

Androgen and prolactin manipulation induces changes in aggressive and nurturing behavior in a fish with male parental care

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ARTICLE INFO

Keywords:

Bluegill
Lepomis macrochirus
 Androgens
 11-ketotestosterone
 Prolactin
 Parental care
 Aggression
 Nurturing
 Trade-off
 Bromocriptine

ABSTRACT

Parental care can include two general types of behavior: (1) aggressive behavior, which is used to defend offspring from predators; and (2) nurturing behavior, which is used to provide offspring with environmental conditions or resources necessary for survival. Many studies have implicated androgens in promoting aggressive behavior and prolactin in promoting nurturing behavior. We experimentally manipulated these hormones to investigate their effects on parental care behavior in bluegill (*Lepomis macrochirus*). Parental males, which provide sole care to the developing eggs and larvae, received an implant with an androgen (11-ketotestosterone [11-KT]), an androgen antagonist (flutamide), prolactin, a prolactin-release inhibitor (bromocriptine), or castor oil (placebo). We found that 11-KT implants led to a significant increase in the frequency of aggressive behavior directed towards a simulated brood predator, and were associated with a nearly significant decrease in the frequency of nurturing behavior directed towards the developing eggs. In contrast, prolactin implants were associated with a significant increase in the frequency of nurturing behavior, but also reduced the frequency of aggressive behavior directed towards the simulated brood predator. These results suggest a hormone-mediated mechanistic trade-off between nurturing and aggressive behavior, whereby parental males are unable to be both highly nurturing and highly aggressive.

1. Introduction

In many animals, males display aggressive behavior during the breeding season when they defend territories and compete for access to females (e.g. Lincoln et al., 1972). Such aggression is often associated with breeding success (e.g. Hegner and Wingfield, 1987), and it is well known that androgens regulate these behaviors (Bouissou, 1983). For example, when rats (*Rattus norvegicus*) are injected with an androgen, their aggression levels towards a conspecific male sharply increase as compared to control males (Farrell and McGinnis, 2003); male European starlings (*Sturnus vulgaris*) castrated at an early age exhibit decreased non-breeding aggression when compared to non-castrated males (Pinxten et al., 2000); nesting bluegill (*Lepomis macrochirus*), after receiving an androgen implant, increase aggression towards a brood predator as compared to control males (Rodgers et al., 2013).

Prolactin is another hormone that regulates behavior related to reproduction. Since its discovery in mammals, prolactin has been linked to milk protein production (Riddle et al., 1933), nursing and other parental care behavior, as well as female receptivity to males (Freeman

et al., 2000). In birds, circulating prolactin concentration increases during the breeding season and is associated with chick brooding, feeding behavior, and other parental behavior (e.g. Angelier et al., 2016; Smiley and Adkins-Regan, 2018). Bromocriptine, a prolactin-release inhibitor, has been used to study the effects of prolactin in mammals and birds, where it has been shown to reduce parental care behavior (Molik and Błasiak, 2015; Smiley and Adkins-Regan, 2018). Studies have also implicated prolactin in regulating nurturing behavior during parental care in fishes (Whittington and Wilson, 2013). Prolactin injected into three-spined sticklebacks (*Gasterosteus aculeatus*) increases fanning, a nurturing behavior of eggs (Páll et al., 2004), and in the discus fish (*Symphysodon aequifasciata*), the number of prolactin receptors in the skin increases during parental care, which suggests that prolactin may be involved in regulating the production of the “discus milk” that the offspring feed on (Khong et al., 2009). Prolactin thus appears to have a conserved role in mediating reproductive traits across animal taxa.

It has been proposed that there is a trade-off between nurturing and aggressive behavior during parental care, and that androgens mediate

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the trade-off. For example, in house sparrows (*Passer domesticus*), males implanted with testosterone exhibit increased aggression, which came at a cost of reduced nurturing behavior (Hegner and Wingfield, 1987). Similar findings have been reported in other birds, including superb fairy-wrens (*Malurus cyaneus*) (Peters, 2002) and dark-eyed juncos (*Junco hyemalis*) (McGlothlin et al., 2007). However, in species with biparental care, this trade-off between aggression and nurturing behavior may be mitigated by females compensating for a male's behavior (i.e. role specialization: Saino and Möller, 1995; Stoehr and Hill, 2000). Such compensation and specialization in bi-parental species can confound analyses intended to determine if there is a trade-off that is actually mediated by androgens. Many fish species, on the other hand, are characterized by uniparental male care (Gross and Sargent, 1985), such that a division of parental care behavior is not possible. Fish thus provide excellent systems to examine a potential androgen-mediated trade-off. Indeed, in an initial study of bluegill (*Lepomis macrochirus*), males implanted with 11-ketotestosterone (11-KT)—the primary androgen in fishes (Borg, 1994)—displayed heightened aggression towards a live brood predator but less nurturing behavior of the developing eggs (Rodgers et al., 2013). In contrast, an earlier study in bluegill found no effect of 11-KT implants on the frequency of aggression directed towards a model of a brood predator (Kindler et al., 1991b). Further study is needed, but together these data suggest that androgens may mediate a trade-off between aggressive and nurturing behavior during parental care.

Here we use bluegill to characterize the effects of both 11-KT and, for the first time in this species, prolactin on parental care. We experimentally manipulated the concentration of these hormones in nesting parental males and then quantified their aggressive and nurturing behavior during the parental care period. We predict that 11-KT will increase aggressive behavior at a cost of reduced nurturing behavior, whereas prolactin will have the opposite effect. Our results allow us to examine the role of androgens and prolactin in any potential trade-off between aggressive and nurturing behavior.

