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Testosterone and 11-ketotestosterone have different regulatory effects on electric communication signals of male *Brachyhypopomus gauderio*

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Abstract

The communication signals of electric fish can be dynamic, varying between the sexes on a circadian rhythm and in response to social and environmental cues. In the gymnotiform fish *Brachyhypopomus gauderio* waveform shape of the electric organ discharge (EOD) is regulated by steroid and peptide hormones. Furthermore, EOD amplitude and duration change on different timescales and in response to different social stimuli, suggesting that they are regulated by different mechanisms. Little is known about how androgen and peptide hormone systems interact to regulate signal waveform. We investigated the relationship between the androgens testosterone (T) and 11-ketotestosterone (11-KT), the melanocortin peptide hormone α -MSH, and their roles in regulating EOD waveform of male *B. gauderio*. Males were implanted with androgen (T, 11-KT, or blank), and injected with α -MSH before and at the peak of androgen effect. We compared the effects of androgen implants and social interactions by giving males a size-matched male stimulus with which they could interact electrically. Social stimuli and both androgens increased EOD duration, but only social stimuli and 11-KT elevated amplitude. However, no androgen enhanced EOD amplitude to the extent of a social stimulus, suggesting that a yet unidentified hormonal pathway regulates this signal parameter. Additionally, both androgens increased response of EOD duration to α -MSH, but only 11-KT increased response of EOD amplitude to α -MSH. Social stimuli had no effect on EOD response to α -MSH. The finding that EOD amplitude is preferentially regulated by 11-KT in *B. gauderio* may provide the basis for independent control of amplitude and duration.

Keywords

11-ketotestosterone (11-KT); alpha-melanocyte stimulating hormone (α -MSH); androgen; communication signal; electric fish, electric organ discharge (EOD); gymnotiform; plasticity; Testosterone (T)

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INTRODUCTION

Hormones co-regulate reproductive condition, behaviors, and, in many cases, the structure of communication signals (Adkins-Regan, 2005; Wilczynski and Chu, 2001). Hormonally mediated correlations between behavior, body condition, and signal structure may enable a receiver to infer the signaler's neuroendocrine state from the signal, and thus predict aggressive response or reproductive performance (Ketterson et al., 2009; McGlothlin et al., 2008; McGlothlin and Ketterson, 2008; Oliveira, 2005). Animals integrate social and physical stimuli in the environment and commonly respond by secreting fast-acting peptide hormones, which rapidly modulate behavior and steroid secretion. While steroid hormones overlap with peptides in these roles, they modulate physical phenotype and behavior over longer time periods through genomic action. The potential interplay between fast-acting peptides and slow-acting steroids enables greater behavioral flexibility over varying temporal ranges and social conditions. Thus, the extent to which steroid hormones may prime or inhibit the actions of peptide hormones can generate versatile behaviors that are best adapted to the individual's environment (Adkins-Regan, 2005). The electric communication signals of gymnotiform fish are regulated by melanocortin peptide hormones and by sex steroids on short and long time horizons, respectively (Stoddard et al., 2006). Because of their structural simplicity, electric signals constitute good models for exploring dual hormone signal regulation and the implication of hormonal interplay for communication.

The weakly electric gymnotiform species *Brachyhypopomus gauderio* (Giora and Malabara, 2009), southern sister species to *B. pinnicaudatus*, produces a sexually dimorphic electric organ discharge (EOD) for electrolocation and communication. The neural circuitry of electrocommunication and electrolocation has been well described (Heiligenberg, 1991; Hopkins, 1995; 1999; Metzner, 1999; Zupanc and Maler, 1997). The EOD pulse rate is regulated centrally in the pacemaker nucleus of the medulla, while waveform amplitude and duration are regulated peripherally (Few and Zakon, 2001; Mills and Zakon, 1991) by the electrocytes, electrically excitable cells of the electric organ (Szabo, 1974). The near simultaneous electric discharge of the anterior and posterior membranes of each electrocyte in *B. gauderio* produces a biphasic signal that varies in amplitude and duration (Fig. 1A). The durations of both phases are nearly equal in females, while males exhibit a longer duration of the second phase (referred to as τ_{p2}) (Franchina, 1997; Hopkins et al., 1990).

The EOD is a dynamic signal that varies on a circadian rhythm, increasing in amplitude and τ_{p2} at night and during social interactions (Franchina et al., 2001; Stoddard et al., 2007) (Fig. 1). This signal plasticity is driven by the slow, genomic action (days-weeks) of steroid hormones (Allee et al., 2009; Dunlap et al., 1998; Stoddard et al., 2006; Zakon, 1993; Zakon and Smith, 2002) and by the rapid, non-genomic (minutes-hours) action of monoamine and peptide hormones (Markham et al., 2009a; Markham and Stoddard, 2005; Stoddard et al., 2003). Androgens masculinize the gymnotiform EOD by increasing waveform duration in peripheral electrocytes (Allee et al., 2009; Bass and Hopkins, 1984; Bass and Volman, 1987; Few and Zakon, 2001; Silva et al., 2002) and altering discharge frequency in the central pacemaker nucleus (Dunlap et al., 1998; Meyer, 1983).

