and circulating 11KT concentrations ($R = 0.21$, $p = 0.10$), but there was a positive relationship in the males in the swap treatment ($R = 0.22$, $p = 0.05$). There was no relationship between the number of opercular flares performed by males and their circulating 11KT in the control treatment ($R = 0.058$, $p = 0.66$), or the swap treatment ($R = 0.064$, $p = 0.57$). Similarly, there was no relationship between the number of bites males performed and their circulating 11KT in the control treatment ($R = 0.18$, $p = 0.16$) or swap treatment ($R = -0.12$, $p = 0.28$).

3.2. Indirect manipulation of perceived paternity

We analyzed the aggressive nest defense behaviour of 62 males ($N_{control} = 30$, $N_{sneaker} = 32$), and the nurturing behaviour of 55 males ($N_{control} = 26$, $N_{sneaker} = 29$). We collected blood from 56 males ($N_{control} = 30$, $N_{sneaker} = 26$). Variation in sample sizes stems from variation in water clarity/video quality, and one male from whom blood coagulation in the needle prevented sample collection.

Parental males in the control and sneaker visual treatments were not significantly different from each other in either egg score (control = 2.4 ± 1.1; mean ± SD; treatment = 2.7 ± 1.5; $t_{50} = -0.91$, $p = 0.37$) or body length (control = 196 ± 9 mm; treatment 193 ± 13 mm; $t_{59} = 1.33$, $p = 0.19$). Based on the analysis of nest defense behaviours, there was no significant difference between the control and sneaker treatments in the number of lateral displays ($t_{59} = -0.12$, $p = 0.90$), opercular flares ($t_{59} = -0.04$, $p = 0.97$) or number of bites ($t_{59} = 1.67$, $p = 0.09$; Fig. 3). Males in the sneaker treatment performed significantly fewer rim circles ($t_{56} = 3.18$, $p = 0.01$), pectoral fans ($t_{56} = 2.57$, $p = 0.02$) and egg consumption motions ($t_{56} = 3.97$, $p < 0.01$) than males in the control treatment (Fig. 4). There was no significant difference in the number of caudal fans performed by males in the control treatment relative to males in the sneaker treatment ($t_{56} = 0.61$, $p = 0.55$). There was no significant difference in the 11KT concentration (ng/mL) of males in the control treatment relative to males in the sneaker treatment ($t_{54} = -0.14$, $p = 0.89$).

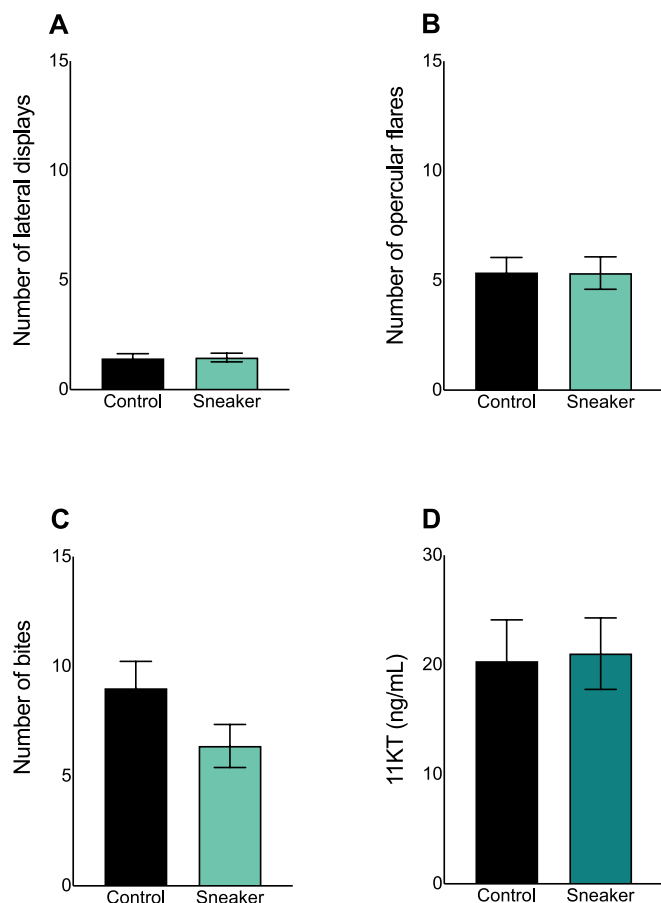There was a positive relationship between the number of rim circles