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 lake is approximately 900 ha and has been a study site for this species since the 1980s (e.g. Gross, 1982). In Lake Opinicon, during the June to July breeding season, parental males enter the littoral zone and build nests side-by-side in colonies. A colony is established over the course of a couple of days, after which females spawn with parental males at that colony during a single day. Multiple colonies are formed at different locations and different times during the breeding season. Parental males remain highly aggressive to each other during colony formation and spawning (Gross, 1982), and during this period, circulating 11-KT concentrations are high (Magee et al., 2006). After spawning, only the parental males remain in the colony to provide parental care to the offspring. The care period consists of about 3 days of caring for eggs, when parental males actively fan the eggs to move oxygenated water across the nest, remove dead or moldy eggs from the nest, and defend the eggs from brood predators in the water column, which include other bluegill, pumpkinseed (*Lepomis gibbosus*), small-mouth bass (*Micropterus dolomieu*), and bluegill × pumpkinseed hybrids (Côté and Gross, 1993; Gross and Macmillan, 1981). After the eggs hatch, the parental males will remain for another 4–7 days while the larvae continue to develop in order to defend the larvae from brood predators. Circulating concentrations of 11-KT tend to be low during the egg period of care, but can rise again during the larval period of care (Magee et al., 2006).

2.2. Hormone manipulation

The experimental protocol used in this study was approved by the University of Western Ontario Animal Care Committee. During the breeding seasons of 2015, 2016 and 2017, swimmers monitored bluegill activity in the lake and identified colony formation and the onset of spawning. On the day after spawning, nests were marked with numbered ceramic tiles, and parental males were captured one at a time using a dip net. A screen was placed over the nest to prevent egg predation during the male's absence. The number of eggs on each nest was estimated using a 5-point scale (see Claussen, 1991). Each parental male was taken to a nearby boat where 200 µl of whole blood was collected from the caudal vein using a 25G needle attached to a 1 ml, heparinized syringe. The time to collect blood never exceeded 2 min from capture. Blood samples were stored on ice until they were brought to the field-based laboratory and centrifuged to separate and extract plasma. The plasma was then stored at –20 °C. Each male was assigned to one of six treatments in a rotating order: (1) placebo (control), (2) 11-KT, (3) flutamide, an 11-KT antagonist, (4) low concentration of prolactin, (5) high concentration of prolactin, and (6) bromocriptine, a prolactin-release inhibitor. In 2015, all treatments were used except the prolactin treatments, in 2016 all treatments were used except the high prolactin treatment, and finally, in 2017, placebo, 11-KT and high prolactin were the only treatments used. The treatments vary among years partly due to logistical constraints. Within a colony, the locations of males assigned to different treatments were distributed haphazardly. As in Rodgers et al. (2012), implants were made with silastic tubing measuring 8 mm in length and 1.47 mm internal diameter. Placebo implants were filled with castor oil, flutamide implants were filled with flutamide powder (Sigma Aldrich, Oakville, ON), 11-KT implants were filled with 11-KT (Steraloids, Newport, RI) dissolved in castor oil (amount = 80 µg KT/implant), bromocriptine implants were filled with bromocriptine powder (Cayman Chemical, Ann Arbor, MI), and prolactin implants were filled with prolactin (Sigma Aldrich, Oakville, ON) dissolved in castor oil at one of two concentrations: low prolactin (amount = 1.25 IU/implant) and high prolactin (amount = 12.5 IU/implant). All implants were sealed at both ends with 1 mm of silicone.

Parental males were anesthetized using clove oil, and implants were inserted by first removing two to three scales, anterior to the pelvic fin, with tweezers. The exposed area was then cut using a scalpel to create a small incision into the peritoneal cavity. Two implants were inserted into the cavity and 1 ml of a saturated solution of the antibiotic oxytetracycline was injected in the wound to help prevent infection. The incision was then sealed with liquid bandage (New Skin; Prestige Brand Holdings, Tarrytown, NY). Fish were given at least 5 min to recover in a water-filled bucket, before being returned to their nest. Once the male resumed active nest-care activities, which usually occurred immediately upon return to the nest, the screen cover was removed.

2.3. Parental care behavior

Parental care behavior was observed on the second and third days after spawning (i.e. one and two days after implantation). Fish were observed by swimmers in the 2015 season. Behavior was recorded using video cameras (GoPro, San Mateo, CA) placed around the colony during the 2016 and 2017 seasons. Parental behavior from two general categories was observed: aggressive and nurturing. To measure nurturing behavior, parental males were observed or filmed for 30 min each day, during the period of 9:00–12:00 EDT. The daily order of behavioral observations was haphazard across individuals to balance any timing effects among treatments. Three nurturing behaviors were counted: (1) pectoral fanning; (2) caudal fanning; and (3) egg removal. Pectoral and caudal fanning bring fresh, oxygenated water across the developing eggs and remove sediment from the eggs (Slijkhuis et al., 1984). Egg removal impedes dead eggs from spreading fungal disease (Côté and Gross, 1993). We also measured rim circling behavior during this

period but analyzed this behavior separately as it is unclear if the behavior is used to oxygenate the eggs similar to fanning, or if it is a nest defensive behavior (see Colgan et al., 1979). To measure aggressive behavior, parental males were observed or filmed for 1.5 min on the same days that nurturing behavior was recorded, during the period of 13:00–15:00 EDT. A pumpkinseed was placed in a transparent plastic bag filled with water attached to a 1.5 m pole and was presented on the border of a parental male's nest for 30 s, followed by a 30 s interval when the pumpkinseed was removed from the nest, and then a final 30 s presentation. Three aggressive behaviors were counted during the two 30 s intervals when the pumpkinseed was present: (1) lateral displays; (2) opercular flares; and (3) bites. A lateral display occurs when the male turns laterally relative to the invading fish, an opercular flare occurs when the male approaches the invading fish and extends his opercula outwards, and a bite occurs when the male makes physical contact with the invading fish using his mouth. All behaviors were turned into counts per minute for analysis.

