

Sex and Species Differences in Neuromodulatory Input to a Premotor Nucleus: A Comparative Study of Substance P and Communication Behavior in Weakly Electric Fish

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ABSTRACT: Many electric fish species modulate their electric organ discharges (EODs) to produce transient social signals that vary in number and structure. In *Apteronotus leptorhynchus*, males modulate their EOD more often than females, whereas in *Apteronotus albifrons*, males and females produce similar numbers of modulations. Sex differences in the number of EOD modulations in *A. leptorhynchus* are associated with sex differences in substance P in the diencephalic nucleus that controls transient EOD modulations, the CP/PPn. These sex differences in substance P have been hypothesized to regulate sex differences in the production of EOD modulations. To comparatively test this hypothesis, we examined substance P immunoreactivity in the CP/PPn of male and female *A. leptorhynchus* and *A. albifrons*. Because the number of EOD modulations is sexually monomorphic in *A. albifrons*, we predicted no sex difference in substance P in the CP/PPn of this species.

Contrary to this prediction, male *A. albifrons* had significantly more substance P in the CP/PPn than females. This suggests that sex differences in substance P are not sufficient for controlling sex differences in the number of EOD modulations. Modulation structure (frequency excursion and/or duration), however, is also sexually dimorphic in *A. leptorhynchus* and is another possible behavioral correlate of the sexually dimorphic distribution of substance P. The present study found pronounced sex differences in the structure of EOD modulations in *A. albifrons* similar to those in *A. leptorhynchus*. Thus, sex differences in substance P may influence sex differences in the structure, rather than the number, of EOD modulations. © 2004

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INTRODUCTION

Reproductive communication behavior is often sexually dimorphic, seasonally variable, and/or hormon-

ally modulated. Studies of sex differences in neural circuits that control such behaviors have provided insight into proximate mechanisms of plasticity in social behavior (Kelley, 1988). The electric organ discharge (EOD) of gymnotiform fish provides an accessible system for studying the neural and hormonal control of sexually dimorphic behavior. The EOD is used for electrolocation and communication and is a remarkably plastic and readily quantifiable behavior. EOD frequency and waveform are species-specific, sexually dimorphic, seasonally variable, and steroid-sensitive (Hopkins, 1988; Zakon, 1993). In addition, the neural circuit that controls the EOD is

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relatively simple and well-characterized (Heiligenberg et al., 1996; Metzner, 1999; Smith, 1999). In most species, the electromotor circuit spans only two to four synapses from thalamic and mesencephalic premotor nuclei to the medullary pattern generator for the EOD, the pacemaker nucleus (PMn), to the electric organ. Furthermore, both the structure of the EOD and the extent and direction of sexual dimorphism in electrocommunication behaviors have diversified across closely related species. This diversity facilitates comparative studies on sexual dimorphism and neural control of communication behavior.

The EOD of many gymnotiform species can be modulated to produce discrete communication signals known as chirps and rises. Characterized by transient changes in EOD frequency and amplitude, chirps and rises can be produced spontaneously, but are most often elicited by conspecific EODs or by playbacks of sinusoidal voltage signals simulating EODs (Larimer and MacDonald, 1968; Dye, 1987; Maler and Ellis, 1987; Zupanc and Maler, 1993; Engler et al., 2000; Engler and Zupanc, 2001). EOD modulations are controlled by the diencephalic central posterior/prepacemaker nucleus (CP/PPn). The CP/PPn sends descending glutamatergic inputs to the PMn. These glutamatergic inputs depolarize relay and pacemaker cells in the PMn, thereby causing a transient increase in EOD frequency (Kawasaki et al., 1988; Zupanc and Heiligenberg, 1992; Heiligenberg et al., 1996; Zupanc and Maler, 1997; Metzner, 1999).

The brown ghost knifefish (*Apteronotus leptorhynchus*) and the black ghost knifefish (*Apteronotus albifrons*) both produce similar chirp-like modulations when stimulated with a playback signal. The two species differ, however, in the degree of sexual dimorphism of chirp behavior. The number of chirps produced under identical stimulus regimes is sexually dimorphic in *A. leptorhynchus* but sexually monomorphic in *A. albifrons* (Dunlap et al., 1998). Male *A. leptorhynchus* produce 20 to 40 times more chirps than females whereas similar numbers of chirps are produced by each sex in *A. albifrons* (Zupanc and Maler, 1993; Dulka and Maler, 1994; Dunlap et al., 1998). Studies investigating the neural control of chirp production in *Apteronotus* have focused only on *A. leptorhynchus*. Results from these studies suggested that sex differences in chirping behavior might be related to sex differences in substance P-containing afferents to the CP/PPn (Zupanc and Maler, 1993; Dulka et al., 1995). The CP/PPn in male *A. leptorhynchus* contains substantially more substance P-like immunoreactive (SPlir) fibers than in females (Weld and Maler, 1992; Dulka et al., 1995). Moreover, treating females with androgens both up regulates SPlir in the

CP/PPn and increases the number of chirps produced (Weld et al., 1991; Dulka et al., 1995).

