Title: Androgens regulate sex differences in signaling but are not associated with male variation in morphology in the weakly electric fish *Parapteronotus hasemani*

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ABSTRACT

Sexually dimorphic signaling is widespread among animals and can act as an honest indicator of mate quality. Additionally, differences in signaling and morphology within a sex can be associated with different strategies for acquiring mates. Weakly electric fish communicate via self-generated electrical fields that transmit information about sex, reproductive state, and social status. The weakly electric knifefish *Parapteronotus hasemani* exhibits sexual dimorphism in body size as well as substantial within-male variation in body size and jaw length. We asked whether *P. hasemani* exhibits hormonally mediated sexual dimorphism in electrocommunication behavior. We also asked whether males with short versus long jaws differed significantly from each other in morphology, behavior, hormone levels, or reproductive maturity. Males produced longer chirps than females, but other signal parameters (electric organ discharge frequency; chirp rate and frequency modulation) were sexually monomorphic. Pharmacologically blocking androgen receptors in males reduced chirp duration, suggesting that this sexually dimorphic trait is regulated at least in part by the activational effects of androgens. Males sorted into two distinct morphological categories but did not differ in circulating 11-ketotestosterone or testosterone. Short-jawed males and long-jawed males also did not differ in any aspects of signaling. Thus, chirping and high levels of 11-ketotestosterone were reliably associated with reproductively active males but do not necessarily indicate male type or quality. This contrasts with other alternative male morph systems in which males that differ in morphology also differ in androgen profiles and signaling behavior.

INTRODUCTION

Sexual selection can act upon numerous traits to produce sexual dimorphism in morphology and behavior. Sexually dimorphic traits that exhibit variation within one sex can serve as honest indicators of quality and may differentially attract potential mates (Badyaev & Hill, 2000; Owens & Hartley, 1998; Zahavi, 1975). Variation within a sex can either be continuous or discrete. Discrete within-sex variation is often associated with the presence of alternate reproductive tactics, whereby individuals of one sex (typically males) use one of two or more mutually exclusive strategies to secure mating opportunities (Godwin, 2010; Gross, 1996; Oliveira et al., 2008). Distinct male reproductive phenotypes may result from either fixed developmental trajectories or plastic responses to internal or external cues including age, size, or energy reserves (Godwin, 2010; Humfeld, 2013). Fish species with alternative reproductive tactics often have two behaviorally distinct male morphs that differ substantially in size. One subset of males (*courting males*) make a relatively large investment in growth and development of secondary sexual characteristics while other males (*noncourting males*) invest more heavily in gonad development and sperm production at the expense of growth. The larger courting males reproduce by attracting females and defending a territory, while the smaller noncourting males rely on “sneak-spawning” or “streak-spawning” to surreptitiously fertilize eggs (Taborsky, 1994). The mechanisms that regulate the development and maintenance of alternative reproductive tactics among fish vary, but there is strong evidence that androgens play a role, often with higher levels of 11-ketotestosterone relative to testosterone in courting males and the reverse in non-courting males (Brantley et al., 1993).

South American weakly electric knifefish are an excellent system in which to study sexually dimorphic morphology and behavior. Weakly electric fish communicate via a

specialized electric organ that continuously emits a weak electrical field. The electric organ discharge (EOD) frequency is a distinguishing characteristic that can indicate sex, species, dominance, and/or individual identity (Zakon & Dunlap, 1999). During social interactions, fish can rapidly increase EOD frequency on short timescales to produce context-specific chirps (Hagedorn & Heiligenberg, 1985; Zakon & Dunlap, 1999). Sexual dimorphism in signaling appears ubiquitous, although there is a great diversity across species in the signal parameters that differ between the sexes. Sex differences in EODs and chirps are regulated by sex steroid hormones (reviewed in Smith, 2013).

