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Sex differences in the electrocommunication signals of the electric fish *Apteronotus bonapartii*

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Abstract

The South American weakly-electric knifefish (Apteronotidae) produce highly diverse and readily quantifiable electrocommunication signals. The electric organ discharge frequency (EODf), and EOD modulations (chirps and gradual frequency rises (GFRs)), vary dramatically across sexes and species, presenting an ideal opportunity to examine the proximate and ultimate bases of sexually dimorphic behavior. We complemented previous studies on the sexual dimorphism of apteronotid communication signals by investigating electric signal features and their hormonal correlates in *Apteronotus bonapartii*, a species which exhibits strong sexual dimorphism in snout morphology. Electrocommunication signals were evoked and recorded using a playback paradigm, and were analyzed for signal features including EOD frequency and the structure of EOD modulations. To investigate the androgenic correlates of sexually dimorphic EOD signals, we measured plasma concentrations of testosterone and 11-ketotestosterone. *A. bonapartii* responded robustly to stimulus playbacks. EODf was sexually monomorphic, and males and females produced chirps with similar durations and amounts of frequency modulation. However, males were more likely than females to produce chirps with multiple frequency peaks. Sexual dimorphism in apteronotid electrocommunication signals appears to be highly evolutionarily labile. Extensive interspecific variation in the magnitude and direction of sex differences in EODf and in different aspects of chirp structure suggest that chirp signals may be an important locus of evolutionary change within the clade. The weakly-electric fish represent a rich source of data for understanding the selective pressures that shape, and the neuroendocrine mechanisms that underlie, diversity in the sexual dimorphism of behavior.

Introduction

Communication signals, particularly those functioning in reproductive contexts, often evolve rapidly and can be highly diverse (Andersson 1994). In many cases, variation occurs not only across species, but also between sexes, making communication behavior a useful model for understanding both the proximate and ultimate origins of sexually dimorphic behavior. Weakly-electric ghost knifefish (family: Apteronotidae) are a promising system for understanding the evolution of species and sex differences in communication behavior for

several reasons: first, they produce electrocommunication signals that are well-defined and readily quantified; second, these signals are highly diverse across species; and third, these signals vary remarkably between sexes.

In apteronotids, electrocommunication signals are produced by a neurogenic electric organ that fires to produce an electric organ discharge (EOD) with a characteristic frequency (EODf). During social interactions, fish also produce brief modulations of the EOD called chirps and gradual frequency rises (GFRs). Both EODf and chirping function as agonistic and reproductive communication signals (Hagedorn & Heiligenberg 1985; Hupe & Lewis 2008; Larimer & Macdonald 1968; Triefenbach & Zakon 2008). The magnitude and direction of sexual dimorphism of EODf and chirping vary across four previously studied apteronotid species (Dunlap et al. 1998; Engler & Zupanc 2001; Kolodziejski et al. 2007; Zhou & Smith 2006). These data suggest that sexual dimorphism in EODf and chirping is pervasive across the apteronotids, and additionally, that these sex differences can be manifested in distinct parameters of EOD and chirp signals.

Differences in sexually dimorphic traits across closely related species also provide a good opportunity to understand how physiological mechanisms evolve to produce species diversity in sexually dimorphic behavior. In electric fish, EOD behavior and the neural circuitry controlling EOD production are profoundly affected by steroid hormones (Zakon & Dunlap 1999; Zakon & Smith 2009). In brown ghosts (*Apteronotus leptorhynchus*) and black ghosts (*Apteronotus albifrons*), masculinizing effects of androgens on EODf and chirping parallel the magnitude and direction of sex differences in these behaviors (Dunlap et al. 1998).

We studied sex differences in EODs and chirping in *Apteronotus bonapartii*, an apteronotid species that is widespread across South American waterways (Cox Fernandes 1998; Hilton & Cox Fernandes 2006). Its head and snout morphology is sexually dimorphic, with males often possessing an elongate head and snout relative to females (Hilton & Cox Fernandes 2006). In *A. leptorhynchus*, a similar sexual dimorphism in snout morphology is associated with pronounced sexual dimorphism in EOD behavior. By contrast, a lack of sexual dimorphism in the head morphology of *Adontosternarchus deventanzii* is correlated with relatively little sexual dimorphism in EOD behaviors (Zhou & Smith 2006). These observations suggest that the magnitude of sexual dimorphism in morphology and behavior may be associated in apteronotids. The prominent morphological sex differences in *A. bonapartii* provide an additional opportunity to test this hypothesis.

We analyzed EOD behaviors of *A. bonapartii* by conducting playback experiments and measured androgen levels to understand the hormonal correlates of sex differences in communication signals. Because some of the fish in this study were caught directly from the field and recorded shortly after collection, we had an unusual opportunity to examine the behavior and endocrine regulation of sexual dimorphism in natural populations of *A. bonapartii* during the breeding season. Few studies have looked at sex differences in EODf and chirping behavior in free-living apteronotids shortly after capture. In this study, our goals were to (1) explore the relationship between sex differences in morphology and sex differences in behavior, and (2) explore the hormonal correlates of sex differences in communication behavior in *A. bonapartii*.

