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Male-typical courtship, spawning behavior, and olfactory sensitivity are induced to different extents by androgens in the goldfish suggesting they are controlled by different neuroendocrine mechanisms.

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ABSTRACT

Male-typical reproductive behaviors vary greatly between different species of fishes with androgens playing a variety of roles that appear especially important in the gonochorist cypriniform fishes. The goldfish is an important model for the cypriniformes and while it is clear that male goldfish are fully feminized by prostaglandin F$\text{2\alpha}$ (PGF$_{2\alpha}$), it is not clear whether females will exhibit normal levels of male-typical reproductive behaviors as well as olfactory function when treated with androgens. To answer this question, we exposed sexually-regressed adult female goldfish to several types of androgen and monitored their tendencies to court (inspect females) and mate (spawn, or attempt to release gametes) while monitoring their olfactory sensitivity until changes in these attributes were maximized. Untreated adult males (intact) were included to determine the extent of masculinization. Treatments included the natural androgens, 11-ketotestosterone and testosterone (KT and T), administered via capsules (KT+T-implanted fish); the artificial androgen, methyltestosterone (MT), administered via capsules (MT-C); and MT administered in the fishes’ water (MT-B). Male-typical olfactory sensitivity to a pheromone (15keto-PGF$_{2\alpha}$) increased in all androgen-treated groups and by week 6 was fully equivalent to that of males. Male-typical courtship behavior increased in all androgen-treated groups although slowly, and only MT-B females came to exhibit levels equivalent to those of males after 18 weeks. In contrast, male-typical mating activity increased only slightly, with MT-B females reaching levels one-third that of males after 30 weeks. We conclude that while androgens fully masculinize olfactory sensitivity and courtship behavior in goldfish, mating behavior is controlled by a different neuroendocrine mechanism(s) that has yet to be fully elucidated.

Keywords: Androgen, spawning, olfaction, pheromone, masculinization, bipotential.
1. Introduction

Unlike mammals and birds, whose sex-specific behaviors are fixed early in life by androgenic steroids (Pfaff et al., 2002), most adult teleost fishes appear capable of exhibiting either male or female behavior if exposed to appropriate hormones in the appropriate contexts (Stacey and Sorensen, 2009; Bass and Grober, 2009; Munakata and Kobayashi, 2010; Pradhan et al., 2015). Bipotentiality, herein defined as the ability of one sex to express the traits of the other, takes many forms in the nearly 500 families of teleost fish. Further, although most species of fish exhibit only a single sexual/gonadal phenotype (e.g. gonochorism), some species of fish exhibit different “reproductive phenotypes” or alternative reproductive strategies in which males either start life as females (hermaphroditism) and/or exist as distinctly different morphs (Grober and Bass, 2002; Bass and Grober, 2009). Gonadal androgens play diverse roles in these reproductive phenotypes and life history strategies (Grober, 1997; Grober and Bass, 2002) which can be organizational, modulatory, and/or activational, and include roles determining secondary sex characteristics, gonadal sex, and expression of reproductive (courtship and mating) behaviors as well as agonistic, parental, and nest-building behaviors (Borg, 1994; Oliveira et al., 2001; Bass and Grober, 2009; Munakata and Kobayashi, 2010). The endocrine basis of male-typical reproductive behaviors has now been examined in approximately half a dozen species of fish with alternative reproductive phenotypes that express high levels of male-male interactions/agonistic displays and social hierarchies (Grober and Bass, 2002; Bass and Grober, 2009; Pradhan et al., 2014). Similar studies have also been conducted in the goldfish (Carassius auratus), a gonochorist cypriniform fish which exhibits little male-male aggression, does not form social hierarchies, and whose males seek out and court females using the pheromones they
release before spawning (i.e. they “scramble” for access to females in open water; Kobayashi et al., 2002). While male-typical agonistic behaviors in the socially complex fishes appear to be mediated by brain neuropeptides with androgenic steroids playing modulatory roles (Bass and Grober, 2009), male-typical reproductive behaviors in the goldfish appear to be directly induced by androgens with neuropeptides playing little role (Munakata and Kobayashi, 2010; Parhar et al., 2001). However, whether these seemingly different functions are taxon-, life-history, and/or behavior-specific is not clear. Further, endocrine mediators of male-typical spawning behavior (i.e. behaviors closely associated with mating and gamete release) in goldfish suggest it may be controlled independently of courtship (Stacey and Kobayashi, 1996; see below).

The goldfish may be the best studied model of androgen-driven male-typical reproductive behaviors in the family cyprinidae, one of the largest families of fishes with well over 2,500 species (Nelson, 2006). Studies have shown that while adult male goldfish behavior can be completely feminized by hormones within minutes by injecting them with prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) (i.e. PGF$_{2\alpha}$-treated males exhibit female-typical reproductive behaviors indistinguishable from those of naturally-receptive females; Kobayashi and Stacey, 1993; Kobayashi et al., 2013; Sorensen et al., 1988; Stacey, 1981), it is not clear whether all aspects of male reproductive behavior (i.e. both courtship and mating) can be completely masculinized by androgenic steroids in female goldfish. Approximately half a dozen studies have shown that while female goldfish treated with various androgens will both court and mate with sexually-active females (Stacey and Kobayashi, 1996; Kobayashi et al., 1997; Belanger et al., 2010; Kobayashi et al., 2013), but these effects take months to develop and spawning behavior is generally low. Stacey and Kobayashi (1996) conducted the only study we know of to examine the effects of androgens on both male-typical courtship and spawning behaviors in female
goldfish which used untreated males as a reference to gauge potency. These authors found that females implanted with capsules containing the natural androgens, 11-ketotestosterone (KT) and testosterone (T), spawned only one-half as frequently as untreated adult males after 3.5 months and about one-quarter as frequently after 4.5 months. The combination of these steroids was more potent than either one alone. In contrast, the male-typical courtship behavior of KT+T-implanted females reached nearly 90% of the levels of untreated males within 4.5 months. However, because this study relied exclusively on androgen implants, and the hormone titers of the implanted fish were dropping by 4.5 months, the question remains whether hormone treatment was long enough. More recently, Saoshiro et al. (2013) implanted female goldfish with an artificial androgen, methyltestosterone (MT), and found that male-typical spawning rates were also only half of female-typical spawning rates in MT-implanted females. When taken together, these results strongly suggest that courtship and mating might be controlled by different neuroendocrine mechanisms that are influenced by androgens in different ways, as has been well documented in mammals (Beach, 1942; Everitt, 1990; Pfaus et al., 2001). Direct (head-to-head) tests of the effects of androgens on all of the different male-typical behaviors that include untreated males as well as a variety of androgens (both natural and artificial) and that extend for an indefinite period of time are needed to address this possibility.

Sex-typical courtship behavior and olfactory function in many fishes including the cyprinids, are intimately linked but the specific relationship(s) between them are not yet well understood. For instance, while it is known that hormonally-derived sex pheromones detected by the olfactory system of goldfish drive both male-typical and female-typical reproductive behaviors (Stacey and Kyle, 1983; Sorensen et al., 2005; Kawaguchi et al., 2014), and that the olfactory system is both sexually dimorphic and bipotential (Sorensen and Goetz, 1993;
Cardwell et al., 1995; Belanger et al., 2010), it is not yet known whether natural androgens can fully masculinize olfactory function in females. It is also unclear whether the rate at which androgens masculinize olfactory function in female fish is the same as the rate of behavioral masculinization, and thus whether the same androgen-driven neuroendocrine mechanism(s) might be responsible for both phenomena. For instance, while Cardwell et al. (1995) found that immature cyprinid, the silver barb (*Puntius schwanenfeldi*) implanted with both MT and 11-ketoandrostenedione exhibited increased olfactory sensitivity to metabolites of PGF$_{2\alpha}$-derived pheromones in as little as 10 days and seemingly normal increases in male-typical courtship behavior (spawning was not examined), they did not directly compare changes in olfactory sensitivity and behavior to those in untreated males to determine if complete masculinization had occurred. Further, while Belanger et al. (2010) found that several cyprinids treated with MT in a bath for three weeks had increased olfactory sensitivity to PGF$_{2\alpha}$-derived pheromones and that MT-treated juvenile zebrafish (*Danio rerio*) (another scramble-spawning cyprinid) had olfactory sensitivity equivalent to that of untreated males, they did not examine the effects of MT on the expression of male-typical spawning behavior or the potency of natural androgens. The specific relationship between androgen-induced olfactory sensitivity to sex pheromones and male-typical reproductive behaviors is thus unclear.

