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Effects of Exogenous Testosterone on Parental Care Behaviours in Male Bluegill Sunfish (*Lepomis macrochirus*)

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Abstract

Androgens are known to mediate aggressive and defensive behaviour in many vertebrate species. However, high concentrations of androgens might also conflict with the expression of nurturing behaviours and therefore a trade-off can exist between aggressive and nurturing behaviours during parental care. We explored the role of testosterone in paternal care in bluegill sunfish (*Lepomis macrochirus*), where males provide both sole defence of the young from predators and sole nurturing behaviour such as fanning of the eggs. At the onset of parental care, we manipulated testosterone levels in males using testosterone propionate implants. We then observed the frequency of nurturing and aggressive behaviours displayed by the males over 6 d of parental care. Testosterone-implanted fish were more aggressive when presented with a brood predator, performing more bites, opercular flares and lateral displays than control males. Testosterone-implanted males, however, were not less nurturing than control fish, performing similar levels of fanning and nest-cleaning behaviours. Thus, our results support a positive relationship between testosterone and paternal aggression but no testosterone-mediated trade-off between paternal nurturing and aggression.

Introduction

There are several different forms of aggression and many of these forms have been linked to androgens (e.g. territorial aggression; Wingfield et al. 1990; dominance aggression; Oliveira et al. 1996; sexual aggression; Schwagmeyer et al. 2005; see Wingfield et al. 2006 for a review). One form, parental aggression, has been studied predominantly in birds (e.g. Silverin 1980; Hegner & Wingfield 1987; see Lynn 2008 for a review), but more recently also in a variety of other taxa including insects (e.g. Scott 2006), fish (e.g. Ros et al. 2004) and mammals, including humans (e.g. Trainor & Marler 2001; Archer 2006; Gettler et al. 2011). In most of these studies, high levels of parental aggression were found to be associated with high androgen levels. Even so, many studies that have investigated the relationship between androgen levels and aggression during parental care have measured changes in androgen levels in response to a nest intruder. These studies generally found that intrusions resulted in an increase in androgen levels in the nest-tending parental individual (e.g. Wingfield 1985; Hirschenhauser et al. 2004). Other studies have manipulated androgens during parental care and examined the subsequent effect on parental aggression (e.g. Ros et al. 2004; Schwagmeyer et al. 2005). However, while high androgen levels consistently seem to result in increases in parental aggression, high androgen levels may also interfere with nurturing behaviour (e.g. Hegner & Wingfield 1987; see Hirschenhauser & Oliveira 2006 for a review). Androgen levels during parental care can thus lead to a parental care trade-off: an individual with high androgen levels will mostly defend but not nurture its offspring, whereas an individual with low androgen levels will mostly nurture but not defend them.

The trade-off between aggressive and nurturing behaviours has been studied extensively in species with biparental care, but studying the trade-off in

these systems may not be ideal because the male and female can assume different parental roles. For example, when a male house sparrow's (*Passer domesticus*) testosterone levels are experimentally elevated, the male typically brings less food to his nestlings but invests more energy in nest defence (Hegner & Wingfield 1987; Schwagmeyer et al. 2005). Meanwhile, the female compensates for the male's low feeding by increasing the amount of food that she brings to the nestlings (Hegner & Wingfield 1987; Schwagmeyer et al. 2005). Likewise, male dark-eyed juncos (*Junco hyemalis*) with elevated testosterone levels feed nestlings less and do so at a slower rate, with females fully compensating for the males' low feeding (Ketterson et al. 1992). In such systems, parental roles can be mostly 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 testosterone, it is less clear whether the observed trade-off in males is entirely because of increased androgens or partly driven by compensation by females. Examining the trade-off in a system where only one parent provides care would overcome this potential confound because the effects of androgens on aggression and nurturing cannot be compensated for by another parent.

Few studies have tested the trade-off in species that provide uniparental care (Kindler et al. 1989, 1991; Ros et al. 2004; Dey et al. 2010). In bluegill (*Lepomis macrochirus*), as in many fish, males provide sole care for the offspring and this system thereby offers an excellent opportunity to explore the effects of androgens on paternal behaviour in a uniparental care system. An additional benefit to studying this question in bluegill is that, in northern latitude populations, parental males conclude spawning and the females leave the vicinity of the colony before the males focus directly on parental care (Gross 1982). Thus, a competitive division of energy between parental care and mating opportunities does not exist (see De Ridder et al. 2000).