The purpose of this study was two-fold. First, to further test the hypothesized relationship between substance P and chirp rate, we investigated whether the association between SPlir in the CP/PPn and chirp rate was conserved in the closely related species, *A. albifrons*. If sex differences in substance P afferents to the CP/PPn underlie sex differences in chirp rate, we predicted no sex difference in SPlir within the CP/PPn of *A. albifrons*, which is sexually monomorphic for chirp rate. Second, we measured structural parameters of chirps, including duration and frequency excursion, to test the hypothesis that sex differences in chirp structure were associated with sex differences in SPlir in the CP/PPn. Previous studies in *A. leptorhynchus* have demonstrated that male and female chirps differ not only in number, but also in structure; male chirps have more extensive frequency and amplitude modulation than female chirps (Hagedorn and Heiligenberg, 1985; Dulka and Maler, 1994; Bastian et al., 2001). Chirp structure in *A. albifrons* may also be sexually dimorphic (Dunlap and Larkins-Ford, 2003). If sex differences in substance P regulate sex differences in chirp structure, we predicted a consistent association between sex differences in chirp structure and sex differences in substance P afferents to the CP/PPn in both *Apteronotus* species.

MATERIALS AND METHODS

Subjects

Twenty reproductively mature *A. leptorhynchus* (9 male, 11 female) and 24 reproductively mature *A. albifrons* (12 male, 12 female) were purchased from commercial suppliers and housed in individual 36- or 64-l tanks within two 2000-l recirculating aquarium systems. The tanks were maintained on a 12:12 light/dark cycle at 26.0–26.4°C, pH 4.5–6.0, and conductivity of 100–300 $\mu\text{S} \cdot \text{cm}^{-1}$. The sex of each fish was initially determined by EOD frequency and later confirmed by postmortem inspection of the gonads. This study was conducted within the guidelines outlined by the National Institute for Health's "Guide for the Care and Use of Laboratory Animals," and all protocols were approved by the Bloomington Institutional Animal Care and Use Committee (BIACUC).

Behavioral Data Collection

Behavioral tests were conducted in the dark in a 38-l tank maintained at 25.8–26.1°C and at a conductivity of 100–200 $\mu\text{S} \cdot \text{cm}^{-1}$. Depending on body size, fish were placed in one of three opaque plastic tubes with removable end-caps

(internal tube diameters: 3.2, 3.8, 5.1 cm). The recording tubes were secured to the bottom of the tank and surrounded by four carbon or Ag/AgCl electrodes: two at the head and tail of the fish to record the fish's own discharge and two on either side of the fish (orthogonal to the recording electrodes) to deliver playback stimuli. To facilitate recordings, plastic mesh was placed over each end-cap and over a 2 cm gap in the middle of each recording tube. The signal from the recording electrodes was amplified and band-pass filtered (Model P-55 preamplifier; Grass Instruments, W. Warwick, RI; gain 100X, 0.1 Hz–10 kHz).

EOD frequency was continuously monitored with a Fluke 187 multimeter, and this frequency reading was used to determine the frequency of playback stimuli (see below). Sinusoidal voltage stimuli, simulating the presence of another electric fish, were produced with a function generator (Instek model GFG 8216A). Stimuli were attenuated to a field intensity of 1.5–2.0 mV cm⁻¹ (measured midway between the stimulating electrodes), passed through a transformer to remove any DC offset, and delivered through the second pair of electrodes. The amplified signal from the recording electrodes was recorded on the left channel of a sound card (SoundBlaster Live; Creative Technologies), and a copy of the stimulus signal from the function generator was recorded on the right channel. These signals were digitized at 44.1 kHz with Cool Edit Pro (Syntrillium, Phoenix, AZ).

During each recording session, a series of five stimuli was presented to the fish in random order. The stimuli varied in their difference frequency (DF) relative to the fish's own EOD frequency. Many studies have used only a single stimulus, or a narrow range of stimuli (i.e., 3–10 Hz below the fish's EOD frequency) during playback experiments because stimuli close to the subject's own EOD frequency elicit more chirps (Dye, 1987; Maler and Ellis, 1987; Engler and Zupanc, 2001; Dunlap, 2002; Dunlap and Larkins-Ford, 2003). We used a wider range of stimuli (–5, ±20, and ±150 Hz), however, because chirp rate and structure can vary with stimulus frequency (Bastian et al., 2001; Engler and Zupanc, 2001; Triefenbach and Zakon, 2003). Stimuli 20 Hz above or below the fish's own EOD frequency (±20 Hz DF) simulated same-sex conspecifics, whereas the +150 and –150 Hz DF stimuli simulated either a conspecific of the opposite sex or a fish of another species. The –5 Hz DF stimulus simulated a same-sex conspecific of almost equal EOD frequency and reliably elicited a jamming avoidance response (Bullock et al., 1972).

Before testing, fish were removed from their home tank, transferred to the testing chamber, and allowed 30 min to acclimate in the dark. Each recording session began with a 4-min baseline recording (no stimulus) followed by 4-min stimulus trials with each of the five stimuli. Each trial consisted of a 1-min “prestimulus” period (stimulus off), a 2-min playback stimulus presentation, and a 1-min “poststimulus” period (stimulus off). The five stimuli were presented in random order with an intertrial interval of 10 min.