Weakly electric species modulate EOD parameters in a species-specific manner in the contexts of competition, aggression, and mating. In *B. occidentalis*, winners of agonistic interaction have lower peak-power frequency and larger amplitude signals than losers. DHT, but not estrogen implants, masculinize these signal parameters, mimicking waveforms of dominant individuals (Hagedorn and Carr, 1985; Hagedorn and Zelick, 1989). Similarly, in *B. gauderio* social competition elevates circulating androgen (T and 11-KT) levels (Salazar and Stoddard, 2009) while in mormyriiform electric fish *Brienomyrus brachyistius*, dominant

males have the highest 11-KT levels (Carlson et al., 2000). In *Apteronotus leptorhynchus*, another gymnotiform species, 11-KT levels are correlated with aggressive EOD modulations, which can be induced by 11-KT implants (Dunlap, 2002; Dunlap et al., 1998).

While androgens masculinize EOD waveform on a long-term timescale of days to months, melanocortins such as adrenocorticotrophic hormone (ACTH) and α -melanocyte stimulating hormone (α -MSH) induce similar changes in the EOD waveform within minutes (Markham et al., 2009a). Melanocortins and their receptors are highly conserved across vertebrates (Metz et al., 2006) and regulate a diverse array of physiological and behavioral processes including social communication, learning, glucocorticosteroid release, and aggressive and sexual behaviors (Brain and Evans, 1977; Ducrest et al., 2008; Gonzalez et al., 1996; Wikberg et al., 2000). In *B. gauderio*, injections of ACTH or α -MSH activate the cAMP/PKA pathway, which extends the 2nd phase of the EOD (τ_{p2}) by extending action potential duration, and increases EOD amplitude by differentially modulating the timing of action potentials of the two excitable membranes of the electrocytes (Markham and Stoddard, 2005). These waveform changes are comparable to those observed during social interactions, suggesting a mechanism for social regulation of EOD (Franchina et al., 2001; Markham et al., 2009a; Markham and Stoddard, 2005; Stoddard et al., 2006). Additionally, in *B. gauderio*, prolonged social isolation causes a decline in EOD amplitude and τ_{p2} that can be partially reversed with melanocortins (Franchina et al., 2001; Franchina and Stoddard, 1998; Stoddard et al., 2007).

The interaction between androgens and melanocortins (and thus the interaction between long-term and rapid EOD modulators) has been less clear. Studies have suggested a bidirectional relationship between steroids and melanocortins, where α -MSH enhances the effects of gonadal steroids centrally and peripherally and steroids upregulate melanocortin receptors, increasing sexual and aggressive behavior in rodents (Ducrest et al., 2008; Wikberg et al., 2000). In *B. gauderio* females, 5 α -dihydrotestosterone (DHT) implants enhance τ_{p2} response to injections of either serotonin or ACTH with little effect on EOD amplitude (Allee et al., 2009). Since both T and 11-KT are important for the development of secondary sexual characteristics and the accompanying sex-specific reproductive behaviors in teleost fish (Oliveira et al., 2005), it is possible that the EOD waveform is differentially regulated by the actions of aromatizable T and the non-aromatizable 11-KT, as is the case in teleost species with alternative reproductive strategies (Bass, 2008; Lee and Bass, 2005; Perry and Grober, 2003; Remage-Healey and Bass, 2006). Subsequently, the absence of amplitude modulation by non-aromatizable DHT observed in a prior study (Allee et al., 2009) could be because amplitude is regulated by aromatizable T.

In this study we test the hypothesis that circulating androgens regulate melanocortin actions on electric signal waveform. To that end, we explored the interactions between α -MSH, T, and 11-KT in generating communication signal plasticity in *B. gauderio*.

Methods

Animals

We randomly selected sexually mature male *Brachyhyopomus gauderio* (total length=17.9 $\pm$ 1.9 cm, weight= 8.7 $\pm$ 2.9g) from our outdoor breeding colony at Florida International University. These captive-reared 15-17th generation fish were maintained in 450-liter outdoor pools, in mixed-sex groups of 5-10 individuals. The water surface was covered with water hyacinths, *Eichhornia crassipes*. Water conductivity was maintained at 70-100 μ S cm⁻¹. For data collection, fish were brought indoors and placed individually in 284-liter aquaria. Aquaria were kept in a light- and temperature-controlled room on a 12:12 L:D light cycle. Water temperature and conductivity were maintained at 27-28°C and

70–100 $\mu\text{S cm}^{-1}$, respectively. Fish were fed oligochaete “blackworms” *ad libitum* every other day. The EOD data collection was continuous from the time each fish was placed into the recording tank until it was removed at the end of the experiment. Experiments were conducted during the breeding season, May–Sep 2008 & 2009. Experiments complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (DHEW Publication 80-23, revised 1985) and were approved by the FIU IACUC (protocol approval no. 08-023).

Data collection

EODs were recorded in an automated, calibrated recording system described in detail elsewhere (Franchina et al., 2001; Stoddard et al., 2003). Briefly, the recording system consists of 12 automated aquaria, 120 × 44 × 44 cm, in an environmentally controlled room. Each aquarium is divided into thirds by plastic mesh screening with plastic mesh funnels. The funnels direct fish through an unglazed ceramic tube that joins the outer two compartments. To obtain repeatable measurements, EODs are amplified and digitized from nichrome wire electrodes at opposite ends of the tank only when the fish is in the ceramic tube, and thus centered in the tank. Electrodes on top of the ceramic tube register when the fish is geometrically centered in the tank, and trigger the computer to record EODs from electrodes on the tank ends.