Parapteronotus hasemani is a species of electric fish in which reproductively mature males vary dramatically in morphology. Short-jawed males have relatively small body sizes with short jaws that make them visually indistinguishable from females. Long-jawed males have longer, heavier bodies and disproportionately larger jaws with pronounced upward curvature (Fig. 1A&B). Indeed, long-jawed males have such a drastically different appearance compared to females and short-jawed males that they were previously misclassified as a separate species (Cox Fernandes et al., 2002). Here, we assess whether the communication signals of *P. hasemani* are hormonally mediated indicators of sex. Additionally, we test whether the two male morphs show differences in signaling or androgen concentration that could be correlated with male quality or differential attractiveness to females.

METHODS

Animals

Sexually mature male (n=18) and female (n=6) *P. hasemani* were collected from the Amazon and Nanay Rivers in Peru in August 2006 and January 2008 by commercial suppliers and transported to Indiana University. Fish were housed separately in 35-L tanks within a 2000-L recirculating aquarium on a 12:12 light/dark cycle. Temperature was maintained at 25-27° C, conductivity at approximately 150 μ S/cm, and pH 5.5-6.5. These housing conditions have been shown to stimulate gonadal recrudescence in captivity (Kirschbaum, 1975; Kirschbaum, 1979). Animal care and experimental protocols were approved by the Indiana University Bloomington Institutional Animal Care and Use Committee.

Morphology

To quantify morphology, we measured body mass, body length, and jaw size. Two measures of jaw size were used: the distance from the anterior tip of the mandible to the meeting point of the mouth (gape length) and the distance from the most anterior point of the premaxilla to the posterior edge of the operculum (jaw length). Males were divided into two groups (short-jawed males and long-jawed males) based on the bimodal distribution of jaw measurements (Fig 1A). Jaw length was not measured for four males. Data from those males were included in analyses of sex differences but not within-male differences. Sex was assessed by measuring plasma 11-ketotestosterone (11-KT) and later confirmed by gonadal inspection (19 out of 24 fish). Sex was always determined by gonadal inspection for any fish that did not provide sufficient samples for 11-KT analysis.

EOD and Chirping Behavior

To test for the presence of sexually dimorphic signaling behavior, we recorded EOD frequency and chirping behavior of male and female *P. hasemani* using a chirp chamber paradigm that has been described elsewhere (Kolodziejski et al., 2005). Briefly, the fish was enclosed in a shelter tube inside a darkened tank. Two carbon electrodes placed parallel to the fish's body were used to record the EOD and chirping. Stimulus signals were generated in audio software (CoolEdit Pro, Syntrillium; Phoenix, AZ) and presented via carbon electrodes placed perpendicular to the fish's body. The stimulus was calibrated midway between the electrodes to a root-mean-square (RMS) amplitude of approximately 1.5 mV/cm. After a thirty-minute acclimation period and a four-minute baseline recording, each fish was randomly presented with five sinusoidal stimuli simulating EODs of other fish +5 Hz, -20 Hz, +20 Hz, -150 Hz, and +150 Hz relative to the fish's EOD frequency. Each recording consisted of a one-minute pre-stimulus period, a two-minute playback period, and a one-minute post-stimulus period. Recordings were interspersed with ten-minute rest periods. These recordings were conducted in February-March of 2011.

EOD and Chirp Analysis

EOD frequency was measured by performing a fast Fourier transform (Blackman-Harris window, size 65536) on the baseline recording and was adjusted to the frequency expected at 26°C using a $Q_{10^{\circ}\text{C}}$ of 1.6 (Dunlap et al., 2000). Chirps were analyzed offline in Igor Pro (Wavemetrics; Lake Oswego, OR) using the custom procedure eFish (efish23e, Brian Nelson, University of Oregon, Eugene, OR; <http://nelsonbs.com/eFish/efish.html>). For details of the

algorithm and processing, see Turner and colleagues (2007). For each fish, we combined data from the six recordings to determine mean chirp rate, duration, and frequency modulation.