Analysis of Electrocommunication Behavior

In *A. leptorhynchus*, transient modulations of the EOD fall into two broad categories that can be distinguished both functionally and mechanistically: chirps and rises. Chirps are relatively rapid (15–100 ms) and have a broad range of frequency modulation (few to hundreds of Hz; Engler et al., 2000; Engler and Zupanc, 2001). Rises are more protracted than chirps (hundreds of milliseconds to tens of seconds), and often have less frequency modulation (few to tens of Hz). Several nomenclatures have been proposed for rises including short, medium, and long rises (Hagedorn and Heiligenberg, 1985; Tallarovic and Zakon, 2002), yodels (Dye, 1987), and gradual frequency rises (GFRs; Engler et al., 2000). Rises are best distinguished from low frequency chirps by duration; however, there is considerable overlap between low frequency chirps and relatively short duration rises (see Fig. 3), which makes precise separation of chirps from rises in this area of overlap difficult. Because of the difficulty in accurately separating chirps from rises along the full extent of their distributions, we will refer to all chirps and rises together as short-term frequency modulations (STFMs). STFMs in this study did not exceed 45 s, distinguishing them from longer-term EOD modulations such as the jamming avoidance response (JAR; Bullock, 1972) and long-term frequency elevations (LTFEs; Oestreich and Zakon, 2002). Although we will refer to chirps and rises collectively as STFMs, we used cluster analyses to define, and independently analyze, different categories of STFMs (see below).

EOD recordings were analyzed offline with customized procedures written by one of the authors (Nelson, 2004) for Igor Pro 4.0 (Wavemetrics). Although the orthogonal placement of the stimulating and recording electrodes substantially attenuated stimulus artifacts, some stimulus contamination was still detected by the recording electrodes. Each recording was therefore subjected to a playback removal procedure. The playback signal from the function generator was recorded directly on the right channel, but the contamination created by the playback at the recording electrodes differed in phase and amplitude from the original playback signal. We therefore estimated the phase and amplitude of the original playback signal relative to the contamination created by the playback at the recording electrodes. This estimation was performed over a short playback removal window, which was typically 0.05–0.2 s in duration and was selected over a region in which the fish's EOD frequency was uniform. Within the playback removal window, the phase of the playback stimulus was advanced and delayed by a variable number of samples and subtracted from the combined waveform. This process was repeated with different phase-shifts until subtracting the playback waveform minimized the amplitude modulation caused by the playback. The amplitude of the playback stimulus was then adjusted to further minimize beating in the recorded waveform. The phase and amplitude parameters calculated from the playback removal window analysis were then used to phase shift and scale the playback signal and subtract it

from the combined waveform over the duration of the entire recording.

The fundamental EOD frequency was measured with an autocorrelation algorithm. Autocorrelations were calculated with a 6-ms Hanning window, advanced 2 ms per iteration. The short-term fundamental frequency within each window was calculated as the inverse of the time of the maximum autocorrelation function after performing a smoothing spline interpolation. The interpolation was performed over a time segment that resulted in a frequency resolution during analysis of less than 1 Hz.

Previous studies of electrocommunication behavior have used different techniques to measure EOD frequency. These techniques include zero-crossings analysis (Dulka et al., 1995; Bastian et al., 2001; Dunlap and Larkins-Ford, 2003) and the Frequency Analysis algorithm of Cool Edit Pro (Syntrillium), which uses the phase shift of peak frequency components in adjacent Fast Fourier Transformations (FFTs) to calculate frequency (Engler et al., 2000; Tallarovic and Zakon, 2002; Triefenbach and Zakon, 2003). Furthermore, different studies used different sampling rates and different FFT window sizes. To calibrate our analysis technique against those used previously, we synthesized simulated chirps of known duration and frequency excursion and analyzed them with the algorithms used in previous studies. The autocorrelation algorithm used in the present study accurately tracked EOD frequency, and was comparable to, or more accurate than, the zero-crossings and Cool Edit Frequency Analysis algorithms. The autocorrelation algorithm was always more accurate than alternative algorithms when noise typical of naturally recorded signals (e.g., noise from playback stimulus, harmonics, etc.) was present.

An automated procedure was used to identify and count STFMs from the frequency trace. Baseline frequency was estimated over a 2-s window, advancing every 0.5 s, flanking the area of interest. Frequency mode, rather than median or mean, was used to estimate baseline frequency to minimize disruption by large, transient frequency modulations (such as nearby chirps or rises). STFMs were noted whenever the EOD frequency differed from the frequency baseline according to the following parameters: >3 Hz above frequency baseline, duration greater than 5 ms but less than 60 s, and minimum inter-STFM interval of 100 ms. STFMs were binned according to when they occurred during each trial: before, during, or after stimulus presentation. The beginning and end of each modulation were defined by the times at which EOD frequency crossed a threshold 1 Hz above or below the EOD baseline. For each STFM detected, the automated Igor procedure then calculated duration, time to peak frequency, and frequency excursion above and below baseline (Fig. 2). Visual inspection of the frequency trace of each STFM confirmed that the automated procedure correctly identified the modulations and accurately quantified relevant parameters.

Tissue Preparation for Immunohistochemistry

Some of the fish used in the behavioral tests were also used for substance P immunohistochemistry: 13 *A. leptorhynchus* (5 males, 8 females) and 15 *A. albifrons* (7 males, 8 females). Five to ten days after behavioral testing, fish were anesthetized by immersion in 0.075% phenoxyethanol and transcardially perfused with a heparinized Krebs solution (in mM: 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 2.0 CaCl₂; 2 units/mL heparin; saturated with 95% O₂/5% CO₂; pH 7.3) followed by Zamboni's fixative (4% paraformaldehyde/0.18% picric acid, in 0.05 M phosphate buffer, pH 7.2; 30–40 min.). Gonads were removed and weighed to assess reproductive condition by calculating the gonado-somatic index ($GSI = \frac{\text{mass}_{\text{gonads}}}{\text{mass}_{\text{body} + \text{gonads}}} * 100$). Brains were removed and postfixed in Zamboni's fixative for 24–48 h and then transferred to a cryoprotectant solution [12% glycerol in 0.1 M phosphate buffered saline (PBS), pH 7.2], which was changed every 24 h until processing.

