Hormones and Behavior 63 (2013) 454–461

Contents lists available at SciVerse ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Androgen-mediated nurturing and aggressive behaviors during paternal care in bluegill sunfish (*Lepomis macrochirus*)

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ARTICLE INFO

Article history:
Received 4 June 2012
Revised 14 December 2012
Accepted 16 December 2012
Available online 23 December 2012

Keywords:
Bluegill sunfish
Lepomis macrochirus
Androgens
Flutamide
Parental care
Aggression
Nurturing
Testosterone
11-Ketotestosterone
Trade-off

ABSTRACT

Male parental care in vertebrates often involves both defensive and nurturing behaviors. Whether androgens differentially mediate these two types of behaviors, or a trade-off exists between them, has been studied by behavioral endocrinologists for years but predominantly in species with biparental care. In such systems, potential detrimental effects of elevated androgens on parental care behaviors are often compensated for by changes in behavior of the unmanipulated parent. In contrast, for species where only one parent provides care, manipulation of androgen levels may more clearly determine if there are differential effects of androgens on these two types of behaviors and whether the proposed trade-off between defensive and nurturing behavior exists. Here, we manipulated androgen levels in two ways in bluegill sunfish (*Lepomis macrochirus*), a species where males provide sole parental care for the young. At the onset of the care period, males were implanted with 11-ketotestosterone, a major teleost androgen, the androgen receptor antagonist flutamide, or a blank implant. A separate control group experienced no manipulation. Males were then observed over several days and tested for their aggressiveness towards an experimentally-presented brood predator and for nurturing behavior (fanning of the eggs, removal of dead or fungal-infected eggs). Males implanted with 11-ketotestosterone displayed 64% more aggressive behaviors and 71% fewer nurturing behaviors than control groups. In contrast, males implanted with flutamide displayed 7% fewer aggressive behaviors and 126% more nurturing behaviors than control males. Taken together, these results show that aggression and nurturing behaviors are mediated by androgens and suggest that there is a trade-off between the two behaviors during parental care in this species.

Introduction

Behavioral endocrinologists have long been interested in the relationship between androgens and aggression. Since 1990, the Challenge Hypothesis proposed by Wingfield et al. (1990) has become a cornerstone for understanding the role that androgens might play in mediating male aggressive behavior in the context of reproduction. The Challenge Hypothesis suggests that high levels of androgens facilitate territorial aggression or mate defense when confronted with an intruder. To date, the hypothesis has been predominately studied in birds. For example, Hau et al. (2000) found that male spotted antbirds (*Hylophylax naevioides*) with exogenously elevated androgen levels displayed more aggression in response to a social challenge by another male than did control males. Conversely, male spotted antbirds implanted with the androgen receptor antagonist-, flutamide-, were less aggressive to a social challenge than were control males. More recently, this phenomenon has been studied in a variety of other taxa including reptiles (e.g., tree lizards, *Urosaurus ornatus*: Weiss and Moore, 2004), insects (e.g., burying beetles, *Nicrophorus*: Scott, 2006) and mammals, including humans (e.g., ring-tailed lemurs, *Lemur catta*: Cavigelli and Pereira, 2000; California mice, *Peromyscus californicus*: Trainor and Marler, 2001; humans: Archer, 2006; Gettler et al., 2011). Overall, the Challenge Hypothesis has been an important contribution to our understanding of hormone–behavior relationships related to territorial aggression (see also Hirschenhauser and Oliveira, 2006).

As an extension of the Challenge Hypothesis, there has been increasing interest in the potential trade-off between aggression and nurturing behaviors during parental care. It has been proposed that this trade-off is also mediated by androgens. In an early study by Hegner and Wingfield (1987), male house sparrows (*Passer domesticus*) were implanted with exogenous testosterone (T) or with an androgen receptor antagonist. They found that T-implanted males performed more acts of nest defense but fewer acts of nurturing behaviors (i.e. feeding the young) than control males. In contrast, males implanted with the androgen receptor antagonist performed less nest defense but more nurturing behaviors. Likewise, Schwagmeyer et al. (2005) found that T-implanted house sparrows were not only more aggressive towards

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http://dx.doi.org/10.1016/j.yhbeh.2012.12.008

conspecifics than were control males, but also spent significantly less time at their nests, incubated the eggs less than half as much and fed the nestlings significantly less than did control males. More recently, McGlothlin et al. (2007) found that male dark-eyed juncos (*Junco hyemalis*) with elevated gonadotropin releasing hormone (and subsequently increased T) displayed more territorial aggression towards an intruder but provided less food to their offspring than control males. These studies on birds, among others (e.g., Ketterson et al., 1992; Silverin, 1980), have provided early evidence that male parental behaviors are affected by androgen levels.

Although an increasing number of studies have investigated the role of androgens on aggressive and nurturing behaviors in parental care systems where only one parent provides care, most previous studies examining this trade-off have done so in species with biparental care. However, these latter studies can be problematic because changes in behavior of the manipulated parent can be, and often are, compensated for by changes in behavior of the other parent. For example, in Hegner and Wingfield's (1987) study, although the T-implanted male house sparrows (*P. domesticus*) provided fewer feeding opportunities to offspring, the females fully compensated for the decrease by increasing their level of feeding. Likewise, T-implanted male dark-eyed juncos (*J. hyemalis*) fed their nestlings less and did so at a slower rate, while their mates compensated for the lower feeding rates (Ketterson et al., 1992). In such systems, parental roles may be divided into one of defender and the other of nurturer. While the trade-off still tends to be found in species with biparental care and appears to be mediated by T, it is not clear to what degree T affects behavior — that is, if the observed trade-off in males is entirely due to increased androgens or only partially due to increased androgens, with compensation by the females driving the remaining effect. Examining the effects of androgens on aggressive and nurturing behavior in a system where only one parent provides care can overcome this problem because the effects of androgens on the behaviors cannot be driven or compensated for by another parent.

