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Androgens enhance plasticity of an electric communication signal in female knifefish, *Brachyhypopomus pinnicaudatus*

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Abstract

Sex steroids were initially defined by their actions shaping sexually dimorphic behavioral patterns. More recently scientists have begun exploring the role of steroids in determining sex differences in behavioral plasticity. We investigated the role of androgens in potentiating circadian, pharmacological, and socially-induced plasticity in the amplitude and duration of electric organ discharges (EODs) of female gymnotiform fish. We first challenged female fish with injections of serotonin (5-HT) and adrenocorticotrophic hormone (ACTH), and with social encounters with female and male conspecifics to characterize females' pre-implant responses to each treatment. Each individual was then implanted with a pellet containing dihydrotestosterone (DHT) concentrations of 0.0, 0.03, 0.1, 0.3, or 1.0 mg 10g⁻¹ body weight. We then repeated all challenges and compared each female's pre- and post-implant responses. The highest implant dose enhanced EOD duration modulations in response to all challenge types, responses to male challenge were also greater at the second highest dose, and responses to ACTH challenge were enhanced in females receiving all but the smallest dose (and blank) implants. Alternatively, amplitude modulations were enhanced only during female challenges and only when females received the highest DHT dose. Our results highlight the differential regulation of EOD duration and amplitude, and suggest that DHT enhanced the intrinsic plasticity of the electrogenic cells that produce the EOD rather than modifying behavioral phenotypes. The relative failure of DHT to enhance EOD amplitude plasticity also implies that factors other than androgens are involved in regulating/promoting male-typical EOD circadian rhythms and waveform modulations displayed in social contexts.

Keywords

5-HT; ACTH; Circadian rhythm; DHT; Gymnotiform; Implants; Knifefish; Sex steroids; Sexual dimorphism; Social behavior

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Females and males frequently differ in their responses to inter- and intrasexual social encounters with conspecifics and in their sensitivities to hormones that regulate social behaviors. These sexual dimorphisms are often the behavioral accompaniment to a species' reproductive system (Andersson, 1994; Kelley, 1988) where they augment sex-typical behavioral responses by exaggerating physiological or morphological trait differences or by imposing limits on such trait expression in one sex or the other. Gonadal steroid hormones play a major role in shaping these behavioral patterns both during development and at sexual maturity (Adkins-Regan, 1981; Adkins-Regan, 1998; Arnold and Breedlove, 1985; Balthazart et al., 1996; Crews and Silver, 1985; Goncalves et al., 2008; Goy and McEwen, 1980; Kelley, 1988; Kendrick and Schlinger, 1996; Kime, 1993; Rhen and Crews, 2000). Artificially altered levels of sex steroids can masculinize or feminize physiology and morphology depending on which hormones are introduced, the sex and developmental stage of the individuals exposed, and sensitivity of particular tissues to the hormones (Brenowitz and Lent, 2002; Cooke et al., 1998; Herfeld and Moller, 1998; Lund et al., 2006; Staub and Beer, 1997; Wilson and Davies, 2007). These changes in structure are often accompanied by changes in behavior. Recent studies on teleost fish, however, suggest that androgens may be more efficient at masculinizing morphologies and physical traits or a fraction of male typical behaviors than they are at activating the totality of male behavioral repertoires (Lee and Bass, 2005; Oliveira et al., 2001; Oliveira et al., 2005). These roles of androgens in sexual differentiation are well documented, but their function in potentiating *behavioral plasticity*, or the range of responsiveness to various stimuli, is an active area of investigation.

Among teleosts, the electric communication systems of electric fish are excellent models for investigating the roles of androgens in potentiating sexually dimorphic behavioral plasticity versus organizing morphology. Gymnotiform and mormyrid fishes produce weak electric organ discharges (EODs) for electrolocation and electrocommunication (Bennett, 1961; Hopkins, 1983; Lissman, 1958) and the ontogenetic development of sexually dimorphic EODs is controlled by sex steroids (Zakon, 2000). Extended durations in male EOD waveforms compared to female EODs is a commonly recurring sexual dimorphism in pulse-type fish (Bass and Hopkins, 1983, 1985; Franchina, 1997; Hagedorn and Carr, 1985; Mills and Zakon, 1991; Stoddard et al., 2006). When sexual dimorphisms in EOD waveform exist, extended durations of male EODs have been linked to high social status, higher levels of circulating androgens, and increased conspicuousness to predators (Carlson et al., 2000; Franchina et al., 2001; Hagedorn and Zelick, 1989; Hanika and Kramer, 1999; Hanika and Kramer, 2000; Kramer, 1997). Additionally, in species with sexually dimorphic EODs, plasma levels of testosterone and 11-ketotestosterone are often correlated with masculine waveforms, estradiol profiles are correlated with female and juvenile waveforms, and sexually mature female EODs can be 'masculinized' by administering exogenous androgens (Bass and Hopkins, 1983, 1985; Dulka and Maler, 1994; Dulka et al., 1995; Dunlap et al., 1997; Dunlap and Zakon, 1998; Hagedorn and Carr, 1985; Hagedorn and Heiligenberg, 1985; Meyer, 1983; Silva et al., 1999; Zakon et al., 1991). The composite literature makes it clear that androgens are correlated with production of male-like EOD waveforms, yet our insight into how higher androgen levels interact with various physiological and social environments is limited.