2.4. Hormone measurements

At the end of the third day after spawning, fish were again collected with dip nets and taken to the boat. Approximately 200 μ l of blood was collected, as described above, for final circulating hormone analysis. For a subset of males (those collected in 2017), enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical, Ann Arbor, MI) were used to determine the concentrations of 11-KT and cortisol in the plasma. The samples were run in triplicate, and the ELISAs were validated by assessing parallelism between a standard curve obtained with a calibrated 11-KT solution and serial dilutions of samples from our fish. These analyses were used to confirm that the 11-KT implants did in fact alter circulating concentrations of 11-KT. The cortisol analysis provided a measure of potential differences in stress level among treatments. We have not yet been able to validate an ELISA kit for analysis of circulating prolactin concentrations. For the 11-KT assay the intra-assay coefficient of variation was 8.1% and the inter-assay coefficient of variation was 6.4%. For the cortisol assay the intra-assay coefficient of variation was 14% and the inter-assay coefficient of variation was 20%. Concentrations of 11-KT and cortisol were within the range expected based on previous studies of bluegill parental males during the breeding season (Knapp and Neff, 2007; Magee et al., 2006).

2.5. Statistical analyses

We used SPSS version 25 (IBM) or JMP version 4.0.4 (SAS Institute Inc.) for all analyses. First, we compared abandonment rates prior to the final collection among treatments using a Chi-Square test. Subsequent analyses focus only on individuals that stayed until the final collection. To compare body length and egg score for fish assigned to different treatments, we used an ANOVA model for body length and an ordinal regression for egg score, with both models including treatment as a fixed factor and year as a random factor. The effects of implants on circulating hormone concentrations (11-KT and cortisol) were assessed using ANOVAs for the initial and final hormone concentrations with treatment as a fixed factor. Effects of hormone manipulation on behavior were assessed using ANOVAs that included treatment as a fixed factor and year as a random factor. When a significant effect of treatment was observed, effect size was calculated as η^2 (sum of squares for the treatment factor divided by the total sum of squares) and post hoc comparisons among treatments were performed using Fisher's least significant difference (LSD). Our main analysis focused on the sum of nurturing behaviors (pectoral fanning + caudal fanning + egg removal) and the sum of aggressive behaviors (lateral display + opercular flare + biting), with each sum expressed per minute of observation. We also present the analysis of the individual behaviors in the Appendix A. Similar patterns of significance were observed when the behavioral data were instead analyzed with non-parametric Wilcoxon

Table 1

Characteristics of parental male bluegill (*Lepomis macrochirus*) assigned to one of six hormone manipulation treatments. Abandonment is presented as the number of males that stayed or abandoned prior to the final collection, whereas body length and egg score are presented as the mean \pm 1 SE. Only fish that stayed on their nests until the final blood collection are included in the body length and egg score data.

Treatments	Abandoned (n)	Stayed (n)	Body length (mm)	Egg score (1–5)
Control	16	58	183 \pm 1	3.2 \pm 0.1
11-KT	24	49	183 \pm 1	2.9 \pm 0.2
Flutamide	13	22	189 \pm 2	3.5 \pm 0.2
Low prolactin	5	11	188 \pm 3	3.2 \pm 0.3
High prolactin	7	33	181 \pm 1	2.9 \pm 0.1
Bromocriptine	15	20	189 \pm 2	3.2 \pm 0.2

Note: There were no significant differences among treatments in any of these metrics (see text for details).

tests followed by pairwise Kruskal-Wallis tests between hormone treatments (data not shown). Finally, we used Spearman's test to correlate nurturing and aggressive behavior. This correlation was run on both the untransformed data (sums of aggressive and nurturing behavior per minute of observation), as well as on the same data after applying a z-score transformation within each treatment to control for any absolute differences across the treatments.

3. Results

3.1. Comparison of parental males by treatment

The rate of nest abandonment did not differ significantly among treatments ($n = 273$, $\chi^2 = 9.42$, $df = 5$, $p = 0.094$; Table 1). Body length did not differ among fish assigned to different treatments ($F_{5,183} = 0.69$, $p = 0.66$; Table 1), but did differ among years ($F_{2,183} = 4.44$, $p = 0.013$). Egg score did not differ among fish assigned to different treatments ($\chi^2 = 2.79$, $df = 5$, $p = 0.73$; Table 1) or among years ($\chi^2 = .87$, $df = 2$, $p = 0.65$). There were no significant differences in circulating cortisol concentration among treatments before implantation ($F_{2,90} = 0.70$, $p = 0.50$; Table 2) or after implantation ($F_{2,89} = 0.77$, $p = 0.46$; Table 2). There were no significant differences in circulating 11-KT concentrations among treatments before implantation ($F_{2,90} = 0.99$, $p = 0.37$; Table 2), but there were significant differences after implantation ($F_{2,89} = 4.40$, $p = 0.015$, $\eta^2 = 0.099$; Table 2); the 11-KT concentrations were significantly higher in the 11-KT treatment than the control and prolactin treatments.

3.2. Parental care behavior

The frequency of aggressive behavior exhibited by the 11-KT treatment males was significantly higher than the other treatments ($F_{5,179} = 22.62$, $p < 0.001$, $\eta^2 = 0.222$; Fig. 1A). Males in the high prolactin treatment displayed the lowest level of aggression, and their level was significantly lower than the bromocriptine-implanted fish. Unexpectedly, flutamide appeared to have no effect on aggressive behavior. There was no effect of year on aggressive behavior ($F_{2,179} = 1.69$, $p = 0.19$).

We found that the frequency of nurturing behavior was highest in males in the high prolactin treatment, and lowest for the males in the bromocriptine treatment ($F_{5,168} = 14.13$, $p < 0.001$, $\eta^2 = 0.381$; Fig. 1B). The males in the 11-KT treatment displayed a trend towards less nurturing behavior than control males, but the difference was not statistically significant ($p = 0.075$). There was a year effect on nurturing behavior ($F_{2,168} = 14.89$, $p < 0.001$); the frequency of nurturing behavior was lower in 2015, when behaviors were observed directly than in 2016 and 2017 when behaviors were filmed. Parental males did not show a clear reaction to the presence of an observer who

Table 2

Hormone concentrations for three treatment groups in parental male bluegill (*Lepomis macrochirus*). Shown are the number of samples analyzed and mean circulating 11-KT and cortisol concentrations (\pm SE) before and after implantation for parental males collected in 2017.