An additional confounding variable in most previous studies of aggression and nurturing during parental care is that mating behaviors are often simultaneous or overlapping with parental care. Many such studies have found an androgen-mediated increase in courtship behavior at the apparent expense of nurturing behavior; i.e. males with increased androgen levels performed less nurturing behavior and, instead, sought out additional mates (e.g., European starlings, *Sturnus vulgaris*: De Ridder et al., 2000; superb fairy-wrens, *Malurus cyaneus*: Peters, 2002; dark-eyed juncos, *J. hyemalis*: McGlothlin et al., 2007). Thus, investigating the role of androgens on different types of parental behaviors in species where mating is discrete from parental care would also be beneficial.

Here, we investigate the role of androgens on the expression of aggressive and nurturing behaviors during parental care in bluegill sunfish (*Lepomis macrochirus*). In this species, fish complete mating and females leave the vicinity of males' nests before sole paternal care of the offspring commences. We have previously investigated the role of T on aggressive and nurturing behaviors during parental care and found that although bluegill parental males implanted with T displayed more aggressive behavior, they did not display less nurturing behavior (Rodgers et al., 2012). However, in many fish species, the unaromatizable 11-ketotestosterone (KT) is the primary androgen and may more directly inhibit nurturing behavior than T or its other metabolites. For example, in the rock-pool blenny (*Parablennius parvicornis*), exposure to KT decreased the frequency of males' egg fanning behavior (Oliveira et al., 2001). Furthermore, in bluegill, KT levels are lowest during the egg phase of paternal care when fanning and other offspring nurturing behaviors are most frequent (Kindler et al., 1989; Magee et al., 2006). Thus, KT may be a better candidate for investigating the role of androgens on aggressive and nurturing behaviors.

In the current study, we manipulated androgen signaling at the onset of paternal care by implanting males with KT, the androgen receptor antagonist flutamide, or a blank implant. We then observed the amount of aggressive and nurturing behaviors the fish displayed. Although flutamide often does not lower the circulating levels of androgens, it has its biological effect by lowering the frequency at which androgens bind to their receptors and subsequently has downstream effects on target tissues (Neri, 1989). This androgen receptor antagonist has not previously been implanted in fish (it has previously been administered via flow-through water in experimental tanks; e.g., Jensen et al., 2004; Sebire et al., 2008; Martinovic-Weigelt et al., 2011). As such, implanting flutamide in our fish represents an important step forward in hormone-based field studies. We predicted that, if an androgen-mediated trade-off exists, male bluegill implanted with KT would be more aggressive towards a brood predator and less nurturing than sham and unmanipulated (control) males, whereas males implanted with flutamide would be less aggressive towards a brood predator but more nurturing than sham and unmanipulated males.

Materials and methods

Study site and animals

Lake Opinicon (44°34′N, 76°19′W) is a 900-ha lake and its bluegill have been studied for over 30 years (e.g., Colgan et al., 1979; Rodgers et al., 2012). The bluegill breeding season begins in late May and lasts until early July during which time there are three or more breeding bouts. A bout begins when parental males move to the littoral zone of the lake and build nests by sweeping their caudal fin from side to side over the substrate (Scott and Crossman, 1973). These males form nests in colonies that can contain up to 300 males (Cargnelli and Neff, 2006). Nest building typically lasts one day before females arrive in groups. A male and female will begin a spawning ritual where they circle the outer rim of the nest until the female dips on her side and releases a few of her eggs, which is quickly followed by the male releasing sperm. Such spawning bouts continue for one day, after which females return to the deeper waters of the lake to feed and replenish their energy. Parental males remain at the nests for another 7–10 days to provide parental care (Scott and Crossman, 1973). For the first three days (egg stage), a parental male must nurture his offspring by fanning the eggs and removing moldy eggs from the nest. However, males must also protect their offspring from brood predators by actively chasing and biting predators as well as performing other aggressive displays (Neff, 2003; Neff and Gross, 2001). Once the eggs hatch, the parental male no longer provides nurturance but continues to provide defense against brood predators. Five to seven days after the eggs hatch, the parental male concludes parental care and returns to deeper waters of the lake to replenish his energy before potentially returning for the next breeding bout (Magee et al., 2006).

Treatment assignment and implants

From June 4 to June 19, 2010, swimmers monitored the shores of Lake Opinicon for formation of bluegill colonies and mating activity. The day after spawning, 98 males were captured from their nests using dip nets and were brought to a nearby boat, where initial blood samples (~300 μL) were taken from the caudal vein. Numbered tiles were placed on the shore side of each nest for fish identification and nest covers were placed on nests to protect the eggs from brood predators while parental males were being handled. Blood collection time, measured from the time the fish was caught until the needle was removed from the caudal vein, averaged 67 s (range: 35–174 s). Fish were then anesthetized using clove oil, weighed (to the nearest gram) and measured for total length (to the nearest mm). Fulton's condition factor was calculated using the equation (mass/length^3)$\times 10^5$, which estimates an individual's energetic state (Neff and Cargnelli, 2004). An egg score was also assigned to each parental

male's nest following Claussen (1991) for a score of 1 (1–4900 eggs), score 2 (4600–29,000 eggs), score 3 (27,000–53,000 eggs), score 4 (49,000–87,000 eggs) or score 5 (82,000–113,000 eggs). The fish were then implanted in the abdominal cavity with either a sham implant filled with castor oil (S; n = 17), a flutamide implant (F; n = 17), one 11-ketotestosterone implant (KT1; n = 20) or two 11-ketotestosterone implants (KT2; n = 23). A fifth group consisted of males that did not undergo surgery or receive an implant, which served as our controls (C; n = 21). Fish were collected randomly from the colony and treatment was assigned by rotation through the five treatments. Sham and KT implants were made with silastic tubing (Konigsburg Instruments, Pasadena, CA, United States) and were 7 mm in length (1.47 mm i.d., 1.96 mm o.d.) with 1 mm silicone sealant on the ends. The KT implants were made using crystalline KT (Steraloids, Newport, RI, United States) dissolved in ethanol and subsequently mixed into castor oil (concentration ~ 8 mg KT/mL oil). Flutamide implants were made by packing the interior of the tubing with crystalline flutamide (Sigma Aldrich, Oakville, ON, Canada). These implants were 8 mm in total length (1.47 mm i.d. and 1.96 mm o.d.) and were sealed with 1 mm silicone sealant on the ends. KT and flutamide doses were calculated based on doses used in previous studies on fish and birds while also calculating for average parental male bluegill body mass (Alonso-Alvarez et al., 2007; Kindler et al., 1991; Ros et al., 2004; Yamaguchi et al., 2004, 2005).