Flutamide Treatment

Male *P. hasemani* (n=8; 3 long-jawed, 5 short-jawed) were treated with the androgen receptor blocker flutamide to test for activational effects of androgens on EOD frequency and chirping. Before treatment, we recorded chirps and took a baseline blood sample from each fish. Then fish were divided into two groups, and treatment was assigned using a balanced within-subjects repeated measures design. One group received flutamide treatment followed by a ten-day washout period and then the control (ethanol vehicle) treatment. The other group received the control treatment, then the flutamide treatment, and then a ten-day washout followed by another control treatment. Thus, all fish received both control and flutamide treatments, but the order was balanced, and all fish received a vehicle washout treatment after the flutamide treatment. This design allowed us to control for possible time effects. Each treatment period (control or flutamide) lasted for three weeks. During flutamide treatments, flutamide (Sigma-Aldrich; St. Louis, MO) dissolved in ethanol was added to tank water (0.5 mg flutamide/L of tank water) in a recirculating aquarium system. In the vehicle control treatments, ethanol without flutamide was added to yield the same tank water ethanol concentration (0.0025%) as in the flutamide treatment. Aquarium water was periodically replenished by adding water treated with the appropriate dose of flutamide or ethanol. We recorded chirps and took a blood sample after each treatment. This experiment was conducted in March-July of 2012.

Hormone Assays

We collected blood samples 1-3 days after each recording in order to measure plasma concentrations of T and 11-KT. Each fish was lightly anesthetized with 0.075% 2-phenoxyethanol (Sigma-Aldrich; St. Louis, MO) in deionized water before 10-15 μ L of blood was collected from the caudal vein using a 1 mL syringe and a heparinized needle. Blood samples were centrifuged for five minutes at 10,000 rpm to extract plasma. Plasma samples were initially frozen at -20° C and then stored at -80° C. T and 11-KT enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI; T kit catalog #582701; 11-KT kit catalog #582751) were performed according to the manufacturer's specifications. Plasma samples were diluted in assay buffer at a range of concentrations to ensure at least one sample fell within the range of sensitivity (T: 1:25 and 1:50; 11-KT: 1:25, 1:50, 1:100, and 1:300). Samples from putative males were diluted more than samples from putative females. Each sample was assayed in duplicate for each dilution. The assay detection limit was 1.6 pg/mL for 11-KT and 3.9 pg/mL for T. Intra-assay variability was calculated using the coefficient of variation of the four replicate wells distributed across the plate and containing the 12.4 pg/mL standard (11-KT) or the 62.5 pg/mL standard (T). The intra-assay variability was 12% for 11-KT and 25% for T. Insufficient plasma was available to conduct 11-KT assays on 3 females and 3 males or to conduct T assays on 3 females and 4 males.

Statistics

Statistical analyses were performed with JMP 11 (SAS Institute, Inc.; Cary, NC) and Statistica 7 (StatSoft Inc.; Tulsa, OK). Comparisons of 11-KT and T concentrations, EOD frequency, and chirp parameters between the sexes and between the two male types were accomplished with two-tailed Welch's t-tests assuming unequal variances. A 2-factor repeated

measures ANOVA was used to compare changes in chirp parameters between fish that received flutamide vs. vehicle as their first treatment. Within-subjects comparisons of chirp parameters in baseline vs. flutamide vs. vehicle washout treatments were made with a single-factor repeated measures ANOVA. Effect size was estimated using Cohen's d for pairwise comparisons and eta squared for the ANOVAs.

RESULTS

Between Sex Comparisons

As expected, jaw morphology was sexually dimorphic and varied between the two male morphs. Because gape length and jaw length were tightly correlated ($R^2=0.95$, $p<0.001$), only comparisons with jaw length are reported here. Males as a group had longer jaws than females (Fig. 1A & 1B; $t(16.9)=4.78$, $p<0.001$, $d=2.31$). Males also had greater total body lengths than females (Table 1; $t(8.8)=3.22$, $p=0.01$, $d=1.52$). Chirp duration was the only sexually dimorphic signal parameter. Chirps produced by males lasted significantly longer than chirps produced by females (Fig. 1C; $t(14.5)=2.63$, $p=0.02$, $d=1.26$). EOD frequency, chirp rate, and chirp frequency modulation did not differ between the sexes (Table 1; t-tests, $p>0.10$). Males did not differ from females in T concentration ($t(3.0)=1.89$, $p=0.15$, $d=1.20$), but males did have higher 11-KT concentrations than females (Table 1; $t(14.3)=5.79$, $p<0.0001$, $d=3.66$).