Treatments	n Before	n After	11-KT (ng/ml) Before	11-KT (ng/ml) After	Cortisol (ng/ml) before	Cortisol (ng/ml) after
Control	37	35	27.9 \pm 4.7	12.6 \pm 2.4 ^b	7.6 \pm 1.7	7.3 \pm 1.4
11-KT	26	25	25.3 \pm 5.2	20.8 \pm 2.2 ^a	5.1 \pm 1.0	6.7 \pm 2.2
High prolactin	30	32	19.1 \pm 3.8	10.4 \pm 2.6 ^b	6.5 \pm 1.4	4.8 \pm 1.0

Note: ANOVAs were used to compare hormone concentrations among treatments. When the treatment effect was significant, the same letter is used to indicate groups that were not statistically different (see text for details). The number of samples analyzed before and after implantation differ because the necessary amount of plasma was not available for a small number of the sample collections.

was floating several meters from their nest, so it is unclear whether or not the variation among years occurred because the observer's presence affected the expression of nurturing behaviors.

There was a negative correlation between nurturing and aggressive behavior (Spearman's $r = -0.318$, $n = 171$, $p < 0.001$; Fig. 2). This negative relationship was also significant when the data were first Z-scored within treatments (Spearman's $r = -0.185$, $n = 171$, $p = 0.015$; data not shown).

4. Discussion

There is a well-established relationship between circulating androgen concentrations and the frequency and intensity of aggressive behavior across animal taxa (Marler et al., 2003). Consistent with this relationship, we found that parental males implanted with 11-KT, the primary androgen in fishes, displayed more aggressive behavior towards a brood predator than individuals that received a placebo implant. Hormone assays validated the effectiveness of our implants: whereas the control males showed a drop in circulating 11-KT concentration between the two measurement days, the 11-KT implanted males maintained a high concentration of the hormone (also see Magee et al., 2006). Flutamide implants appeared to have no effect on the frequency of aggressive behaviors displayed by the parental males. This result matches a previous study in bluegill (Rodgers et al., 2013), and is consistent with studies in mammals, lizards and other fishes that have shown no effect of flutamide on aggression, despite effects of flutamide on other reproductive traits in these taxa (e.g. Heilman et al., 1976; Sebire et al., 2008; Tokarz, 1987; van Breukelen, 2013). Indeed, strong evidence linking flutamide to aggression has largely come from studies of birds (e.g. Canoine and Gwinner, 2002; Sperry et al., 2010). Interestingly, at least one study in a fish has identified a nuclear androgen receptor that mediates the effects of 11-KT on a reproductive trait (production of spiggin, a glue used by male stickleback during nest construction), and has also shown that flutamide blocks the receptor and action of 11-KT on that trait (Olsson et al., 2005). Yet another study on the same species has shown that flutamide has no effect on aggressive behavior (Sebire et al., 2008). Thus, it is possible that aggression is mediated by a different androgen receptor than the one mediating reproductive traits (also see Li and Al-Azzawi, 2009). However, the mechanism linking circulating androgens to aggressive behavior is unknown and it may differ among taxa.

Prolactin is another hormone that is linked to behavior during reproduction. The hormone has been linked to parental care behavior in mammals (e.g. Brown et al., 2017) and birds (e.g. Smiley and Adkins-Regan, 2018). The effect of prolactin on parental care behavior in fishes is less well-studied (reviewed by Whittington and Wilson, 2013). We administered implants containing prolactin in parental male bluegill and observed more than a doubling in the frequency of nurturing behavior exhibited towards the eggs. Prolactin implants also resulted in a two-fold increase in fanning in three-spined sticklebacks (Páll et al., 2004). Our results thus contribute to the evidence that prolactin promotes nurturing behavior during parental care in many vertebrates.

The amino acid sequence of prolactin differs substantially between

tetrapods and teleost fishes (Wallis, 2000). In our study, we used ovine prolactin to produce the implants. Ovine prolactin is 199 amino acids long, in comparison to a range of 177–185 in teleost fishes, with identities of about 30–40% (Manzon, 2002; Noso et al., 1993; Whittington and Wilson, 2013). Despite the differences in amino acid sequence and length, the prolactin-implanted group in our study showed significantly more nurturing behavior than the control group, suggesting sufficient conservation in prolactin structure across mammals and fishes to preserve its effect on behavior. Páll et al. (2004) compared the effects of ovine prolactin and salmon prolactin on a parental care behavior (fanning) and a courtship behavior (zigzag) in three-spined sticklebacks. Both ovine and salmon prolactin increased fanning behavior, whereas only salmon prolactin affected courtship behavior. This finding suggests some limitation in the conservation and efficacy of mammal prolactin in fishes. Indeed, mammal prolactin may be effective in stimulating nurturing behavior but not courtship behavior in fishes. Why there would be a differential effect based on the type of behavior is unclear.

Bromocriptine acts by inhibiting the release of prolactin from the anterior pituitary (McComb et al., 1982). Bromocriptine has been used to assess the role of prolactin in several species. In Adélie penguins (*Pygoscelis adeliae*), individuals implanted with bromocriptine, during the parental care stage, shifted their behavior back to those typically displayed earlier in breeding (Thierry et al., 2013). In mammals such as the rat (*Rattus norvegicus*), bromocriptine implants disrupt the onset of parental behavior (Bridges and Ronsheim, 1990; Donhoffner et al., 2017) and in zebra finches, bromocriptine almost completely eliminates parental care behavior (Smiley and Adkins-Regan, 2018). In bluegill, Kindler et al. (1991a) showed that bromocriptine implanted males decreased nurturing behavior during parental care. We found similar results in our study, notably with a significantly lower dose of bromocriptine than Kindler et al. (1991a) used (0.35 mg vs. 2.5 mg per 100 g of fish); albeit, neither our study nor the Kindler et al. (1991a) study were able to verify the actual circulating concentration of prolactin in the treated and control fish. Nevertheless, these studies add further support that prolactin affects the frequency of nurturing behavior during parental care.