Males that underwent implant surgery were administered with 50 μL of antibiotic solution (oxytetracycline; Sigma-Aldrich, Oakville, ON, Canada) into the incision to prevent infection and the wound was sealed with New Skin (Prestige Brand Holdings, Inc., Irvington, NY United States). Fish were placed in a bucket filled with lake water and allowed to recover for 5 min before they were placed back on their nests, where they typically resumed care within a few minutes.

Behavioral observations

On the first and second days after implantation, males were observed by one of six swimmers for aggressive and nurturing behaviors between 0900 and 1700 EST. The presence of observers does not tend to affect the behavior of bluegill (e.g., Coleman et al., 1985; Gross, 1982). All swimmers were blind to the treatment group of each fish. Aggressive behaviors were assessed by placing a ca. 16-cm pumpkinseed sunfish (*Lepomis gibbosus*) on the edge of the parental male's nest. The pumpkinseed was held in a plastic bag attached to a 1.5-m pole. On each of the two days, the number of bites, opercular flares and lateral displays directed towards a brood predator were recorded over two 30-second periods, with a 30-second break away from the nest in between presentations (see Neff and Knapp, 2009). These numbers were later summed as a measure of total aggressive behavior (i.e. based on the full 2 min).

Nurturing behaviors were assessed based on 15 min of observation on each of the two days (30 min total). The number of caudal sweeps, pectoral fans and egg removal behaviors was recorded and later summed for a measure of total nurturing behavior. These aggressive and nurturing behaviors are described in Rodgers et al. (2012). The order of recording for aggressive and nurturing behaviors was switched each day.

On the third day after implantation, males were again captured from their nests and final blood samples were taken from the caudal vein to measure changes in hormone levels. Mean collection time for these samples was 73 s (range: 39–150 s). All blood samples were kept on ice until they were returned to the lab, where the plasma was separated using a centrifuge and then transferred to a new tube that was then frozen at −20 °C until hormone assays were conducted. A total of 47 males were included in the statistical analyses, of which 11 were control fish, 8 were shams, 9 were flutamide fish, 9 were KT1 fish, and 10 were KT2 fish. Fifty-one fish were not included in analyses due to abandonment of their nest before final capture (n = 49) or uncharacteristic behavior, such as leaving the nest for long periods of time during the behavioral observations (n = 2).

11-Ketotestosterone implant effectiveness

We used a different set of parental males to determine the effect of KT implants on plasma T and KT levels. Importantly, these males were not exposed to an experimental brood predator prior to the final blood sample collection. This validation involved implanting additional groups of parental male bluegill with one or two KT implants (each implant having a concentration of ~8 mg KT/mL castor oil) and taking blood samples from a subsample of each group of males 24 h, 48 h and 7 days after the implants were in place. A total of 29 males were used; 15 males were implanted with one KT implant (KT1) and 14 males were implanted with two KT implants (KT2). Within the KT1 group, 7 males had blood collected 24 h after implantation, 6 different males had blood collected 48 h after implantation, and 2 males had blood collected 7 days after implantation. Within the KT2 group, 5 males were sampled 24 h after implantation, 6 males 48 h after implantation, and 3 males 7 days after implantation. Sample sizes were small due to some logistical constraints in the field.

Radioimmunoassay

Plasma levels of KT, T and estradiol were determined using radioimmunoassay (RIA) following chromatographic separation as described in Magee et al. (2006). We measured estradiol to verify that our KT treatment, through a negative feedback loop, did not affect estradiol levels in the event that KT manipulation resulted in increased T levels. Briefly, we used 100 μL of plasma for each sample and added 2000 cpm of each titrated hormone to allow for correction for losses during extraction and chromatography. Samples were extracted twice with 2 mL diethyl ether and the combined ether extraction was dried down under nitrogen. Samples were resuspended in 10% ethyl acetate in iso-octane and run through diatomaceous earth-glycol columns. Collection of pure fractions of T, estradiol and KT was achieved by sequential elutions with increasingly polar solutions of ethyl acetate in iso-octane. Each fraction was collected, dried down under nitrogen, resuspended in 500 μL phosphate-buffered saline containing 0.1% gelatin and stored overnight at 4 °C. The T antibody used (Wien T-3003 from Research Diagnostics, Flanders, NJ, now Fitzgerald Industries, Acton, MA, United States) has high cross-reactivity with KT and could thus be used to assay both T and KT. The estradiol antibody we used was from Biogenesis (7010–2650, Kingston, NH, United States). RIAs were conducted in duplicate and were counted on a Beckman Tri Carb scintillation counter. Samples from the various treatments were randomly distributed among three assays, while keeping a given male's initial and final samples within the same assay to avoid introducing interassay variation to pre- versus post-analyses. Intra-assay coefficients of variation were based on six standard samples of known hormone concentration and ranged from 8.3 to 22.5% and 18.3 to 25.5%, for T and KT, respectively. Inter-assay coefficients of variation were 17.4% and 7.6% for T and KT, respectively. All estradiol levels were below our assay's level of detectability (approximately 8 ng/mL) and thus are not discussed further and were not analyzed statistically.

Cortisol was also measured to assess the stress response in our fish and help verify that the surgery itself, and subsequently cortisol levels, did not influence the behaviors we observed. Cortisol was extracted from a separate 100-μL aliquot of plasma using diethyl ether as above and then assayed via RIA, but without prior chromatographic separation. The cortisol antibody used was purchased from Esoterix Endocrinology (F3-314, Calabasas Hills, CA, United States). Cortisol samples were run in two assays with intra-assay coefficients of variation of 13.6% and 11.4% and an inter-assay coefficient of variation of 12.5%.