Materials and Methods

Animals

A total of fifty-eight *Apteronotus bonapartii* were obtained from Brazil and Peru (females=21, males=32, immature=5). Forty-three fish (male n=22, female n=16, immature

n=5) were seine netted (described in (Cox Fernandes et al. 2002)) during the rainy season of 2007 near Manaus, Brazil (Marchantaria Island). These fish were transported back to the Instituto Nacional de Pesquisas da Amazônia (INPA, Manaus) for behavioral recording and housed separately in aerated tanks filled with water from the collection locale. Recordings were done within two days of capture. The other fifteen individuals (male n=10, female n=5) were collected from the Rio Amazonas near Iquitos, Peru, by Ornamental Amazon Fish Aquarium in 2005 and 2007, and were imported to Indiana University for behavioral recordings. These fish were maintained in the laboratory within a 2000-l recirculating freshwater aquarium system on a 12:12 light/dark cycle at 25.5-27.5°C, pH 6.0-7.0. The fish were housed at low conductivity (100-400 μ S/cm) and fed live black worms to maintain reproductive condition. Standard lengths were measured from the tip of the snout to the posterior margin of the caudal fin, along the anterior-posterior axis of each fish. The sex of each fish was determined at the end of the experiment by post-mortem inspection of the gonads. Animal care and experimental protocols followed the NIH Guide for Care and Use of Laboratory Animals and were approved by the Indiana University Bloomington Institutional Animal Care and Use Committee.

Stimulus presentation and electrical recordings

EODs from Brazilian fish were recorded at INPA within a day of collection from the field. EODs were amplified ($\times 100$ -1000, model 3000, A-M Systems (Sequim, WA), digitized (Digidata 1440, Axon Instruments) and recorded on a computer running Axoscope (version 10.0 Axon Instruments). EODf was measured from these recordings with Clampex 9.0 (Axon Instruments). EOD frequencies were temperature compensated at 26°C using a $Q_{10}^{\circ C}$ of 1.6, a value determined empirically by recording EODf over a range of temperatures for a subset of the fish. A similar $Q_{10}^{\circ C}$ governs the temperature-dependence of EODf in *A. leptorhynchus* (Dunlap et al. 2000).

EODf, chirps, and GFRs were recorded by using a previously described playback paradigm (Turner et al. 2007). Individuals were held in perforated PVC tubes with plastic mesh at either end and placed in a darkened 38-l tank maintained at 25.3-27.0°C. Temperatures within a typical recording session did not vary by more than 0.1°C. A pair of carbon electrodes was positioned at the head and tail of the fish to record the fish's own discharge, and another pair was placed on either side of the fish's tube to deliver playback stimuli. Fish were acclimated for 15-30 minutes before recordings.

Stimuli and Recordings

Simulated EOD playback stimuli that consisted of sinusoidal voltage signals were generated using Cool Edit Pro (Syntrillium; Phoenix, AZ, USA) and calibrated using a Fluke multimeter (model 187; Everett, WA, USA) to a field-intensity between 0.6-0.7mV/cm at a point midway between and collinear to the stimulus electrodes. This signal intensity was intended to approximate the EOD of a medium-sized conspecific. We split the stimulus signal, routing one copy into the experimental tank and digitizing the other on the right channel of the recording sound card. Each fish was presented, in random order, with a series of five stimuli designed to cover the range of conspecific EOD frequencies: -150Hz, -20Hz, -5Hz, +20Hz, and +150Hz, relative to the fish's own EODf. After a baseline recording with no stimulus, each stimulus was presented as a four minute trial separated by ten minute intervals. Each trial included a one minute pre-stimulus period (stimulus off), two minutes of playback stimulus (stimulus on), and one minute of post-stimulus recording (stimulus off). Signals from the recording electrodes were differentially amplified (gain 100-1000 $\times$; Grass model P-55 or A-M Systems model 3000), band-pass filtered (0.1 Hz – 10 kHz) and digitized at 44.1 kHz on the left channel of a sound card in a computer running Cool Edit Pro (Syntrillium, Phoenix, AZ, USA).

Behavioral analysis

EOD signals were analyzed using customized procedures (Brian Nelson, University of Oregon, Eugene, OR) running in Igor Pro as detailed in an earlier study (Kolodziejwski et al. 2005). Briefly, any contamination from the playback stimulus was removed by subtracting a copy of the playback, appropriately scaled and phase-shifted, from the recording. An autocorrelation algorithm with a 6ms Hanning window advanced 2 ms per iteration was used to determine the fundamental EOD frequency. Baseline frequency was defined as the mode frequency over a sliding 1s window. The procedure detected EOD modulations (EODms: chirps, GFRs) when a frequency elevation of 3Hz or greater over the baseline persisting for a duration of at least 10ms but less than 30s, and a minimum inter-EODm interval of 100ms. The beginning and end of each detected EODm was defined by the time points at which EODf came within 1Hz of the baseline frequency. Duration and peak frequency parameters were automatically quantified for each EODm. Frequency traces were visually inspected following analysis to ensure accurate identification and quantification of EODms. Some chirps had more than one distinct frequency peak. To quantify these peaks and to determine whether there were sex differences in the production of multi-peaked chirps, an observer blind to the sex of the fish visually counted the number of peaks for each chirp. Chirps and GFRs were readily distinguished, and were separated visually based on the degree of FM (described in Turner et al. 2007). Jamming avoidance responses (JARs) and JAR-like (JARl) behaviors were quantified by taking the difference between the baseline frequency prior to stimulus onset and the plateau frequency during the stimulus playback. In recordings where the JARl behavior shifted between discrete frequency plateaus, the higher frequency value was used.

Hormone analysis

After behavioral recordings, fish were anesthetized using 0.004% clove oil or 0.075% 2-phenoxyethanol, and blood was drawn from the vertebral sinus into a heparinized syringe. Samples were stored on ice until centrifugation. Plasma was drawn off following centrifugation and stored at less than minus 20°C until assayed. Blood samples were taken within a day after behavioral recordings.