The weakly electric gymnotiform fish, *Brachyhypopomus pinnicaudatus* (Hopkins, 1991) produces sinusoidal biphasic EODs with prominent sexual dimorphism in the duration of the second phase (P2) (Hagedorn, 1988; Hopkins et al., 1990; Westby, 1988). Waveform amplitude and duration of P2 oscillate with circadian rhythms that free-run under constant light or constant dark and both EOD parameters increase over minutes in response to social interactions (Franchina et al., 2001; Franchina and Stoddard, 1998; Hagedorn, 1995; Silva et al., 1999; Stoddard et al., 2003; Stoddard et al., 2007). Previous studies of short-term waveform flexibility and circadian oscillations have focused on male waveforms and their responses to social and pharmacological challenges (Allee et al., 2008; Hagedorn and Zelick, 1989; Markham and

Stoddard, 2005; Stoddard et al., 2003); however both sexes modulate their EODs in response to stressors and social stimuli (Franchina et al., 2001; Markham and Stoddard, 2005; Salazar and Stoddard, 2007; Silva et al., 1999; Stoddard et al., 2003) and express circadian rhythms in EOD waveform structure (Franchina and Stoddard, 1998; Hagedorn, 1995; Silva et al., 2007; Stoddard et al., 2007).

In every context studied to date, females' EOD waveform modulations are smaller and less consistent than those of males, suggesting androgens could be responsible for the sexually dimorphic EOD flexibility expressed in this fish. Social isolation attenuates EOD amplitude and P2 duration and social stimulation by the addition of a conspecific stimulus fish can restore waveform characters to pre-isolation levels (Franchina et al., 2001). Male tank-mates are more potent than females in restoring the magnitudes of circadian oscillations of previously isolated males. These findings, combined with literature showing males of many taxa respond to same-sex social challenges by increasing circulating androgens (Oliveira et al., 2002; Wingfield et al., 1990), suggest that androgens might regulate differences in behavioral responses to social interactions as well as the magnitude of circadian oscillation in EOD waveform (Stoddard et al., 2003).

However, sex steroids are not the sole humoral regulators of the EOD waveform, which suggests plasticity in behavioral displays may be controlled entirely by other factors or by interactions between androgens and these factors. Melanocortin peptides act directly on electrocytes to increase amplitude and P2 duration of the EOD waveform over a time scale of minutes (Markham et al., 2009; Markham and Stoddard, 2005). Melanocortin injections produce waveform changes identical in shape and time course to modulations initiated by social interactions (Franchina et al., 2001; Hagedorn, 1995). Injections of three upstream regulators of circulating melanocortin levels, serotonin, CRF, and TRH, also elicit these changes in EOD waveform (Markham et al., 2009). Our goals in this study were: (1) to determine whether androgens enhance circadian rhythmicity in female EOD waveform amplitude and/or duration and (2) to determine the role of androgens in regulating behavioral plasticity in the EOD waveform in response to social challenges and to pharmacological challenges known to elicit responses mimicking normal social responses.

Methods

Animals and measurement system

Animals were adult female *B. pinnicaudatus* maintained in mixed-sex groups in 450-liter outdoor stock pools (dimensions: 185 × 95 × 26 cm) on Florida International University grounds in Miami, Florida. We randomly selected mature fish from the outdoor pools, brought them indoors, and held females individually in 284 liter automated EOD measurement tanks (120 × 44 × 44 cm) throughout each experiment. Test fish were fed oligochaete blackworms *ad libitum* under constant photoperiod (12L: 12D) and temperature (28 °C ± 1 °C). Experiments were approved in advance by the FIU IACUC and complied with the "Principles of Animal Care" publication No. 86-23, revised 1985, of the National Institutes of Health.

We recorded calibrated EODs from the freely swimming females at intervals of ~1 min around the clock for the *entirety* of each trial using an automated system described in detail elsewhere (Stoddard et al., 2003). Briefly, the system amplifies and digitizes EOD waveforms only when the fish swims through an unglazed ceramic tube centered in the tank between two recording electrodes. The EOD waveform of *B. pinnicaudatus* is a sinusoidal wave that varies in its amplitude and in the duration of the second phase (P2; Fig 1A); waveform features that reach their maxima during the nighttime hours. We measured the amplitude of the EOD waveform peak-to-peak and P2 duration as τ_{P2} , the time constant of an inverse exponential function fit to the decay segment of the 2nd phase of the EOD waveform (Fig 1A).

Design Overview

Experimental data were collected during January 2003 - December 2008 in twelve separate trials (n=3–8 females per trial; median trial duration = 28d; mean trial duration = 28.6d). A total of 58 female subjects (not including female social challengers) were used; however data for all female-challenge combinations are not necessarily included in statistical analyses. Complications with individual fish (e.g. illness) as well as technical problems (e.g. power outages, electrode failure, etc.) sometimes resulted in missing data for particular females and/or female-challenge combinations. We therefore continued running new trials with new fish until we collected data from six females for each DHT dose and challenge type.

For each trial, we acclimated females to the testing tanks for a minimum of 24 hours then ran a series of pharmacological and social challenges known to elicit EOD modulations. Each challenge was presented on a different day and provided the individual's pre-DHT implant responses to each challenge. After all pre-implant challenges had been administered; we implanted females with silicone pellets containing the non-aromatizable androgen 5 α -dihydrotestosterone (DHT) and resumed continuous EOD recordings while leaving the subjects undisturbed. We re-challenged the females with the same series of challenges as before, starting on the day after the nightly EOD τ_{p2} had crested (3–7 days depending on DHT implant dose administered). Implant doses of 0.0, 0.03 mg, 0.1 mg, 0.3 mg, or 1.0 mg 10 g⁻¹ body weight were pseudo- randomly assigned to individuals until we had minimum sample sizes of six females per DHT dose with complete before- and after-implant challenge data.

The order of challenge presentation was randomly assigned for both pre-implant and post-implant portions of the trial series prior to the start of each trial. We shuffled challenge orders when necessary to prevent unnecessary interruptions to the time-sensitive trial. Florida is the lightning capital of the United States and if a social challenge was scheduled for a day when it was unsafe to sample challengers from the pools located on the roof, we substituted a pharmacological challenge that day. A total of 11 pre-implant challenge and 10 post-implant orders were used across the 12 trials.