We explored the effect of testosterone (T) in parental male bluegill using T implants to experimentally elevate circulating levels of this androgen. Past studies have found that under normal conditions, T and 11-ketotestosterone (11KT) levels of parental male bluegill are typically lower during parental care periods, compared to levels shortly prior to and during spawning (Kindler et al. 1989; Magee et al. 2006). We hypothesised that exogenously elevating T in parental males when nurturing behaviours begin would modulate nurturing and aggressive behaviours. We predicted that administered T would increase the frequency of parental aggressive behaviours (defence of the young) and decrease the frequency of nurturing behaviours.

Materials and Methods

Study Species and Site

Bluegill are native to North America and are found in freshwater lakes ranging from Northern Mexico to Southern Canada (Scott & Crossman 1973). We conducted our study in the 900-hectare Lake Opinicon (44°34′N, 76°19′W) in Ontario, Canada, where bluegill have been studied for over 30 yr (e.g. Colgan et al. 1979; Neff & Knapp 2009). In Lake Opinicon, males of the reproductive phenotype known as 'parentals' mature at the age of 7 yr and then spawn with females from late May to early July in several bouts (Gross 1982). A bout begins when a group of parentals form a colony and build individual nests in the littoral zone. An established colony can contain up to 300 males (Cargnelli & Neff 2006). Spawning at a colony typically lasts only a single day, after which females leave the colony and only the parental males care for the offspring in their nests. For the first 3 d after spawning, a parental male defends his nest from brood predators, fans his eggs to increase oxygen availability and removes moulding eggs from the nest (Côté & Gross 1993). The eggs then hatch and the parental male focuses on protecting the young from brood predators until the young leave the nest 4–7 d later. A parental male will then return to deeper waters of the lake to replenish his energy supplies before possibly returning to the littoral zone for another breeding bout (Cargnelli & Neff 2006).

Behavioural Observations

The field portion of our study was conducted from June 14 to 21, 2009. Using daily snorkelling surveys, swimmers located nests with spawning parental males and females. The day after spawning, 56 parental males were captured between 1000 and 1700 EST and were brought to a nearby boat, where initial blood samples (approx. 300 μl) were taken from the caudal vein. Blood collection time (measured from the time the fish was caught until the needle was removed from the caudal vein) averaged 117 ± 47 s. A numbered tile was placed at each male's nest for fish identification, and while the male was on the boat, a nest cover was placed over his nest to protect the eggs. After blood sample collection, males were anesthetised using clove oil. Length and mass were taken,

from which we later calculated Fulton's condition factor [(mass/length^3) $\times$ 10^5], which estimates the energetic state of an individual (Neff & Cargnelli 2004). Individuals were implanted in the abdominal cavity with either one placebo silastic implant (P) filled with silicone sealant, one testosterone implant (T1) or two testosterone implants (T2). Males in a fourth group (control, C) were handled but had no surgery. Fourteen males were assigned to each of the four treatments through haphazard collection and subsequent rotation through the treatments. Silastic implants (i.d., 1.47 mm, o.d., 1.96 mm; Konigsburg Instruments, Pasadena, CA, USA) were packed with 8-mm T propionate (Sigma-Aldrich, Oakville, ON, Canada), and each end was sealed with 1-mm silicone sealant. Following implant placement, 50 μl of an antibiotic solution (oxytetracycline; Sigma-Aldrich, St. Louis, MO, USA) was injected into the wound to prevent infection, New Skin (Prestige Brand Holdings, Inc., Irvington, NY, USA) was applied to the wound, and fish were placed in a bucket of lake water for a 5-min recovery period. Males were then returned to their nests where they resumed care within a few minutes.

On days 2 and 3 after spawning, when all males had unhatched eggs in the nest, three swimmers observed and recorded the frequency of nurturing behaviours (Table 1) for each parental male for 15 min. All swimmers were blind to the fish's treatment. On each of days 5 and 6 after spawning, parental males guarding fry were presented with a potential brood predator, a ca. 160-mm pumpkinseed sunfish (*Lepomis gibbosus*), in a clear bag attached to a pole. On each of these 2 d, the predator was placed on the edge of each nest for two 30-s periods, with a 30-s break in between (see Neff 2003), totalling a 2-min recording over the 2 d. We recorded the frequency of aggressive behaviours (Table 1) displayed during these presentations and used the sum in the analyses.