Plasma was assayed for 11-ketotestosterone (11-kT) (fish from Brazil and Peru) and testosterone (T) (fish from Brazil only) using enzyme immunoassay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's directions. Dilutions ranged from 1:25 to 1:100 for 11-kT and 1:20 for T. The intraassay variations over four 11-kT assays were 2.5, 8.4, 13.9 and 27.2%; and the interassay variation was 17.7%. Intraassay variation for the T assay was 19.5%.

Statistical analysis

Statistical analyses were performed with Statistica 7 (StatSoft Inc.; Tulsa, OK, USA). Sex and population differences in morphometry, androgens and EOD were analyzed using a factorial ANOVA with sex and population as factors. Hormonal and morphometric correlates of EOD behavior were analyzed using a general linear model with sex as a categorical predictor. Effects of stimulus presence (pre-stimulus, stimulus-on, post-stimulus) and stimulus frequency (± 150 , ± 20 , -5) were analyzed using repeated measures analyses of variance (RMANOVA). Stimulus condition and sex were used as factors. Tukey's Honestly significant difference (HSD) test was used to test for differences among stimulus conditions. To avoid pseudoreplication, statistical tests on sex and population differences in chirp parameters were performed on the average chirp parameters of each fish, rather than on each chirp. Statistical significance was set at an alpha level of 0.05. One individual with an outlying T value of 17ng/mL, 3.5 standard deviations above the mean, was excluded from the analysis.

Results

EOD frequency

EODf in *A. bonapartii* varied substantially across individuals and ranged from 973Hz to 1673Hz. However, EODf was not sexually dimorphic (Factorial ANOVA Sex $F(1,48)=0.004$, $p=0.95$; table 1). EODf was not correlated with body length in either males or females ($F(2,33)=2.79$, $p=0.104$).

Structure of EOD modulations

Like other apteronotid species, *A. bonapartii* produced two distinct types of EODms: chirps and gradual frequency rises (GFRs). GFRs had little frequency modulation (individual average range: 3.5Hz-30Hz) and lengthy, variable durations (individual average range: 34.8ms-10.2s), while chirps had greater FM and short durations (fig.1a). In contrast with *A. leptorhynchus* and *A. albifrons*, where bimodal distributions of FM and duration suggest distinct chirp types (Bastian et al. 2001;Engler et al. 2000;Hagedorn & Heiligenberg 1985;Kolodziejski et al. 2005), the variation in chirp FM and duration in *A. bonapartii* was continuous. *A. bonapartii* chirps comprised a single category with frequency modulations (FM) ranging from 105Hz to 657Hz, and durations ranging from 8.6ms to 159.9ms (fig.1b). Chirps were accompanied by decreases in EOD amplitude and followed by small frequency undershoots. Interchirp intervals ranged between 300ms and 90s, averaging approximately 11s in both sexes (fig.2). Consistent with previous descriptions of *A. bonapartii* EOD modulations, a subset of chirps showed multiple peaks (Turner et al. 2007) (fig.1b inset), with two distinct frequency peaks being the most common. Single-peaked chirps did not differ in duration from multi-peaked chirps ($F(1,1817)=2.88$, $p>0.05$), but did have greater amounts (approximately 50Hz) of FM than multi-peaked chirps peaks ($F(1,1817)=111.65$, $p<0.001$). Chirp FM and duration were highly variable and spanned a wide range of values. However, although chirp parameters varied substantially between individuals, the chirps produced by any individual fish were quite consistent in structure (fig.1b). Variation across individuals explained substantially more of the variance in chirp parameters than variation within individuals (FM: mean square (MS) within individuals =2422, MS between individuals = 565073, $F(1,29)=233$, $p<0.001$; DUR: MS within individuals =0.000037, MS between individuals = 0.005238, $F(1,29)=140$, $p<0.001$).

Effect of stimulus presence and stimulus frequency on EOD modulations

Fish chirped robustly in response to playback stimuli. Chirp rates were greatest during the stimulus presentation than before or after (RMANOVA $p<0.001$). In contrast, the rate of GFR production was greater before the stimulus was presented than during or after (RMANOVA $p<0.005$). This is consistent with chirp and GFR production in other apteronotids (Kolodziejski et al. 2007). Fish did not chirp differently in response to EOD playbacks of different frequencies: stimulus frequency did not significantly affect chirp rate, duration, or FM (RMANOVA $p>0.1$).

Effect of stimulus frequency on the Jamming Avoidance Response (JAR) and JAR-like (JAR-I) modulations

Fish responded to the -5Hz difference frequency with a reliable jamming avoidance response (fig.3). Small, sustained increases in EODf were also produced in response to the -20Hz stimulus and the -150Hz stimulus. Because these responses were similar to JARs, but produced in response to difference frequencies that do not typically elicit JARs in other species, we called these behaviors JAR-like increases (JARIs). Apteronotids can also produce non-selective rises (NSR) in EOD frequency. These elevations in EOD frequency resemble JARs and JARIs; however, the NSR effectively brings the fishes' EODf closer to

that of the jamming fish rather than moving it away (Dye 1987). In *A. bonapartii*, NSRs occurred in response to the +20Hz, and +150Hz stimulus frequencies. In the +20Hz condition, these NSRs often brought the fishes' EOD close to the frequency of the stimulus. Within individuals, the magnitudes of frequency elevation across all difference frequencies was highly correlated, such that fish with relatively robust JARs also had high JARs and NSRs (correlations of JAR/JAR/NSR magnitudes between stimulus frequencies ranged from 0.52-0.81, $p < 0.05$ for all pairwise comparisons). JAR magnitude did not differ between the sexes ($F(1,29)=1.13$ $p > 0.1$).