Brains were rinsed in PBS, freeze-mounted on a sliding microtome, and sectioned at 50 μm . Every fourth section was mounted on a gelatin-coated slide and stained with thionin. Sections containing the central posterior/prepacemaker nucleus (CP/PPn) were identified by using the *A. leptorhynchus* brain atlas (sections 17–19; Maler et al., 1991). Because of nearly identical cytoarchitecture, neuronal landmarks used to locate the CP/PPn in *A. leptorhynchus* were also used to identify the CP/PPn in *A. albifrons*.

Substance P Immunohistochemistry

To control for variation in immunohistochemical processing, most brains were batch-processed in parallel: one brain from each species and sex was used in each immunohistochemical procedure whenever possible. Sections containing CP/PPn were processed for substance P immunofluorescence with a tyramide signal amplification kit (TSA; Molecular Probes T-20922 or PerkinElmer NEL-701A). Free-floating sections were rinsed in PBS-TX (pH 7.2, 0.1% Triton X-100) and immersed for 1 h at room temperature in a 4:1 solution of methanol and 3% H₂O₂ to quench endogenous peroxidase. Sections were then rinsed in PBS-TX and transferred to a 1% blocking reagent (provided in TSA kit) diluted in PBS-TX for 1 h. Sections were incubated (4°C, 36 h) in substance P antibody (rabbit αSP , 1:1000 or 1:5000; Peninsula Laboratories, San Carlos, CA; cat. no. IHC 7451) diluted in 1% blocking reagent with 0.01% thimerosal. Control sections were treated similarly except that the primary antibody was omitted and replaced with blocking reagent. Sections were then rinsed and incubated (room temperature, 1 h) in horseradish peroxidase (HRP)-conjugated goat antirabbit IgG (1:150; Molecular Probes G-21234) diluted in 1% blocking reagent. Sections were rinsed in PBS-TX and transferred to a fluorescein-conjugated tyramide solution (provided in TSA kit) diluted 1:100 in amplification buffer (provided) for 10 min, and protected

from light. Sections were rinsed in PBS-TX and counterstained with a 15 μM propidium iodide solution (Molecular Probes P-1304) diluted in PBS-TX for 30 min, protected from light. Sections were then rinsed, mounted on gelatin-coated slides, and dried overnight in the dark. Slides were coverslipped with Vectashield soft- or hard-set mounting medium for fluorescence (Vector H-1000 or H-1400). When the soft-set medium was used, the edges of the coverslips were secured using quick-dry white nail polish. Slides were stored in the dark at -20°C .

SPIir Quantification

Sections were analyzed with a confocal microscope attached to a computer running Leica TCS-NT software (version 1.0; Leica Microsystems, Heidelberg, Germany). Sections with propidium iodide staining most closely resembling sections 17–19 in the *A. leptorhynchus* brain atlas (Maler et al., 1991) were selected from each animal for imaging. Under a 10X objective, one side of the bilaterally symmetrical CP/PPn was focused in the center of the field of view. A 40X objective was then used, and the image was centered on the area of the CP/PPn closest to the ventricle. A vertical array of optically sectioned confocal images (z-series) was then captured in each of three different locations along the dorsomedial-ventrolateral axis of the CP/PPn. The propidium iodide counterstaining was used to ensure that sampled images lay within the CP/PPn. A fourth z-series with the same confocal laser and illumination settings was collected from the same section within the dorsal subdivision of the torus semicircularis (TSd), which contained no SPIir. The image stack from the TSd was used to quantify the nonspecific background fluorescence (see below). Each z-series consisted of eight 512×512 pixel images, and each image in the stack was averaged over four scans.

To quantify specific SPIir in the CP/PPn, three optical sections were selected in a randomly systematic manner from each z-series stack of eight images. We randomly selected one of the first three optical sections of the z-series and then selected every third successive optical section. SPIir fluorescence in these images was then measured relative to a background fluorescence intensity estimated from the TSd control images. For each animal, three optical sections from TSd were first processed in Scion Image (Windows version 4.0.2) to determine the threshold pixel intensity for above-background immunofluorescence. A histogram of pixel intensity was generated for each of the three TSd images selected from the z-series stack and the above-background intensity threshold was arbitrarily defined as the intensity of the brightest 1% of pixels in TSd. The above-background threshold from the TSd images was then used to determine the number of pixels brighter than background threshold in the nine images of CP/PPn from each animal.

Statistical Analysis

A *k*-means cluster analysis was used to categorize STFMs from each species (Tallarovic and Zakon, 2002; Dunlap and

Larkins-Ford, 2003). Previous studies have demonstrated that *A. leptorhynchus* EOD modulations can be clustered into relatively robust categories based on parameters such as duration and frequency excursion (Zupanc and Maler, 1993; Engler et al., 2000; Engler and Zupanc, 2001; Tallarovic and Zakon, 2002). *A. albifrons* modulations also appear to fall into distinct categories (Dunlap and Larkins-Ford, 2003). Within each species, male and female spontaneous, evoked, and poststimulus STFMs were combined before clustering. Measurements of positive frequency excursion and duration of STFMs were used in the cluster analysis. Duration values were log transformed, and frequency and log duration values were standardized as z-scores. Because the fish often moved within the recording tube during testing, amplitude modulations could not be measured accurately and were therefore not used to categorize STFMs.