To quantify the effects of androgens and α -MSH on the waveform, we measured changes in the peak-to-peak amplitude and τ_{p2} , the time constant of an inverse exponential function fitted to the decay segment of the second EOD phase (Fig. 1).

Experimental design

Experiment 1: Effect of androgens on EOD waveform—To verify that testosterone and 11-ketotestosterone modulate EOD waveform (amplitude and τ_{p2}) in *B. gauderio*, 18 sexually mature males were placed in EOD recording tanks and isolated for 10 days. Social isolation causes a decline in EOD amplitude baseline and τ_{p2} , stabilizing by the end of the first week (Franchina et al., 2001). At the end of the isolation period, males were randomly divided into three implant groups (n=6 per group), removed from their respective tanks and implanted with silicone implants containing either testosterone (T), 11-ketotestosterone (11-KT), or a blank (see implant protocol below). Fish were returned to the recording tanks and their EODs were monitored for seven days for changes in response to the implant, and to determine the time when the androgen effects peak, for a subsequent experiment. We discarded data from one male in the T-implanted group because he exhibited an anomalously reversed circadian rhythm, so that group had only 5 males.

Experiment 2: Effect of androgens on melanocortin responsiveness—Males were randomly assigned to one of three implant groups (11-KT, T, or blank) with 6 individuals per group. All males were placed into individual EOD recording tanks and isolated for 6 days (Fig. 2). During this time their EODs were recorded and monitored. On days 7 and 8 of social isolation, before steroid treatment, each fish was injected with either 25 $\mu\text{M g}^{-1}$ body weight α -MSH or saline control, in counterbalanced random order. This concentration of α -MSH was chosen based on previous studies showing that 25 $\mu\text{M g}^{-1}$ fully enhances the EOD (Markham et al., 2009a; Markham and Stoddard, 2005). On the ninth day of recording, individuals were removed from their recording tanks, implanted with silicone implants (T, 11-KT, or blank), and quickly returned to their tanks to resume recording.

The effect of androgen implants on EOD amplitude was monitored daily. When the EOD amplitude reached a maximum, determined by the lack of increase for 24 hours (usually 2–4

days after the implant), fish were re-injected with 25 μM α -MSH and saline, as explained above. To verify that androgen implants elevated circulating androgen levels, all fish were bled 4–5 days post-implant for hormone analyses at a later time (see Blood collection and analyses below). Fish were returned to recording tanks and monitored for another three days to ensure full recovery before returning to outdoor pools.

Experiment 3: Effect of a social interaction on melanocortin responsiveness—

We used a social challenge treatment for comparison purposes to examine whether androgen implants fully mimic the effect of a social companion on waveform recovery from social isolation. Eight males were isolated for 6 days and implanted with blank implants on day 9, as in Experiment 2. The following day, a size-matched male was placed into the middle compartment of each recording tank to serve as a social stimulus. This placement allowed electrical interaction between the experimental and stimulus males but not physical contact. A PVC hiding tube was placed in the middle compartment, oriented perpendicularly to the ceramic tube of the experimental male to reduce signal interference from the social stimulus. As in Experiment 2, males were injected with saline and α -MSH prior to implants (days 7 and 8) and then re-injected 2 and 3 days after the beginning of the social challenge.

Implants

11-ketotestosterone (Sigma, K8250) and testosterone (Sigma, T6147) were each mixed with silicone (Dow Corning 3140 MIL-A-461 45 RTV coating) in a 1:4 ratio and shaped into thin lines. The mixtures were allowed to cure for about a week and the implant concentrations were recalculated to adjust for mass lost through evaporation. The resulting implants release all of the steroids contents within 2–4 days, which is evident by the steady decline in EOD amplitude and τ_{p2} 5 days after implanting (unpublished observations). Implants were custom cut for each fish based on its weight, for a final concentration of 0.05mg of 11-KT or T per 10 grams of fish. This concentration was based on previous studies (Allee et al., 2009)

To place the implants, we anesthetized the fish by immersion in a 0.075% solution of 2-phenoxyethanol (Sigma, P-1126FD) or 0.008% clove oil (80mg l^{-1} eugenol, Sigma, E51791). We used an 18-gauge needle to cut a small hole through the skin into the fatty area on the trunk dorsal to the anal fin rays into which we inserted an implant. The incision was closed with surgical glue and the fish was returned to the recording tank. EOD amplitude and duration were monitored for any changes.

MSH injections

We dissolved α -melanocyte stimulating hormone (α -MSH) (Sigma, M4135) in physiological saline: NaCl 114 mM l^{-1} , KCl 2mmol l^{-1} , $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 4 mM l^{-1} , $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 2 mM l^{-1} , HEPES 2 mM l^{-1} , pH adjusted to 7.2 with NaOH (modified from Ferrari and Zakon, 1993), for a final concentration of 100 μM . Aliquots were frozen until day of use and then diluted with saline to 25 μM prior to injection. Physiological saline injections controlled for handling and injection. Experimental and control solutions were injected at 1 $\mu\text{l g}^{-1}$ of fish intramuscularly using a Hamilton syringe fitted with a disposable 31.5 gauge needle. All injections were administered between 11:00 and 14:00.