Sex Differences in EOD modulations

In contrast to previous descriptions of sex differences in EOD behavior of other apteronotid species (including *A. albifrons*, *A. leptorhynchus*, *A. rostratus*), many chirp parameters in *A. bonapartii* were sexually monomorphic. Chirp rates ($F(1,28)=0.98$, $p=0.33$) and GFR rates ($F(1,28)=0.57$, $p=0.45$) were highly variable and did not differ between males and females (fig.4a, table 1). Neither chirp FM ($F(1,21)=0.21$, $p=0.65$) nor chirp duration ($F(1,21)=1.31$, $p=0.26$) differed significantly between males and females (fig.4bc, table 1). Interchirp intervals were also not sexually dimorphic ($F(1,19)=0.02$, $p=0.88$) (table 1). In addition, males and females showed similar chirp rates in response to different stimulus frequencies ($F(4,120)=1.34$ $p=0.26$). Males and females did differ, however, in the production of chirps with multiple frequency peaks. On average, males produced chirps with more frequency peaks than females ($F(1,18)=29.77$, $p < 0.001$) (fig.4d, table 1). This was due to males producing a greater proportion of multi-peaked chirps than females (fig.5).

Hormonal regulation of EOD behavior

Plasma 11-kT levels were approximately six to seven times higher in males than in females ($F(1,38)=5.469$, $p < 0.05$) (table 1). Plasma T levels were not sexually dimorphic ($F(1,26)=0.75$ $p > 0.1$) (table 1). Plasma levels of 11kT and T were not correlated with each other in either males (multiple regression $F(1,17)=0.22$, $p > 0.5$) or females (multiple regression $F(1,6)=0.94$, $p > 0.1$). Circulating androgens (11-kT, T) were not correlated with any EOD behavior whether populations were analyzed together (GLM $p > 0.05$ for all analyses; EODf (fig.6), chirp rate, chirp duration, chirp FM, chirp peaks, JAR magnitude) or separately (GLM $p > 0.05$ for all analyses).

Population differences

Peruvian fish tended to produce higher frequency EODs than Brazilian fish, but this trend was not significant ($F(1,48)=3.90$, $p=0.054$). Chirp duration ($F(1,21)=3.35$ ($p > 0.05$), chirp FM ($F(1,21)=0.10$ $p > 0.05$), and chirp peaks ($F(1,18)=0.63$ $p > 0.05$) also did not differ between populations. There were no significant differences in the magnitude of sexual dimorphism of EODf, chirp rate, chirp duration, chirp FM and peak production between populations ($p > 0.05$ for all interactions between sex and population). Chirp rates, however, were higher in fish caught from Brazil than in fish from Peru ($F(1,28)=6.41$, $p < 0.05$).

Discussion

Sex differences in electrocommunication behavior have been studied in only a few apteronotid species. We analyzed the EOD signals from *A. bonapartii* to understand how the magnitude and direction of sexual dimorphism in communication signals vary across the apteronotid clade. Most parameters of electrocommunication signals in *A. bonapartii* did not differ between sexes: EODf, chirp rate, chirp FM and chirp duration were all sexually monomorphic. There were also no sex differences in the response to different stimulus frequencies or in the magnitudes of the jamming avoidance response and the non-specific response. The production of complex chirps with multiple frequency peaks, however, was

sexually dimorphic in *A. bonapartii*, and males were more likely to produce chirps with a greater number of peaks than females.

In *A. bonapartii*, individual variability in EODf and chirp parameters was high, paralleling data from Turner et al. (2007). EODf and chirp FM, in particular, spanned ranges which placed them among the widest documented in the Aptereronotidae. Variation in EODf and most chirp parameters (chirp rate, chirp FM and chirp duration), however, did not segregate by sex as it does in many apteronotids. In other apteronotids, sex differences in EODf and chirping are prominent, with values that do not overlap even at sample sizes much smaller than those in our present study (Dunlap et al. 1998; Kolodziejski et al. 2005). The lack of sex differences in EODf, and chirp rate, FM and duration in *A. bonapartii* is further supported by the lack of a correlation of EODf and these chirp parameters with 11-kT. Because circulating hormone levels can be influenced by social environment (Dunlap et al. 2002; Wingfield et al. 1990), it is possible that variation in the duration of captivity, or in exposure to conspecific playbacks, may obscure relationships between 11-kT and behavior. Despite this, 11-kT levels did not differ significantly across males from Brazil, whose blood was sampled within a few days of capture, and males from Peru, whose blood was sampled after months in the laboratory ($F(1,29)=0.143$, $p>0.1$). In addition, 11-kT did not differ between fish that had been given chirp playbacks, and fish that had not ($F(1,26)=1.17$, $p>0.1$, data not shown). Our results suggest that sexual monomorphism in EOD signal parameters may be more widespread among apteronotids than has been previously demonstrated, and that spectrottemporal complexity (e.g. differences in FM rates and/or the number of peaks in a chirp) may be a locus of diversification in EOD signals.

Species variation in sexual dimorphism of EOD behavior

Sex differences in electrocommunication behavior are common across the apteronotid family. In *A. leptorhynchus*, *A. rostratus*, *A. albifrons*, *Adontosternarchus devenanzii* and *A. bonapartii*, the magnitude and direction of sexual dimorphism in EOD parameters, including EODf, chirp rate, chirp duration, chirp FM, and FM peak production, varies greatly across species (table 2).