Finally, one of the difficulties in interpreting existing studies on bipotentiality and masculinization in fish, and cyprinids in particular, has been the near-exclusive use of the artificial androgen, MT (Belanger et al., 2010; Saoshiro et al., 2013), the actions and fate of which are poorly understood. Studies have yet to directly compare the effects of MT with those of KT and/or T across time on either behavior or olfactory function in any fish. High doses of MT have been shown to feminize gonadal development in some species (Rinchard et al., 1999;
Pawlowski et. al., 2004), calling into question whether MT’s effects are purely androgenic. Additionally, while studies of the effects of MT on masculinization have applied it using either implanted capsules or via the water (in a bath), they have not directly compared the efficacy of these two treatment protocols or compared them to KT-treatments. In this study we directly examined the tendencies of both natural (KT and T) and artificial (MT) androgens to masculinize courtship and mating behavior as well as olfactory sensitivity in sexually-regressed female goldfish, a model cyprinid and a gonochorist. We tested both natural and artificial steroids administered via implants and via a bath until maximal effects were seen and responses had plateaued (i.e. as long as required). Both female and mature male goldfish were included as controls in our experiments to directly gauge the strength of observed responses and determine whether complete masculinization (i.e. expression of attributes fully equivalent in magnitude to those of natural [normal] males) had occurred. Plasma steroids and secondary sex characteristics were measured at regular intervals. A wide range of male-typical reproductive behaviors was evaluated. We asked the following questions: 1) Do androgens induce both completely normal male-typical courtship and mating behaviors in females given enough time, and at similar rates which might reflect a common underlying neuroendocrine mechanism(s)? 2) Do androgens induce completely normal male-typical olfactory sensitivity in females given enough time and at rates that match those seen for male-typical behavior(s)? 3) Does MT have the same effects as natural androgens on the expression of all male-typical reproductive behaviors and olfactory sensitivity and is MT equally effective whether administered via either an implanted capsule or via a bath?

2. Materials and methods
2.1 Animals

Goldfish were purchased from Hunting Creek Fisheries (Thurmont, MD, USA) in early spring prior to spawning and shipped to the University of Minnesota where they were sexed by examining them for secondary sex characteristics and gametes, after which males and females were placed into separate 1000-L flow-through circular stock tanks. These tanks were supplied with 20°C well water and maintained on a 16h:8h (L:D) photoperiod. We knew from personal experience that this treatment would facilitate gonadal regression in females but not males, and we wanted to reduce basal steroids in females used for treatments. Fish were fed *ad libitum* (Romet medicated flake fish food; Florida Tropical Fish Farms Association, Wimauma, FL).

Two weeks prior to the start of experiments, 80 female (body wt [mean ± S.D]: 54 ± 18.2 g) and 15 male (body wt [mean ± S.D]: 55 ± 5.7 g) goldfish were selected at random from stock tanks and placed into five 230-L round flow-through tanks (4 L/min) maintained on a 16h:8h (L:D) photoperiod. When the experiment started, males were spermiating and had tubercles (a secondary sexual characteristic) on both their pectoral fins and opercula, and females were immature (i.e. their oocytes were not vitellogenic). Both testosterone (T) and estradiol (E2) were very low in females at the start of the experiment and all sampling times (see results). All procedures were approved by the University of Minnesota IACUC (#1302-30339A) and conducted in accordance with the Guide for the Care and Use of Laboratory Animals.

2.2 Experimental design
This experiment had five groups that included natural and artificial steroids as well as controls. We used sexually-regressed females for 4 treatments which included: 1) Blank capsule-implanted female, control female, (F); 2) females implanted with capsules containing KT and T (KT+T; the most effective natural steroid mixture identified by Stacey and Kobayashi, 1996); 3) females implanted with capsules containing MT [MT-C; a treatment known to induce male-typical reproductive behavior (Saoshiro et al., 2013)]; and 4) females maintained in a bath containing MT [MT-B; a treatment known to masculinize female behavior and olfactory function (Cardwell et al., 1995; Belanger et al., 2010)]. We also included untreated sexually-mature, intact males (M) as reference controls in this experiment so we could directly gauge the extent of any possible masculinization. By using a treatment group which added MT to holding water (via a bath) we were able to continue this experiment indefinitely until responses had plateaued and beyond the time that steroid release from the capsules might be dropping. At the start of the experiment, 20 females were marked with unique fin clips so we could track them, and then randomly assigned into one of the 4 treatment groups. Each treatment had three sub-groups: one sub-group of 5 fish was used to measure plasma steroids, another sub-group of 5 was used to measure olfactory responses, and a third sub-group of 8 was used for behavior assays. Two fish were held as extras in case of mortality. The start times for treatments were staggered to avoid overlap. Fish were then tested or bled at 5-6 week intervals. The experiment continued until both the olfactory and behavioral responses of all treatment groups had either plateaued for at least a month or dropped; this was 24 weeks for both the MT-C and KT+T groups, and 30 weeks for the MT-B, control male and control female groups. At the end of the experiment, all fishes were euthanized (0.1% MS222; Western Chemicals, Ferndale, WA) and their gonads removed to assess maturity and size (Gonadosomatic index [GSI], gonad weight /body weight x 100).
2.3 Steroid treatments

The protocol for steroid implants followed that of Stacey and Kobayashi (1996) who developed this technique to create optimal natural androgen levels in goldfish that might last several months. Briefly, Dow Corning medical grade Silastic tubing (Dow Corning, Midland, MI) was cleaned with 70% ethanol and cut into either 20 mm (for KT and T) or 30 mm lengths (MT), and then sealed at one end with silicone adhesive (Dow Corning, Midland, MI). To prepare KT capsules, KT (Steraloids, Newport, RI) was dissolved in ethanol then placed into castor oil at a 1:9 ratio and evaporated overnight, after which 70 µl of the oil was added to empty capsules (350 µg of KT per capsule, ~6.3 µg/g body weight), which were sealed with silicone adhesive and allowed to dry. For T, 10 mg of crystalline T (Steraloids, Newport, RI) was added directly to capsules (10 mg/capsule, ~180 µg/g body weight), which were also sealed with silicone adhesive. MT capsules were made in the same manner as T capsules and we added 20 mg of MT (~360 µg/g body weight) into each capsule following Saoshiro et al. (2013). Control capsules (30 mm) were left empty and sealed with adhesive. Filled capsules were then inserted into the body cavities of anesthetized fishes (0.01% MS-222; Western Chemicals, Ferndale, WA) through incisions (8 mm) made 1 cm anterior to their urogenital apertures, following Stacey and Kobayashi (1996). For fish treated with water-borne MT in a bath (MT-B), a 10⁻³ Molar (M) solution of MT dissolved in ethanol was added daily to their 230L holding tanks to create a concentration of approximately 10⁻⁸ M following established protocols (Belanger et al., 2010). The inflow of water to each holding tank was 4 L/min so the steroid concentration dropped 10⁻
fold an hour. Fish holding tanks were cleaned every 10 days to minimize algae buildup stimulated by ethanol addition.