Fig. 4. Nurturing behaviours (A. Rim circling, B. Caudal fan, C. Pectoral fan, D. Egg consumption) by parental male bluegill (*Lepomis macrochirus*) during the egg stage (Mean ± SEM). Control males with higher perceived paternity are denoted by black bars. Treatment males with lower perceived paternity (sneaker visual cue) are denoted by green bars.
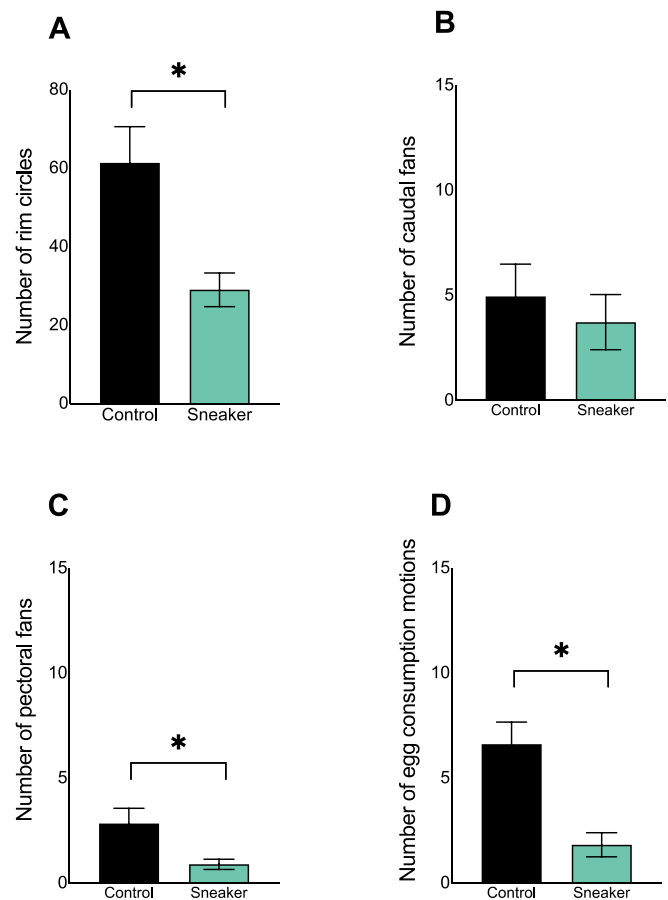
Fig. 3. Nest defense behaviours (A. Lateral Display, B. Opercular Flare, C. Bite) and 11-ketotestosterone concentration (D) by parental male bluegill (*Lepomis macrochirus*) during the egg stage in response to a natural predator (Mean ± SEM). Control males with higher perceived paternity are denoted by black bars. Treatment males with lower perceived paternity (sneaker visual cue) are denoted by green bars.

performed by males and circulating 11KT in the control treatment (R = 0.41, $p = 0.05$), but not in the sneaker treatment (R = 0.082, $p = 0.70$). In comparison, there was no relationship between number of caudal fans and 11KT for males in the control treatment (R = 0.3, $p = 0.16$), while there was a positive relationship in the sneaker treatment (R = 0.54, $p = 0.01$). There was a positive relationship between the number of pectoral fans males performed and their 11KT concentrations for both the control (R = 0.49, p = 0.02) and sneaker treatment males (R = 0.53, p = 0.01). Finally, there was no relationship between egg consumption and 11KT in either the control (R = 0.031, $p = 0.89$) or sneaker treatment males (R = 0.073, $p = 0.73$).

4. Discussion

Our results show parental males adjust both nurturing and defensive behaviours in response to perceived paternity. Males with higher perceived paternity defended their nests more aggressively and provide a higher quality of nurturing care than those with lower perceived paternity. Indeed, when paternity was directly manipulated by swapping eggs between nests, males with higher paternity more aggressively defended their nests than males with lower paternity. Our results support and expand on the experiment by Neff (2003), in which the author also subjected parental male bluegill to indirect and direct reductions in perceived paternity and observed that males with experimentally reduced paternity reduced their level of care. Neff (2003) analyzed overall nest defensive behaviour, which our study expands upon by both supporting increased defensive behaviour by control males, and by

analyzing each behaviour separately to determine that biting drives the observed changes. Our study also quantified four nurturing behaviours. We determined differences in nurturing parental care are also observable prior to eggs hatching and, in line with Neff (2003), persist after eggs hatch. Taken together, our research replicates and expands upon the behavioural differences by parental males in response to perceived paternity.