In *A. leptorhynchus* and *A. rostratus*, males produce higher EODfs than females (Hagedorn & Heiligenberg 1985; Kolodziejski et al. 2005). In *A. albifrons*, this pattern is reversed with males producing lower EODfs than females (Dunlap et al. 1998; Kolodziejski et al. 2005). In *A. devenanzii* and *A. bonapartii*, there are no sex differences in EODf (Zhou & Smith 2006). Furthermore, the magnitude of sex differences in EODf is evolutionarily labile and differs even within species, across different populations of *A. albifrons* (Ho & Smith 2009; Rack & Smith 2009).

Chirp rate is also sexually dimorphic in *A. leptorhynchus* and *A. rostratus*, with males chirping at greater rates than females (Zupanc & Maler 1993). However, chirp rate does not differ between the sexes in *A. albifrons*, *A. devenanzii*, or *A. bonapartii*.

Finally, chirp structure is sexually dimorphic in every species that has been studied, but different aspects of chirp structure differ between species: in *A. rostratus* and *A. leptorhynchus*, males produce “bigger” chirps with greater amounts of FM (type-1 chirps) (Bastian et al. 2001; Hagedorn & Heiligenberg 1985) than females; in *A. albifrons*, males produce chirps with longer duration than females (Dunlap & Larkins-Ford 2003; Kolodziejski et al. 2005). Chirps in *A. bonapartii* were not sexually dimorphic in FM or duration. Nevertheless, *A. bonapartii* generates multi-peaked chirps which are produced in a sex-specific manner: males, but not females, produced chirps containing multiple frequency peaks. Although single-peaked chirps tended to have greater amounts of FM than multi-peaked chirps, variation was high, and chirp FM was not sexually dimorphic. Time intervals

between peaks within a multi-peaked chirp were much shorter than those between individual chirps – less than half that of the briefest interchirp interval, and more than two orders of magnitude less than the average interchirp interval. Thus, multi-peaked chirps represent changes in the spectrotemporal structure of individual chirps, rather than clustering due to changes in the timing of multiple chirps. This sex difference in the production of chirps with multiple frequency peaks is similar to sex differences in chirping in *Adontosternarchus devenanzii* (Zhou & Smith 2006). As in *A. bonapartii*, *A. devenanzii* males were much more likely than females to produce multi-peaked chirps (table 2). Because current apteronotid phylogenies provide differing placements for *A. bonapartii* relative to *A. devenanzii* (Turner et al. 2007), it is unclear whether the evolution of multi-peaked chirp represents a single event, or whether the production of multi-peaked chirp peaks arose multiple times. However, in *Porotergus gimbeli*, *Sternarchogiton nattereri* and *Adontosternarchus balaenops*, taxa which are consistently positioned more closely to *Adontosternarchus devenanzii* than to *A. bonapartii*, multi-peaked chirps are not produced (Turner et al. 2007), providing some evidence that multiple peaks evolved independently within *Adontosternarchus* and in *A. bonapartii*.

Are EODf and chirping subject to different selective pressures?—A survey of sexually dimorphic electrocommunication behavior in apteronotids reveals that while the EOD can either be sexually monomorphic or dimorphic, chirping behavior is always sexually dimorphic. It is instructive to consider how sexual selection acts on these two traits, which are both involved in reproductive behavior, but which have distinct functions and production mechanisms. The EOD is a continuously produced signal, with dual functions in communication and electrolocation. By contrast, chirping is a dynamic signal, produced only transiently during social interactions, and is used only for communication. The relatively low degree of interspecific variation in sexual dimorphism of EODs relative to chirping may result from constraints that prevent its elaboration. These constraints may be functional (e.g. the demands of electrolocation prevent modifications in EOD) or ecological (e.g. changes in EOD incur a survival cost through increased energetic demands (Salazar & Stoddard 2008) or conspicuousness to predation (Stoddard 1999)).

Are different chirp parameters subject to different selective pressures?—

Chirping behavior is sexually dimorphic in all apteronotids that have been studied. However, there are species differences in which parameters are sexually dimorphic. Sex differences may be variously encoded in chirp FM, chirp duration, chirp rate, and/or number of chirp peaks, depending on the species (table 2). There are several hypotheses which could explain this species-level divergence in male chirp signals.

For instance, the signal content of each chirp parameter may vary across species, as could female preferences for different indicators of quality, and direct or indirect benefits. The costs of particular signal parameters are not well studied in apteronotids; however, differential predation pressures and/or the metabolic costs of producing different chirp parameters could lead to trait diversity across species. Alternatively, variation in signal parameters could result from phylogenetic inertia. Although the range of variation in electrocommunication signals across currently studied apteronotids suggests that chirp traits are evolutionarily labile, comprehensive sampling of chirping behavior across a robust apteronotid phylogeny will be required to test this hypothesis.

Another possibility is that signal elaboration results from optimizing signal transmission. Weakly-electric fish species are highly diverse and span a range of social systems and ecological niches in which communication and electrolocation must occur (e.g. (Hagedorn & Heiligenberg 1985; Winemiller & Adite 1997)). These differences in social and ecological environment, in conjunction with the sensory characteristics of each species, will

produce differing sets of conditions under which EOD signals are propagated and detected (Endler 1992). Thus, a sensory drive model of chirp diversification predicts that differences in receiver physiology and/or habitat characteristics across species may drive the elaboration of different chirp parameters by optimizing detectability. In *Apteronotus leptorhynchus*, where chirp FM is sexually dimorphic, high- and low-frequency chirps are encoded by different mechanisms that maximize detectability in particular social contexts. For instance, the large difference in EOD frequency during interactions between males and females and the resulting high-frequency beat pattern is disrupted most effectively by large chirps (Benda et al. 2005). This is consistent with the hypothesis that the high-frequency of chirps produced by males in reproductive contexts promotes signal detection by females. Conversely, low-frequency chirps are more conspicuous, and are also produced more often, in same-sex contexts where the interaction of similar EOD frequencies produces a low-frequency beat pattern. The social environment in which chirps occur (i.e. the proximity, number, and sex of interacting individuals), the variation of EOD frequency across individuals and sexes, and the mechanisms by which electrosensory systems encode complex signals created by multiple interfering EODs, are thus likely to influence the diversification of sexually dimorphic chirp parameters in different species. Further comparative studies of electrosensory mechanisms and how they encode chirping behavior are necessary to test this hypothesis.