The loss of some subjects due to incomplete data precluded a truly randomized order of implant assignment (i.e. if a female implanted with 0.3mg 10g⁻¹ DHT had to be removed from the study, we replaced her with another individual in a subsequent trial to achieve our goal of n=8 females/DHT dose/challenge type). Female EODs were recorded at all times for the duration of the experiment with the singular exception of when females were removed from their tanks and DHT implants were inserted (described below) providing a time course of DHT effects on waveform characters. Sham implants (no DHT) provided a measure of control for the possibility that observed post-implant effects were the result of typical confounds such as the passage of time, repeated handling, and multiple challenges, rather than increased levels of circulating androgens.

Androgen Implants

We chose the non-aromatizable androgen DHT to provide comparable results with prior studies using DHT (e.g. Dunlap and Zakon, 1998; Ferrari et al., 1995; Few and Zakon, 2001; Few and Zakon, 2007; Hagedorn and Carr, 1985; Keller et al., 1986; Meyer, 1983; Meyer and Zakon, 1982; Mills and Zakon, 1991; Mills et al., 1992). In addition, recent evidence that aromatase expression in the brain is critical to the organization of sexually dimorphic behaviors across vertebrates, including teleosts (Goncalves et al., 2008; Lephart, 1996; Morris et al., 2004), we wanted to ensure any changes we observed in female EODs were limited to androgenic activity at androgen receptors. We tested four doses of DHT to ensure detection of any androgen effects on female responsiveness, not to quantify dose-responses to any challenge type.

We fabricated androgen implants from Dow Corning 3140 silicone mixed with DHT crystals following methods of Elsaesser et al. (1989). A 3:2 mixture of silicone and DHT was extruded through a 20-gauge needle in straight lines onto a piece of weigh paper. Blank implants of plain silicone were created using the same protocols at the same time, then weighed and cured under a chemical fume hood next to the DHT implants provide an estimate of solvent evaporation. After one week, the plain silicone sample was reweighed to calculate mass lost through evaporation. We applied this evaporation percentage to the DHT implants and recalculated the ratio of silicone to DHT as 2.43:1 mg. Implants were stored in airtight containers at room temperature until use. On the day implants were inserted, we reweighed each female and cut a length of implant to a mass that would deliver the assigned dose of DHT appropriate for individual body weight (e.g. a 5.9 g female assigned to the 0.03 mg 10g⁻¹ DHT dose group would be implanted with a length of implant weighing precisely 0.0430 mg).

To insert the implants, we deeply anesthetized the fish by immersion in 2-phenoxyethanol (750 $\mu\text{l l}^{-1}$), used a sterile 18-gauge hypodermic needle to create a small opening between two ribs ventral to the lateral line and dorsal to the electric organ, and inserted the implant into the peritoneal cavity with forceps. We closed the incision with surgical glue and returned the fish to her tank. The time females were outside of their tank to undergo this procedure marked the only time EODs were not being continuously recorded in each trial. The implant procedure took less than 5 minutes and all fish recovered quickly.

Circadian Oscillations

At the conclusion of each trial, we extracted EOD data for analysis from the comprehensive file for each female. For the remainder of this paper, the moniker ‘pre-implant’ refers to any data collected before DHT implants were inserted and ‘post-implant’ refers to all data collected after implants were inserted.

We characterized the magnitude of each female’s circadian oscillation by collecting daily minimum (baseline) and nightly maximum (peak) amplitude (mV cm^{-1}) and τ_{p2} (ms) from a single 24 hr period. The duration of each trial ranged from 19 to 42 days; therefore, we standardized the values used to characterize DHT effect on female circadian rhythms by measuring each individual’s peak and baseline EOD values on the day immediately preceding the first pre-implant challenge (pretest oscillation) and then again on the day immediately preceding the first post-implant challenge (posttest oscillation) for both amplitude and τ_{p2} (Fig 1B). The log-ratios of these values were then analyzed via mixed-model restricted maximum likelihood analysis described in detail below. Changes in the time course of female EOD circadian oscillation as a result of DHT is outside of the scope of this analysis and not reported in this paper.

Pharmacological challenges

Pharmacological challenges consisted of intramuscular injections of serotonin (5-HT; 5-hydroxytryptamine creatinine sulfate complex, Sigma-Aldrich) or adrenocorticotrophic hormone (synthetic porcine ACTH, Sigma-Aldrich), two compounds known to elicit rapid increases in EOD amplitude and τ_{p2} in male fish (Allee et al., 2008; Markham and Stoddard, 2005; Stoddard et al., 2003). Females were quickly netted from their tanks and injected in the hypaxial muscle with 2.5 mM 5-HT (2.5 $\mu\text{M g}^{-1}$) or 25.0 μM ACTH (25.0 nM g⁻¹), at a volume of 1 $\mu\text{l g}^{-1}$ body weight (bw), midday between 1000 and 1500 hours. Fish were returned to their tanks immediately after the injection, generally in less than 30 s from capture. Automated recording of the EOD was interrupted only during the injection process.

Social Challenges

In captivity, gymnotiform electric fish readily hide in narrow tubes and males have been particularly noted for defending these hiding tubes against intruders (Dunlap and Oliveri, 2002; Hagedorn and Zelick, 1989). To determine how females respond toward conspecifics in competitive social situations, we challenged each female by introducing a sexually mature male or female into her ceramic hiding tube. Challengers were randomly selected from outdoor stock pools for use as social challengers and their weights and lengths recorded immediately prior to challenge. We paired challengers with focal females randomly with regard to size to reduce potential covariation between relative size and female response to challenge. All female subjects were challenged with both a male and a female conspecific presented on separate days.