Blood collection and analysis

Males were removed from the recording tanks, anesthetized by immersion in 0.075% 2-phenoxyethanol and bled from the subvertebral sinus. Blood was collected into 1ml polypropylene tube containing 5 μl of 10% EDTA and centrifuged for 15 minutes at 7000 rpm at -4°C . Plasma samples were separated and transferred into a separate polypropylene tube and stored at -20°C until steroid extraction and analysis. Plasma samples were extracted according to a steroid extraction protocol validated for this species (Salazar and

Stoddard, 2009). Briefly, 250 μ l plasma samples were extracted using a mixture of hexane:ethyl acetate (90:10 for 11-KT and 70:30 for T) and resuspended in the phosphate buffer provided in the enzyme immunoassays (EIA) kits (Cayman Chemical, Inc.). Concentrations were quantified in duplicate according to the specification of the T and 11-KT EIA kits.

The detection limits of the EIA kits were 1.3 pg/ml for 11-KT and 6 pg/ml for T. To verify extraction recovery, 11-KT and T EIA kit standards were extracted using 5 ml of hexane:ethyl acetate alongside the plasma samples. Extraction efficiency was assessed by comparing kit standards for each hormone with the standards that have been extracted using hexane:ethyl acetate. All plasma samples were run in two 11-KT and T assays. For 11-KT, the intra-assay coefficients of variation were 4.04% and 8.16%, while the inter-assay coefficient of variation was 1.44%. For T, the intra-assay coefficient of variation was 3.17% and 10.75% and the inter-assay coefficient of variation was 2.78%.

Data treatment and analyses

In *B. gauderio*, all treatment effects are superimposed on the individual's circadian rhythm (Franchina and Stoddard, 1998). To isolate the effects of treatments (implants, social challenge, and injections) from circadian oscillations, we used a previously described MATLAB (Mathworks, Natick, MA) data analysis program (Stoddard et al., 2003) to fit a smoothing spline function (MATLAB Spline Toolbox) to minute-by-minute EOD parameter data. We used the resulting functions to extract treatment effects. To examine the effect of androgen implants and social challenge on the waveform amplitude and τ_{p2} , we measured the daytime minima and nighttime maxima before and after the treatment and then calculated a relative change in both parameters. To isolate the effects of α -MSH and saline injections from circadian oscillations, we fit the smoothing spline function to the data one more time, but this time omitting the effects of the injections, which are clearly visible in the data. We took the difference between the two fit lines (with and without injection effects) to represent the effects of injections on the waveform, independent of circadian oscillations. We assessed the effect of implants and social challenges on α -MSH response by calculating the difference in α -MSH injection effects before and after implant/social treatment.

Statistical analyses

We combined data from the first two experiments to examine the effect of androgen implants on EOD waveform. We used a two-way MANCOVA (GLM multivariate procedure in SPSS 14.0, Model III) with two fixed factors: (1) treatment [11-KT, T, blank] and (2) Year [2008 (Expt. 1), 2009 (Expt. 2)]. To account for the possible influence of body size on the response to steroid treatment and year, we used body weight and length as covariates. To examine the effects of androgen implants on α -MSH responsiveness (Expt. 2), we compared the differences in the magnitude of amplitude and τ_{p2} response to α -MSH injection between the three implant groups using one-way MANCOVA (GLM multivariate procedure in SPSS 14.0, Model III) with implant [11-KT, T, blank] as a fixed factor and body weight and length as covariates. Paired t-tests analyzed the effects of a social challenge on amplitude and τ_{p2} , as well as on α -MSH responsiveness (Expt. 3). We log-transformed plasma steroid levels to meet the assumptions of normality and analyzed using one-way ANOVA. Where MANCOVA differences were significant ($p < 0.05$), we used the protected LSD post hoc analysis to determine significant pairwise differences. Data are reported as mean $\pm$ SEM. All analyses were performed using MATLAB and SPSS (version 14.0), $\alpha = 0.05$ two tailed.

RESULTS

Androgen effects on blood levels

Androgen implants elevated plasma levels only for the particular androgen implanted and did not alter levels of the other androgen (ANOVA T: $F_{2, 11} = 22.09$, $p < 0.01$; 11-KT $F_{2, 11} = 6.42$, $p = 0.01$) (Table 1). For example, even though T is a precursor for biosynthesis of 11-KT, the T implants only elevated plasma T levels, without affecting plasma 11-KT, which did not differ between T and blank implanted males ($p = 0.78$).

Effect of androgens on EOD waveform

Androgen implants increased τ_{p2} both in the day (Fig. 3A and B) and at night (Fig. 3C and D) relative to blank implanted males (LSD post-hoc: T day $p < 0.01$; T night $p < 0.01$; 11-KT day $p < 0.01$; 11-KT night $p < 0.01$). 11-KT had a larger effect than T on τ_{p2} at night (Fig. 3C), but not during the day (Fig. 3A; post-hoc LSD; day $p = 0.31$; night $p < 0.01$). However, only 11-KT elevated EOD amplitude at night (Fig. 3D; post-hoc LSD: T $p = 0.23$; 11-KT $p = 0.02$), while neither androgen changed daytime amplitude (Fig. 3B; post-hoc LSD: T $p = 0.51$; 11-KT $p = 0.97$). Body length, but not weight, significantly predicted daytime τ_{p2} , but not amplitude (univariate between-subject tests; daytime τ_{p2} $p = 0.02$, amplitude $p = 0.70$).