Coevolution of sexual dimorphism in EOD and morphology—The effect of sexual selection on trait evolution has long been a subject of interest to evolutionary biologists (Partridge & Harvey 1986); however, the mechanisms by which different sexually-selected traits may interact, or may diversify in different ways across taxa are still under investigation. Studies predict several possibilities for elaboration in different secondary sexual traits. For example, traits under similar intensities of sexual selection may show positive correlations in elaboration, or may interact to produce a negative correlation (Gilliard 1956).

While a number of empirical studies (e.g. (Badyaev et al. 2002; Shutler & Weatherhead 1990)), often in birds, have been done in the context of auditory and visual signals, these questions have not been extended to sensory modalities such as electroreception. The diversity in the magnitude and direction of sexual dimorphism of signaling traits in the Apterontidae provides a chance to consider how sexual selection affects the evolution of multiple characters, as well as the generality of these effects across sensory modalities.

Here, we consider a scenario involving the effects of sexual selection on interactions between signals in separate sensory modalities – specifically, we focus on EOD and morphology, which are roughly analogous to song and plumage in avian taxa. *A. bonapartii* displays prominent sex differences in morphology, and males can possess dramatically elongated snouts (Hilton & Fernandes 2006). In the absence of much data concerning the reproductive behavior and natural history of this species, the causes of snout-length dimorphism remain unclear. However, several examples of sexually dimorphic head morphology are documented in the apteronotids. These species include *A. leptorhynchus* where males have longer snout lengths than females (Hagedorn & Heiligenberg 1985), *Sternarchorhynchus cf. roseni* and *Sternarchogiton nattereri*, in which males develop external dentition (Rapp Py-Daniel & Cox Fernandes 2005), and *Parapteronotus hasemani* and *Compasaraia samueli* (Albert & Crampton 2009; Cox Fernandes et al. 2002), in which males develop extreme snout and jaw elongation. Behavioral observations of *A. leptorhynchus* (Hagedorn & Heiligenberg 1985; Triefenbach & Zakon 2008) and *C. samueli* (Albert & Crampton 2009) suggest that sex differences in head morphology may result from intra-sexual competition between males. Intraspecific variation in snout morphology may

also be associated with differences in ecological specialization, as in the trophic morphs of the mountain whitefish (Whiteley 2007).

The prominent sexual dimorphism in snout length coupled with the relative lack of sex differences in most EOD behavior in *A. bonapartii* raises further questions about the relationship between different signaling traits. Do trade-offs exist between EOD and morphological traits? Theory suggests that in the presence of multiple condition-based male ornaments, female preference may displace one trait in favor of another more prominent or 'honest' signal (Schluter & Price 1993), and could in part explain why increases in song ornamentation are correlated with decreases in carotenoid-based male plumage ornamentation in finch species (Badyaev et al. 2002). In addition, if the associated costs are high, selection should favor "transference" to a less costly alternative, as has been hypothesized for bowerbird plumage and bowers (Gilliard 1956). Thus transference to sexual dimorphism in EOD may occur, for example, if that is less costly than developing sexual size dimorphism.

A brief survey of the apteronotids reveals conflicting phylogenetic evidence for the transference hypothesis in the clade as a whole. In *A. bonapartii* sexual dimorphism in snout morphology is coupled with a relatively low degree of sexual dimorphism in electrocommunication signals. This inverse correlation is paralleled in the extremely snout dimorphic *P. hasemani*, which shows little sexual dimorphism in EODf, although chirping has not yet been studied (Cox Fernandes et al. 2002; Cox Fernandes & Podos 2004). In contrast, *A. leptorhynchus* has relatively high degrees of sexual dimorphism in both morphology and EOD signals, while *A. albifrons* and *A. devenanzii* have both low to moderate levels of sexual dimorphism in morphology and EOD signals. It would be of interest to determine the nature of this relationship given additional taxon sampling and data on phylogenetic relationships between apteronotid species.

Steroid regulation of EOD behaviors

Circulating androgen (T, 11-kT) levels were not correlated with EODf nor with any aspect of chirping. In *A. albifrons* and *A. leptorhynchus*, sexually dimorphic EOD traits are sensitive to androgens, whereas sexually monomorphic traits are androgen-insensitive (Zakon & Dunlap 1999). Specifically, 11-kT implants masculinize the sexually-dimorphic EODf of both species, as well as sexually-dimorphic chirping in *A. leptorhynchus*, but do not affect sexually monomorphic chirp rates in *A. albifrons* (Dunlap et al. 1998; Schaefer & Zakon 1996). Although male *A. bonapartii* had higher 11-kT and produced chirps with more peaks than females, 11-kT levels within males were not correlated with the number of peaks in their chirps. Direct manipulations of 11-kT will be needed to determine whether the production of multi-peaked chirps in apteronotid electric fish is androgen-sensitive. Alternative pathways such as estradiol, non-steroidal mechanisms, or organizational effects, could also contribute to sex differences in chirp peaks. In *A. leptorhynchus*, sex differences in EODf are regulated by estrogens as well as by androgens (Schaefer & Zakon 1996). Furthermore, androgen treatment of females *A. leptorhynchus* only partially masculinizes chirping (Zakon & Dunlap 1999). These results suggest that although activational effects of androgens are important regulators of sex differences in apteronotid signals, additional hormonal and non-hormonal mechanisms also contribute to sexual dimorphism in EODs and chirping.