Researchers have argued that there is a trade-off between aggressive and nurturing behavior during parental care (e.g. Buchanan et al., 2007; Ros et al., 2004; Rosvall, 2013). Our data suggest that a clear trade-off between aggressive and nurturing behavior is present in bluegill. We observed a significant negative relationship between the frequency of aggression and the frequency of nurturing behavior at the level of individual fish. This relationship was independent of behavioral differences among treatments, and suggests that individuals may be constrained in their capacity to concurrently exhibit high levels of both aggression and nurturing. Importantly, because aggressive and nurturing behaviors were measured in different contexts—aggression during a simulated brood predator incursion and nurturing during nest care in which predator incursions were infrequent—this tradeoff cannot be explained by competing allocations of time to these different behaviors (i.e. time spent on aggressive behavior did not reduce the time available for nurturing behavior). Because parental male bluegill

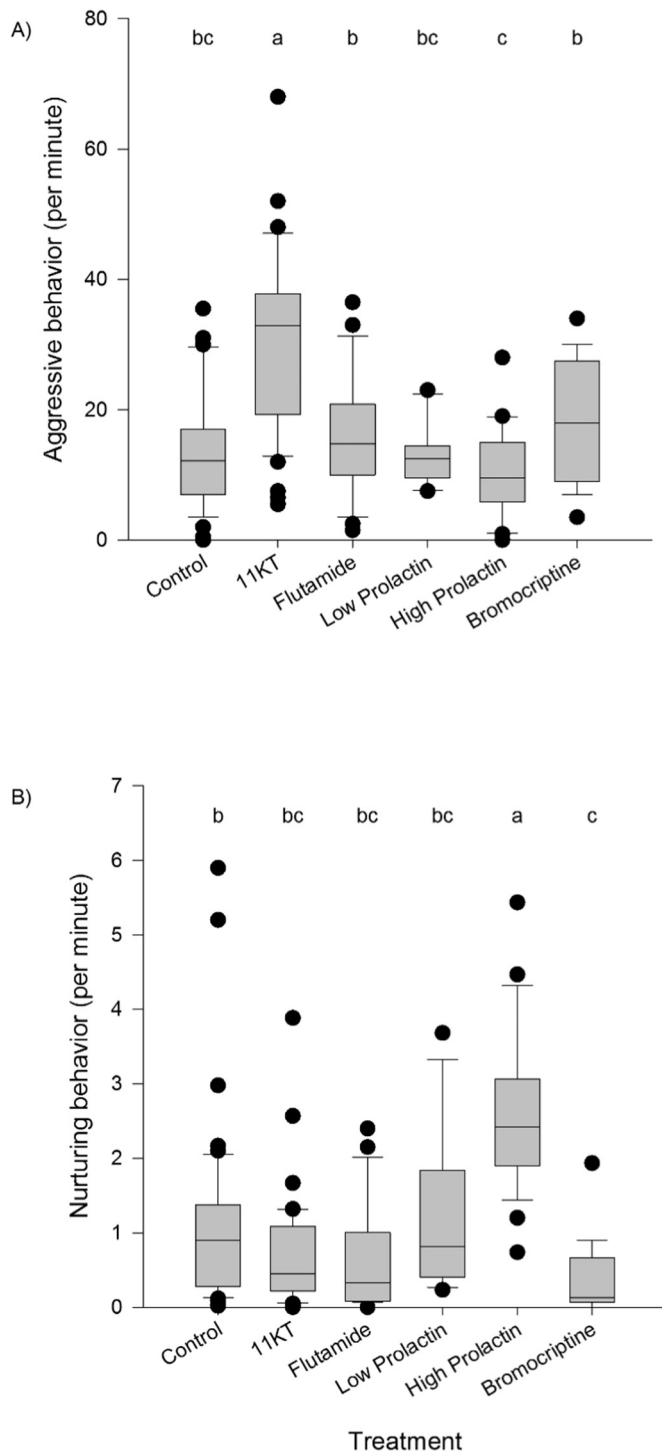


Fig. 1. Aggressive and nurturing behavior performed by nest-tending male bluegill (*Lepomis macrochirus*). The sum of aggressive behaviors (lateral display + opercular flare + biting) and the sum of nurturing behaviors (pectoral fanning + caudal fanning + egg removal) are expressed per minute of observation. Fish were assigned to one of six hormone manipulation treatments. The boxplots display median, 25th, and 75th percentiles, with the whiskers representing the 10th and the 90th percentile. Data outside this range are shown. Treatments with the same letters are not statistically different (see text for details).

provide uniparental care, there is no opportunity for compensation from female parents as in biparental species, which can obscure trade-offs between aggressive and nurturing behaviors (e.g. [Stoehr and Hill, 2000](#)). In bluegill this trade-off is mediated by both 11-KT and prolactin levels. 11-KT implanted bluegill showed increased aggressive behavior and reduced nurturing behavior (also see [Rodgers et al., 2013](#)), whereas prolactin implanted bluegill showed increased nurturing behavior and reduced aggressive behavior. This latter effect was most pronounced when comparing our high prolactin and bromocriptine groups. However, the underlying mechanistic pathways through which these two hormones interact to shape behavior remain unresolved. Future work could further examine the nature of this trade-off by implanting bluegill with both 11-KT and prolactin to determine if parental males can maintain high levels of both aggressive and nurturing behavior during parental care, or if the apparent hormone-mediated trade-off means an individual cannot be both hyper-aggressive and hyper-nurturing.

Acknowledgements

We thank S. Garner for his valuable comments on the manuscript, and V. Broughton, L. Silveira, S. Colborne, M. Stefan, N. Zathe and K. Mitchell for their assistance in the field. We thank Queen's University Biological Station for the use of their facilities. The research was support by grants from CNPq Brazil, the University of Oklahoma, and NSERC of Canada. We dedicate this paper to the memory of Rosemary Knapp, a great colleague and dear friend.