On day 7 after spawning, males were re-captured and brought to a boat where a second blood sample was immediately collected. Mean time from re-capture to completion of blood sampling was 88 ± 35 s. Males were then weighed and measured for length for final calculation of Fulton's condition factor, euthanised using clove oil and dissected to ensure implants had stayed in place. Overall, 34 males were included in analyses, comprising seven controls, 10 placebos, nine T1 males and eight T2 males. Nineteen males were excluded from the behavioural analyses because they abandoned their nests before a complete set of behavioural observations could be recorded or before final blood samples could be collected. An additional three males (one control and two T1's) were excluded from the analyses due to unusual behaviour such as swimming away from the nest for long periods of time, where we were unable to obtain an accurate recording of their behaviour.

Radioimmunoassays

Plasma levels of T, 11KT and estradiol were determined using radioimmunoassay (RIA) following chromatographic separation as described in Magee et al. (2006). From each plasma sample, we used 100 μl of plasma and added approx. 2000 cpm of each titrated hormone to allow for correction for losses during extraction and chromatography. Plasma samples were extracted twice with 2 ml diethyl ether. The ether was dried down under nitrogen, and 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 11KT was achieved by sequential elution with 10%, 20% and 30% ethyl acetate in iso-octane, respectively. 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, USA) has high cross-reactivity with 11KT and could thus be used to assay both T and 11KT. The estradiol antibody we used was from Biogenesis (7010–2650, Kingston, NH, USA). Samples were assayed in duplicate, and a charcoal–dextran solution in phosphate-buffered saline (without gelatin) was used to separate bound and unbound hormone fractions.

Table 1: Behaviours quantified during paternal care in bluegill sunfish (*Lepomis macrochirus*)

Type of behaviour	Behaviour observed	Description of behaviour
Nurturing	Pectoral fanning	Rapid synchronous movement of the pectoral fins outwards from the side of the body
	Caudal sweep	Movement of the caudal fin from side to side at a 45° angle from the nest
	Egg removal	Removing moulding eggs from the nest with the mouth
Aggressive	Biting	Nipping at the predator with the mouth and teeth
	Opercular flare	Extending the opercula laterally while facing the predator
	Lateral display	Presenting body lengthwise to the predator

The bound fraction was counted on a Beckman Tri-Carb scintillation counter. Samples were run in two assays. Intra-assay coefficients of variation for T were 4.8% and 6.6%, for estradiol were 19.7% and 14.2%, and for 11KT were 9.3% and 13.7%. Inter-assay coefficients of variation were 3.5%, 5.3% and 9.2% for T, estradiol and 11KT, respectively.

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, USA). Cortisol samples were run in duplicate in a single assay with an intra-assay coefficient of variation of 16.5%.

Statistical Analyses

All data were analysed in JMP version 9.0.2 (SAS Institute Inc., Cary, NC, USA). First, we used a chi-square test to determine whether the males that abandoned were specific to any particular treatment. We used a MANOVA to analyse differences in initial hormone levels, Fulton's condition factor, and body length between males that stayed and males that abandoned. For the males that stayed throughout the experiment, we used two ANOVAs to examine differences among treatments for Fulton's condition factor and body length. Comparison of initial hormone levels among treatments was first analysed using a MANOVA. Differences in individual hormones were then analysed using repeated measures ANOVAs, one for each hormone and each treatment. The repeated measure was the hormone concentration in the first and second blood samples (i.e. before and after the implantation). Initial Fulton's condition factor and body length were originally included as covariates in all analyses; however, these covariates had no significant effect on the overall models and were removed from the final analyses.

Twenty-six (77%) of all initial estradiol levels and 14 (83%) of the final estradiol samples from control and placebo males were below the sensitivity of the assay (approx. 0.35 pg/tube; approx. 0.9 ng/ml plasma), and thus, these data were not analysed statistically. However, we present descriptive statistics for the second estradiol samples from the T-implanted males because only one (from a T2 male) of the 17 samples was non-detectable. For that male, we used the value calculated from the standard curve in our analyses as an estimate of his very low estradiol levels. All other hormone samples were detectable and were used in the analyses.