2.4 Measuring hormones and secondary sexual characteristics (tubercles)

To determine the effects of different hormones on masculinization, we measured plasma T, KT, and MT in all groups once every 5-6 weeks. Estradiol (E2) was also measured in all groups at weeks 1 and 24. Blood samples (300-400 µl) were drawn from the caudal vasculature of anesthetized (0.01% MS222, Western Chemical, CO) fish from each treatment’s sub-group using 25-gauge syringes with heparinized needles. Blood was centrifuged to collect plasma (10,000 g) and frozen (-20°C) until it could be assayed. Tubercles on the pectoral fins and opercula were noted at the same time following Stacey and Kobayashi (1996). Plasma steroid levels were measured by Enzyme Linked Immuno-Sorbent Assay (ELISA) using commercial kits for KT (Item # 582701, Cayman Chemical Company, Ann Arbor, MI), T (Item # 5872751, Cayman Chemical Company, Ann Arbor, MI), Estradiol (Item # EA 70, Oxford Biomedical Research, MI) and MT (a custom assay was developed by Cayman Chemical Company, Ann Arbor, MI). The detection threshold of each ELISA was determined using two methods. The first method used serial dilutions of a known concentration of standard androgen, while the second used serial dilutions of female plasma spiked with a known concentration of standard androgen. Both dilution series were then assayed using the optimum concentration of antibody suggested by the company to generate a standard curve. The lowest concentration in the linear range of the standard curve was considered as the detection threshold for each assay. Both methods produced the same detection thresholds which were: 1.3 pg/ml for KT, 6 pg/ml for T, 0.95 pg/ml for MT,
and 0.02 ng/ml for E2. Intra- and inter-assay coefficients of variation for androgen assays were determined by repeated measurements of high and low value quality controls and found to be 7.5% and 14.0% for KT, 4.0% and 18.5% for T, and 4.0% and 18.5% for MT. The cross-reactivity for the KT antiserum was: 5.0% for MT, 2.5% for T, 0.5% for androstenedione, and 0.5% for 5α-androstene-3β,17β-diol. The cross-reactivity for the T antiserum was: 5.0% for MT, 0.0% for KT, 1.2% for androstenedione and 1.5% for 5α-androstene-3β,17β-diol. The cross-reactivity of the MT antiserum was: 10.0% for T, 3.5% for KT, 3.0% for androstenedione and 0.5% for 5α-androstene-3β, 17β-diol. In this manuscript, we consider (and label) MT as immunoreactive MT (irMT) due to the relatively high cross-reactivity of the MT antiserum. The cross-reactivity of the E2 antiserum was determined to be 1.0% for T.

2.5 Measuring behavioral responsiveness: courtship, mating, and feeding

Behavioral tests were conducted every 5-6 weeks following protocols of Stacey and Kobayashi (1996) with a few changes made to allow for more detailed quantification of courtship following DeFraipont and Sorensen (1993) and Appelt and Sorensen (2007) who monitored both the time-taken-to-start-courtting as well as the frequency of courting (vs. percent time). Briefly, tests were conducted in 70L flow-through (1 L/min) glass aquaria, which contained two resident females as well as spawning substrate (floating yarn balls which mimicked spawning weeds). Reproductive behavior in goldfish, like that of many scramble spawners (Stacey, 1987; Kobayashi et al., 2002), is largely driven by males that nudge (or inspect females — a form of courtship) females, which they discern using a PGF2α-derived sex pheromone the females release in their urine and via their gills. Spawning (mating) occurs when
males and females enter spawning substrate where females release sticky eggs with a flip of their tails in the company of males that release sperm by also flipping their tails (Kobayashi et al., 2002). Spawning (mating) can occur frequently (every few min in fully responsive fish) and involves little aggression although males may push each other as they jostle in small groups to gain access to females. When goldfish are reproductively inactive they will often search for food by sampling in the bottom substrate with their mouths and this behavior is useful to measure because it generally shows the opposite trends of reproductive behaviors (Stacey and Kobayashi, 1996). For our tests, reproductive activity was induced in resident females by injecting them with PGF\(_2\alpha\) (0.1 µg/g body wt), i.m. using a microsyringe, a treatment that elicits full sexual activity and pheromone release (Sorensen et al., 1988; Stacey and Kobayashi, 1996). Injected fish were immediately placed back in their aquaria and 15-min later (when PGF\(_2\alpha\) exerts its influence) a test (treated) fish was added. For the next 45 minutes, an observer who was not blind to the treatment condition noted several behaviors including: 1) Courtship activity: nudging — a type of inspection behavior in which males contact females around their urogenital apertures and gills where pheromones are released; 2) Time-taken-to-court — time taken for fish to nudge a sexually-receptive female the first time, a measure known to decrease following pheromone exposure in males and thought to reflect sensory alertness (DeFraipont and Sorensen, 1993); 3) Mating (spawning) activity — when males and females align themselves in spawning weeds and flip their tails, including both: a) complete spawning acts when tail flips were observed and b) incomplete spawning acts when males entered weeds with females but did not flip their tails. We also noted feeding behavior — instances in which goldfish dug into the bottom substrate using their mouths. Behaviors were recorded using a handheld Noldus Observer 5.0 (Noldus Information Technology, Leesburg, VA).
2.6 Measuring olfactory sensitivity

Once every 5-6 weeks, the peripheral olfactory sensitivity of 5 fish from each treatment group and the untreated males was measured using extracellular multi-unit electro-olfactogram (EOG) recording following established protocols (Irvine and Sorensen, 1993; Sorensen et al., 2005). This extracellular multiunit technique has been shown to faithfully reflect the sensitivity of the goldfish olfactory epithelium to sex pheromones as confirmed by behavioral assays (Sorensen et al., 1988, 1990). For EOG recording, fish were anesthetized (0.1% MS222), placed onto a stand that continuously perfused their gills with anesthetic (0.01% MS222), the flap of skin overlying an olfactory capsule trimmed, and the exposed naris perfused with 18°C well water via a capillary tube (10 ml/min) into which 5-sec pulses of odorant were added using a custom-built switching device (Sorensen et al., 1988). Fish were grounded using a syringe needle while an indifferent (reference) electrode made from a gelatin-filled (8%) capillary glass pipette bridged to an Ag-AgCl electrode (World Precision Instruments, Tampa, FL) filled with 3 M KCl was placed on their skin surface near the perfused naris. An identical recording electrode was then placed and positioned immediately above the surface of the olfactory epithelium at a location which we determined from 30 min of pilot tests to evoke maximal responses to our standard odorant (10⁻⁵M L-Serine; Sigma Co., St. Louis, MO) while yielding no responses to well water control (blank). Voltage transients were amplified with a DC preamplifier (Grass Co, Woodshole, MA, USA) and saved on a computer (AD Instruments, Colorado Springs, CO, USA). Responses were recorded to the following stimuli: 1) Blank well water control; 2) 10⁻⁵ M L-Serine standard (a food odor); 3) concentrations of 10⁻¹²M - 10⁻⁷M 15Keto-ProstaglandinF₂α
(15K-PGF$_{2\alpha}$; Cayman Chemical Company, Ann Arbor, MI, USA — a potent female spawning pheromone; Sorensen et al., 1988), and $10^{-12}$ - $10^{-8}$M 17,20β-dihydroxy-4-pregnen-3-one (17,20βP [Sigma Co, MO] — a potent female priming pheromone; Dulka et al., 1987; Sorensen et al., 1990). Odorant solutions were made up from stocks each day along with well water controls. All stimuli were tested at least twice with two min recovery times and then a third time if inconsistencies were apparent, after which responses were averaged. Stimuli were tested in a prescribed order from low to high concentrations with well water (blank) controls and the standard being tested at both the start and finish. Responses were measured from baseline to peak, after which possible responses to well water control were subtracted from the responses to the test stimuli (there were none), and the resulting values expressed in terms of absolute responses (mv) as well as responses relative to the average L-Serine response for that series.