Statistical analyses

All statistical analyses were performed in JMP version 4.0 (SAS Institute Inc., Cary, NC, United States). First we tested for implant effectiveness by assessing if plasma T or KT levels changed across days (1, 2 and 7) in males using four ANOVAs, with day as the fixed factor and T or KT levels for KT1 and KT2 groups as dependent variables.

With the behavioral data, a Chi-Square test was performed to determine if there was a difference in the proportion of males that abandoned from each treatment group. Next, a MANOVA was used to determine differences between males that stayed and males that abandoned in initial blood-sampling time, body length, body mass, Fulton's condition factor, and egg score. Where significance was found in MANOVAs, t-tests were performed to determine differences between males that stayed and males that abandoned for each of the dependent variables. For the males that stayed, a MANOVA was also used to analyze differences among treatment groups in initial and final blood-sampling times, body length, body mass, Fulton's condition factor, and egg score. We then used Spearman correlations to analyze first, the relationships between blood-sampling time and hormone levels, and second, the relationship between the time of day we took blood samples and hormone levels.

Three ANOVAs were conducted to determine differences in initial hormone levels (KT, T and cortisol) between treatment groups. Repeated-measure ANOVAs (one for each hormone analyzed: KT, T and cortisol) were then performed to analyze changes in hormone levels over time, across treatments, and in the interaction between treatment and time. The repeated measures were hormone levels before implantation and three days after implantation. We also used an ANOVA to compare the percent difference in KT levels [(post value minus pre value) / pre value × 100] across the treatment groups.

Behavior data were analyzed for treatment differences using two ANOVAs: one for summed aggressive behaviors and one for summed nurturing behaviors. ANOVAs were also conducted to determine treatment differences within the individual aggressive and nurturing behaviors (aggressive behaviors: bites, opercular flares, lateral displays; nurturing behaviors: caudal sweeps, pectoral fanning, egg removal). Observer (4 for aggression measurements and 3 for nurturing behavior measurements) was added as a random factor in these REML models. We then assessed if a trade-off between aggressive and nurturing behaviors exists among individuals using a Spearman correlation. Initially, body length, body mass, Fulton's condition factor, and egg score were included in all analyses as covariates, but these factors did not have a significant effect and were excluded from all final analyses. For all ANOVA analyses, Tukey's post-hoc test was used for pairwise comparisons.

Results

11-Ketotestosterone implant effectiveness

11-Ketotestosterone implants appeared to prevent the typical decline in androgen levels that takes place shortly after the onset of paternal care in parental male bluegill (Kindler et al., 1989; Magee et al., 2006) (Fig. 1). However, likely due to a small sample size, there were no statistically significant differences in KT levels among days 1, 2 and 7 (after implantation) in KT1 males ($F_{2,12} = 0.31$, $p = 0.74$) or KT2 males ($F_{2,11} = 0.35$, $p = 0.71$) (Fig. 1a). T levels also did not differ among days 1, 2 and 7 in KT1 males ($F_{2,12} = 0.74$, $p = 0.51$) or KT2 males ($F_{2,11} = 1.8$, $p = 0.21$) (Fig. 1b). Although androgen levels in KT2 males were higher on day 2 than KT1 males, these differences were not statistically significant (KT: $t = 2.0$, $df = 10$, $p = 0.19$; T: $t = 1.9$, $df = 10$, $p = 0.20$).

Comparison of males that abandoned nests versus males that stayed after treatment manipulation

There was no difference in the proportion of males from each treatment group that abandoned their nests after manipulation ($n = 8$–13 per treatment; $\chi^2 = 0.29$, $df = 4$, $p = 0.99$). Because we could not obtain post-manipulation behavioral and hormonal data from these males, they were not included in analyses where post-manipulation measurements were required. There was a significant difference between males that abandoned and those that stayed in the overall model of initial measures of time to collect the blood sample, body length, body mass, Fulton's condition factor and egg score (MANOVA: $F_{4,91} = 3.17$, $p = 0.02$) (Table 1). Males that abandoned tended to be shorter in length ($t = 1.83$, $df = 96$, $p = 0.07$), in better body condition ($t = 1.83$, $df = 96$, $p = 0.07$), have lower egg scores ($t = 1.93$, $df = 96$, $p = 0.06$) and take longer to collect blood samples from ($t = 1.73$, $df = 96$, $p = 0.09$) than males that remained on their nests after treatment manipulation (Table 1). Males that abandoned also had significantly higher T on the day after spawning than males that stayed ($t = 2.12$, $df = 96$, $p = 0.04$) but there was no significant difference in KT levels ($t = 1.42$, $df = 96$, $p = 0.16$), cortisol levels ($t = 1.41$, $df = 96$, $p = 0.16$) or body mass ($t = 0.81$, $df = 96$, $p = 0.42$) between these two groups of males (Table 1).