Baseline EOD amplitude differed between the two years of the study (GLM multivariate tests: $F_{4, 24} = 4.99$, $p < 0.01$). Pre-implant day and night EOD amplitudes of the fish sampled in 2009 were significantly higher than those in 2008 (univariate between-subjects tests: day amplitude $p = 0.02$; night amplitude $p = 0.03$). Pre-implant day and night τ_{p2} , however, did not differ between years (univariate between-subjects tests: day τ_{p2} : $p = 0.85$; night τ_{p2} : $p = 0.90$). The interaction between implants and year was not significant for any of the EOD parameters tested (GLM multivariate tests: $F_{8, 50} = 1.86$, $p = 0.09$). Neither body weight nor body length differed between the two years (GLM multivariate tests: weight $F_{4, 24} = 0.52$, $p = 0.72$; length $F_{4, 24} = 1.15$, $p = 0.36$).

Effect of social stimuli on EOD waveform

Addition of a conspecific male to the tank significantly increased EOD amplitude of the focal male both by day and night (Fig. 4b) (paired t-test: day t: $p = 0.01$; night $p = 0.01$). However, social stimulation only increased τ_{p2} during the night (Fig. 4a; paired t-test: day t = 0.09, $p = 0.93$; night t = -8.73, $p < 0.01$).

Androgen effects on α -MSH challenge

As reported previously (Stoddard et al., 2006), α -MSH enhanced both τ_{p2} (Fig. 5b; $t = 3.23$, $df = 25$, $p < 0.01$) and amplitude (Fig. 5c; $t = 8.87$, $df = 25$, $p < 0.01$). Overall, 11-KT imparted the most pronounced increase in EOD waveform responsiveness to α -MSH, while T only enhanced responsiveness of τ_{p2} (Fig. 6). Neither body length nor weight predicted the magnitude of α -MSH injection response to implant treatments (GLM multivariate tests: weight $F_{2, 12} = 0.41$, $p = 0.67$; length $F_{2, 12} = 0.16$, $p = 0.86$). Implant type had a significant effect on EOD response to α -MSH (GLM multivariate tests, $F_{4, 26} = 4.05$, $p = 0.01$) While both androgen implants increased τ_{p2} response to α -MSH (Fig. 6a; post-hoc LSD; 11-KT $p < 0.01$; and T $p = 0.03$), only 11-KT increased amplitude responsiveness to α -MSH (Fig. 6b; post-hoc LSD: 11-KT $p = 0.02$; T $p = 0.78$). Social interactions had no effect on response to α -MSH for either EOD parameter (Fig. 7; amplitude $t = -0.19$, $df = 7$, $p = 0.86$; τ_{p2} $t = -1.21$, $df = 7$, $p = 0.27$).

The combined 11-KT and α -MSH treatments enhanced the waveform multiplicatively, inducing a significantly larger increase than expected from the summation of the individual androgen and peptide treatments (paired t-test; τ_{p2} $t = -5.56$, $df = 5$, $p < 0.01$; amplitude $t = 2.42$, $df = 5$, $p = 0.06$). Combined T and α -MSH effects did not deviate significantly from the

summation of individual hormone treatments (paired t-test; τ_{p2} $t=-0.80$, $df=5$, $p=0.46$; amplitude $t=1.84$, $df=5$, $p=0.13$).

DISCUSSION

Of the two dominant teleost androgens, only 11-KT increased EOD amplitude and increased the response of amplitude to α -MSH (Fig. 6, Table 2). It is important to note that both androgen implants elevated circulating T and 11-KT levels above normal physiological levels, compared to the baseline levels of Blank implanted males (Table 1) and the levels reported by Salazar and Stoddard (2009). However, despite the supraphysiological levels, T failed to elevate EOD amplitude or increase amplitude responsiveness to α -MSH (Fig. 6, Table 2), suggesting that the effects we observed are 11-KT specific. To our knowledge, this is the first study to show that EOD amplitude can be modulated by a non-aromatizable androgen, but not its precursor testosterone. In a previous study conducted on female *B. gauderio*, none of the DHT implant doses ($0.03-1\text{mg } 10\text{g}^{-1}$ of fish) significantly elevated EOD amplitude or enhanced the effects of melanocortins on amplitude (Allee et al., 2009). However, all androgen implants, DHT (Allee et al., 2009), T, and 11-KT enhanced baseline τ_{p2} (Fig. 3A, C) and the responsiveness of τ_{p2} to injected melanocortins (Fig 6a).

In other gymnotiforms, androgens differentially affect the EOD signal. While 11-KT masculinizes the EOD frequency and duration in all species tested to date (Dunlap et al., 1998; Dunlap and Zakon, 1998), the effects of T and DHT seem to be species-specific (Dunlap et al., 1998; Zakon et al., 1990). DHT lowers EOD frequency of *Apteronotus albifrons*, but has no effect on frequency of *A. leptorhynchus*. Conversely, T has no effect on EOD frequency of *A. albifrons*, while feminizing (lowering) the frequency of *A. leptorhynchus* and masculinizing (lowering) it in *Sternopygus macrurus* (Dunlap et al., 1998; Meyer, 1983; Zakon, 1999). In *B. gauderio* and *B. occidentalis* T and DHT implants masculinize female EODs by increasing duration of the EOD's second phase (Hagedorn and Carr, 1985; Silva et al., 1999). While the differential sensitivity to T has been attributed to disparate aromatase activities in the brain (Zakon, 1999), the differential effect of DHT and 11-KT on EOD suggests that different androgens activate different pathways regulating waveform shape.