K-means clustering requires an *a priori* assumption of the number of clusters (categories) in a data set. In the present study, we used a cluster value of $k = 3$ after visual inspection of multiple cluster options clearly showed that three clusters produced the most distinct categories (see Fig. 3). Cluster analysis allowed us to categorize STFMs, which collectively varied nonuniformly in duration and frequency modulation, into more discrete subsets. In *A. leptorhynchus*, chirp structure changes as a function of stimulus frequency (e.g., male-like vs. female-like stimuli), which suggests biological relevance for chirp categories (Bastian et al., 2001; Triefenbach and Zakon, 2003).

SPSS 11.5.0 for Windows was used to transform and cluster data. Statistical analyses of STFM parameters and SPIir in CP/PPn were performed with a two-factor ANOVA protocol in Statistica (StatSoft Inc., Tulsa, OK). Data sets were subjected to the Levene's test of homogeneity of variance in SPSS. Data sets that failed this test were logarithmically transformed prior to statistical analysis. Significance was defined by an α value of 0.05.

RESULTS

Reproductive Condition

Gonadal condition and a robust sex difference in EOD frequency demonstrated that all fish were in reproductive condition at the time of testing and perfusion [Fig. 1(A); Table 1]. EOD frequency differed between males and females in both *A. leptorhynchus* [$F(1, 18) = 118.12$; $p < 0.0001$] and *A. albifrons* [$F(1, 22) = 73.04$; $p < 0.0001$]. Consistent with a previous report (Dunlap et al., 1998), the sex difference in EOD frequency was in opposite directions in the two species. EOD frequency was higher in males than in females in *A. leptorhynchus*, but higher in females than in males in *A. albifrons* [Fig. 1(A); Table 1]. In both species, female abdomens were distended with masses of yolking eggs, and male testes were fully recrudesced. Female GSIs ranged from 1.58 to 9.80

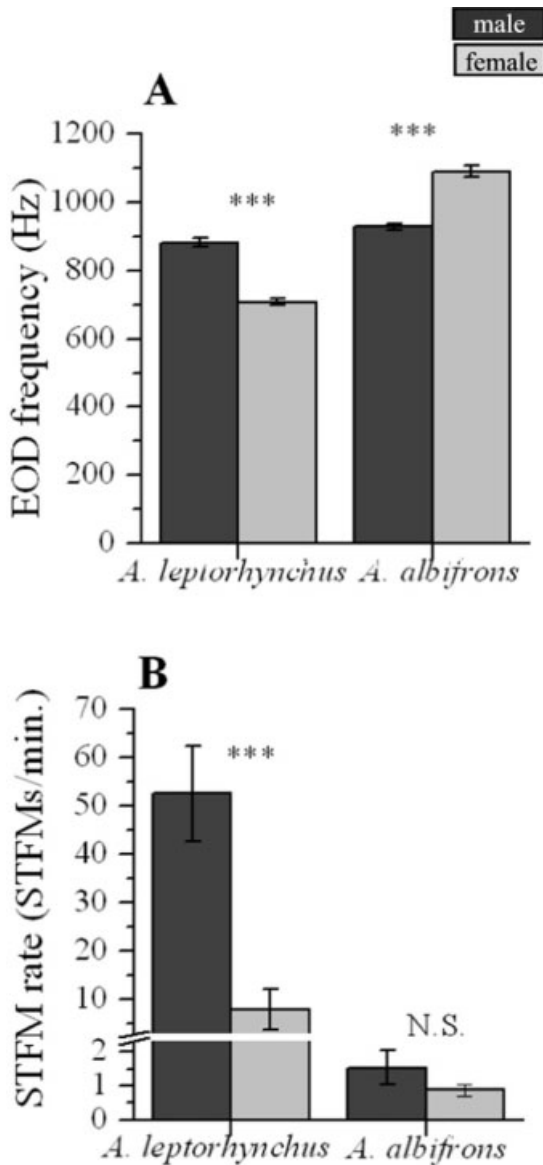


Figure 1 Sex and species differences in EOD frequency and STFMs production rate in *A. leptorhynchus* and *A. albifrons*. (A) EOD frequency was sexually dimorphic in both species, but in opposite directions. In *A. leptorhynchus*, EOD frequency was higher in males ($n = 9$, dark bars) than females ($n = 11$, light bars), whereas in *A. albifrons*, EOD frequency was higher in females ($n = 12$) than males ($n = 12$). (B) *A. leptorhynchus* males produced more STFMs than females; there was no significant sex difference in STFMs rate in *A. albifrons*. Bars represent mean \pm SEM. *** $p < 0.001$, N.S. not significant.

(mean: 6.81) in *A. leptorhynchus* and 0.93 to 8.21 (mean: 4.06) in *A. albifrons*. Male GSIs ranged from 0.31 to 0.95 (mean: 0.53) in *A. leptorhynchus* and 0.32 to 1.18 (mean: 0.75) in *A. albifrons*. GSI values in the present study were two to eight times greater

than those reported in a previous comparative study of sexually dimorphic electrocommunication in these species (Dunlap et al., 1998).