Behavioural data were first $\log_{10}$+1-transformed to achieve normality. We used principal component analysis (PCA) to construct two composite indices, one index of nurturing behaviours and one index of aggressive behaviours. We used the first axis for both indices because they had positive loadings (nurturing behaviour: caudal sweeps = 0.591, pectoral fanning = 0.594, egg removal = 0.545 and Eigenvalue = 1.62; aggressive behaviour: biting = 0.708, lateral displays = 0.705, opercular flare = 0.039 and Eigenvalue = 1.40) and captured 58% of the variance within the nurturing behaviours and 49% of the variance within the aggressive behaviours. These PC1 scores were compared among treatments using one-way ANOVAs with observer added as a random effect. When overall significance was found in the ANOVAs, we used a Tukey's *post hoc* test to determine pairwise differences. When significance was found in the PC1 score ANOVAs, we also used a MANOVA to assess if any individual behaviour was driving the significant effect.

Results

The number of males that abandoned their nests (n = 3–6 per treatment) did not differ significantly among treatments (χ^2=0.66, df = 3, p = 0.88). Because a full set of behavioural and hormonal data could not be obtained from these males, they were not included in hormone and behaviour analyses. There also were no significant differences in initial hormone concentrations, body length or Fulton's condition factor between males that stayed vs. males that abandoned their nests (MANOVA: $F_{4,38}$=0.62, p = 0.65).

For the 34 males that remained for the duration of the experiment, there were slight differences in both body length and Fulton's condition factor among the treatments, with control males being significantly smaller (ANOVA: $F_{3,30}$=3.49, p = 0.03) but in better condition (ANOVA: $F_{3,30}$=3.48, p = 0.03) than the other males (Table 2). Overall, mean (±SD) initial levels of T, 11KT and cortisol were 2.3 ± 1.7, 7.5 ± 4.9 and 23.6 ± 31.6 ng/ml, respectively (initial estradiol levels were below the sensitivity of the RIA). Initial hormone levels did not differ significantly among treatments (MANOVA: $F_{6,44}$=1.13, p = 0.36). Seven days after implant placement, T concentrations were significantly higher in T-implanted males compared to initial levels (ANOVA, T1 males: $F_{1,8}$=23.0, p < 0.001; T2 males: $F_{1,7}$=169.3, p < 0.001; Table 2). Typical mean levels of hormones in bluegill at the onset of care are 1–8 ng/ml for T, 5–15 ng/ml for 11KT and 25

Table 2: Mean (±SD) for body length, Fulton's condition factor and hormone concentrations of parental males during parental care in bluegill (*Lepomis macrochirus*). For each treatment group, upper values indicate before manipulation and lower values indicate 7 d after manipulation

Treatment	n	Body length (mm)	Fulton's condition factor ($g/mm^3 \times 10^5$)	Testosterone (ng/ml)	11-Ketotestosterone (ng/ml)	Estradiol (ng/ml)	Cortisol (ng/ml)
Control	7	194 ± 10	2.0 ± 0.1	2.3 ± 0.5	9.8 ± 5.7	ND	26.6 ± 29.3
		194 ± 9	1.8 ± 0.1	2.4 ± 2.2	7.0 ± 6.6	ND	25.8 ± 30.0
Placebo	10	203 ± 6	1.8 ± 0.1	2.8 ± 2.6	7.4 ± 5.2	ND	9.9 ± 13.8
		202 ± 6	1.8 ± 0.1	1.8 ± 0.7	5.4 ± 2.2	ND	51.5 ± 62.3
Testo (T1)	9	201 ± 5	1.8 ± 0.1	2.2 ± 1.3	7.2 ± 5.0	ND	18.9 ± 11.5
		199 ± 6	1.8 ± 0.1	93.4 ± 57.5	8.1 ± 5.8	7.6 ± 11.2	39.8 ± 35.5
Testo (T2)	8	204 ± 5	1.8 ± 0.1	1.6 ± 1.2	5.7 ± 3.7	ND	44.1 ± 56.9
		201 ± 7	1.8 ± 0.1	125.1 ± 26.5	7.7 ± 8.4	12.4 ± 13.2	8.6 ± 7.4

ND, non-detectable (see text for details).

–150 ng/ml for cortisol (Kindler et al. 1989; Magee et al. 2006). Thus, the elevated T levels in our study were about 20-fold higher than is typical of nesting parental males and about 10-fold higher than levels seen in parental males in the days immediately prior to spawning (Kindler et al. 1989; Magee et al. 2006). For all treatments, cortisol and 11KT levels did not differ significantly between the two sampling points (all $p > 0.09$). Although not analysed statistically, mean final estradiol levels were notably higher in the T-implanted groups compared to the control and placebo groups.