2.7 Statistical analyses

Courtship (nudging) activity, time-taken-to-court, frequency of complete spawning (mating), frequency of incomplete spawning (mating), EOG, and hormone data were all analyzed using the R statistics package (version 3.0.3) and Graphpad Prism (version 5.0, San Diego, CA, USA). Behavioral data were expressed as a rate (frequency per minute). Responses shown by all treatment groups including untreated males were evaluated together. Data for each attribute was first tested for normality (Shapiro-Wilk test) as well as homogeneity of variance (Levene’s test). If the data was not normally distributed, we attempted to normalize it by log transformation after which it was analyzed. If data could not be normalized, nonparametric analyses were performed (see below); otherwise normally distributed data was analyzed by two-way ANOVA with
repeated measures. If normally distributed data was subsequently found to contain significant
differences by ANOVA (P<0.05), we followed up this finding by both performing comparisons
between all treatment groups (including untreated males) using Tukey’s post hoc tests to look for
overall treatment effects, and also by using separate repeated measures one-way ANOVAs and
Tukey’s post hoc tests to identify specific time points where values changed within treatments.
Non-normal data were evaluated using a similar approach but in two steps because there is no
practical equivalent of a two-way ANOVA. For these tests, we first used a non-parametric
Kruskal-Wallis ANOVA along with Dunn’s post hoc tests to test for differences between all	reatment groups and control males at matched time points. Next, we used Friedman tests with
Dunn’s post hoc tests to test for changes between different time points within each treatment
group. The significance level for all tests was set at P<0.05. Post hoc tests were adjusted for
multiple comparisons using Bonferroni corrections. For all parametric analyses, we report the F
statistic as F (dfn, dfd). For non-parametric analyses, we report the H statistic for the Kruskal-
Wallis test and the Friedman statistic for the Friedman test, with degrees of freedom expressed as
the number of groups less one. Effect size estimates were calculated as eta squared (η²,
calculated as R² equals to between group sum-of-squares divided by the total sum-of-squares,
Graph Pad Prism ver. 6) for repeated measures one way ANOVA; standard omega squared
(std.ω², calculated as % of total variation, Sheskin, 2004) for repeated measures two way
ANOVA; and eta squared based on H statistic (η²_H, Cohen 2008) for the Kruskal-Wallis test.

3. Results

3.1 Gonadal development and tubercles
All female goldfish including controls had small non-vitellogenic gonads (GSI < 2.0%) at the conclusion of the experiment (Table 1). There was no difference between treatment groups (F_{4,95} = 0.9; P>0.05, one-way ANOVA) nor were there any intersex fish (Table 1). While control males were fully tuberculated throughout the experiment and control females never developed tubercles, nearly half of females in all three androgen-treated groups were tuberculated at week 1 and all were tuberculated at week 18 (Table 1). Control males had well-developed testes (GSI = 2.95 ± 0.1%) as well as expressible milt (sperm).

3.2 Hormones

Plasma estradiol (E2) was very low (i.e. <0.2 ng/ml) at both week 1 and 24 in all treatment groups including control females and control males (Supplementary Fig. 1). Similarly, plasma levels of all three androgens (KT, T and irMT) in control females were consistently below 1 ng/ml throughout the experiment and did not change with time (Tukey’s post hoc test, P>0.05). Plasma levels of KT, T and irMT in control females were always below those of all androgen-treated females and control males (Tukey’s post hoc test, P>0.05). In contrast, plasma levels of all three androgens in control males were relatively high and ranged from 2-7 ng/ml, with T being the highest throughout the experiment (Fig. 1A). These values did not change with time (Tukey’s post hoc test, P>0.05) and were consistently higher than those of control females at all time points (Tukey’s post hoc test, P<0.05) (Fig 1E). Greater complexity was seen in the androgen levels measured in the three groups of androgen-treated females. These data were normally distributed and are described below.
Analysis of the KT data using repeated measures two-way ANOVA showed overall differences (P<0.05) with significant treatment (F_{4,80} = 153.52, P<0.05, std.ω^2 = 65.75), time (F_{4,80} = 21.71, P<0.05, std.ω^2 = 12.4), and interaction effects (F_{16,80} = 322 9.03, P<0.05, std.ω^2 = 15.47) (Fig 1). MT-B fish had relatively low levels of KT (<3 ng/ml) that were always equivalent to the levels measured in control males and did not change with time (Fig. 1B; Supplementary Fig 3A). In contrast, KT+T-implanted fish had relatively high levels of KT (>5 ng/ml), which were greater than the levels measured in control males at weeks 1, 6 and 12, (Tukey’s post hoc tests, P<0.05) and then dropped at week 18 (F_{4,16} = 59.57, P<0.05, η^2 = 0.93, repeated measures one-way ANOVA) and became equivalent to levels in control males (Tukey’s post hoc tests, P>0.05) (Fig. 1C; Supplementary Fig. 3B). MT-C females showed different patterns than did KT+T fish (P<0.05); these fish had modest levels of KT (>2 ng/ml) that did not differ from values measured in control males and did not change across time (Tukey’s post hoc tests, P>0.05) (Fig. 1D; Supplementary Fig 3B).

Analysis of T data using repeated measures two-way ANOVA showed overall differences (P<0.05) with significant treatment (F_{4,80} = 96.80, P<0.05, std.ω^2 = 35.82), time (F_{4,80} = 12.57, P<0.05, std.ω^2 = 6.20) and interaction effects (F_{16,80} = 23.57, P<0.05, std.ω^2 = 45.68) (Fig 1). MT-B fish had relatively high levels of T (4-8 ng/ml) equivalent to the levels in control males at all times except week 1 when they were greater (Tukey’s post hoc tests, P<0.05) (Fig. 1B; Supplementary 3C). KT+T-implanted females had very high levels of T (>20 ng/ml) that were greater than those measured in control males at weeks 1, 6 and 12 (Tukey’s post hoc tests, P<0.05), after which their T levels dropped (F_{4,16} = 47.06, P<0.05, η^2 = 0.92, repeated measures one-way ANOVA) and became equivalent to the levels of control males (P>0.05; Fig. 1C; Supplementary 3D). MT-C females had high levels of T (3-10 ng/ml) greater than the levels...
measured in control males at all time points except week 1 and week 24 (Tukey’s post hoc tests, P<0.05) (Fig. 1D; Supplementary 3D).

Analysis of irMT data using repeated measures two-way ANOVA also showed overall differences (P<0.05) with significant treatment (F_{4,80} = 95.96, P<0.05, std.\omega^2 = 42.92), time (F_{4,80} = 11.43, P<0.05, std.\omega^2 = 6.82), and interaction effects (F_{16,80} = 18.94, P<0.05, std.\omega^2 = 33.88) (Fig 1). MT-B fish had a low level of irMT (<5 ng/ml) equivalent to that measured in control males (Tukey’s post hoc tests, P<0.05) and that did not change with time (Fig 1B; Supplementary Fig. 3E). KT+T-implanted females had a modest level of irMT (2-4 ng/ml) equivalent to irMT levels measured in control males at all time points and that did not change with time (Tukey’s post hoc test, P>0.05; Fig. 1C; Supplementary Fig. 3F). MT-C females had high levels of irMT (>10 ng/ml) greater than those of control males (Tukey’s post hoc test, P<0.05) at all time points until week 18 when their plasma irMT dropped (F_{4,16} = 27.48, P<0.05, \eta^2 = 0.85, repeated measures one-way ANOVA) to less than 5 ng/ml and became equivalent to levels measured in control males and the other androgen-treated groups (Fig. 1D; Supplementary 3F).

3.3 Courtship behavior

Nudging activity was not normally distributed and could not be normalized by log transformation, so it was evaluated using separate Kruskal-Wallis tests that found treatment differences at all time points (KW test: [week 1: KW statistic [4] =10.02, P<0.05, \eta^2_H = 0.17; week 6: KW statistic [4] = 18.26, P<0.05, \eta^2_H = 0.40 ; week 12: KW statistic [4] = 8.29, P<0.05, \eta^2_H = 0.12; week 18: KW statistic [4] = 14.57, P<0.05, \eta^2_H = 0.30; week 24: KW statistic [4] =
13.24, P<0.05, η^2_H = 0.26 and week 30: KW statistic [2] = 11.25, P<0.05, η^2_H = 0.20] using all 5 groups). Control females did not show any nudging activity at any time and their nudging rate was thus consistently lower than those of all androgen-treated groups and control males at all time points (Dunns’ post hoc tests, P<0.05). In contrast, control males consistently nudged at rates that started at about 10 nudges/min at week 1 and increased with time (Friedman statistic [4] = 25.06, P<0.05) with increases at both week 12 (Dunns’ post hoc tests, P<0.05) and week 24 (Dunns’ post hoc tests, P<0.05), after which their activity leveled off at a median rate of approximately 40 nudges/min (Fig. 2A; Supplementary Fig. 4A). MT-B fish exhibited a modest initial nudging rate (<5 nudges/min) at week 1 that also increased with time (Friedman statistic [4] = 25.47, P<0.05) and showed an increase at week 18 (Dunns’ post hoc tests, P<0.05) when it became fully equivalent to the nudging rate of control males (Dunns’ post hoc tests, P>0.05), after which neither rate changed further (Dunn’s post hoc tests, P>0.05, Fig. 2B; Supplementary Fig. 4A). KT+T-implanted and MT-C fish showed activity patterns that did not differ from each other (Dunns’ post hoc tests, P>0.05); both groups showed modest initial nudging rates (<5 nudges/min) that peaked at about 10 nudges per min at week 12 when this rate was greater than the rate of control females (Dunns’ post hoc tests, P<0.05) but less than that of control males (Dunns’ post hoc tests, P<0.05) (Fig 2C, D). No further changes were noted after week 12 in either KT+T-implanted or MT-C fish (Supplementary Fig. 4B).