Immediately prior to the onset of the social challenge, we blocked one end of the focal female's recording tube with polyester filter fiber while she remained inside. We guided the challenger into the open end of the tube then blocked the second opening with filter fiber and continued recording EODs. We unblocked both ends of the tube after 45 minutes and returned the challenger to its outdoor pool. Identification and removal of male challengers was unambiguous due to the clear sexual dimorphism in tail morphology (Hopkins, 1991; Hopkins et al., 1990). For any same-sex dyads where female identity could not be determined from natural markings or obvious differences in body size we differentiated the female subject and challenger by their measured weights and lengths.

Responses to both pharmacological and social challenges were calculated by determining peak challenge responses and subtracting circadian baseline values from challenge responses (Fig 1C) using methods and data analysis programming described in detail by Stoddard et al. (2003).

Statistical analysis

Circadian data and responses to challenges were calculated as relative measures: circadian compared diel peak relative to baseline EOD values over the course of a single 24 h period, and responses to challenge measured the peak response to a challenge relative to that individual's EOD baseline immediately preceding the challenge (Fig 1C). Comparing the differences between relative scores can be complicated by scaling issues and regression toward the mean (Bonate, 2000); accordingly, we calculated log-ratio scores for all data (Eq. 1 & Eq. 2) adding a constant ($c=1$) to all values to manage negative numbers (i.e. decreases in EOD in response to challenge). Log ratio scores for each challenge type were calculated twice: once for pretest responses and again for posttest responses.

While we conserved females used in the study by testing each female's responses to all challenge types, we did not take repeated measures (>2) on responses to each independent challenge, nor did we use females as their own control (i.e. implant each female with each dose of DHT). As such, our experiment exploring the effects of the exogenous, non-aromatizable androgen DHT on female EOD responsiveness was a pretest-posttest statistical design (Bonate, 2000). We performed two separate mixed-model restricted maximum likelihood analyses (RMLA) on the adjusted log-ratio scores, one for amplitude and one for τ_{p2} . Time (pretest/posttest), challenge type (circadian oscillation, 5-HT, ACTH, female challenger, and male challenger), and DHT dose (blank, 0.03, 0.1, 0.3, and 1.0 mg $10g^{-1}$) were categorized as fixed effects, while subject was classified as a random effect. All main and possible interaction effects were analyzed using the linear mixed-models procedure in SPSS, evaluating Type III sums of squares in tests for significance. All possible pairwise comparisons based on estimated marginal means for time*subject* DHT dose combinations were compared to a Bonferroni-corrected alpha value of 0.002 to determine whether responses to each challenge type differed

pretest versus posttest while maintaining experiment-wise alpha at 0.05. All data analyses were performed with SPSS [version 16.0].

The longitudinal experimental design may have been influenced by challenge order effects; however the replacement of fish with incomplete experimental data precluded systematic analysis of order effects with appropriate parametric linear models. We did, however, explore our data with Kruskal-Wallis ANOVA to look for evidence of order effects on female responses. We grouped females based on the DHT dose they ultimately received (i.e. pooled all pretest responses from females ultimately implanted with $0.1 \text{ mg } 10 \text{ g}^{-1}$ DHT), which grouped together females from multiple trials, years, seasons, and different challenge orders. We then compared their pre-implant challenge responses and we found no significant differences (Table 1). We selected this analysis strategy as the most liberal approach in testing for significant order effects.

Results

Effect of DHT on female response to challenge

The omnibus RMLA analysis evaluating females' EOD amplitude responses revealed a significant two-way interaction of time (pretest vs. posttest) X DHT dose ($F_{4, 314.02} = 9.09$, $p < 0.001$). We decomposed this interaction by post-hoc pairwise comparisons of pretest vs. post-test EOD amplitude response within each challenge type within each dose level. Females' amplitude response to female social challengers was higher after the largest DHT dose ($1.0 \text{ mg } 10 \text{ g}^{-1}$) than before DHT treatment ($p < 0.002$, Bonferroni correction). In no other condition did DHT change females' amplitude response to the challenges (Fig. 2A–E).

All main and interaction effects for τ_{p2} responses were significant by RMLA analysis ($p < 0.001$), including a significant three-way interaction of time (pretest vs. posttest) X challenge X dose ($F_{16, 336.0} = 3.701$, $p < 0.001$), we further analyzed this interaction by post-hoc pairwise comparisons of pretest vs. posttest EOD τ_{p2} responses within each challenge type within each DHT dose level. The highest dose of DHT ($1.0 \text{ mg } 10 \text{ g}^{-1}$) increased females' τ_{p2} circadian oscillations and responses to all challenges (Fig. 2F–J) ($F_{1,336}$; $p < 0.002$ for all conditions). Females' τ_{p2} responses to ACTH injections were enhanced also by intermediate DHT doses of 0.10 and $0.3 \text{ mg } 10 \text{ g}^{-1}$ ($F_{1,336}$; $p < 0.001$, for both contrasts) and females' τ_{p2} responses to male challengers were enhanced by $0.3 \text{ mg } 10 \text{ g}^{-1}$ DHT ($F_{1,336}$; $p < 0.001$). We report the sample sizes for each comparison in Table 2.

Sexual dimorphisms in response to challenges

Given the varied effects of DHT on females' EOD responses, a question that naturally arises is whether sex differences in responsiveness to the various challenges were present before the DHT implants. And, in cases where DHT enhanced females EOD responses, the question arises of the degree to which DHT treatment made females' responsiveness more like males'. To address these issues we first analyzed conditions where DHT did not alter female responsiveness by comparing males EOD responses to pooled female pre-implant EOD responses (t-test). Male circadian oscillation data were extracted from untreated males from datasets unrelated to this experiment, male responses to 5-HT were drawn from data published by Stoddard et al. (2003), and male responses to ACTH from experiments reported by Markham and Stoddard (2005). We also exposed six males to the same social challenges described above.