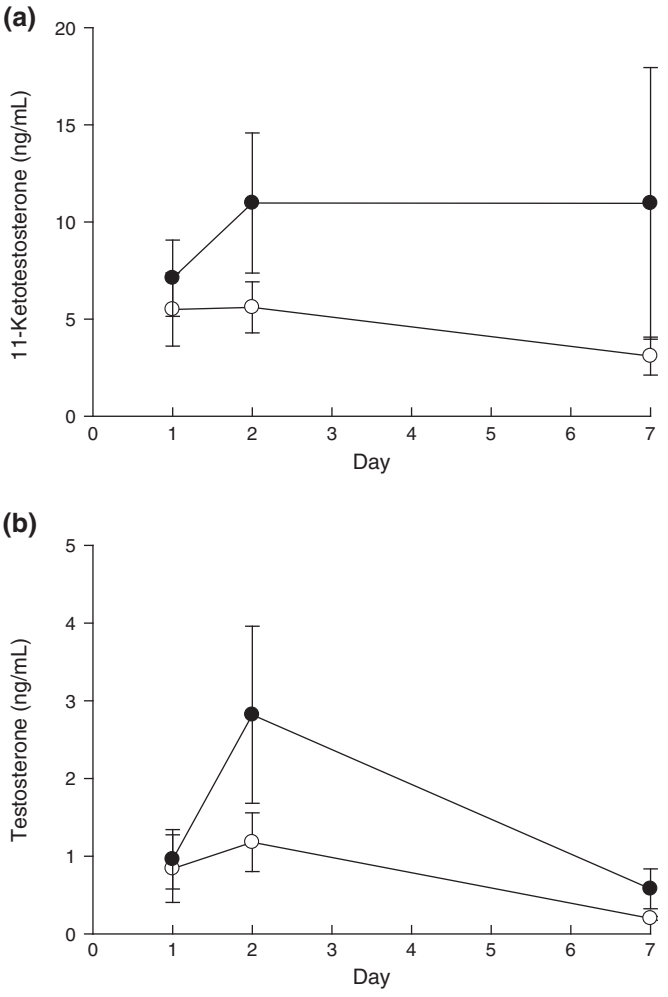

Fig. 1. Effectiveness of 11-ketotestosterone implants in male bluegill sunfish (*Lepomis macrochirus*). Shown are means (±SE) of circulating (a) 11-ketotestosterone and (b) testosterone levels in the plasma 24 h, 48 h and 7 days after implantation of one (KT1; open circles) or two (KT2; filled circles) 11-ketotestosterone implants containing 8 mg KT/mL castor oil. Sample sizes: KT1: day 1 = 7, day 2 = 6, day 7 = 2; KT2: day 1 = 5, day 2 = 6, day 7 = 3.

Table 1
Summary metrics for males that stayed or abandoned their nest after experimental manipulation in bluegill (*Lepomis macrochirus*).

	Stayed	Abandoned
n	47	51
Blood sampling time (s)	63 ± 19	71 ± 24
11-Ketotestosterone (ng/mL)	12.8 ± 9.6	17.4 ± 15.9
Testosterone (ng/mL)	2.0 ± 1.2*	2.9 ± 2.7
Cortisol (ng/mL)	11.9 ± 13.6	7.5 ± 15.0
Body length (mm)	201 ± 11	198 ± 8
Body mass (g)	157 ± 26	153 ± 22
Fulton's condition factor ($g/mm^3 \times 10^5$)	1.9 ± 0.1	2.0 ± 0.1
Egg score	2.3 ± 1.2	1.9 ± 1.0

Shown are means ± SD. Egg score is an estimate of brood size. The asterisk denotes a significant difference between the two male types (*t*-test, $p = 0.04$).

For the 47 males that stayed throughout the experiment, there were no differences among treatment groups in initial or final time to collect the blood sample, body length, body mass, Fulton's condition factor or egg score (MANOVA: $F_{34,131} = 0.80$, $p = 0.76$) (Table 2). Mean initial (day 1 after spawning) hormone levels (±SD) for the males that remained throughout the experiment were 12.8 ± 9.6 ng KT/mL plasma, 2.0 ± 1.2 ng T/mL plasma and 11.9 ± 13.6 ng cortisol/mL plasma. Initial levels of hormones did not differ significantly among treatment groups for KT (ANOVA: $F_{4,46} = 1.38$, $p = 0.26$), T (ANOVA: $F_{4,46} = 0.36$, $p = 0.83$) or cortisol (ANOVA: $F_{4,46} = 1.71$, $p = 0.17$). For these males, neither KT, T nor cortisol levels correlated with either time to collect the blood sample (Spearman correlation: $p > 0.09$ for all) or time of day that we collected blood samples (Spearman correlation: $p > 0.17$ for all).

Effect of treatment on changes in hormone levels

KT levels did not differ significantly among treatments (repeated measures ANOVA; $F_{4,42} = 1.21$, $p = 0.32$) or between the sampling time points (pre- and post-manipulation) ($F_{1,42} = 0.23$, $p = 0.64$), and there was no significant interaction between treatment and time ($F_{4,42} = 1.67$, $p = 0.18$) (Table 2). It is important to note, however, that although the day 3 levels of KT were similar among treatments, the direction of change between sampling time points differed: while the mean KT levels of control groups decreased over the three days (Mean ± SE; Control: −6.60 ± 3.55 ng/mL, Sham: −5.20 ± 5.17 ng/mL), mean KT levels of all experimental groups increased (Flutamide: +2.58 ± 5.08 ng/mL KT1: +4.66 ± 2.67 ng/mL, KT2: +0.48 ± 2.33 ng/mL) (Table 2). This difference in pattern of change in KT levels across treatments is particularly noticeable when comparing the percent difference in KT levels calculated as [(post-manipulation − pre-manipulation)/pre-manipulation × 100] (Fig. 2). Although there was no significant overall effect of treatment (ANOVA: $F_{4,46} = 2.11$, $p = 0.10$) in this latter analysis, a *t*-test confirmed that control and sham males combined had significantly lower KT levels than the KT1 and KT2 males combined ($t = 2.07$, $df = 36$, $p = 0.04$).

Overall, mean T levels were higher on day 3 than on day 1 (repeated measures ANOVA: $F_{1,42} = 9.49$, $p < 0.01$), but there was no significant difference among treatments ($F_{4,42} = 0.81$, $p = 0.61$), nor was there an interaction between time and treatment ($F_{4,42} = 1.99$, $p = 0.11$) (Table 2). Of all groups, KT1 males had the highest T levels on day 3 (Table 2).

Cortisol levels did not differ significantly over time (repeated measures ANOVA: $F_{1,42} = 0.33$, $p = 0.57$) and there was no significant interaction between time and treatment ($F_{4,42} = 1.08$, $p = 0.38$) (Table 2). However, there was a trend for cortisol levels to differ among treatment groups ($F_{4,42} = 2.50$, $p = 0.06$), with controls having higher cortisol levels than the other groups, particularly when compared to KT2 males (Tukey: $p = 0.04$) (Table 2).