Analysing PC1 for nurturing behaviours revealed no significant difference across treatments (ANOVA: $F_{3,30}=1.03$, $p = 0.39$; Fig. 1a). However, aggressive behaviours did differ significantly among treatments as measured by the PC1 (ANOVA: $F_{3,30}=3.53$, $p = 0.03$; Fig. 1b). Specifically, T1 males were more aggressive than control ($p = 0.02$) and placebo males ($p < 0.01$). Tukey's pairwise comparisons revealed no significant differences between the remaining groups (all $p > 0.34$). There was no significant difference in the individual aggressive behaviours examined (MANOVA: $F_{6,58}=1.43$, $p = 0.22$; Table 3).

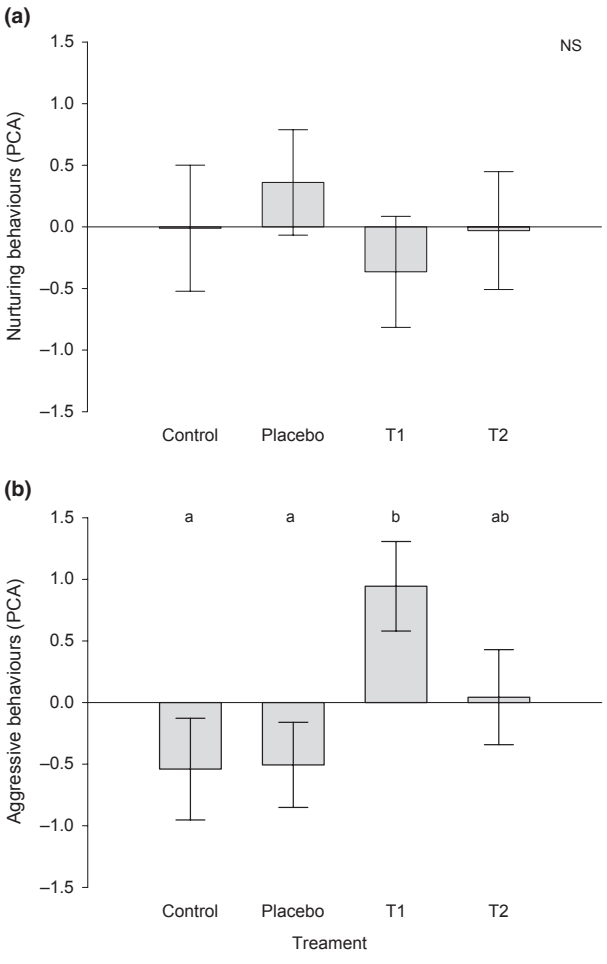

Fig. 1: Paternal behaviour in response to experimentally manipulated levels of testosterone in male bluegill (*Lepomis macrochirus*). Shown are means (±1 SE) of the first axis for a principal component analysis of (a) nurturing behaviours and (b) aggressive behaviours. Treatments include males given no implant (control, n = 7), a placebo implant (n = 10), one testosterone implant (T1, n = 9) or two testosterone implants (T2, n = 8). Bars with different letters are significantly different from each other ($p < 0.05$).

Discussion

Our results suggest that while T does not affect nurturing behaviours, it does, either directly or via its metabolite estradiol, mediate aggressive behaviours during parental care in bluegill. Consistent with our data, a positive relationship between androgen levels and aggression has been reported in birds (Hegner & Wingfield 1987), humans (Archer 2006), chimpanzees (*Pan troglodytes schweinfurthii*; Muller & Wrangham 2004), ring-tailed lemurs (*Lemur catta*; Cavigelli & Pereira 2000), lizards (*Anolis carolinensis*; Greenberg & Crews 1990) and some other fish species (see Olive-

Table 3: Means (±SD) for nurturing and aggressive behaviours by parental male bluegill (*Lepomis macrochirus*) during parental care. Nurturing behaviours were recorded over 30 min and aggressive behaviours directed towards a potential nest predator were recorded over 2 min (see text for details)

		Nurturing behaviour			Aggression towards predator		
Treatment	n	Caudal sweeps	Pectoral fanning	Egg removal	Bites	Opercular flares	Lateral displays
Control	7	13 ± 32	8 ± 16	6 ± 5	46 ± 16	11 ± 8	7 ± 3
Placebo	10	1 ± 2	14 ± 11	9 ± 7	50 ± 10	9 ± 4	5 ± 3
Testo (T1)	9	8 ± 23	11 ± 13	4 ± 4	61 ± 12	10 ± 9	11 ± 4
Testo (T2)	8	2 ± 3	9 ± 7	5 ± 5	52 ± 14	12 ± 7	8 ± 4

ira et al. 2002 for a review; *Parablennius parvicornis*, Ros et al. 2004). Some caution is warranted when interpreting our data, however, because our implants induced pharmacologically high T levels. Nevertheless, a large body of evidence indicates that testosterone plays a part in mediating several forms of aggression in a broad range of animals.