In the case of EOD amplitude responses in conditions where DHT did not enhance females responses, we found that male amplitude circadian oscillations are greater than females ($t_{64}=3.645$; $p<0.00$) as are their amplitude responses to 5-HT ($t_{46}=2.465$; $p=0.02$) and ACTH

($t_{40}=5.420$; $p<0.00$) challenge (Table 3). A marginal difference ($t_{48}=1.851$; $p=0.07$) exists between male and female amplitude responses to male challengers.

For conditions where DHT enhanced female responsiveness to challenges, we compared pretest-female (pooled for all females), posttest-female (high DHT dose), and male responses to those challenges to determine if sexual dimorphisms exist under normal conditions and how the increased DHT-female responsiveness compares to male responses. Significant omnibus ANOVAs ($p<0.000$) were followed by Dunnett's T3 multiple comparisons assuming unequal variance and significance level $\alpha=0.008$ (Table 4). We found no differences between males and pre-implant females in their EOD amplitude or τ_{p2} responses to female challengers ($p=0.12$ and $p=1.00$, respectively). Following the highest dose DHT treatment, τ_{p2} female responses surpass male responses ($p<0.00$), while amplitude responses remain equivalent to male responses ($p=0.35$).

No sexual dimorphisms are evident in τ_{p2} response to 5-HT or male challengers ($p=0.027$ and $p=0.35$, respectively). The high dose of DHT increased female τ_{p2} responses to 5-HT; yet posttest-female responses and male responses remain comparable ($p=0.01$). Posttest female τ_{p2} responses were significantly greater than male responses to male challengers ($p=0.003$).

We did find naturally occurring sexual dimorphisms in τ_{p2} circadian oscillation ($p=0.000$) and in the responses to ACTH ($p=0.001$). In the first case, the high dose of DHT masculinizes female circadian oscillations such that they are comparable to males ($p=0.352$). In contrast, DHT induces supramasculine τ_{p2} responses to ACTH ($p=0.000$) such that posttest-female responses far exceed male responses to this challenge.

Discussion

Our most important findings are that the non-aromatizable androgen DHT enhances or exaggerates EOD plasticity in circadian oscillations and in response to pharmacological and social challenges. These androgenic actions of DHT on the EOD are essentially limited to enhancements of waveform τ_{p2} , and DHT does not always masculinize female responses to challenges where sex differences generally exist.

Response to Pharmacological Challenges

Serotonin and ACTH modulate the EOD waveform in male *B. pinnicaudatus* and melanocortin peptides generally elicit more striking changes than 5-HT or any of the 5-HT agonists/antagonists we have tested (Allee et al., 2008; Markham et al., 2009; Markham and Stoddard, 2005; Stoddard et al., 2003). Therefore we were not surprised that in this study, the effects of DHT on EOD waveform plasticity in response to pharmacological challenge were most pronounced for τ_{p2} responses to ACTH injection (e.g. Fig 1C). All females except those in the control (sham implants) and 0.03 mg $10g^{-1}$ dose groups showed dose-related enhancement of posttest magnitudes of τ_{p2} responses to ACTH challenge. The change in τ_{p2} response to ACTH was the only instance where we saw an effect of DHT at the 0.1 mg $10g^{-1}$ dose which suggests androgens appreciably increase sensitivity to ACTH. Ongoing studies of hormone profiles in *B. pinnicaudatus* (Salazar and Stoddard, 2007) suggest some of our DHT implant doses delivered supra-physiological levels of androgens, hence caution is warranted when interpreting the relative pharmacological versus physiological significance of our results at this time.

Following DHT implants, females' τ_{p2} responses to 5-HT injection were enhanced only for the group receiving the highest DHT dose and no changes in amplitude response to 5-HT were detected. DHT also has little effect on female amplitude responses to ACTH. Post-hoc analysis shows us that amplitude responses to both pharmacological challenge types were sexually

dimorphic where males produced more exaggerated responses than females, yet even the highest DHT dose did not increase female amplitude responses to either challenge. The posthoc analyses also provided evidence that while the amplitude response to 5-HT is sexually dimorphic where male responses are larger, we found no sex difference in τ_{p2} response to 5-HT. Contrarily, DHT enhanced female τ_{p2} and not amplitude response to 5-HT. These results suggest two things about the influence of exogenous androgens on female responsiveness to 5-HT: (a) they by themselves do not activate male typical amplitude responses to 5-HT and (b) they enhance the magnitude of EOD τ_{p2} response where responsiveness was always present and not sexually dimorphic. This scenario supports and extends our hypotheses that 5-HT regulates EOD plasticity indirectly by activating the hypothalamic-pituitary-interrenal axis (HPI) axis (Markham et al., 2009) such that DHT could have different effects on central serotonergic systems versus its effects on peripheral melanocortin responsiveness, perhaps even moderating central 5-HT responsiveness while strongly enhancing peripheral ACTH responsiveness.

Serotonin exerts opposing effects on the EOD via central interaction with at least two different classes of 5-HT receptors that likely regulate release of melanocortins such as ACTH into circulation (Allee et al., 2008; Markham et al., 2009; Markham and Stoddard, 2005). Males show a moderate increase in EOD amplitude after 5-HT injections (Stoddard et al., 2003), whereas in this study, 5-HT challenge often caused pre-implant females' to reduce their EOD amplitudes and the within dose group responses were quite variable (Fig 2B). The reduction in female EOD amplitude (pretest mean = -0.0103 ; median = -0.0123 mV cm $^{-1}$) raises interesting questions about the effects of 5-HT in female *B. pinnicaudatus*.