JAR and JAR-like modulations

The jamming avoidance response (JAR), a short-term elevation of EODf in response to a jamming stimulus close to the fishes' own EODf, is typically produced in response to small DFs (Bullock et al. 1972a). This elevation may be unique to apteronotids which, unlike

Eigenmannia sp., are only able to respond to jamming stimuli by increasing EODf (Heiligenberg et al. 1996). In *A. bonapartii*, we not only recorded robust JARs responses to -5 stimuli, but also JARl responses to -20 and -150Hz stimuli. Fish also made frequency elevations in response to stimulus frequencies above their own EODf (+20, +150Hz), a response consistent with communication signals called non-selective responses (NSR) in other apteronotid species (Bastian et al. 2001; Dye 1987). The JAR, JARl, and NSR present qualitatively similar features: each response is a sustained frequency increase of similar magnitudes, and which remain elevated throughout the duration of the stimulus. The NSR and JARl, like the JAR, begins with the onset of the stimulus playback and varies with the magnitude of the DF (Dye 1987).

Eigenmannia sp. and *A. leptorhynchus*, produce JARs in response to difference frequencies up to 20Hz, with the strongest responses elicited between 2 and 6Hz. As DF increases, the amplitude of the JAR decreases progressively, until almost no response is observed at a DF of 20Hz (Bullock et al. 1972b; Dye 1987). The presence of a sustained EODf elevation to unexpectedly large difference frequencies in *A. bonapartii* suggests that the sensitivity of the electrosensory circuits that trigger the JAR in this species may have expanded, responding to beat frequencies as fast as 150Hz.

The significance of JARls and NSRs however, remains unclear. It is unlikely that these responses function in jamming avoidance for two reasons. First, it is not known whether the beat pattern created by 150Hz difference frequencies has any jamming effect on the ability of *A. bonapartii* to electrolocate. Second, the small change in EODf produced by the JARl (1-2Hz) relative to the difference frequency (up to 150Hz) would alter DF negligibly and is unlikely to affect electrolocation ability. In *Eigenmannia* and *Sternopygus*, electrolocation performance deteriorates when a jamming signal approaches a fishes' own EOD (Heiligenberg 1973; Matsubara & Heiligenberg 1978). Furthermore, the direction of frequency change during the NSR is in the opposite direction of that expected for jamming avoidance. It is possible that JARls and NSRs in *A. bonapartii* have a communication function. For example, in *A. albifrons*, slow-rising modulations function as submissive signals (Serrano-Fernandez 2003), and in *Eigenmannia*, the JAR may function as a social signal (Kramer 1987). Alternatively, NSR-like modulations produced by freely-interacting dyads of *A. leptorhynchus* may represent intentional jamming of higher frequency rivals (Tallarovic & Zakon 2005). JARl behavior in *A. bonapartii* may thus signal aggressive challenges; JARl elevations during the +20Hz DF occasionally came within several hertz of matching the stimulus frequency.

Population differences in EOD behavior

Population differences in morphology, pigmentation and EOD behavior have been observed in several apteronotid species. In particular, population differences in chirp structure are apparent in *P. hasemani* and *Porotergus gimbellei* (Turner et al. 2007). *A. bonapartii* populations also differed in chirp rates, with fish from the Brazilian population producing chirps at higher rates than fish from the Peruvian population. Because Brazilian fish were recorded within a day of capture rather than following a longer period of housing in captivity, variation in chirp rate might be explained by differences in environmental conditions. However, despite these population differences in the rate of EOD modulations, there remained no sex differences in either population.

Information content of EODf and EOD chirp structure

EODf in *A. bonapartii* spanned a range of more than 700Hz (943Hz-1673Hz), despite the fact that EODf was sexually monomorphic. This intraspecific variation in EODf exceeds that of apteronotid species with sexually dimorphic EODs (e.g EODf ranges from 824Hz to

1110Hz in *A. albifrons* and 600Hz to 1000Hz in *A. leptorhynchus*) (Kolodziejski et al. 2005; Meyer et al. 1987). Sexual monomorphism in the EOD of *A. bonapartii* results from a wide variability of EODf in both sexes, rather than from a compression of frequency range. The magnitude of chirp FM was also highly variable and not sexually dimorphic. *A. bonapartii* has the broadest range of EODf and chirp FM of any apteronotid species studied (Turner et al. 2007) (present study); however, the wide range of 'signal space' does not appear to convey sex-specific information. Instead, EODf and chirp structure in *A. bonapartii* may communicate other information such as individual identity or social status.

Effects of stimulus frequency on chirping behavior

Playback frequency had no effect on chirping behavior in *A. bonapartii*. Chirping was also unaffected by the frequency of the stimulus in *A. devenanzii*, another species with a sexually monomorphic EODf. By contrast, in *A. leptorhynchus* and *A. albifrons*, both species with sexually dimorphic EODfs, chirp rate depends strongly on stimulus frequency (Bastian et al. 2001; Engler & Zupanc 2001; Kolodziejski et al. 2007). Thus, our results combined with those in other species, support the hypothesis that the dependence of chirping on the relative EODfs of interacting fish depends on whether the EODf is sexually dimorphic. DF does not affect chirp rates in sexually monomorphic species, but does influence chirp rate in sexually dimorphic species. In sexually dimorphic, but not sexually monomorphic species, receivers may be extracting and responding with chirps to salient, sex-specific, information about signalers based on EODf.