Within-Male Comparisons

Long-jawed males had significantly longer jaws than short-jawed males, which is consistent with our visual morphological categorizations ($t(12.7)=16.86$, $p<0.0001$, $d=10.98$). Long-jawed males and short-jawed males did not differ in 11-KT concentration ($t(5.8)=0.68$,

p=0.43, d=0.28), T concentration ($t(2.7)=0.05$, $p=0.97$, $d=0.03$) or 11-KT/T ratio ($t(3.2)=0.51$, $p=0.64$, $d=0.33$). The two male types also did not differ in EOD frequency, chirp rate, chirp duration, or degree of chirp frequency modulation ($t<1.9$; $p>0.08$).

Flutamide Treatment

Chirp duration decreased significantly in fish that received flutamide as their first treatment, whereas vehicle treatment did not significantly affect chirp duration relative to baseline (baseline chirp duration for flutamide group: 1.16 ± 0.03 sec; post-flutamide: 0.96 ± 0.03 sec; baseline chirp duration for vehicle group: 0.96 ± 0.06 sec; post-vehicle: 0.90 ± 0.05 sec; treatment x time interaction, $F(1,6)=20$, $p<0.01$, $\eta_p^2=0.77$; PLSD, baseline vs. post-treatment in the flutamide group, $p<0.001$). Fish in the initial vehicle control group subsequently received flutamide, and we also used within-subjects comparisons to examine the effects of flutamide and subsequent washout treatments across all males in the experiment. Among all males, flutamide treatment decreased chirp duration relative to pre-treatment baseline recordings (Fig. 2; $F(2,14)=5.2$, $p=0.02$, $\eta_p^2=0.43$; PLSD, baseline vs. flutamide, $p<0.05$). This effect was reversible, as chirp duration in post-flutamide washout/vehicle recordings was not significantly different from baseline (PLSD, baseline vs. washout, $p=0.21$) and was marginally greater than chirp duration post-flutamide treatment (PLSD, flutamide vs. washout, $p=0.07$). This effect was specific to the sexually dimorphic chirp duration parameter; flutamide treatment did not affect EOD frequency, chirp rate, or chirp frequency modulation ($F(2,14)<2.1$, $p>0.15$).

DISCUSSION

Sexually dimorphic signaling can be critical for identifying and attracting a high-quality mate (Zahavi, 1975). Chirping in electric fish is an important social signal used during courtship and aggressive interactions (Hagedorn & Heiligenberg, 1985). The sexual dimorphism observed here suggests that chirp duration could serve as an indicator of sex in *P. hasemani*. Blocking androgen receptors with flutamide partially de-masculinized male chirp duration in the direction and approximate magnitude predicted by sex differences, suggesting that this feature of the EOD is at least partially controlled by the activational effects of androgens. This effect was specific to chirp duration and did not alter other aspects of signaling. Further work should examine whether other gonadal steroids such as estradiol affect this sexually dimorphic signaling parameter.

Animals that employ alternative reproductive tactics show within-sex differences in morphology that are associated with different tactics for acquiring mates (Taborsky, 1994). Interestingly, the striking morphological variation among male *P. hasemani* was not correlated with any detectable differences in EOD frequency or chirping that could indicate functional differences in mate quality or social status. Likewise, the similar levels of 11-KT and T among long-jawed and short-jawed male *P. hasemani* are in stark contrast with the hormonal variation between male morph types exhibited by fish species with alternative reproductive tactics such as bluegill sunfish and midshipman fish (Brantley et al., 1993; Kindler et al., 1989). Short-jawed and long-jawed *P. hasemani* males have androgen levels that are similar to each other and dissimilar from those of females. The hormone levels reported here are comparable to those seen among reproductively mature individuals in breeding condition in other species of weakly electric fish within the family Aptereronotidae (Cox Fernandes et al., 2010; Dunlap et al., 1998; Ho et al., 2010). This hormonal evidence combined with previous descriptions of gonadal state among males of varying sizes indicates that both male types are reproductively competent and