Androgen administration reproduced the enhancing effect of social interaction on the EOD τ_{p2} . However, the EOD amplitude elevation induced by 11-KT is not comparable to the large increase elicited by social stimulation (Fig. 3, Table 2), suggesting that another unidentified hormone is also involved in regulating EOD amplitude. Social stimulation did not affect EOD responsiveness to α -MSH treatment (Fig. 7), possibly because endogenous melanocortins saturated melanocortin receptors during social interactions.

Differential effects of 11-KT and T on behavior have been reported mainly in species with alternative reproductive tactics (Grober and Bass, 2002; Hirschenhauser et al., 2004; Lee and Bass, 2005; Oliveira et al., 2005). In these species, 11-KT levels are highly correlated with aggressive and territorial behaviors of the "bourgeois" phenotypes, while T maintains the female and socially parasitic (sneaker and satellite) male phenotypes. For example, in the batrachoid fishes, T regulates only the vocalizations of females and type II sneaker males, while 11-KT regulates the vocalizations of type I, territorial, paternal males; vocal activity of type I males are insensitive to T (Bass, 2008). Interestingly, while some female gymnotiforms such as *B. gauderio* and members of the genus *Apteronotus* have higher plasma concentrations of T than males (Dunlap et al., 1998; Salazar and Stoddard, 2009), these patterns have not been associated with any female-specific behaviors.

Among teleosts with fixed reproductive strategies the specific role of each androgen has been hard to elucidate. Androgen responses to aggressive interactions, territorial intrusions and females seem to reflect species-specific reproductive and parental strategies (Hirschenhauser et al., 2004). In general, 11-KT levels in all species are highly responsive to territorial intrusions, while T levels, do not vary significantly in response to social challenges in various species of cichlids (Hirschenhauser et al., 2004; Parikh et al., 2006). In the three-spined stickleback, *Gasterosteus aculeatus*, 11-KT levels are greatest during the beginning stages of the courtship phase (Pall and Borg, 2002) and 11-KT is needed for the production of spiggin, a proteinacious glue for nest building (Olsson et al., 2005). Our findings demonstrate that EOD amplitude, which is also associated with competition intensity (Franchina et al., 2001; Salazar and Stoddard, 2009), is modulated preferentially by 11-KT and is less sensitive to T manipulations, in *B. gauderio*, another species with fixed reproductive strategies.

Behavioral significance of signal modulation

Previous studies in our lab have found that EOD amplitude response to social challenge is specific to the sex of the challenger. Male challengers induce a rapid (10-20 min) amplitude increase, as well as a long-term amplitude increase, peaking within 24 hours. Female challengers, on the other hand, only elicit a long-term amplitude increase on the scale of days but no rapid increase on the scale of minutes comparable to that elicited by males (Franchina et al., 2001). As in our study (Fig. 4), Franchina et al. (2001) found that compared to EOD amplitude, duration was much more responsive to social challenge on both time scales and in response to either sex. In *B. gauderio*, increased male-male competition causes an increase in amplitude and τ_{p2} (Salazar and Stoddard, 2009). EOD amplitude and the variances of circulating 11-KT levels track competition intensity, increasing as the male density in a pool is increased. On the other hand, τ_{p2} and T levels seem to track the presence or absence of competition (Salazar and Stoddard, 2009). In all of the studies on *B. gauderio*, τ_{p2} is always more responsive than amplitude to any stimulus (Allee et al., 2009; Franchina et al., 2001; Markham and Stoddard, 2005; Salazar and Stoddard, 2009).

The differential time course in response to different types of social stimuli might reflect the reproductive behaviors of *B. gauderio*, which breed seasonally in temperate areas (Quintana et al., 2004; Silva et al., 2002) in an exploded lek polygyny breeding system (Miranda et al., 2008). The transition from non-breeding to breeding season is likely preceded by increasing androgen levels, which stimulate the development of secondary sexual characteristics (Adkins-Regan, 2005), and expression of androgen receptors in the pacemaker nucleus (Pouso et al., 2010). Increased androgen levels masculinize EOD waveform (Hagedorn and Carr, 1985; Pouso et al., 2010; Silva et al., 2002) and likely increase melanocortin sensitivity (see below). As males compete for and establish territories during the breeding season, rapid EOD enhancement can signal quality and competitive ability to nearby competitors and potential mates. These short-term responses are driven by melanocortins (Markham et al., 2009a; Markham and Stoddard, 2005).