Apteronotids as a model for studying the origins of sexual dimorphism in behavior

Although phyletic sampling remains sparse, continued study of the apteronotids should reveal an important source of behavioral, ecological, molecular and physiological data for understanding the origins of sexually dimorphic behavior. This study raised questions about the relationship between sexual dimorphism and sexual diethism, and showed that extreme sex differences in morphology are not necessarily correlated with sex differences in EOD behavior. Our data also contribute to an emerging picture of remarkable evolutionary lability in the sexual dimorphism of EOD behaviors. Across the Apteronotidae, species differences in EODf and chirp responsiveness to gonadal steroids often explain species divergence in the magnitude and direction of sexual dimorphism. Further studies using hormone removal and replacements are required to clearly determine whether sexual dimorphism of multi-peaked chirps in *A. bonapartii* is mediated by steroid sensitivity. The striking species diversity in the magnitude and direction of sexual dimorphism suggests that electrocommunication behavior evolves rapidly and may be important in apteronotid speciation. Studies aimed at understanding the selective pressures that act on, and the corresponding neuroendocrine mechanisms that underlie sexually dimorphic EOD behaviors, will be useful for understanding how diversity in sexually dimorphic behavior evolves.

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Fig.1.

(a) Scatterplot of frequency modulation (FM) and duration for all chirps and gradual frequency rises (GFRs) in males and females. Each data point represents a single chirp or GFR. Note the large variation in chirp FM and the lack of clustering of chirps into discrete types. (b) FM and duration of chirps produced by individual fish in this study (median $\pm$ interquartile range). Note the large between-individual variation in chirp FM, but the relatively small within-individual variation (interquartile bars) in chirp FM. Chirp characteristics can be individually distinct and are distributed over a wide range. GFRs are not included in this plot. (inset) Traces show EODf over time during chirps with single and multiple peaks.

Fig.2.

Histogram of chirps from all individuals, showing distributions of interchirp intervals. Note changes in bin size.

Fig.3.

(a) Magnitudes of JAR (-5) / JARI (-20, -150) / NSR (+20, +150) in response to each stimulus frequency (mean $\pm$ SE). More robust responses were elicited by stimulus frequencies closer to the fishes' own EOD. (b) Representative JAR, JARI, and NSRs from one individual. Traces show EODf over time. Presence of playback stimulus indicated by dark bar between one and three minutes.

Fig.4.

Chirp parameters in males and females (mean $\pm$ SE). No sex differences were found in (a) the rate of chirp production during playbacks (b) the amount of frequency modulation or (c) duration. (d) The number of peaks per chirp was sexually dimorphic (mean $\pm$ SE; “*”, $p < 0.05$).

Fig.5. Percent of chirps with one, two, three, or more peaks, averaged by individuals. Males are more likely to produce chirps with greater numbers of peaks than are females.

Fig.6.

(a) Relationship between 11-kT and EODf in males and females. Data were analyzed in a general linear model, which yielded no significant correlations in either sex. (b) Relationship between T and EODf in males and females. Data were analyzed in a general linear model, which yielded no significant correlations in either sex.

Table 1Sex differences in size, signal parameters, and hormone levels¹

Parameter	Males	Females
Total length (mm)	164±6.6 (21)	161±11.6 (16)
EODf (Hz)	1279.9±22.3 (31)	1258.9±35.8 (21)
Chirp Rate (no. per min) ²	26±8 (21)	15±7 (10)
Chirp Duration (ms) ²	30±3 (16)	25±1 (8)
Chirp FM (Hz) ²	276±31 (16)	273±30 (8)
Peaks per chirp (no.) ^{2*}	2.2±0.15 (11)	1.2±0.11 (6)
Interchirp Interval (ms) ²	10.8±1.7 (7)	11.6±3.3 (14)
11-kT (ng/mL) [*]	0.651±0.126 (30)	0.105±0.014 (12)
T (ng/mL)	7.788±0.641 (20)	8.769±0.786 (8)

¹ mean +/- SE, sample size in parentheses² chirp parameters are presented based on averages for individual fish

* significant sex difference, p<0.01

Table 2

Comparison of sex differences in different apteronotid species

Species	EOdf	Chirp Rate	Structure
<i>A. leptorhynchus</i> ^{1,2,3,4}	M>F	M>F	<i>Duration</i> M>F <i>FM</i> M>F <i>Peaks</i> M=F ⁸
<i>A. albifrons</i> ^{3,5,6}	M<F	M=F	M>F M=F M=F M>F ⁸
<i>A. deventanzii</i> ⁷	M=F	M=F	M=F M=F M=F M>F
<i>A. bonapartii</i>	M=F	M=F	M=F M=F M>F

¹ Dye 1987.

² Bastian et al. 2001. (males produce a greater proportion of high-frequency chirps (type-1) than females; type-1 chirps have more FM and longer durations than low-frequency (type-2) chirps, which are produced by both sexes)

³ Kolodziejewski et al. 2005.

⁴ Zupanc & Maler 1993.

⁵ Dunlap et al. 1998.

⁶ Dunlap & Larkins-Ford 2003.

⁷ Zhou & Smith 2006.

⁸ These species do not typically produce multi-peaked chirps.