that short-jawed males are not simply immature long-jawed males (Cox Fernandes et al., 2002). Nevertheless, over a three-year period we saw one short-jawed male develop the distinct curvature and jaw length of a long-jawed male while other similar-sized males did not. Thus it remains unclear how the development of long jaws is initiated and maintained and why some males develop this striking morphology while others do not.

Further behavioral experiments in more naturalistic environments are needed to observe courtship behaviors, mate preference, and intrasexual aggression. These observations could provide insight into whether males that vary in jaw morphology have different strategies for attracting mates or defending limited resources. For example, exaggerated male body mass and jaw size could correlate with differential levels of aggression that allow long-jawed males to limit short-jawed males' access to territories and females. There may also be contextual differences in how chirping is used during social interactions that were not apparent in our recordings of chirps elicited by playbacks. These observations of social behaviors would help link the observed morphological variation among *P. hasemani* with its functional and evolutionary contexts.

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FIGURE LEGENDS

Fig. 1. Comparison of female and male morphology and chirping behavior in *Parapteronotus hasemani*. A) Long-jawed males (top) have larger, more curved jaws than short-jawed males (middle) and females (bottom). B) Mean jaw length is greater for males than females. Males fell into two categories: long-jawed males (>50 mm) and short jawed (< 45 mm). Individual data points shown with crosses. C) Chirps produced by males were longer in duration (mean $\pm$ S.E.M.) than chirps produced by females. Asterisks indicate a statistically significant difference (t-test, $p < 0.05$)

Fig. 2. Male chirp duration before, immediately after, and three weeks after flutamide treatment. A) Males treated with flutamide showed decreased chirp duration relative to baseline. Short-jawed males are indicated with dashed lines; long-jawed males are indicated with solid lines. B) Mean ($\pm$ S.E.M) change in chirp duration relative to baseline. Chirp duration after a post-flutamide three-week vehicle/washout treatment was marginally greater than chirp duration immediately following flutamide treatment ($F(2,14)=5.2$, $p=0.07$).

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Table 1

	Between Sexes		Within Males	
	Females	All males	Short-jawed male	Long-jawed males
Body length (mm)	228.0±14.0 ^a (6)	280.4±8.3 (18)	272.6±6.1 ^b (12)	317.3±19.1 (3)
Jaw length (mm)	29.7±1.5 ^a (6)	40.7±1.8 (15)	37.7±.08 ^b (12)	53.0±0.4 (3)
Electric organ discharge frequency (Hz)	877.5±23.7 (6)	875.0±15.8 (18)	887.4±19.1 (12)	842.5±34.9 (3)
Chirp duration (sec)	0.65±0.10 ^a (6)	1.01±0.10 (16)	1.08±0.13 (11)	0.96±0.13 (3)
Chirp rate (chirps/min)	3.7±1.4 (6)	6.7±1.1 (16)	7.4±1.5 (11)	4.0±0.13 (3)
Chirp FM (Hz)	549.8±37.9 (6)	541.2±15.7 (16)	554.9±18.5 (11)	494.7±40.3 (3)
11-KT (ng/mL)	0.14±0.05 ^a (3)	2.56±0.42 (15)	2.69±0.55 (11)	2.34±0.61 (3)
T (ng/mL)	0.76±0.19 (3)	0.35±0.09 (14)	0.35±0.10 (11)	0.36±0.26 (3)

^a significant difference between sexes, p<0.05, t-test
^b significant difference between long vs. short jawed males, p<0.05, t-test
Some data points were not available for all fish; see Methods for details. Sample sizes are shown in parentheses. All values are mean ± S.E.M.

5-Figure 1

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