Electrocytes in *B. pinnicaudatus* are remarkable excitable cells that generate two independent action potentials which sum to produce single-cell discharges that closely resemble the EOD recorded *in vivo*. In contrast to 5-HT, melanocortin peptides such as ACTH directly increase the amplitude and τ_{p2} of the single-cell discharge within minutes by activating a G-protein coupled receptor and its corresponding second messenger pathway to reshape the waveform and timing of the electrocytes' action potentials (Markham and Stoddard, 2005). The clear enhancement of ACTH-induced τ_{p2} plasticity by DHT treatment in three dose groups suggests that DHT somehow amplifies the electrocytes' response and/or sensitivity to circulating melanocortins. This magnification may be due to DHT increasing the surface expression of melanocortin receptors or initiating expression of different melanocortin receptors, which in turn alters plasticity in the magnitude of response to ACTH challenge. Alternatively or additionally, DHT might act anywhere downstream of the melanocortin receptor, including the corresponding second messenger pathways and ion channels that generate the EOD. Discovering how DHT amplifies the single-electrocyte discharge would provide valuable insights into the mechanisms by which steroid hormones regulate the intrinsic plasticity of excitable cells. Recent studies in a wave-type gymnotiform fish have shown that DHT acts over days to weeks to slow Na $^{+}$ current inactivation (Ferrari et al., 1995) and delayed rectifying K $^{+}$ current activation in electrocytes (McAnelly and Zakon, 2007).

Differential peripheral versus central effects of DHT on EOD plasticity could be mediated by the expression patterns of distinct androgen receptors. Teleosts have at least two distinct nuclear androgen receptors, AR α /AR1 and AR β /AR2, which exhibit different tissue distributions and binding affinity characteristics for natural and synthetic androgens (Ikeuchi et al., 1999; Pasmanik and Callard, 1988; Pottinger, 1987; Sperry and Thomas, 1999a, 1999b; Takeo and Yamashita, 1999, 2000; Todo et al., 1999). DHT and testosterone bind to the same androgen receptors in electric fish (Bass et al., 1986), and although specific binding affinity and disassociation profiles vary between species, studies agree that DHT shows high affinity for teleost AR (Fitzpatrick et al., 1994; Pasmanik and Callard, 1988; Sperry and Thomas, 1999a; Takeo and Yamashita, 2000). It is possible that our implants were more effective at one AR

type and/or tissue than another, resulting in differences in DHT effect on waveform amplitude compared to τ_{p2} . In Atlantic croakers, for example, AR β /AR2 shows a greater binding affinity for DHT than T or 11-ketotestosterone (11-KT) and is more abundant in peripheral tissues such as the gonads, whereas AR α /AR1 is most abundant in brain tissues and is more specific for T than 11-KT or DHT. AR have been identified in the electric organs of electric fish (Dunlap and Zakon, 1998; Few and Zakon, 2001), but receptor profiles for *B. pinnicaudatus* have yet to be determined. While it is likely that our species also have multiple AR isoforms with different physiological profiles, we cannot yet determine the site(s) of action of our DHT implants.

Another possibility is that each female's particular steroidal milieu, influenced by social history, may have affected 5-HT system function, contributing to the disparate plasticity of responses to 5-HT and ACTH. Estrogens have been shown to decrease 5-HT release, reduce the number of 5-HT receptor binding sites, down regulate 5-HT autoreceptor activity/ expression, and alter behavioral response by activating different classes of 5-HT receptors (Bethea et al., 2002). Furthermore, other steroid hormones, including testosterone, 11-ketotestosterone, estradiol, and cortisol often play significant roles in shaping the behavior of teleost fish, including other species of electric fish (Zakon, 1993). New research indicates enzyme activity, such as aromatase activity, and estrogens are key components in an individual's ability to produce male-typical behaviors (Ball and Balthazart, 2004; Baum, 2003; Goncalves et al., 2008; Hallgren et al., 2006). Activity of aromatase in the teleost brain is reportedly 100–1000 times greater than in birds and mammals (Callard et al., 2001) and has already been shown to be a critical component of organizing suites of male behaviors in some fish (Goncalves et al., 2008). If conversion of androgens to estrogens in the brain is likewise necessary for stimulating male-typical behaviors in *B. pinnicaudatus*, the non-aromatizable property of DHT could explain why we did not observe changes in all behaviors examined in this study. Although a full hormonal profile for *B. pinnicaudatus* is not currently available, female *B. pinnicaudatus* have as much or more circulating testosterone than males (Salazar and Stoddard, 2007), a common androgen profile in teleosts (Borg, 1994). It is reasonable to postulate that females also have higher levels of circulating estrogens than males and that these hormones may temper responses to 5-HT challenge in both pre- and post-implant females. It is likely that factors such as social status, sex ratio of pool populations, season, and other factors influenced female serotonergic responsiveness in this study. We harvested subjects from mixed-sex pools where individuals freely interacted and we did not control for social status or environment for each female. Any number of these mechanistic differences could explain why we saw such dramatic responses to ACTH after DHT was implanted, yet responses to 5-HT were less sensitive.

Response to Social Challenges

While measuring response to pharmacological challenges informs us about the mechanistic capacity of electrocytes to generate an enhanced social signal, measuring response to social challenge addresses the higher-level question of how steroid hormones influence the expression of behavior within social contexts.

Amplitude response to male challengers was marginally greater in males than in females, and DHT had no significant effect on this EOD parameter in response to this challenge. Similarly, males and females showed no difference in magnitudes of amplitude response to female challengers or τ_{p2} response to either male or female challengers. DHT did increase female responsiveness to these challenges, particularly in EOD τ_{p2} , yet the behavioral significance of these results remains unclear.