Our other objective was to determine the role of 11KT in the regulation of parental care behaviour and the possibility of this hormone serving as a mechanism for how parental males alter their behaviour in response to perceived paternity. The role of 11KT in the regulation of teleost behaviour has been extensively tested using experimental manipulations (Cunha et al., 2019; Rodgers et al., 2012; Ros et al., 2004; Kindler et al., 1991) while the response of naturally circulating plasma concentrations has rarely been examined. The few studies that have quantified plasma 11KT concentrations in response to stimuli yield conflicting results: 11KT increases in response to territorial intrusion in *Sarotherodon galilaeus* and *Sparisoma viride* (Ros et al., 2003; Cardwell & Liley, 1991) but did not increase in *Neogobius melanostomus* or *Acanthochromis polyacanthus* (Sokolowska et al., 2013; Hay and Pankhurst, 2005). At a species specific level, prior research has demonstrated that bluegill increase their nest defense behaviour when subjected to artificially elevated 11KT delivered via subcutaneous implants (Rodgers et al., 2012; Cunha et al., 2019). Our results from the direct manipulation of paternity experiment showed that males with higher paternity actually had lower circulating 11KT concentrations after the eggs hatched. During the egg stage of care, as with males in the visual manipulation of sneakers, there was no difference in circulating 11KT concentration between treatments. Furthermore, circulating 11KT was positively related only to lateral displays in one group of males. All other aggressive defense behaviours were not related to circulating 11KT concentrations. Our data thus suggest that 11KT is not responding to changes in paternity or perceived paternity and the observed differences in parental care are regulated by another mechanism. Instead, prior work has shown that males that renest increase circulating androgen concentrations towards the end of the parental care period (Specker & Kishida 2000; Pankhurst & Peter 2002; Magee et al., 2006). Thus, it is conceivable that the elevated 11KT levels in males in our egg swap treatment were associated with renesting potential in response to low paternity.

The difference between our findings and those of previously-published work may be interpreted as a difference between response and regulation of 11KT. In prior studies, bluegill had subcutaneous implants inserted to administer varying concentrations of 11KT and in one study, implanted males had 60% higher concentrations of 11KT relative to the control males (see Cunha et al., 2019). In these implant studies, bluegill with higher circulating levels of 11KT exhibited more aggressive behaviour, so clearly circulating levels can affect parental behaviour (Rodgers et al., 2012; Cunha et al., 2019). Felix et al. (2020) suggest that androgen response varies between individuals and is related to their scope for response (maximum physiological level – baseline level). In the implant studies, the bluegill 11KT baseline levels were elevated by implantation and the natural scope for response was presumably reduced. In our study, we observed the physiological response to paternity in which 11KT was not directly manipulated, and so changes in hormone concentrations were attributed to their natural baseline and response to paternity cues. In particular, bluegill in our study should have had more flexibility in their androgen concentration changes due to a lower baseline level and thus higher scope for response. In the context of perceived paternity, males do not appear to differentially regulate circulating levels of 11KT during the egg phase, and seemingly only elevate the androgen in response to low paternity once the eggs hatch. This indicates paternity may influence regulation of 11KT in the context of renesting, and not in terms of regulation of aggressive parental behaviour.

Considering our 11KT results contradicted our prediction, we looked further into the relationship between individual circulating 11KT and parental care behaviours. Interestingly, we found no relationship between most of the nest defense behaviours and 11KT concentrations. The one exception was a positive relationship between lateral displays and 11KT concentration for the males in the egg swap treatment. We also found several positive relationships between nurturing behaviours and 11KT concentrations. It is possible that bluegill maintain sufficiently high levels of 11KT that they do not respond to challenges by elevating circulating concentrations. Goymann et al (2019) proposed the "Challenge Hypothesis 2.0" that posits males in promiscuous mating systems are not constrained by a trade-off between nurturing and aggressive behaviours. Rather, males can maintain high levels of androgens during the parental care season without impacting the quality of care. They also suggest that androgen concentrations may be high enough to respond to challenges without requiring any additional elevation in circulating androgen concentrations. Our findings support this idea, by providing empirical evidence that male bluegill are not constrained by an androgen-parental care trade-off.

Future research should examine other potential mechanisms underlying adaptive parental care behaviour in bluegill. While males with higher perceived paternity perform a higher quality of parental care, it does not appear that 11KT is the key hormone driving this response. That said 11KT receptors can be activated in<10 min and may be involved in the observed differences in behaviour through differences in density or ligand binding efficiency (Goymann et al., 2019; Moore et al., 2020; Borg, 1994). Outside of androgenic activity, we suggest future investigations consider nonapeptides as a potential mechanism to mediate parental care given their role in care modulation (DeAngelis et al., 2020; Cunha-Saraiva et al., 2019) and potential linkage to paternity (Stiver et al., 2019). Our study highlights the importance of increasing our understanding of the neuroendocrine and neurogenomic mechanisms that regulate behaviour, as it is clear they are complex and challenging to generalize across taxa.

CRediT authorship contribution statement

Emma K.L. Churchman: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Timothy J.A. Hain:** Methodology, Investigation, Writing – review & editing, Supervision, Project administration. **Rosemary Knapp:** Conceptualization, Methodology, Resources, Supervision, Project administration. **Bryan D. Neff:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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