Appendix A

The individual aggressive behaviors were examined using ANOVA models that included treatment as a factor and year as a random effect. For the frequency of biting, we observed a significant effect of treatment ($F_{5,179} = 17.36$, $p < 0.001$; [Table A1](#)); males in the 11-KT treatment performed significantly more bites than males in the other treatments, while males in the bromocriptine treatment performed significantly more bites than males in both the control and high prolactin treatments. The frequency of biting also showed a significant effect of year ($F_{2,179} = 13.08$, $p < 0.001$), with fewer bites observed in 2015 than in either 2016 or 2017. For the frequency of lateral displays, we observed a significant effect of treatment ($F_{5,179} = 3.89$, $p = 0.002$; [Table A1](#)); males in the 11-KT treatment performed significantly more lateral displays than males in all treatments except flutamide. The frequency of lateral displays also showed a significant effect of year ($F_{2,179} = 51.52$, $p < 0.001$), with more lateral displays observed in 2015 than in either 2016 or 2017. For the frequency of opercular flares, we observed a significant effect of treatment ($F_{5,179} = 7.04$, $p < 0.001$; [Table A1](#)); males in the 11-KT treatment performed significantly more opercular flares than males in all treatments except low prolactin, while males in the flutamide treatment also performed significantly more opercular flares than males in the high prolactin treatment. The frequency of opercular flares also showed a significant effect of year ($F_{2,179} = 10.01$, $p < 0.001$), with more opercular flares observed in 2017 than in either 2015 or 2016.

Examining the individual nurturing behaviors, the frequency of caudal fanning did not differ significantly among treatments ($F_{5,168} = 0.33$, $p = 0.89$; [Table A1](#)) or among years ($F_{5,168} = 2.65$, $p = 0.074$). For the frequency of pectoral fanning, we observed a significant effect of treatment ($F_{5,168} = 15.67$, $p < 0.001$; [Table A1](#)); males in the high prolactin treatment performed significantly more pectoral fanning than males in the other treatments. The frequency of pectoral fanning also showed a significant effect of year ($F_{2,168} = 13.08$, $p < 0.001$), with significantly more fanning observed in 2017 than in either 2015 or 2016. For the frequency of egg removal, we observed a significant effect of treatment ($F_{5,168} = 2.61$, $p = 0.026$; [Table A1](#)); males in the high prolactin treatment performed significantly more egg removal than males in the bromocriptine and 11KT

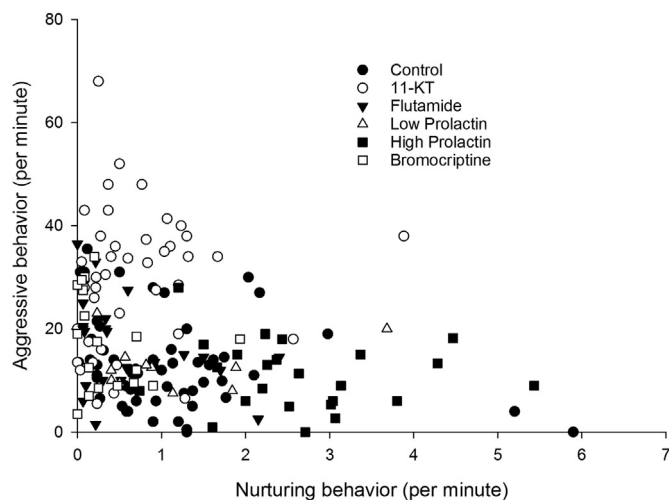


Fig. 2. Relationship between aggressive and nurturing behavior performed by nest-tending male bluegill (*Lepomis macrochirus*). The two variables were negatively correlated: Spearman's $r = -0.318$, $p < 0.001$, $n = 176$. Number of behaviors performed per minute is shown.

treatments. The frequency of egg removal also showed a significant effect of year ($F_{2,168} = 13.17$, $p < 0.001$), with more egg removal observed in 2016 than in either 2015 or 2017.

The frequency of rim circling performed by males was not significantly different among treatments ($F_{5,168} = 1.21$, $p = 0.34$; Table A1), although there was a significant effect of year ($F_{2,168} = 3.26$, $p = 0.041$), with less rim circling observed in 2015 than in 2016 or 2017.

Table A1

Parental care behavior performed by parental male bluegill (*Lepomis macrochirus*) assigned to one of six hormone manipulation treatments. Means ± 1 SE are presented for each behavior as the number of observations per minute.

Treatment	Bites	Lateral display	Opercular flare	Caudal fanning	Pectoral fanning	Egg removal	Rim circling
Control	6.60 \pm 0.78 ^{cd}	3.35 \pm 0.76 ^b	3.47 \pm 0.39 ^{bc}	0.19 \pm 0.05	0.46 \pm 0.09 ^b	0.43 \pm 0.11 ^{ab}	1.36 \pm 0.19
11-KT	16.53 \pm 1.40 ^a	6.31 \pm 0.72 ^a	7.18 \pm 0.99 ^a	0.18 \pm 0.08	0.29 \pm 0.06 ^b	0.23 \pm 0.04 ^b	1.31 \pm 0.16
Flutamide	7.05 \pm 0.99 ^{bc}	6.66 \pm 1.51 ^{ab}	2.86 \pm 0.94 ^b	0.20 \pm 0.12	0.11 \pm 0.05 ^b	0.32 \pm 0.09 ^{ab}	0.98 \pm 0.22
Low Prolactin	9.95 \pm 1.79 ^{bcd}	1.14 \pm 0.27 ^b	1.86 \pm 0.52 ^{abc}	0.24 \pm 0.17	0.27 \pm 0.14 ^b	0.61 \pm 0.14 ^{ab}	1.98 \pm 0.54
High Prolactin	6.25 \pm 0.91 ^d	0.71 \pm 0.15 ^b	3.36 \pm 0.60 ^c	0.30 \pm 0.10	1.72 \pm 0.20 ^a	0.58 \pm 0.11 ^a	1.86 \pm 0.31
Bromocriptine	9.53 \pm 1.57 ^b	5.97 \pm 1.12 ^b	2.34 \pm 0.50 ^{bc}	0.08 \pm 0.06	0.06 \pm 0.04 ^b	0.22 \pm 0.06 ^b	1.76 \pm 0.42

Note: ANOVAs were used to compare hormone concentrations among treatments. When the treatment effect was significant, the same letter is used to indicate groups that were not statistically different.