The mechanism by which T could have its effects on increasing parental aggressive behaviours warrants additional studies. Although our T manipulation did not increase circulating levels of 11KT, another behaviourally important androgen in many fish species (e.g. Oliveira et al. 2001), it did elevate circulating estradiol levels. Thus, the T we implanted may have had its effect via activation of estrogen receptors in relevant brain areas. Indeed, several studies in birds and rodents suggest that T does not influence aggression directly, but rather has its effects via metabolism to estradiol in the brain (e.g. Hau et al. 2000; Soma et al. 2000; Silverin et al. 2004; Trainor et al. 2006). For example, male California mice (*Peromyscus californicus*) treated with an inhibitor of aromatase, the enzyme that converts T to estradiol, displayed a lower frequency of aggressive behaviours towards an intruder than did control males (Trainor et al. 2006). Without having blocked the conversion of T to estradiol, we cannot determine the specific mechanism by which T-implantation increased levels of aggression in our T1 fish.

Interestingly, our results revealed that T1 individuals were more responsive in terms of aggression to increases in T than individuals with double the dose (T2). This result suggests an inverted-U-shaped dose response curve as is commonly seen in hormone manipulation studies both within and above the normal physiological range (Hews & Moore 1997; Adkins-Regan 2005). For example, although prolactin can induce egg fanning in cichlid fish, higher doses can inhibit the behaviour (Blüm & Fiedler 1965). Similarly, rats placed in an escapable shock situation and administered various doses of corticosterone displayed an inverted-U-shaped response in learned helplessness behaviour: in the low and high dose treatments, rats exhibited few attempts to escape, whereas rats given a moderate dose maintained high escape attempts and success (Kademian et al. 2005). Interestingly, our results contrast those of Kindler et al. (1991) who found no effect of elevated T on aggression in a study of similar design on bluegill. However, the elevated level of androgens in this earlier study may have still been at the lower end of an inverted-U-shaped response curve. In addition, that study used a model of a bluegill to elicit predator defence behaviour rather than a live non-conspecific predator as we did here, which might also explain the difference in results. Moreover, Kindler et al. (1991) did not measure estradiol levels, so we cannot address whether the difference in results between the two studies could be related to differences in estradiol levels.

Although we found some support that parental aggressive behaviours (defence of the young) increased with increasing T levels, our results are not entirely consistent with the proposed trade-off between parental nurturing and aggressive behaviours. The frequency of nurturing behaviours was not lower in T-treated males as we had predicted. This result could be due to a number of factors, such as the pharmacologically high dose of T or relatively small sample size. However, the maintenance of nurturing behaviour in our T-implanted males may have been a result of their elevated estradiol levels as has been found in other studies. For example, exposure to 17α-ethinyl estradiol increased the time male sand gobies (*Pomatoschistus minutus*) spent fanning their eggs compared to controls (Saaristo et al. 2009). Thus, the higher estradiol levels in our T-implanted fish could have compensated for any T-induced decrease in nurturing behaviours, even as estradiol might also have played a role in the increased nest defence. Alternatively, nurturing behaviour may follow a U-shaped dose response curve, where in our study, changes in

behaviour were not detected because of our low (control) and high pharmacological levels of T, but would be detected by high physiological levels of T. Future studies in bluegill could utilise an aromatase inhibitor or estrogen and androgen receptor blockers, as well as lower doses of T to tease apart the roles of estradiol and androgens in mediating nurturing and aggressive behaviours during paternal care.

In conclusion, although some caution is warranted when interpreting our data because of the pharmacological levels induced by our implants, the data implicate that T directly, or indirectly via estradiol, mediates parental aggression in this species. However, no androgen-mediated trade-off appeared to exist between parental aggression and nurturing behaviour in these fish.

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