Proposed mechanism for androgen regulation of melanocortin-driven EOD signaling

Androgens might increase melanocortin sensitivity through different mechanisms. In the distantly related gymnotiform *Sternopygus macrurus*, the sexually dimorphic EOD waveform duration is regulated by the kinetics of Na^+ and K^+ currents in the electrocytes (Ferrari et al., 1995; Few and Zakon, 2001; 2007; Schaefer and Zakon, 1996). The kinetics of voltage-gated ion channels in *S. macrurus* electrocytes are regulated by steroid hormones, and accordingly, electrocytes express androgen receptors (Dunlap and Zakon, 1998; Few and Zakon, 2001; 2007; Liu et al., 2008). In addition, circadian rhythmicity and short-term EOD amplitude modulations of *B. gauderio* and *S. macrurus* are regulated by melanocortins

through the activation of the cAMP/PKA pathway (Markham and Stoddard, 2005; McAnelly et al., 2003; Stoddard et al., 2006). Recent findings show that the melanocortin ACTH activates the cAMP/PKA pathway, promoting exocytosis of preformed voltage-gated Na^+ and inward rectifying K^+ channels from vesicles into the electrocyte membrane, thus increasing electrocyte sodium flux during the action potential, causing EOD amplitude to increase (Markham et al., 2009b).

Androgens might enhance melanocortin sensitivity of electrocytes by either affecting the expression of melanocortin receptors or the density and kinetics of voltage-gated ion channels. Data from other vertebrates have shown that androgens can stimulate melanocortin receptor expression (Ducrest et al., 2008; Wikberg et al., 2000). The expression of the recently described, $\text{Na}_v1.4\text{bL}$ splice variant in the electric organ of *S. macrurus* is positively correlated with EOD frequency and is lowered by androgen treatment. An alternate splice variant, $\text{Na}_v1.4\text{a}$ is constitutively expressed in the electric organ and muscle. Possibly, androgens selectively alter Na^+ channel splicing in favor of decreased $\text{Na}_v1.4\text{bL}$ levels, thereby reducing the rate of Na^+ current inactivation and masculinizing the waveform (Liu et al., 2008). A similar mechanism might operate for the sexually dimorphic expression of the potassium channels $\text{Kv}1.1\text{a}$ and $\text{Kv}1.2\text{a}$ (Few and Zakon, 2007). Thus, androgens might increase EOD sensitivity to melanocortins either by increasing melanocortin receptor synthesis or by selectively changing the ratio of Na^+ channel splice variants synthesized. Melanocortins, in turn, could activate the cAMP/PKA pathway shuttling the newly formed channels into the electrocyte membrane to produce EOD waveform enhancement or could alter kinetics by directly phosphorylating existing membrane channels.

In our study only 11-KT enhanced EOD amplitude in response to α -MSH injections, while both androgens increased EOD τ_{p2} response to MSH (Fig. 6, Table 2). 11-KT implants significantly elevated circulating 11-KT levels (Table 1) and increased nighttime amplitude (Fig. 2; $p = 0.012$). Since the types of androgen receptors found in some teleosts (AR1 and AR2) have the highest affinities for T and DHT (Larsson et al., 2002; Sperry and Thomas, 1999), differential androgen receptor expression on the electrocyte surface cannot account for the 11-KT specific, multiplicative enhancement of amplitude and τ_{p2} . Since AR2 affinity for T is still higher than for 11-KT, it is not likely that α -MSH amplitude response is specific to a particular receptor. However, since androgen receptor affinities have not been examined in gymnotiforms, it is possible that unlike the AR1 and AR2 found in Atlantic croaker (Sperry and Thomas, 1999), androgen receptors in gymnotiforms have higher affinity for 11-KT than T. The recent finding that one of the androgen receptors has been lost in the *Otophysi* lineage (Douard et al., 2008) suggests that the remaining receptor might mediate the actions of T and 11-KT differently than in species with two functional receptors. Preliminary findings in our lab suggest that the electrocytes of *B. gauderio* express only one type of AR receptor (Villinger and Stoddard, unpublished observations). Thus, the AR receptor in *B. gauderio* might be preferentially activated by 11-KT, independent of binding affinity, as has been reported in three-spined stickleback (Olsson et al., 2005). Preferential sensitivity to 11-KT could also explain the differential magnitude of the combined androgen-melanocortin treatments, with 11-KT upregulating expression of melanocortin receptors or ion channel densities in active membranes of the electrocyte more effectively than T.

The finding that 11-KT but not T enhances EOD amplitude and the melanocortin action on amplitude suggest that EOD amplitude is regulated by a different mechanism or pathway than EOD duration, allowing different information to be communicated by the two signal parameters.

Conclusions

Both sexually dimorphic EOD parameters are regulated by short-term and long-term modulators. 11-KT modulates EOD amplitude in the long-term while melanocortins modulate it in the short-term. Likewise, T and 11-KT modulate τ_{p2} in the long-term while melanocortins modulate it in the short-term. Changing social environment, competition for mates, and aggressive interactions all can stimulate a behavioral and motivational response within minutes, which would be reflected on the EOD via melanocortins. Continuous stimulation or seasonal changes would enhance the EOD via long-term action of androgens. We suspect that increased androgen levels during the breeding season stimulate ion channel synthesis in the electrocytes, in anticipation of increased social interactions. The synergistic and differential effects of steroid and peptide hormones on modulation of EOD parameters allows changes in the signal to track long-term and short-term physiological responses. Because fish can increase T without increasing 11-KT, the differential sensitivity of EOD amplitude and 2nd phase duration to the two androgens (only 11-KT modulates EOD amplitude, while both androgens modulate signal duration) provides a potential mechanism for independent regulation of these two sexually dimorphic signal traits.

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Figure 1.