A previous study in which *B. pinnicaudatus* swam freely and interacted through a mesh divider revealed that males produce larger amplitude modulations of EOD waveform in response to

males than in response to females (Franchina et al., 2001). The current study found no evidence of naturally occurring sexually dimorphic responses to either inter- or intrasexual forced social challenges. Our protocol used forced social challenges where focal females were trapped inside their recording tubes with challengers for 45 minutes and numerous features of this challenge could explain the disparity between our results and Franchina et al. (2001). Perhaps rapid modulations in EOD waveform directed toward conspecifics is a deterrent to further intrusion and blocking escape routes nullified EOD enhancements as an appropriate behavioral response. Perhaps leaving challengers in the focal female's tank for 45 minutes was too long or too short of a time period observe the expected results. It is interesting that response to female challengers was the only situation where DHT enhanced EOD amplitude modulations, which suggests true inter- and intrasexual behavioral patterns exist that our protocol was unable to detect.

We observed qualitative behavioral differences in focal female behavior toward female versus male challengers that statistical analysis of their waveforms did not capture. Focal females were more likely to avoid close contact with female challengers and females frequently exhibit minor cuts and scrapes following female challenge. Females seemed to more readily share their tubes with males, signs of agonistic interactions were rare after male challenges, and males often had to be chased out of the recording tubes. These observations lead us to suspect that the differences we observed in female reaction to other females are indicative of real differences in sex-directed behavior and could be investigated further using protocols that more accurately mimic unimpeded social interactions. Preliminary research supports this idea (Salazar and Stoddard, 2007).

Evidence suggests that androgens may masculinize morphology or exaggerate existing traits rather than inducing the expression of masculine behaviors, per se (Oliveira et al., 2005). When masculine behaviors are induced by increasing androgen levels, the entire repertoire of male-typical behaviors is not necessarily reproduced (e.g. Lee and Bass, 2005). Results from our experiments lend support to this emerging picture. Overall, androgens appear to increase responsiveness of the EOD to social stimuli and relevant neurotransmitters and hormones. When changes were observed, higher concentrations of DHT elicited greater changes in challenge response, which supports our interpretation that androgens facilitate the electrocytes' level of responsiveness rather than orchestrating a suite of male-typical social behaviors. However, this increase in responsiveness should be distinguished from a true 'masculinization' of sex-directed behavior because we did not uncover natural sexual dimorphisms in response to challengers of either sex.

The results from this study lead to interesting questions that must be addressed before we can fully understand the behavioral significance and hormonal control of EOD modulations in our fish. The clear dissociation between amplitude and τ_{p2} responses lend further support to previous studies that suggest disparate systems control modulations of EOD amplitude and waveform (Allee et al., 2008; Franchina and Stoddard, 1998; Markham et al., 2009). Accordingly, we speculate that the EOD might be a complex, multicomponent signal comparable to the coqui call of Puerto Rican tree frogs *Eleutherodactylus coqui* (Narins and Capranica, 1980), fiddler crab waves (Backwell and Passmore, 1996), grouse-calls (Gibson, 1996; Pope, 2000), and cricket chirps (Scheuber et al., 2003a, 2003b, 2004). If the EOD is indeed a complex signal, it is possible that τ_{p2} and amplitude broadcast different information about the signaler, possibly aimed at different receivers, and providing variable information to different audiences. The EOD modulations characteristic of *B. pinnicaudatus* males could be for mate attraction although this has yet to be demonstrated experimentally (Hagedorn and Heiligenberg, 1985). Females exhibit positive taxis toward longer males with greater EOD amplitude and longer τ_{p2} (Curtis and Stoddard, 2003) although any relationship between EOD parameters and either male or female reproductive success has yet to be quantified. In addition to mate attraction, EOD waveforms may be important for intrasexual competition, given that

both males and females engage in competitive or hostile intrasexual social interactions (Crampton, pers. comm., Salazar and Stoddard, 2007). Carlson et al. (2000) proposed androgen-facilitated plasticity in EOD duration functions as rapid-response indicator of male quality in intrasexual interactions under unstable social circumstances. Field observations of *B. pinnicaudatus* suggest high male mortality, particularly early in the breeding season, which suggest male dominance hierarchies are constantly shifting (Miranda et al., 2008). If this is the case, dominant males would benefit from consistently high levels of circulating androgens that mediate enhanced durations of EODs when the make-up of social groups can rapidly change in order to acquire or maintain dominant status. This would be particularly true if predation and physiological pressures on males are such that they do not survive past one reproductive season, as field observations of sex ratios indicate (Miranda et al., 2008). Additional behavioral studies are needed to identify the real-time behavioral and social significance of changes in EOD amplitude and τ_{P2} .

Finally, the present experiments raise the question of what cellular-level changes DHT initiates in the electrocyte to increase its responsiveness to circulating melanocortin peptides. The pronounced effect of DHT on female EOD τ_{P2} and not on amplitude is somewhat unexpected since both parameters are sexually dimorphic, both are modulated within minutes by the actions of serotonin and melanocortins in males and in response to social encounters (Franchina and Stoddard, 1998; Hagedorn and Zelick, 1989; Hopkins et al., 1990; Markham et al., 2009; Stoddard et al., 2003). The results from our experiments demonstrate that DHT did not simply enhance the excitability of electrocytes and their membranes because we found virtually no DHT effect on waveform amplitude, even in responses to challenges where sexual dimorphisms are evident. The possibility that amplitude cannot be increased or is 'maxed out' due to female body size limitations is belied by the dramatic amplitude enhancements we found in response to female challengers (Fig 2). Androgen-induced changes limited to EOD τ_{P2} beg clarification of what information is conveyed by enhanced amplitude and τ_{P2} and to what audience(s). The ability to develop masculine electrocytes and to maintain a level of responsiveness to stimuli does appear to be androgen dependent; however the production of socially relevant behaviors using these enhanced structures appears to incorporate other as of yet undetermined factors in *B. pinnicaudatus*. A future direction to explore is whether high levels of *aromatizable* androgens interact with 5-HT in the brain or other tissues to regulate EOD plasticity while non-aromatizable androgens such as DHT and 11-ketotestosterone (11-KT) are more influential in the periphery at the level of electrocytes.