Effect of treatment on changes in parental behavior

There was a significant difference in the total number of aggressive behaviors performed among treatments (ANOVA: $F_{4,37} = 3.2$, $p = 0.02$) (Table 3, Fig. 3a). Specifically, males treated with two KT implants (KT2 group) performed more aggressive behaviors than males in the flutamide, control and sham groups (Tukey: all $p < 0.01$). All other comparisons were not statistically significant (Tukey: all $p > 0.21$). Among the individual aggressive behaviors, there was a significant difference in the number of bites performed across treatments (ANOVA: $F_{4,37} = 3.70$, $p = 0.01$), with KT2 males performing significantly more bites than sham (Tukey: $p = 0.05$) and flutamide-implanted (Tukey: $p = 0.01$) males (Table 3). There was no significant difference among treatments for opercular flares (ANOVA: $F_{4,37} = 1.92$, $p = 0.13$) or lateral displays (ANOVA: $F_{4,37} = 1.62$, $p = 0.19$) (Table 3).

There was also a significant difference in the total number of nurturing behaviors performed among treatments (ANOVA: $F_{4,34} = 9.0$, $p < 0.001$) (Table 3, Fig. 3b). The number of nurturing behaviors performed by KT2 males was significantly lower compared to control males (Tukey: $p = 0.01$). KT1 males also performed fewer nurturing behaviors than control males, but this difference was not significant (Tukey: $p = 0.14$). Males treated with flutamide performed significantly more nurturing behaviors compared to all other groups (Tukey: all $p < 0.02$). All other post-hoc comparisons were not statistically significant (Tukey: all $p > 0.26$).

Assessing each nurturing behavior independently revealed a significant difference across treatments in caudal sweeps (ANOVA: $F_{4,34} = 3.86$, $p = 0.01$) and pectoral fanning (ANOVA: $F_{4,34} = 4.40$, $p = 0.01$), with the flutamide group performing significantly more of these behaviors compared to the KT2 group (Tukey: $p = 0.01$, $p = 0.03$, respectively) (Table 3). Although both KT-treated groups

Table 2
Summary metrics of males assigned to one of five treatments that stayed at their nest throughout the experiment in bluegill (*Lepomis macrochirus*).

Treatment[a]	n	Pre- or post-manipulation[b]	Blood sampling time (s)	11-Ketotestosterone (ng/mL)	Testosterone (ng/mL)[c]	Cortisol (ng/mL)	Body length (mm)	Body mass (g)	Fulton's condition factor ($g/mm^3 \times 10^5$)	Egg score
Control	11	Pre	59 ± 14	14.3 ± 10.0	2.1 ± 1.3	13.5 ± 13.4	197 ± 8	145 ± 17	1.9 ± 0.2	2 ± 1
		Post	77 ± 27	7.7 ± 6.3	2.0 ± 1.6	33.7 ± 52.4	196 ± 9	140 ± 16	1.9 ± 0.1	
Sham	8	Pre	65 ± 17	17.4 ± 13.6	1.9 ± 1.4	9.6 ± 7.2	206 ± 10	167 ± 25	1.9 ± 0.1	2 ± 1
		Post	73 ± 25	12.2 ± 7.9	2.9 ± 2.7	11.5 ± 11.7	204 ± 10	159 ± 22	1.9 ± 0.1	
Flutamide	9	Pre	65 ± 28	14.3 ± 11.2	1.9 ± 0.4	19.5 ± 21.5	199 ± 7	155 ± 18	2.0 ± 0.1	2 ± 1
		Post	73 ± 30	16.9 ± 13.6	3.2 ± 2.2	14.1 ± 13.9	198 ± 8	149 ± 15	1.9 ± 0.1	
KT1	9	Pre	64 ± 22	7.8 ± 5.2	1.7 ± 1.6	11.5 ± 11.3	206 ± 13	165 ± 32	1.9 ± 0.2	3 ± 1
		Post	72 ± 25	12.4 ± 7.9	4.6 ± 3.2	7.5 ± 8.0	206 ± 14	163 ± 33	1.9 ± 0.1	
KT2	10	Pre	63 ± 18	10.5 ± 5.2	2.3 ± 1.0	4.6 ± 7.1	199 ± 12	155 ± 32	1.9 ± 0.1	2 ± 1
		Post	69 ± 18	11.0 ± 6.2	2.9 ± 2.7	6.2 ± 6.5	198 ± 13	148 ± 31	1.9 ± 0.1	

Shown are means ± SD for measured variables pre- and post-experimental manipulation. Egg score is a measure of brood size.
[a] KT1 = one 11-ketotestosterone implant; KT2 = two 11-ketotestosterone implants.
[b] Pre = one day after spawning and immediately before manipulation; Post = three days after manipulation.
[c] There was a significant effect of sampling period (pre- versus post-manipulation) across all treatments (see text for details).

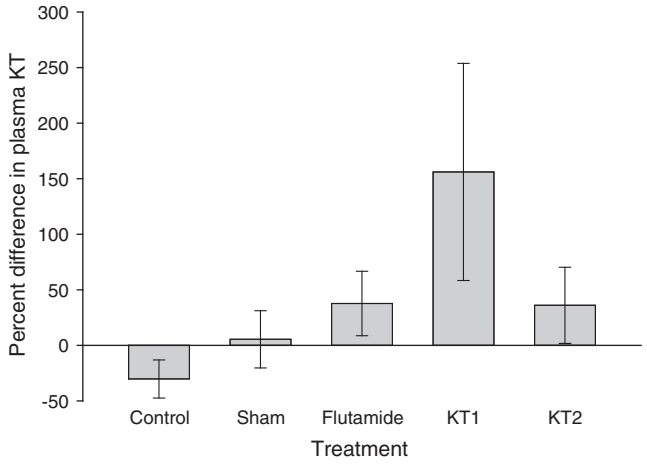

Fig. 2. Changes in circulating levels of 11-ketotestosterone after exposure to one of five treatments in male bluegill sunfish (*Lepomis macrochirus*). Shown are the mean (±SE) percent difference in circulating 11-ketotestosterone calculated as (post-manipulation minus pre-manipulation)/(pre-manipulation × 100). Treatments comprised no manipulation (control, n = 11), sham implant (n = 8), flutamide implant (n = 9), one KT implant (KT1, n = 9), and two KT implants (KT2, n = 10). The means were not significantly different (ANOVA: $F_{4,46} = 2.11$, $p = 0.10$; see text for details).

performed fewer egg removal behaviors, the difference was not significant across treatments ($F_{4,34} = 2.31$, $p = 0.08$) (Table 3).