Time-to-court data was normally distributed (Shapiro Wilk test, P>0.05) and a two way ANOVA test with repeated measures showed both an overall treatment (F_{4,140} = 9.88, P<0.05, std.ω^2 = 18.01), time (F_{4,140} = 2.72, P<0.05, std.ω^2 = 9.18) and an interaction effect (F_{16,140} =2.92, P<0.05, std.ω^2 = 16.81) (Fig. 3). Control females were never observed to court (so their time-to-court values were infinite and transformed for analysis) while control males were consistently
seen to court and had time-to-court values below three min (Fig. 3A). The time-to-court values of control females were consistently greater than those of all other treatment groups at all time points (Tukey’s post hoc tests, P<0.05). Control males consistently took an average of about three min to court throughout the entire experiment and this rate, unlike that noted for nudging for this group, did not change with time but was always less than those for all three androgen-treated groups at all time points (Tukey’s post hoc tests, P<0.05) (Fig. 3A). In contrast, while MT-B fish initially took about 15 min to court at week 1, this value decreased with time (F4,28 = 4.04, P<0.05, η² = 0.44, repeated measures one way ANOVA with Tukey’s post hoc test) and at week 18 it dropped to just under 6 min (P<0.05; Fig 3B; Supplementary Fig. 5A), a value which did not change further and, unlike that noted for nudging behavior, never reached a rate on par with that of control males (Tukey’s post hoc tests, P<0.05). The average time that KT+T-implanted fish took to court also started at about 15 min at week 1; it decreased (F4,28 = 3.05, P<0.05, η² = 0.37, repeated measures one-way ANOVA with Tukey’s post hoc tests) to just over 6 min at week 6 when it was greater than the time taken by control males (Tukey’s post hoc tests, P<0.05) and then increased at week 18 (Tukey’s post hoc tests, P<0.05) (Fig. 3C; Supplementary Fig. 5B). MT-C fish took a very long time to court (>20 min) and this did not change during the experiment (repeated measures one-way ANOVA, P>0.05) (Fig. 3D; Supplementary Fig. 5B).

3.4 Mating (spawning) behavior

Complete spawning activity was not normally distributed and could not be normalized by transformation so it was analyzed by Kruskal-Wallis and Friedman tests with selected follow-up tests. Kruskal-Wallis analysis showed treatment differences at all time points (P<0.05). While
control males consistently exhibited complete spawning acts, control females never spawned
and differences were noted between treatment groups (KW test [week 1: KW statistic [4] = 4.82,
P<0.05, η²_H = 0.02; week 6: KW statistic [4] = 6.37, P<0.05, η²_H = 0.06; week 12: KW statistic
[4] = 6.77, P<0.05, η²_H = 0.07; week 18: KW statistic [4] = 6.76, P<0.05, η²_H = 0.07; week 24:
KW statistic [4] = 7.24, P<0.05, η²_H = 0.09 and week 30: KW statistic [2] = 13.51, P<0.05, η²_H =
0.27] using all the 5 groups) (Fig. 4). Follow-up tests showed that control males spawned more
often (median values ranged from 0.10 - 0.18 for complete spawns/min over the course of the
experiment) than all three androgen-treated groups at all time points (Dunns’ post hoc
comparisons, P<0.05). Further, unlike nudging behavior, spawning behavior exhibited by control
males did not change with time (Fig. 4A). Although all three androgen-treated groups spawned
more frequently than control females at all time points including week 1 (Dunns’ post hoc tests,
P<0.05), there were no statistical differences among the treated groups and their rates did not
change over time. These rates were low; MT-B fish spawned the most of the three groups but at
rates that were variable and only about one-tenth of those of control males (median values
ranged from 0.01 - 0.02 for complete spawns/min over the course of the experiment) (Fig 4B;
Supplementary Fig 6A). KT+T-implanted and MT-C fish showed very little spawning activity
(median values ranged from 0.0 - 0.01 spawns/min over the course of the experiment) at all time
points and these rates did not change during the course of the experiment (Fig. 4C, D;
Supplementary Fig. 6B).

Similar trends were noted for incomplete spawning activity, with control males
consistently attempting to spawn much more often than fish from all treatment groups including
control females, which once again showed no activity at all time points. This data was not
normally distributed and could not be normalized by log transformation. Kruskal-Wallis tests
showed a treatment effect at each time point (KW test [week 1: KW statistic [4] = 6.37, P<0.05, \(\eta^2_H = 0.06\); week 6: KW statistic [4] = 8.02, P<0.05, \(\eta^2_H = 0.11\); week 12: KW statistic [4] = 7.77, P<0.05, \(\eta^2_H = 0.10\); week 18: KW statistic [4] = 9.76, P<0.05, \(\eta^2_H = 0.16\); week 24: KW statistic [4] = 9.51, P<0.05, \(\eta^2_H = 0.15\) and week 30: KW statistic [2] = 12.98, P<0.05, \(\eta^2_H = 0.25\) using all the 5 groups) (Fig. 5; Supplementary Fig. 7). The rate of incomplete spawning by control males did not change across time and was about 8-9 times greater than the number of completed spawns by this group (median values ranged from 0.91-1.44 incomplete spawns/min over the course of the experiment) and consistently greater than that of all three androgen-treated groups. Both the MT-B and KT+T groups exhibited more incomplete spawning activity (median values ranged from 0.17-0.6 incomplete spawns/min over the course of the experiment) than complete spawns, and these rates were also only about one-third the rate of incomplete spawns observed in control males (Dunns’ post hoc tests, P<0.05) and did not change throughout the experiment (Friedman test, P>0.05). The MT-C fish showed very little incomplete spawning activity at all time points (Fig. 5).

3.5 Feeding behavior

Feeding activity was not normally distributed and could not be normalized by transformation, so it was evaluated by separate Kruskal-Wallis tests and showed treatment effects at all time points (KW test [week 1: KW statistic [4] = 12.75, P<0.05, \(\eta^2_H = 0.25\); week 6: KW statistic [4] = 10.04, P<0.05, \(\eta^2_H = 0.17\); week 12: KW statistic [4] = 9.55, P<0.05, \(\eta^2_H = 0.15\); week 18: KW statistic [4] = 17.51, P<0.05, \(\eta^2_H = 0.38\) and week 24: KW statistic [4] = 18.26, P<0.05, \(\eta^2_H = 0.40\) using all the 5 groups) (Fig. 6). Trends were roughly the inverse of
those seen for courtship activity although they did not change with time. Control males had very low levels of feeding activity (median values ranged from 0.01-0.09 feeds/min over the course of the experiment) while control females consistently showed relatively high feeding activity (median values ranged from 1.0-5.2 feeds/min over the course of the experiment) which was greater than that of control males at all time points (Dunns’ post hoc test, P<0.05) (Fig. 6A, E).

The feeding activity of MT-B fish was also low (median values ranged from 0.5-1.0 feeds/ min over the course of the experiment) and was higher than that of control males at week 1 and 6 (Dunns’ post hoc test, P<0.05) and lower than that of control females at weeks 12, 18 and 24 (Dunns’ post hoc test, P<0.05) (Fig. 6B). The feeding activity of KT+T-implanted fish was also modest (medians ranged from 0.5-1.0 feeds/min over the course of the experiment) and had a similar trend to that of MT-B fish (Fig. 6C). The feeding rate of MT-C fish was relatively high (median values ranged from 1.4-5.9 feeds/min over the course of the experiment) and greater than that of control males (Dunns’ post hoc tests, P<0.05) but equivalent to that of control females at all time points (Dunns’ post hoc test, P>0.05) (Fig. 6D).