Quantitative Analysis of STFMs: Production Rate

STFMs were binned according to when they were produced: *spontaneous* (4-min baseline recording or 1-min prestimulus period), *evoked* (during stimulus presentation), or *poststimulus* (1-min poststimulus period). Only STFMs produced under evoked conditions (e.g., during electrical stimulation) were used to calculate the production rate of STFMs because previous investigations of sex differences in chirp rate have focused on evoked chirp rates (Dunlap et al., 1998; Bastian et al., 2001; Dunlap and Larkins-Ford, 2003). Including STFMs produced under all conditions (spontaneous, evoked, and poststimulus) in the analysis, however, did not affect the direction or statistical significance of any of the sex and species differences reported.

We did not analyze evoked STFMs as a function of difference frequency because the effects of difference frequency on chirp rate and chirp structure have been described previously (Bastian et al., 2001; Engler and Zupanc, 2001). Pooling all STFMs together allowed us to characterize and analyze modulations produced under a variety of conditions and provided a more complete picture of the range of STFMs structure. In addition, partitioning the data according to difference frequency would have lowered our sample sizes and therefore our statistical power. Sex and species differences in behavioral responses to various stimulus frequencies and environmental conditions will, however, be analyzed as part of a larger comparative study of apteronotid electrocommunication signals.

All 20 *A. leptorhynchus* (9 males, 11 females) produced STFMs spontaneously and in response to at least one of the five electrical stimuli. A total of 7950 STFMs were recorded in *A. leptorhynchus*: 97.4% of these were produced during electrical stimulation (evoked), 1.9% were produced in the minute following stimulation (poststimulus), and 0.7% were produced during baseline recording or prestimulus recording periods (spontaneous). As shown previously (Dye, 1987; Zupanc and Maler, 1993; Dunlap et al., 1998), males produced significantly more STFMs than females in response to a playback stimulus [$F(1, 18) = 20.01$; $p < 0.001$; Fig. 1(B)]. A cluster analysis performed on the STFMs data found three categories of STFMs (see below): high frequency, low frequency, and long duration STFMs. High and low STFMs resembled modulations previously described

Table 1 Sex Differences in EOD Frequency, STFM Production Rate, GSI, and SPLir in *A. leptorhynchus* and *A. albifrons*

	<i>A. leptorhynchus</i>		<i>A. albifrons</i>	
	Male	Female	Male	Female
EOD freq. (Hz)	881 ± 13	707 ± 10	927 ± 9	1090 ± 17
STFM rate (evoked STFM/min)	52.5 ± 9.8	7.97 ± 4.2	1.53 ± 0.5	0.88 ± 0.2
GSI	0.46 ± 0.06	6.8 ± 0.95	0.75 ± 0.1	4.1 ± 0.87
SPLir (1k pixels >1% background)	50.4 ± 6.4	9.4 ± 4.3	44.1 ± 4.0	2.1 ± 0.4

Data are presented as mean ± SE; <<, >> denote direction of significant (i.e., $p < 0.05$) sex differences within each species; = indicates no significant sex difference.

as chirps, whereas long STFM resembled rises. Sexual dimorphism in STFM production rate was due primarily to a significant sex difference in the combined rate of high and low (i.e., “chirp-like”) STFM production [$F(1, 18) = 20.01$; $p < 0.001$]; there was no sex difference in the rate of long (i.e., “rise-like”) STFM production [$F(1, 18) = 0.623$; $p = 0.44$].

All *A. albifrons* produced at least one STFM during electrical stimulation. A total of 647 STFMs were recorded in *A. albifrons*: 48.4% of these were evoked, 25% were poststimulus, and 26.6% were spontaneous. As described previously (Dunlap et al., 1998; Dunlap and Larkins-Ford, 2003), the number of evoked STFMs in *A. albifrons* did not differ between males and females [$F(1, 22) = 1.62$; $p = 0.22$; Fig. 1(B)]. Partitioning data into chirp-like (low + high) and rise-like (long) STFMs also failed to reveal a sex difference in the number of STFMs produced in *A. albifrons*.

Comparison of STFM Structure in *A. leptorhynchus* and *A. albifrons*

STFMs varied widely in duration, positive and negative frequency excursion, rise time, and fall time (Fig. 2). Previous studies have shown that the distributions of the frequency and duration of EOD modulations (chirps and/or rises) covary in a nonrandom manner resulting in distinct “types” (Hagedorn and Heiligenberg, 1985; Dye, 1987; Engler et al., 2000; Bastian et al., 2001; Engler and Zupanc, 2001; Talarovic and Zakon, 2002; Triefenbach and Zakon, 2003). We therefore used a k -means cluster analysis to divide STFMs produced by each species into three categories based on positive frequency excursion and duration (see Materials and Methods). The cluster analysis categorized modulations into three comparable groups in each species: high, low, and long STFMs (Figs. 3 and 4; Table 2).

The high STFMs of *A. leptorhynchus* were relatively short in duration and were distinguished from other STFMs based on their pronounced positive and negative frequency excursions (Fig. 4; Table 2). Most high STFMs were qualitatively similar to modulations previously described as SP-1 and type 1 chirps, although some of the longer duration (>70 ms) high frequency modulations resembled the relatively rare SP-3 and type 3 chirps (Engler et al., 2000; Engler and Zupanc, 2001). Low STFMs in *A. leptorhynchus* were slightly shorter in duration than high STFMs and had much less frequency excursion in both positive and negative phases (Fig. 4; Table 2). Low STFMs were qualitatively similar to modulations previously described as SP-2 and type 2 chirps, although the frequency excursion of low STFMs in the present study