Finally, there was a significant negative correlation between the total number of aggressive behaviors and the total number of nurturing behaviors across all males (Spearman correlation coefficient = −0.31, n = 47, p = 0.03).

Discussion

Our results suggest that androgens differentially affect aggression and nurturing behaviors during parental care in bluegill sunfish. As a group, males implanted with KT displayed 64% more aggressive behaviors and 71% fewer nurturing behaviors than males in the control groups (control and sham males combined). In contrast, males implanted with the androgen receptor antagonist flutamide displayed 7% fewer aggressive behaviors and 126% more nurturing behaviors than males in the control and sham groups combined. Additionally, across all males, there was a negative relationship between the two types of behaviors. Such a trade-off between aggressive and nurturing behaviors has also been examined in other taxa (e.g., birds: De Ridder et al., 2000; Hegner and Wingfield, 1987; Ketterson et al., 1992; insects: Scott, 2006; rodents: Trainor and Marler, 2001), but has been studied only rarely in uniparental care systems (e.g., Kindler et al., 1989, 1991; Rodgers et al., 2012; Ros et al., 2004). As in the present study, Kindler et al. (1991) investigated aggression and nurturing in nesting bluegill, yet did not find any overall effect of their KT implants or the androgen-receptor antagonist that they used (cyproterone acetate). Those authors, however, used rim circling (continuous swimming around the edge of the nest) as their measure of nurturing behavior. Rim circling may actually be a territorial behavior as it is most frequent prior to spawning when males first set up their nests in colonies (Colgan et al., 1979). Furthermore, those authors used a plastic model of a conspecific

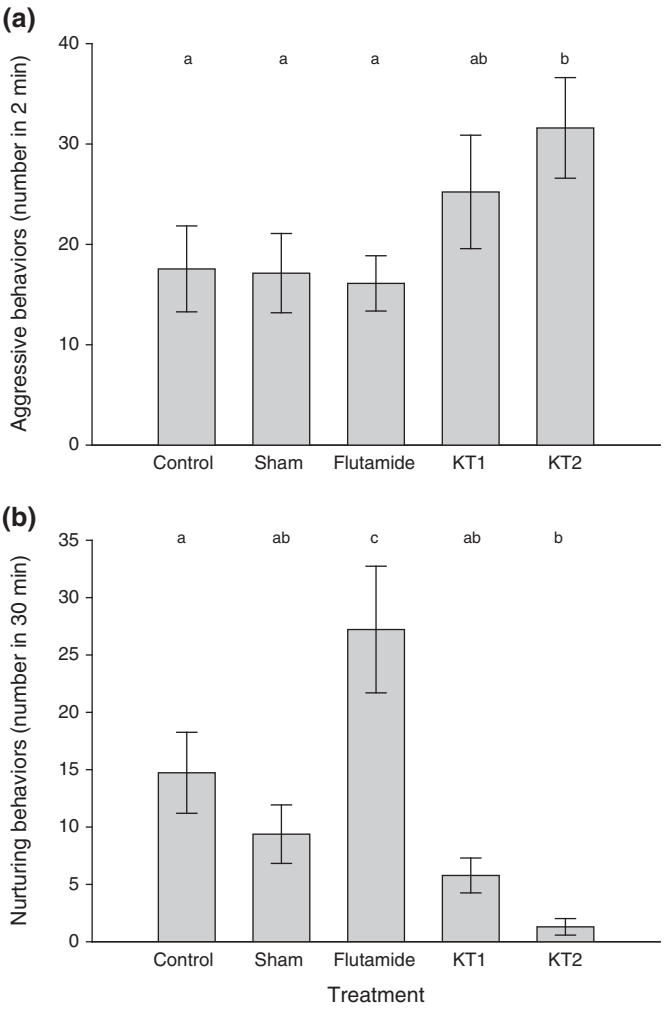

Fig. 3. Paternal care behavior in response to experimentally manipulated levels of 11-ketotestosterone and to the androgen receptor antagonist fluatamide in parental male bluegill sunfish (*Lepomis macrochirus*). Shown are means (±SE) for (a) aggressive behaviors and (b) nurturing behaviors. Aggressive behaviors comprised biting at a brood predator, opercular flares and lateral displays directed towards the predator. Nurturing behaviors comprised caudal sweeps, pectoral fanning and egg removal. Treatments comprised males with no implant (control, n = 11), a sham implant (n = 8), one flutamide implant (n = 9), one 11-ketotestosterone implant (KT1, n = 9), or two 11-ketotestosterone implants (KT2, n = 10). Letters denote homogeneous subsets as determined by ANOVA and Tukey's post-hoc analyses (see text for details).

Table 3
Summary of aggressive and nurturing behaviors displayed during parental care by males assigned to one of five treatments in bluegill (*Lepomis macrochirus*).