3.6 Olfactory sensitivity

Initial analysis of EOG (mv) responses to our standard, L-Serine, did not show an effect of either treatment or time (P>0.05; Supplemental Table 1), so responses to pheromones were expressed relative to this standard to reduce variance (and increase power) following established protocols (Irvine and Sorensen, 1993; Sorensen et al., 1995). We examined only responses elicited by nanomolar concentrations of these pheromones because these concentrations elicited maximal responses (Sorensen et al., 1988, 1991). Relative responses ([response to odor/response
to the standard]*100) to nanomolar concentrations of both pheromones were normally
distributed (Shapiro Wilk test, P>0.05). Two way repeated measures ANOVA to the priming
pheromone, 17,20βP, found no overall differences in sensitivity by treatment (F_{4,80} = 1.05,
P>0.05, std.ω^2 = 17.1), or time (F_{4,80} = 1.2, P>0.05, std.ω^2 = 4.7), and there was no interaction
(F_{16,80} = 1.21, P>0.05, std.ω^2 = 1.5) (Supplementary Fig. 2). In contrast, two way repeated
measures ANOVA to the spawning pheromone, 15KPGF_{2α}, found overall differences (P<0.05)
for treatment (F_{4,80} =12.87, P<0.05, std.ω^2 = 39.18), time (F_{4,80} = 15.81, P<0.05, std.ω^2 = 24.07)
and an interaction (F_{16,80} = 1.10, P<0.05, std.ω^2 = 6.7) (Fig. 7). Tukey’s post hoc tests showed
control males to be consistently more sensitive than control female goldfish to 15KPGF_{2α}
(Tukey’s post hoc tests, P<0.05) at all time points with no changes in the relative sensitivities of
either group over time (P>0.05, repeated measures ANOVA) (Fig. 7A, E). MT-B fish
experienced an immediate increase in sensitivity to 15KPGF_{2α} by week 1 (F_{4,16} = 6.85, P<0.05,
η^2 = 0.55 repeated measures ANOVA with Tukey’s post hoc tests) when their sensitivity to this
pheromone was on par with that of control males (P>0.05, Tukey’s post hoc tests; Fig. 7B), after
which their sensitivity did not change further (P>0.05; Supplementary Fig. 8A). The EOG
sensitivity of KT+T-implanted females to 15KPGF_{2α} had also increased by week 1 and then rose
(P<0.05) to match that of control males by week 6 (F_{4,16} =7.81, P<0.05, η^2 = 0.73, repeated
measures ANOVA; P>0.05, Tukey’s post hoc tests) (Fig. 7C; Supplementary Fig. 8B). MT-C
fish showed a trend identical to that of KT+T-implanted fish (Fig. 7D; Supplementary Fig. 8B)
and matched the sensitivity of untreated males and all androgen-treated groups by week 6
(Tukey’s post hoc tests, P>0.05). No changes were noted in any group after week 6. The average
EOG responses of control males to 15KPGF_{2α} was over twice that of control females and did not
change with time (Tukey’s post hoc tests, P>0.05).
4. Discussion

The results of this study describe a new picture of the bipotential adult goldfish brain, one in which male reproductive behavior is comprised of several distinct components, including courtship and mating, that are controlled by different neuroendocrine mechanisms which are influenced by androgens to differing extents. While we found that the olfactory sensitivity of goldfish to sex pheromones could be fully masculinized by exposure to both natural and artificial androgens within just a few weeks, courtship behavior took a full 18 weeks, and neither mating behavior, nor time-to-court, could be fully masculinized after exposure to any androgen(s) even after over 30 weeks. Each of these attributes thus appears to be influenced by androgens to different extents which suggests that bipotentiality in goldfish is more complex and specialized than previously suspected (Kobayashi et al., 2013). Androgen treatments also increased tubercle counts, confirming that they are responsible for secondary sexual characteristics, as noted previously (Bass and Grober, 2009; Stacey and Sorensen, 2009). Finally, our study described new evidence that courtship behavior in the male goldfish is enhanced by previous sexual experience while mating is not – again alluding to the presence of multiple neuroendocrine mechanisms associated with different types of androgens driving the expression of male-typical reproductive behaviors in this important model species. The scenario in which some aspects of male-typical behavior in a model cyprinid take weeks to be fully expressed in androgen-treated females and other behaviors are never fully masculinized, contrasts dramatically with that for feminization in the cyprinids, in which prostaglandin F$_{2\alpha}$ treatment fully feminizes all aspects of male behavior within minutes (Stacey, 1981; Stacey and Sorensen, 2009). Thus, male goldfish
having functional but incomplete brain bisexuality are different from the plainfin midshipman fish, *Porichthys notatus*, (Remage-Healey and Bass, 2004; 2007) and masu salmon, *Oncorhynchus masou*, (Munakata, 2012) in which hormonal treatment cannot induce heterotypical sexual behavior, and are also different from the sex changing fishes, which exhibit complete brain bisexuality (Kobayashi et al., 2013). The goldfish has served as a robust and relevant model for hormone and behavior function in cypriniform fishes (Stacey and Sorensen, 2009) to date so we expect these findings to be widely applicable to cypriniformes, and likely other gonochoristic fish.

Our observation that mating and courtship in male goldfish represent distinct behaviors controlled by different brain processes may be new for fish, but it finds considerable support in studies of mammal behavior. It is also consistent with the suggestion that male agonistic and reproductive behaviors are decoupled from each other and androgen influence is limited in fishes, such as in the Mozambique tilapia, *Oreochromis mossambicus*, a species that exhibits strong territorial and nest guarding behaviors (Almeida et al., 2014). Beach (1942) first proposed that the complex nature of copulatory behavior in male mammals might be explained by the “dual nature of sexual arousal and performance” which he tested using behavioral experiments. A series of brain lesioning and chemical manipulation experiments in the male Syrian hamster, *Mesocricetus auratus*, have since shown that while the medial preoptic area is involved with consummatory aspects of male sexual behavior including arousal and preferences for sex odors (i.e. courtship), the ventral strial area is involved with appetitive behaviors associated with mating (ejaculation, etc.) (Been and Petruslis, 2012). Hormones appear to exert different types of influences on these brain regions. Similarly, more recent studies of the rhesus monkey *Macaca mulatta*, show that prenatal androgens determine the propensity of males to
court, but not their actual mounting behavior (Wallen, 2001). Together, these studies suggest that control of male-typical reproductive behaviors is both sophisticated and complex.

Our findings are consistent with those of all previous studies on this topic; the rates with which androgen-treated goldfish and control males courted, spawned, and fed are all similar to those described previously (Stacey and Kobayashi, 1996; Saoshiro et al., 2013). Additionally, although our fish were held in just a few tanks, it seems unlikely that tank-specific phenomena were present because control males and females behaved normally and all fish fed and grew to equal extents. Although our study did not examine the specific effects of context on the expression of male-typical behaviors, we tested a context already known to be optimal for spawning in goldfish (Kobayashi et al., 2002). Finally, while we did not address all possible sources and fates of androgens [Pradhan et al. (2014, 2015) found high levels of androgens in the local tissue and the brain of KT-implanted blue-banded goby, *Lythrypnus dalli*], this deficiency should be addressed by future work and does not bear directly on our main conclusion that different aspects of male reproductive behaviors are controlled by different neuroendocrine mechanisms in the goldfish. In sum, our study, like others (Oliveira et al. 2001; Almeida et al 2014; Pradhan et al., 2014, 2015), clearly shows that the relationship between androgenic steroid hormones and male-typical sex behaviors is not straightforward in fish but is multi-faceted and likely life history specific.