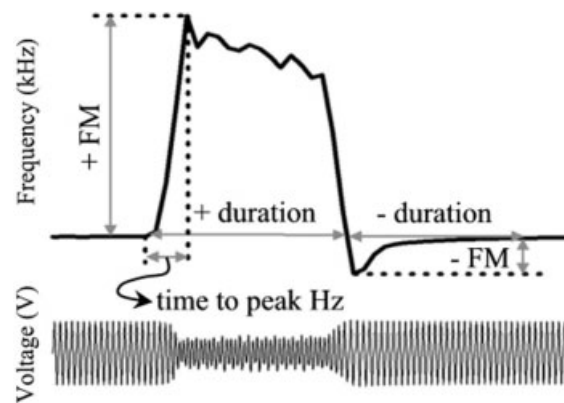


Figure 2 Structural parameters measured from STFMs. Top trace shows EOD frequency (kHz) over time during an STFM; bottom trace shows amplitude (V) over time. Qualitative measurements that varied among STFMs include positive frequency modulation (+FM), negative frequency modulation (−FM), positive duration, negative duration, and time to peak frequency. Amplitude modulation also varied between STFMs but was not analyzed in this study (see Materials and Methods).

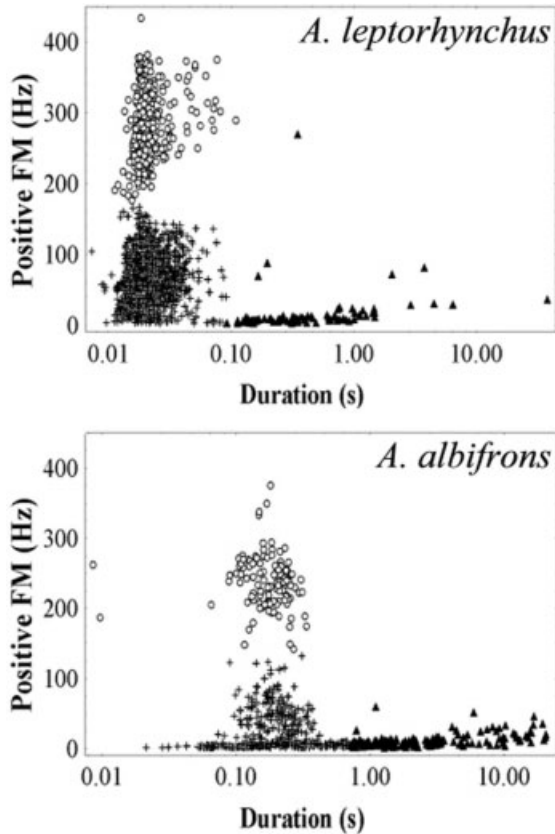


Figure 3 Scatterplots of frequency excursion and duration of STFM recorded from *A. leptorhynchus* and *A. albifrons*. Positive duration and positive frequency modulation (+FM) were used to cluster STFM from each species into three categories: high frequency STFM (open circles), low frequency STFM (plus signs), and long duration STFM (closed triangles). Duration is expressed on a log axis. Note that the three STFM types are comparable in the two species, but that the STFM in *A. albifrons* are significantly longer in duration than those of *A. leptorhynchus*.

spanned a greater range (3–167 Hz) than previously described SP-2 chirps (36–65 Hz; Engler et al., 2000). In addition, over 25% of low STFM in this study had a negative frequency excursion of at least 5 Hz after the positive phase, whereas SP-2 and type 2 chirps were not reported to dip below frequency baseline (Engler et al., 2000; Engler and Zupanc, 2001). Long STFM in *A. leptorhynchus* had smaller frequency excursions, were much longer and more variable in duration than high or low STFM, and unlike high or low STFM, never had a frequency undershoot (Fig. 4; Table 2). Long STFM were qualitatively similar to modulations previously described as medium and long rises (Hagedorn and Heiligenberg, 1985; Tallarovic and Zakon, 2002), yodels (Dye, 1987), and gradual frequency rises (Engler et

al., 2000). In addition, one male produced a single high frequency, long duration modulation (270 Hz, 350 ms) that was similar to the extremely rare SP-4 chirp (Engler et al., 2000).

A. albifrons STFM fell into three categories comparable to those of *A. leptorhynchus*. *A. albifrons* modulations, however, were longer in duration and never had the frequency undershoot characteristic of many *A. leptorhynchus* modulations (Fig. 4; Table 2). *A. albifrons* high STFM were approximately eight times longer in duration than those of *A. leptorhynchus* but exhibited comparable positive frequency excursions. *A. albifrons* low STFM were longer and more variable in duration, and exhibited lower frequency excursions, compared to those of *A. leptorhynchus*. The long STFM of *A. albifrons* had smaller frequency excursions than all other modulations in both species, but were much longer in duration. A recent investigation of evoked electrical behavior in *A. albifrons* (Dunlap and Larkins-Ford, 2003) described four distinct chirp types. The STFM types identified in *A. albifrons* in this study, like those identified by Dunlap and Larkins-Ford (2003), were all longer in duration than comparable STFM types in *A. leptorhynchus*. Most of the modulations identified in the present study, however, differed in structure from those described by Dunlap and Larkins-Ford (2003) and did not fit into comparable categories.