Treatment[a]	n	Aggression toward predator[b]				Nurturing behavior[b]			
		Bites	Opercular flares	Lateral displays	Total aggression	Caudal sweeps	Pectoral fanning	Egg removal	Total nurturing
Control	11	9 ± 12^{AB}	5 ± 4	3 ± 2	18 ± 14^{A}	2 ± 4^{AB}	4 ± 5^{AB}	9 ± 13	15 ± 12^{A}
Sham	8	6 ± 7^{A}	7 ± 5	5 ± 4	17 ± 11^{A}	3 ± 5^{AB}	1 ± 1^{AB}	5 ± 4	9 ± 7^{AB}
Flutamide	9	4 ± 3^{A}	9 ± 5	3 ± 2	16 ± 8^{A}	11 ± 16^{B}	5 ± 5^{B}	11 ± 8	27 ± 17^{C}
KT1	9	10 ± 9^{AB}	11 ± 10	4 ± 3	25 ± 17^{AB}	1 ± 3^{AB}	1 ± 1^{AB}	3 ± 4	6 ± 5^{AB}
KT2	10	14 ± 10^{B}	12 ± 8	7 ± 4	32 ± 16^{B}	0 ± 0^{A}	1 ± 1^{A}	1 ± 2	1 ± 2^{B}

Shown are means ± SD of behaviors recorded on the first and second days after hormone manipulation.
Upper case letters denote homogenous subsets for any behavior where there were significant differences across treatments based on Tukey posthoc tests ($p < 0.05$ for differences; see text for details).
[a] KT1 = one 11-ketotestosterone implant; KT2 = two 11-ketotestosterone implants.
[b] Aggression was recorded over four 30-second periods, nurturing behavior was recorded over two 15-min periods.

bluegill to assess aggression, whereas we used a live potential predator, which may have better simulated a threat to the nesting male bluegill. These differences in methods may explain the apparently disparate results between the two studies. Regardless, our study adds to growing literature supporting differential androgen effects on aggressive and nurturing behaviors during parental care.

Behaviors may respond rapidly to changes in circulating androgen levels. Yamaguchi et al. (2004) used KT implants that were similar to ours in seabream fish (*Pagrus major*) and found that KT levels were initially high, but dropped three days after implantation, likely due to a negative feedback response. We conducted our behavioral observations on days 1 and 2 after implantation, but took final blood samples on day 3 after implantation of the KT implants. We thus speculate that KT levels were higher in our KT-implanted males during the behavioral observation period, but had declined by day 3 when the post-implant blood samples were taken. Even so, while the differences in mean day-3 KT levels did not differ significantly between control and KT-implanted groups, the direction of change in KT concentrations in the KT-implanted males was opposite of that for control and sham males. Mean KT levels increased in KT-implanted groups whereas they decreased in the control groups, as is typical for natural KT levels in bluegill over the egg stage of care (Kindler et al., 1989; Magee et al., 2006). Our data thus suggest that behavioral responses to changes in KT may occur quickly during parental care.

Aggressive and nurturing behaviors may differ in their sensitivity to circulating androgen levels. Flutamide-implanted males displayed more nurturing behaviors than any other group, but, contrary to our expectations, they did not show significantly less aggression than the control groups. A possible explanation for this result is that the dose of flutamide we used may have blocked enough androgen receptors to have an effect on downstream influences on nurturing behavior, but was insufficient to have an effect on downstream influences on aggression. Indeed, a study of house sparrows that used more than double our dose of flutamide found both an increase in nurturing behaviors and a decrease in aggression in flutamide-implanted males compared to controls (Hegner and Wingfield, 1987). Taken together, these results suggest that processes downstream of the androgen receptors that mediate the two types of behavior are differentially sensitive to circulating androgen levels with those affecting nurturing behaviors being more sensitive than those affecting aggressive behaviors.

Another possibility is that flutamide is not as effective an androgen receptor antagonist in fishes as it is in mammals. To answer this question definitively would require a study designed specifically for this purpose. At this point, however, we can point to the inhibitory effects of flutamide treatment on spiggin production as has been shown in male three-spined sticklebacks (*Gasterosteus aculeatus*) (Katsiadaki et al., 2002; Olsson et al., 2005). This male-specific protein produced by the kidney is used in nest construction and is known to be strongly androgen-dependent. Spiggin protein production and mRNA levels are often reduced by flutamide treatments (see Katsiadaki et al., 2002; Olsson et al., 2005), suggesting that flutamide does indeed have androgen antagonist effects in fish species. Nevertheless, further research on this matter is warranted.

Our data support an androgen-mediated trade-off between aggressive and nurturing behaviors during parental care. The current knowledge about the physiological mechanism that mediates such a trade-off, however, is limited. In general, it is known that androgens typically exert their effects on behavior by binding to intracellular or membrane-bound steroid hormone receptors in the brain (Steinman and Trainor, 2010). Subsequently, the hormone–receptor complex acts as a direct transcription factor, which can result in transcription of genes and translation into proteins that then influence behavior, or the complex can activate a secondary messenger (Nelson, 2011; Steinman and Trainor, 2010). Gene knockout experiments in mice have identified over 30 genes that affect aggression, including the androgen receptor gene (reviewed by Maxon and Canastar, 2003). Territorial and dominance types of aggression in three-spined sticklebacks were found to be heritable (Barker, 1994), but the specific genes affecting the behaviors have yet to be identified. Genes that affect nurturing behavior in mice may include the immediate early gene *FosB*, an antagonist of fibroblast growth factor signaling (*SPRY1*) and the Ras-related GTPase *Rad* (Kuroda et al., 2008); however, like aggression, studies identifying the genes that affect nurturing behavior in other taxa are lacking. Overall, little is known about the full complement of genes that underlie the trade-off between aggressive and nurturing behavior during parental care. Such studies are needed to further our knowledge on how hormones affect these behaviors.

In conclusion, our study is among the first to investigate the trade-off between aggression and nurturing during paternal care in a uniparental care system and provide support for an apparent trade-off in these behaviors that is mediated at least partially by circulating androgen levels. Our findings indicate that exploration of the mechanistic details of how androgens influence paternal behavior will be a productive area for future research.

Acknowledgments

We thank Scott Colborne, Tim Hain, Jessica Van Zwol, Adrianna Clapp, Michael Roubakha and Kristal Proulx for their assistance in the field, as well as the staff of Queen's University Biological Station for providing access to their field facilities. We also thank two anonymous reviewers, Karrianne DeBaeremaeker and Kim Wallen for their helpful comments that improved the final paper. The work done here was approved by the Canadian Council of Animal Care and Use Committee. Financial support for this study was provided by the Natural Sciences and Engineering Research Council of Canada (awarded to BDN) and the University of Oklahoma Department of Biology (to RK).

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