References

- Angelier, F., Wingfield, J.C., Tartu, S., Chastel, O., 2016. Does prolactin mediate parental and life-history decisions in response to environmental conditions in birds? A review. *Horm. Behav.* 77, 18–29.
- Borg, B., 1994. Androgens in teleost fishes. *Comp. Biochem. Physiol. C* 109, 219–245.
- Bouissou, M.F., 1983. Androgens, aggressive behaviour and social relationships in higher mammals. *Horm. Res. Paed.* 18, 43–61.
- Bridges, R.S., Ronsheim, P.M., 1990. Prolactin (PRL) regulation of maternal behavior in rats: bromocriptine treatment delays and PRL promotes the rapid onset of behavior. *Endocrinology* 126, 837–848.
- Brown, R.S.E., Aoki, M., Ladyman, S.R., Phillipps, H.R., Wyatt, A., Boehm, U., Grattan, D.R., 2017. Prolactin action in the medial preoptic area is necessary for postpartum maternal nursing behavior. *Proc. Natl. Acad. Sci. U. S. A.* 114, 10779–10784.
- Buchanan, K.L., Goldsmith, A.R., Hinde, C.A., Griffith, S.C., Kilner, R.M., 2007. Does testosterone mediate the trade-off between nestling begging and growth in the canary (*Serinus canaria*)? *Horm. Behav.* 52, 664–671.
- Canoine, V., Gwinner, E., 2002. Seasonal differences in the hormonal control of territorial aggression in free-living European stonechats. *Horm. Behav.* 41, 1–8.
- Claussen, J.E., 1991. Annual Variation in the Reproductive Activity of a Bluegill Population: Effect of Clutch Size and Temperature (MSc thesis). University of Toronto, Toronto.
- Colgan, P.W., Nowell, W.A., Gross, M.R., Grant, J.W.A., 1979. Aggressive habituation and rim circling in the social organization of bluegill sunfish (*Lepomis macrochirus*). *Environ. Biol. Fish* 4, 29–36.
- Côté, I.M., Gross, M.R., 1993. Reduced disease in offspring: a benefit of coloniality in sunfish. *Behav. Ecol. Sociobiol.* 33, 269–274.
- Donhoffner, M.E., Al Saleh, S., Schink, O., Wood, R.I., 2017. Prosocial effects of prolactin in male rats: social recognition, social approach and social learning. *Horm. Behav.* 96, 122–129.
- Farrell, S.F., McGinnis, M.Y., 2003. Effects of pubertal anabolic-androgenic steroid (AAS) administration on reproductive and aggressive behaviors in male rats. *Behav. Neurosci.* 117, 904–911.
- Freeman, M.E., Kanyicska, B., Lerant, A., Nagy, G., 2000. Prolactin: structure, function, and regulation of secretion. *Physiol. Rev.* 80, 1523–1631.
- Gross, M.R., 1982. Sneakers, satellites and parentals: polymorphic mating strategies in North American sunfishes. *Z. Tierpsychol.* 60, 1–26.
- Gross, M.R., Macmillan, A.M., 1981. Predation and the evolution of colonial nesting in bluegill sunfish (*Lepomis macrochirus*). *Behav. Ecol. Sociobiol.* 8, 163–174.
- Gross, M.R., Sargent, R.C., 1985. The evolution of male and female parental care in fishes. *Am. Zool.* 25, 807–822.
- Hegner, R.E., Wingfield, J.C., 1987. Effects of experimental manipulation of testosterone levels on parental investment and breeding success in male house sparrows. *Auk* 104, 462–469.
- Heilman, R.D., Brugmans, M., Greenslade, F.C., DaVanzo, J.P., 1976. Resistance of androgen-mediated aggressive behavior in mice to flutamide, an antiandrogen. *Psychopharmacology* 47, 75–80.
- Khong, H.K., Kuah, M.K., Jaya-Ram, A., Shu-Chien, A.C., 2009. Prolactin receptor mRNA is upregulated in discus fish (*Symphysodon aequifasciata*) skin during parental phase. *Comp. Biochem. Physiol. B* 153, 18–28.
- Kindler, P.M., Bahr, J.M., Gross, M.R., Philipp, D.P., 1991a. Hormonal regulation of parental care behavior in nesting male bluegills: do the effects of bromocriptine suggest a role for prolactin? *Physiol. Zool.* 64, 310–322.
- Kindler, P.M., Bahr, J.M., Philipp, D.P., 1991b. The effects of exogenous 11-ketotestosterone, testosterone, and cyproterone acetate on pre-spawning and parental care behaviors of male bluegill. *Horm. Behav.* 25, 410–423.
- Knapp, R., Neff, B.D., 2007. Steroid hormones in bluegill, a species with male alternative reproductive tactics including female mimicry. *Biol. Lett.* 3, 628–632.
- Li, J., Al-Azzawi, F., 2009. Mechanism of androgen receptor action. *Maturitas* 63,