We show that natural androgens (KT and T) completely masculinize olfactory sensitivity to a female sex pheromone in just a few weeks. The fact that this change occurred more quickly than did changes to courtship behavior (nudging) suggests that, while olfactory input trigger male-typical courtship behavior in goldfish (Stacey and Sorensen, 2009), courtship is probably not exclusively driven by olfactory input and that the two phenomena are not intimately coupled.
Notably, our study, like others (Belanger et al., 2010), found that androgen-mediated changes in olfactory sensitivity are restricted to PGF-derived pheromone. It is interesting to speculate what cellular processes might be responsible for this specific olfactory response but neither the specific mechanisms underlying EOG responses nor the identity of sex pheromone receptors are known in any vertebrate (Green and Zielinski, 2013). The changes we observed in olfactory sensitivity could reflect the expression of new olfactory receptors, second messenger systems, new olfactory receptor cells, or some combination thereof. A recent study in maturing Anguillid eels (Anguilla anguilla) suggests olfactory receptor expression could have a role (Churcher et al., 2015). Only one study that we know of has examined the olfactory histology of androgen-treated fish and it found that female goldfish fed with MT-laced food had thicker olfactory epithelia (Yamazaki and Watanabe, 1979), although the biological relevance of epithelial thickness is unknown. It is interesting that olfactory responsiveness appeared most sensitive to water-borne MT; perhaps this was because the fishes’ olfactory epithelia was exposed to it directly and it might have transport mechanisms similar to the gills (Randall et al., 1998; Scott and Ellis, 2007). Now that the biological relevance of androgen-induced changes in peripheral olfactory sensitivity to sex pheromones has been established, future studies of the cellular and endocrine mechanisms underlying these changes are warranted.

This study produced new insight on the effects of experience on courtship in fishes. Remarkably, nudging rates of untreated male goldfish increased nearly four-fold over the first 18 weeks of our study and this change was not reflected in spawning activity, again suggesting independent neuroendocrine control of these behaviors. Likely, the nudging activity of MT-B fish was also influenced by experience but effects cannot be separated from exposure time to MT. Similar findings showing that reproductive experience can promote courtship activity in a
male fish has been documented in other fishes with different spawning strategies including the
guppy (Poecilia reticulata) (Guevara-Fiore, 2012), the Siamese fighting fish (Betta splendens)
(Rainwater and Miller, 1966), and the three-spine stickleback (Gasterosteus aculeatus) (Bakker
and Rowland, 1995). Studies of the goldfish brain after spawning have shown increased
neurogenesis in regions that contain GnRH (Chung-Davidson et al., 2008), demonstrating one
possible mechanism for the effect of experience on courtship. It is well established that
experience can affect brain/neuronal structures in mammals; for example, Breedlove (1997)
found that copulatory experience in early adulthood affects the size of spinal neurons and
behavioral responsiveness in rats. Similar effects of experience on courtship are seen in many
other mammals including humans (Pfaus et al., 2001). The existence of an experience-driven
mechanism that enhances male courtship and possibly competitive performance in cyprinid fish
would presumably be evolutionarily adaptive.

Finally, our study sheds new light on the action and fate of the artificial androgen, MT in
fishes. MT-B and KT+T-implanted treatments had similar effects on olfactory sensitivity,
courtship, and spawning until week 12 after which circulating levels had dropped in KT+T-
implanted fish, strongly suggesting these steroids bind to similar receptor(s) and activate similar
neuroendocrine mechanism(s) and that MT may bind to both T and KT receptors. While it is
possible that the effects of MT in our study may have been confounded by aromatization, we did
not observe increases in E2 so this seems unlikely. The levels of MT we used (3μg/L was added
to the bath and dropped 10 fold an hour) were much less than the 20 μg/L of continuous
exposure that Hornung et al. (2004) and ≥5μg/L that Pawlowski et al. (2004) used to stimulate
aromatization and feminization in the fathead minnow. Rainbow trout (Oncorhynchus mykiss)
fed with MT-laced food also did not aromatize MT, which they released through their gills along
with other metabolites (Cravedi et al., 1993). Nevertheless, the high levels of T we measured in MT treated goldfish suggest that conversion to other steroids was occurring. Although KT and T levels were higher in implanted fish than the levels in untreated males, we did not observe any pharmacological effects (for example, mortality, gonadal abnormalities and/or intersex fish [Jobling et al., 1998; Arcand-Hoy and Benson, 1998]). Given the large number of experiments that have used MT and its common use in aquaculture (because of its low cost), further study of MT’s precise fates and actions, and how they might relate to those of KT (whose receptors have not yet been identified) would be very interesting.

5. Conclusions

This study has produced new insight into the neuroendocrine basis of male-typical reproductive behavior in scramble-spawning cyprinid fishes, and likely many other gonochorists, and the role of androgens mediating it. Our study showed that female goldfish seem to have functional brain bisexuality but not complete. In contrast to female-typical behavior which appears to be controlled by a single PGF$_{2\alpha}$-based neuroendocrine system, male-typical reproductive behavior in the goldfish appears to have multiple components that are controlled by several neuroendocrine mechanisms which are influenced by androgens in different manners. Previous findings of androgen receptors and aromatase activity in many regions of the goldfish brain (Gelinas and Callard, 1997; Kyle and Peter, 1982; Kyle et al., 1982) describe possible neural substrates for control of complex male reproductive behaviors. The goldfish represents an excellent model to determine how hormones and behavior have come to precisely control each other in flexible yet adaptive ways in an important group of gonochorists.
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References


Fig. 1. Changes in plasma steroid levels in goldfish across time (weeks since the start of treatment). Broken line (— — — ) represent 11-ketotestosterone (KT); solid line (——) represent testosterone (T); dotted line (............) represent immunoreactive methyltestosterone (irMT). Panels represent A) control males (intact); B) females continuously bathed in MT (MT-B); C) females implanted with KT and T (KT+T); D) females implanted with MT (MT-C); E) control females. Symbols and bars indicate mean (ng/ml) and the SEM (N=5). → indicates time points at which hormone levels were significantly different (P<0.05) from the previous sampling point for that particular hormone; * indicates the noted value differed from that of untreated males (P<0.05) at that time point while the lack of a symbol indicates that it did not differ from that of untreated males (P>0.05) at that time point.

Fig. 2. Courtship (nudging) behavior of goldfish across time (weeks since the start of treatment). Panels represent A) control males (intact); B) females continuously bathed in MT (MT-B); C) females implanted with KT and T (KT+T); D) females implanted with MT (MT-C). Values for control females were zero because these fish did not exhibit any courtship behavior. Symbols and bars indicate median nudges/min and the range (N=8). → indicates time points at which values were different (P<0.05) from the previous sampling point for that group; * indicates the noted value differed from that of untreated males (P<0.05) at that time point while the lack of a symbol indicates that it did not differ from that of untreated males (P>0.05) at that time point.

Fig. 3. Time-to-court data of goldfish across time (weeks since the start of treatment). Panels represent A) control males (intact); B) females continuously bathed in MT (MT-B); C) females implanted with KT and T (KT+T); D) females implanted with MT (MT-C). Values for control
females were zero because these fish did not exhibit any courtship behavior. Symbols and bars indicate mean time (min) and the SEM (N=8). → indicates time points at which values were different (P<0.05) from the previous sampling point for that group; * indicates the noted value differed from that of untreated males (P<0.05) at that time point.

**Fig. 4.** Complete mating (spawning) behavior of goldfish across time (weeks since the start of treatment). Panels represent A) control males (intact); B) females continuously bathed in MT (MT-B); C) females implanted with KT and T (KT+T); D) females implanted with MT (MT-C). Values for control females were zero because these fish did not exhibit any spawning behavior. Symbols and bars indicate median complete spawns/min and the range (N=8). * indicates the noted value differed from that of untreated males (P<0.05) at that time point.

**Fig. 5.** Incomplete mating (spawning) behavior of goldfish across time (weeks since the start of treatment). Panels represent A) control males (intact); B) females continuously bathed in MT (MT-B); C) females implanted with KT and T (KT+T); D) females implanted with MT (MT-C). Values for control females were zero because these fish did not exhibit any spawning behavior. Symbols and bars indicate median incomplete spawns/min and the range (N=8).* indicates the noted value differed from that of untreated males (P<0.05) at that time point.

**Fig. 6.** Feeding activity of goldfish across time (weeks since the start of treatment). Panels represent A) control males (intact); B) females continuously bathed in MT (MT-B); C) females implanted with KT and T (KT+T); D) females implanted with MT (MT-C); E) control females.
Symbols and bars indicate median feeds/min and the range (N=8). → indicates time points at which values were different (P<0.05) from the previous sampling point for that group; * indicates the noted value differed from that of untreated males (P<0.05) but did not differ from that of control females (P>0.05) at that time point; while lack of a symbol indicates the noted value did not differ from that of untreated males (P>0.05) but differed from that of control females (P<0.05) at that time point.