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Figure 1.

(A) *Brachyhypopomus pinnicaudatus* produces a biphasic sinusoidal EOD that is characterized by the peak-to-peak amplitude (mV cm⁻¹ at 10 cm) and duration (ms). The duration of the second phase (P2) is particularly variable, as quantified by the parameter τ_{P2} , the time constant of an inverse exponential function fit to the decay segment of P2. (B) Our automated recording system collects approximately nine EODs per minute whenever the focal fish is centered in the measurement tank. We extracted daily baseline and nightly peak values from a 24-hour period to calculate the magnitude of day-night oscillation in EOD amplitude and τ_{P2} . Each dot represents an individual EOD which wobbles relative to immediately preceding and subsequent EODs. We took representative values of peak and baseline EODs, shown here in boxes, rather

than taking the lowest low and highest high values. As the figure depicts, baseline and peak values trend downward over time, therefore circadian oscillation data used to explore DHT effects were collected one day before the first pretest challenge and one day before the posttest challenge. (C) Responses to all challenges for both amplitude and τ_{p2} were calculated by determining peak (P) challenge responses and subtracting circadian baseline (B) values using methods and data analysis programming described in detail in Stoddard et al. (2003). τ_{p2} response to 5-HT and ACTH are shown here to illustrate changes in the waveform in response to challenge before and after DHT implants.

Figure 2.

DHT effects on females' EOD responses to challenge and relation to male EOD responses. Solid circles and bolded lines indicate DHT dose groups where RMLA uncovered significant differences pretest versus posttest. Gray lines and open circles indicate no significant difference pretest versus posttest. Dotted gray lines indicate sham-implanted females. Diamonds indicate mean male responses: solid diamonds indicate male responses were significantly greater compared to pooled pretest female responses and open diamonds indicate we found no evidence of sexual dimorphisms in response to that challenge. Posttest female responses that ANOVA indicated were significantly greater than male responses are marked with **. DHT did not enhance the magnitude of amplitude circadian oscillation (A), amplitude response to 5-HT

(**B**), ACTH (**C**), or male challengers (**E**). Amplitude responses to both pharmacological challenges and magnitudes of circadian oscillation were sexually dimorphic. No sexual dimorphisms were apparent in amplitude responses to either social challenge, yet the highest dose of DHT enhanced female amplitude response to female challengers (**D**). We found a similar pattern of sexually dimorphic responses in waveform τ_{p2} : males produced greater circadian oscillations (**F**) and response to ACTH challenge (**H**), but not to 5-HT (**G**) or either social challenge (**I & J**). The highest DHT dose enhanced female τ_{p2} responsiveness to all challenge types. Posttest female τ_{p2} responses to ACTH were supra-masculinized and all implant doses produced a response except the sham implants and the $0.03\text{mg } 10\text{g}^{-1}$ dose (**H**). The high DHT dose also supra-exaggerated posttest female τ_{p2} responses to both female and male challengers despite our findings that no natural sexual dimorphisms exist in response to these challenges.

Table 1

Kruskal-Wallis test for challenge order effects on EOD waveform amplitude and τ_{P2} (repolarization time constant of 2nd phase) responses to challenge. Pretest female responses grouped by DHT dose.

	amplitude	τ_{P2}
	df, χ^2 , p	df, χ^2 , p
5-HT	4, 6.54, 0.1624	4, 6.19, 0.1851
ACTH	4, 4.7, 0.3196	4, 6.8, 0.1467
Female challenger	4, 3.66, 0.4544	4, 4.33, 0.3628
Male challenger	4, 7.57, 0.2574	4, 8.52, 0.0742

Table 2
Sample sizes for each treatment group analyzed in the mixed model restricted maximum likelihood analysis (RMLA). Sample sizes for amplitude and τ_{p2} were the same unless a sample size for τ_{p2} is noted in parentheses.

DHT dose	Sample sizes							
	Circadian		5-HT		ACTH		Female	Male
	amp	(τ_{p2})	amp	(τ_{p2})	amp	(τ_{p2})	amp	(τ_{p2})
Blank	8		8		6		8	8
0.03	8		8		7		8	7
0.10	8		8		7		8	7
0.30	10		9		7		10	8
1.0	18		9		6		11	10
male	15	(17)	6	(7)	8		6	(6) 5

Table 3

Results of t-test comparisons of male and pretest female responses to challenges where RMLA detected DHT had no significant pretest vs. posttest effect.

Challenge Type	EOD amplitude	Sex difference
circadian oscillation	0.00	Male > Female
5-HT	0.01	Male > Female
ACTH	0.00	Male > Female
male challenger	0.07	Male = Female

Table 4

Results of ANOVA comparing pretest female, posttest female, and male responses to challenges where RMLA detected significant DHT effects.

Challenge Type	Dunnett T3 comparisons	Effect of DHT on female response
female challenger (amp)	DHT-Female _A = Male _{AB} = Female _B	enhanced
5-HT (τ_{p2})	DHT-Female = Male = Female	enhanced
female challenger (τ_{p2})	DHT-Female > Male = Female	exaggerated
male challenger (τ_{p2})	DHT-Female > Male = Female	exaggerated
circadian oscillation (τ_{p2})	DHT-Female = Male > Female	masculinized
ACTH (τ_{p2})	DHT-Female > Male > Female	supra-masculinized