- 142–148.
- Lincoln, G.A., Guinness, F., Short, R.V., 1972. The way in which testosterone controls the social and sexual behavior of the red deer stag (*Cervus elaphus*). *Horm. Behav.* 3, 375–396.
- Magee, S.E., Neff, B.D., Knapp, R., 2006. Plasma levels of androgens and cortisol in relation to breeding behavior in parental male bluegill sunfish, *Lepomis macrochirus*. *Horm. Behav.* 49, 598–609.
- Manzon, L.A., 2002. The role of prolactin in fish osmoregulation: a review. *Gen. Comp. Endocrinol.* 125, 291–310.
- Marler, C.A., Bester-Meredith, J.K., Trainor, B.C., 2003. Paternal behavior and aggression: endocrine mechanisms and nongenomic transmission of behavior. In: *Advances in the Study of Behavior*. 32. Academic Press, San Diego, CA, US, pp. 263–323.
- McComb, D.J., Kovacs, K., Croxford, R., Milligan, J.V., 1982. Bromocriptine suppression of dispersed pituitary lactotrophs from estrogen-pretreated rats: a quantitative electron microscopic study. *Can. J. Physiol. Pharmacol.* 60, 154–159.
- McGlothlin, J.W., Jawor, J.M., Ketterson, E.D., 2007. Natural variation in a testosterone-mediated trade-off between mating effort and parental effort. *Am. Nat.* 170, 864–875.
- Molik, E., Blasiak, M., 2015. The role of melatonin and bromocriptine in the regulation of prolactin secretion in animals – a review. *Ann. Anim. Sci.* 15, 849–860.
- Noso, T., Nicoll, C.S., Kawauchi, H., 1993. Lungfish prolactin exhibits close tetrapod relationships. *Biochim. Biophys. Acta* 1164, 159–165.
- Olsson, P.-E., Berg, A.H., von Hofsten, J., Grahn, B., Hellqvist, A., Larsson, A., Karlsson, J., Modig, C., Borg, B., Thomas, P., 2005. Molecular cloning and characterization of a nuclear androgen receptor activated by 11-ketotestosterone. *Reprod. Biol. Endocrinol.* 3, 37.
- Páll, M.K., Liljander, M., Borg, B., 2004. Prolactin diminishes courtship behaviour and stimulates fanning in nesting male three-spined sticklebacks, *Gasterosteus aculeatus*. *Behaviour* 141, 1511–1519.
- Peters, A., 2002. Testosterone and the trade-off between mating and paternal effort in extrapair-mating superb fairy-wrens. *Anim. Behav.* 64, 103–112.
- Pinxten, R., Elke De, R., Balthazart, J., Luc, B., Eens, M., 2000. The effect of castration on aggression in the nonbreeding season is age-dependent in male European starlings. *Behaviour* 137, 647–661.
- Riddle, O., Bates, R.W., Dykshorn, S.W., 1933. The preparation, identification and assay of prolactin - a hormone of the anterior pituitary. *Am. J. Phys.* 105, 191–216.
- Rodgers, C.M.C., Neff, B.D., Knapp, R., 2012. Effects of exogenous testosterone on parental care behaviours in male bluegill sunfish (*Lepomis macrochirus*). *Ethology* 118, 636–643.
- Rodgers, C.M., Neff, B.D., Knapp, R., 2013. Androgen-mediated nurturing and aggressive behaviors during paternal care in bluegill sunfish (*Lepomis macrochirus*). *Horm. Behav.* 63, 454–461.
- Ros, A.F.H., Bruinijes, R., Santos, R.S., Canario, A.V.M., Oliveira, R.F., 2004. The role of androgens in the trade-off between territorial and parental behavior in the Azorean rock-pool blenny, *Parablennius parvicornis*. *Horm. Behav.* 46, 491–497.
- Rosvall, K.A., 2013. Life history trade-offs and behavioral sensitivity to testosterone: an experimental test when female aggression and maternal care co-occur. *PLoS One* 8, e54120.
- Saino, N., Møller, A.P., 1995. Testosterone-induced depression of male parental behavior in the barn swallow: female compensation and effects on seasonal fitness. *Behav. Ecol. Sociobiol.* 36, 151–157.
- Sebire, M., Allen, Y., Bersuder, P., Katsiadaki, I., 2008. The model anti-androgen flutamide suppresses the expression of typical male stickleback reproductive behaviour. *Aquat. Toxicol.* 90, 37–47.
- Slijkhuis, H., de Ruiter, A.J., Baggerman, B., Wendelaar Bonga, S.E., 1984. Parental fanning behavior and prolactin cell activity in the male three-spined stickleback *Gasterosteus aculeatus* L. *Gen. Comp. Endocrinol.* 54, 297–307.
- Smiley, K.O., Adkins-Regan, E., 2018. Lowering prolactin reduces post-hatch parental care in male and female zebra finches (*Taeniopygia guttata*). *Horm. Behav.* 98, 103–114.
- Sperry, T.S., Wacker, D.W., Wingfield, J.C., 2010. The role of androgen receptors in regulating territorial aggression in male song sparrows. *Horm. Behav.* 57, 86–95.
- Stoehr, A.M., Hill, G.E., 2000. Testosterone and the allocation of reproductive effort in male house finches (*Carpodacus mexicanus*). *Behav. Ecol. Sociobiol.* 48, 407–411.
- Thierry, A.-M., Brajon, S., Masméjan, S., Handrich, Y., Chastel, O., Raclot, T., 2013. Decreased prolactin levels reduce parental commitment, egg temperatures, and breeding success of incubating male Adélie penguins. *Horm. Behav.* 64, 737–747.
- Tokarz, R.R., 1987. Effects of the antiandrogens cyproterone acetate and flutamide on male reproductive behavior in a lizard (*Anolis sagrei*). *Horm. Behav.* 21, 1–16.
- van Breukelen, N.A., 2013. Androgen receptor antagonist impairs courtship but not aggressive behavior in the monogamous cichlid, *Amatitlania nigrofasciata*. *Horm. Behav.* 63, 527–532.
- Wallis, M., 2000. Episodic evolution of protein hormones: molecular evolution of pituitary prolactin. *J. Mol. Evol.* 50, 465–473.
- Whittington, C.M., Wilson, A.B., 2013. The role of prolactin in fish reproduction. *Gen. Comp. Endocrinol.* 191, 123–136.