(**A**) The EOD waveform of *B. gauderio* varies in peak-to-peak amplitude and in duration of the second phase (parameterized as τ_{p2}), (**B**) We recorded EODs around the clock for the duration of the experiment. We selected for analysis the amplitude and τ_{p2} when the EOD reached a daytime minimum and nighttime maximum.

Figure 2. Design of Experiment 2
See methods section for details.

Figure 3.

Effect of androgen implants on daytime (A, B) and nighttime (C, D) EOD. (A) 11-KT and T implants increased τ_{p2} during the day (11-KT $p < 0.01$; T $p < 0.01$), but neither androgen implant increased daytime amplitude (B. 11-KT $p = 0.09$; T $p = 0.51$). (C) 11-KT caused higher elevation of τ_{p2} than T did ($p < 0.01$ for 11-KT; $p < 0.01$ for T), but only 11-KT elevated EOD amplitude (D. 11-KT $p = 0.02$; T $p = 0.23$). Relative change in τ_{p2} and amplitude following social stimulation are shown separately because the data were collected the following year. Circles and crosses represent individual data points represented as relative change. Horizontal and vertical bars depict mean and SEM, respectively. Open and shaded circles represent the two years, 2008 and 2009, respectively, over which these experiments were conducted. For comparison, EOD response to social challenges is presented as pluses next to the implant data. Different letters represent significant differences between treatments ($p < 0.05$), based on LSD pairwise comparisons.

Figure 4.

Effect of social challenge on the EOD waveform. Social challenge significantly increased daytime amplitude (**B.** paired t-test, $t = -3.47$, $p = 0.01$), but not τ_{p2} (**A.** paired t-test, $t = 0.09$, $p = 0.93$). However, at night, τ_{p2} was significantly higher after the social challenge (**A.** paired t-test, $t = -8.73$, $p < 0.01$) as was amplitude (**B.** paired t-test, $t = -3.39$, $p = 0.01$). “Before” refers to the amplitude and τ_{p2} measured before a social challenger was placed in the tank. “After” indicates the peak of the EOD waveform caused by the presence of the social stimulus. Triangles = data points, horizontal bars = means, and vertical bars = SEM

Figure 5.

Effect of α -MSH on EOD waveform. (A) α -MSH injections increase the amplitude and τ_{p2} in the biphasic EOD of *B. gauderio*. Compared to saline, α -MSH injections increase τ_{p2} (B. paired t-test, $t=3.23$, $df=25$, $p<0.01$) and amplitude (C. paired t-test, $t=8.87$, $df=25$, $p<0.01$). Different letters represent significant differences between treatments ($p<0.05$). Pluses, horizontal bars and vertical bars represent individual data points, mean, and SEM, respectively.

Figure 6.

Androgen effect on EOD responsiveness to α -MSH injections. **(A)** 11-KT and T implants increased the τ_{p2} of the MSH response relative to blank implants ($p < 0.01$ for 11-KT, $p = 0.03$ for T). Only 11-KT increased amplitude responsiveness to α -MSH injections **(B)**. 11-KT $p = 0.02$; T $p = 0.78$). Social challenge had no effect the magnitude of α -MSH response in τ_{p2} (paired t-test, $t = -1.21$, $p = 0.86$) or amplitude (paired t-test, $t = -0.19$, $p = 0.27$). Pluses, horizontal bars, and vertical bars represent individual data points, mean, and SEM, respectively. Different letters represent significant differences between treatments ($p < 0.05$), based on LSD pairwise comparisons.

Figure 7.

Effect of social challenge on melanocortin responsiveness. Social challenge had no effect the magnitude of α -MSH response in τ_{p2} (A, paired t-test, $t=-1.21$, $p=0.85$) or amplitude (B, paired t-test, $t=-0.19$, $p=0.26$). Horizontal bars and vertical bars represent mean, and SEM, respectively.

Table 1

Androgen implants significantly elevated plasma T and 11-KT levels compared to blank implanted males (T implant: $p<0.001$; 11-KT implant: $p=0.01$).

Implant Type	Plasma androgen levels (mean $\pm$ SEM)	
	T (ng/ml)	11-KT (ng/ml)
Blank	1.23 $\pm$ 0.09 (n=4)	2.44 $\pm$ 2.27 (n=4)
T	16.58 $\pm$ 3.79 (n=5)	1.66 $\pm$ 0.98 (n=5)
11-KT	2.19 $\pm$ 0.91 (n=4)	11.0 $\pm$ 2.63 (n=4)

Table 2

Androgen implants and social challenge significantly enhanced EOD τ_{p2} and the effects of α -MSH on τ_{p2} . However, only 11-KT elevated EOD amplitude and increased the effects of α -MSH on amplitude. Upward arrows indicate elevation and the horizontal bar (–) indicates no significant change. Arrow number reflects the magnitude of the change. Effects of steroid implants and social challenge on α -MSH response are compared directly to α -MSH effects alone.

Treatment	EOD amplitude	EOD τ_{p2}
α -MSH	↑	↑
T	–	↑↑↑↑
11-KT	↑	↑↑↑↑
Social challenge	↑↑↑↑	↑↑↑↑
T → α -MSH	≈ α -MSH	> α -MSH
11-KT → α -MSH	> α -MSH	>> α -MSH
Social → α -MSH	≈ α -MSH	≈ α -MSH