**Fig. 7.** Relative olfactory sensitivity of goldfish across time from week 0 to week 24. Panels represent A) control males (intact); B) females continuously bathed in MT (MT-B); C) females implanted with KT and T (KT+T); D) females implanted with MT (MT-C); E) Control females. Symbols and bars indicate mean percent and the SEM (N=5). Week 0 is the week before the start of the treatment. → indicates time points at which values were different (P<0.05) from the previous sampling point for that group; * indicates the noted value differed from that of untreated males (P<0.05) but did not differ from that of control females (P>0.05) at that time point; while lack of a symbol indicates the noted value did not differ from that of untreated males (P>0.05) but differed from that of control females (P<0.05) at that time point.

**Supplementary Fig. 1.** Plasma estradiol levels in goldfish for weeks 1 and 24. Panels represent A) females continuously bathed in MT (MT-B) and control males (intact); B) females implanted with KT and T (KT+T), females implanted with MT (MT-C) and control females. Symbols and bars indicate mean (ng/ml) and the SEM (n=5).

**Supplementary Fig. 2.** Relative EOG responses to the priming pheromone, 17α,20β-dihydroxy-
4-pregen-3-one (17,20βP) for week 1 and 24. Panels represent A) females continuously bathed in MT (MT-B) and control males (intact); B) females implanted with KT and T (KT+T), females implanted with MT (MT-C) and control females. Symbols and bars indicate mean percent and the SEM towards increasing concentrations of 17,20βP (10^{-13}M - 10^{-9}M). No difference in responses was noted across all groups for any concentration (P>0.05).

Supplementary Fig. 3. Changes in plasma steroid levels in goldfish across time (weeks since the start of treatment). Panels represent A, B) 11-ketotestosterone levels; C, D) testosterone levels and E, F) immunoreactive methyltestosterone levels in androgen-treated female goldfish (MT-B, KT+T and MT-C), and control males (intact) and control females. Symbols and bars indicate mean (ng/ml) and the SEM (N=5). * indicates the noted value differed from that of untreated males (P<0.05) at that time point while the lack of a symbol indicates that it did not differ from that of untreated males (P>0.05) at that time point.

Supplementary Fig. 4. Courtship (nudging) behavior of goldfish across time (weeks since the start of treatment). Panels represent A) females continuously bathed in MT (MT-B) and control males (intact); B) females implanted with KT and T (KT+T) and females implanted with MT (MT-C). Values for control females were zero because these fish did not exhibit any courtship behavior. Symbols and bars indicate median nudges/min and the range (N=8). * indicates the noted value differed from that of untreated males (P<0.05) at that time point while the lack of a symbol indicates that it did not differ from that of untreated males (P>0.05) at that time point.

Supplementary Fig. 5. Time-to-court data of goldfish across time (weeks since the start of
treatment). Panels represent A) females continuously bathed in MT (MT-B) and control males (intact); B) females implanted with KT and T (KT+T) and females implanted with MT (MT-C). Values for control females were zero because these fish did not exhibit any courtship behavior. Symbols and bars indicate mean time (min) and the SEM (N=8). * indicates the noted value differed from that of untreated males (P<0.05) at that time point.

**Supplementary Fig. 6.** Complete mating (spawning) behavior of goldfish across time (weeks since the start of treatment). Panels represent A) females continuously bathed in MT (MT-B) and control males (intact); B) females implanted with KT and T (KT+T) and females implanted with MT (MT-C). Values for control females were zero because these fish did not exhibit any complete spawning behavior. Symbols and bars indicate median complete spawns/min and the range (N=8). * indicates the noted value differed from that of untreated males (P<0.05) at that time point.

**Supplementary Fig. 7.** Incomplete mating (spawning) behavior of goldfish across time (weeks since the start of treatment). Panels represent A) females continuously bathed in MT (MT-B) and control males (intact); B) females implanted with KT and T (KT+T) and females implanted with MT (MT-C). Values for control females were zero because these fish did not exhibit any incomplete spawning behavior. Symbols and bars indicate median incomplete spawns/min and the range (N=8). * indicates the noted value differed from that of untreated males (P<0.05) at that time point.
Supplementary Fig. 8. Relative olfactory sensitivity of goldfish across time from week 0 to week 24. Panels represent A) females continuously bathed in MT (MT-B) and control males (intact); B) females implanted with KT and T (KT+T), females implanted with MT (MT-C) and control females. Symbols and bars indicate mean percent and the SEM (N=5). Week 0 is the week before the start of the treatment. * indicates the noted value differed from that of untreated males (P<0.05) but did not differ from that of control females (P>0.05) at that time point; while lack of a symbol indicates the noted value did not differ from that of untreated males (P>0.05) but differed from that of control females (P<0.05) at that time point.
**Table 1**: Body weight, gonadosomatic index and tubercle development in each group of goldfish from week 1 to 24

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Wt (Initial)</th>
<th>Body Wt (Final)</th>
<th>GSI (Mean ± SEM, %)</th>
<th>Tubercles (% of fish)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>wk 1</td>
<td>wk 6</td>
</tr>
<tr>
<td>Control male</td>
<td>55±5.7</td>
<td>60±11.7</td>
<td>2.95±0.1</td>
<td>100</td>
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<tr>
<td>Control female</td>
<td>52±2.9</td>
<td>65±15.9</td>
<td>0.72±0.17</td>
<td>0</td>
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<tr>
<td>MT-B¹</td>
<td>58±2.3</td>
<td>68±3.7</td>
<td>1.67±0.34</td>
<td>25</td>
</tr>
<tr>
<td>MT-C³</td>
<td>45±5.0</td>
<td>52±1.9</td>
<td>1.1±0.78</td>
<td>50</td>
</tr>
<tr>
<td>KT+T²</td>
<td>60±1.2</td>
<td>68±2.4</td>
<td>1.5±0.5</td>
<td>30</td>
</tr>
</tbody>
</table>

*Body wt = Body weight (Mean ± SEM, g)

¹GSI = Gonadosomatic index (Mean ± SEM, %) (Taken at the end of the experiment)

¹MT-B = females administered with MT (methyltestosterone) via a bath

²KT+T = females implanted with KT (11-ketotestosterone) and T (testosterone) capsules

³MT-C = females implanted with MT capsules
Fig. 1.

A

Control Male

Plasma Conc. (ng/ml)

B

MT-B

Plasma Conc. (ng/ml)

C

KT+T

Plasma Conc. (ng/ml)

D

MT-C

Plasma Conc. (ng/ml)

E

Control Female

Plasma Conc. (ng/ml)
Fig. 2.
Fig. 3.

A

Control Male

B

MT-B

C

KT+T

D

MT-C

Time to court (min)

WK 1  WK 6  WK 12  WK 18  WK 24  WK 30
Fig. 4.

A. Control Male

B. MT-B

C. KT+T

D. MT-C

Complete spawns/min

* * * * * * *

WK 1 WK 6 WK 12 WK 48 WK 24 WK 30
**Fig. 5.**

A. Control Male

B. MT-B

C. KT+T

D. MT-C

Graphs showing incomplete spawns per minute for different conditions and weeks.
Fig. 6.

A. Control Male

B. MT-B

C. KT+T

D. MT-C

E. Control Female
Fig. 7.

A) Control Male

B) MT-B

C) KT+T

D) MT-C

E) Control Female

The figures illustrate the relative response (% of control) over weeks (0, 1, 6, 12, 18, 24) for different conditions:

- **Control Male**
- **MT-B**
- **KT+T**
- **MT-C**

Each graph shows a trend line with error bars indicating variability. The x-axis represents the weeks, and the y-axis represents the relative response (%). Significant differences are indicated by asterisks (*) on the graphs.
- Male-typical reproductive behaviors in goldfish are controlled by multiple mechanisms
- Courtship behavior can be completely masculinized by androgens in female goldfish
- Mating behavior cannot be completely masculinized by androgens in female goldfish
- Olfactory response to pheromones can be completely masculinized by androgens in goldfish
- Sexual experience increases courtship behavior in goldfish