Sex Differences in STFM Structure

Sex differences in STFM structure were common to both *A. leptorhynchus* and *A. albifrons*. The most obvious difference was in the proportion of high STFM produced. Males of both species were more likely to produce high STFM than females. All 9 *A. leptorhynchus* males produced at least one high STFM whereas only 2 of the 11 females produced any high STFM. Furthermore, male *A. leptorhynchus* produced a greater proportion of high STFM than females [$F(1, 18) = 12.1$; $p < 0.01$; Fig. 5]. Similarly in *A. albifrons*, 7 of the 12 males produced high STFM compared to only 3 of the 12 females. Male *A. albifrons* also produced a greater proportion of high STFM than female *A. albifrons* [$F(1, 22) = 4.65$; $p = 0.042$; Fig. 5].

Within STFM categories, males of both species also produced modulations that differed in structure from female modulations. In *A. leptorhynchus*, low STFM had significantly greater frequency modulation in males than in females [$F(1, 18) = 9.69$; $p < 0.01$; Fig. 6(A)]. Long STFM did not differ between the sexes in *A. leptorhynchus* in any measured parameter, and sex differences in the structure of high

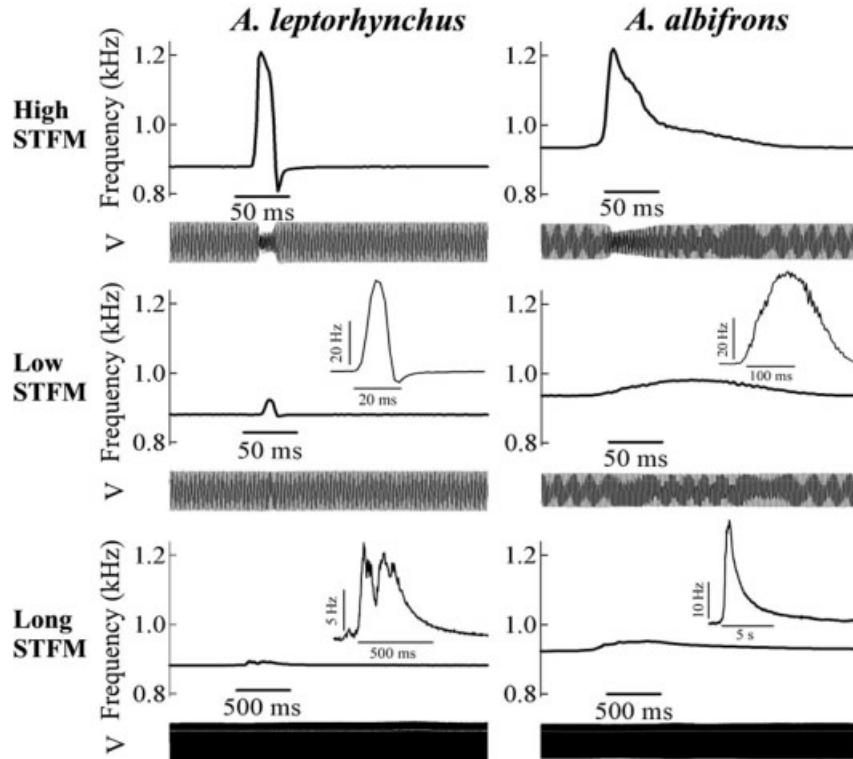


Figure 4 Frequency (kHz) and waveform (V) traces of representative STFMs from each category. High and low STFMs were significantly longer in duration in *A. albifrons* than *A. leptorhynchus*. Long STFMs in *A. leptorhynchus* and high, low, and long STFMs in *A. albifrons* never had frequency undershoots characteristic of all high, and some low, STFMs in *A. leptorhynchus*. Note the pronounced amplitude modulation in high STFMs. The frequency scale of all panels is the same to allow for comparison of frequency excursion across species and STFMs type. The time scale for high and low STFMs in both species is also the same, which demonstrates the species difference in STFMs duration. The time scale for long STFMs is 10-fold slower compared to other STFMs types. Insets are plotted on expanded time and/or frequency scales to reveal detailed structure of low and long STFMs.

STFMs were not assessed because only two females produced these high-frequency modulations.

STFMs produced by *A. albifrons* also differed qualitatively between males and females. Both low

and high STFMs were longer in duration in males than in females [$F(1, 22) = 14.97$; $p < 0.001$; $F(1, 8) = 6.86$; $p < 0.05$; Fig. 6(B,C)]; sexually dimorphic durations were also reported in a previous study of *A.*

Table 2 Frequency and Duration Measurements for STFMs Categories

	<i>A. leptorhynchus</i> STFMs			<i>A. albifrons</i> STFMs		
	High	Low	Long	High	Low	Long
Mean +FM (Hz)	275 ± 10.1	61 ± 5.2	17.4 ± 7.3	237 ± 7.6	24.5 ± 5.6	11.3 ± 1.9
Range +FM (Hz)	176 to 433	3 to 167	3 to 270	142 to 375	2 to 132	2 to 60
Mean dur. (s)	0.023 ± 0.003	0.019 ± 0.001	1.16 ± 0.95	0.186 ± 0.012	0.228 ± 0.028	4.1 ± 0.98
Range dur. (s)	0.011 to 0.11	0.007 to 0.091	0.092 to 37	0.086 to 0.337	0.021 to 0.673	0.71 to 20.7
Mean -FM (Hz)	-42 ± 3.4	-3 ± 0.98	N/A	N/A	N/A	N/A
Range -FM (Hz)	-10 to -95	0 to -42	N/A	N/A	N/A	N/A

Abbreviations: (+FM) positive frequency modulation, (dur) positive duration, (-FM) negative frequency modulation, (N/A) not applicable because these STFMs categories had no frequency undershoot. Data are presented as mean ± SE; ranges are combined for males and females.

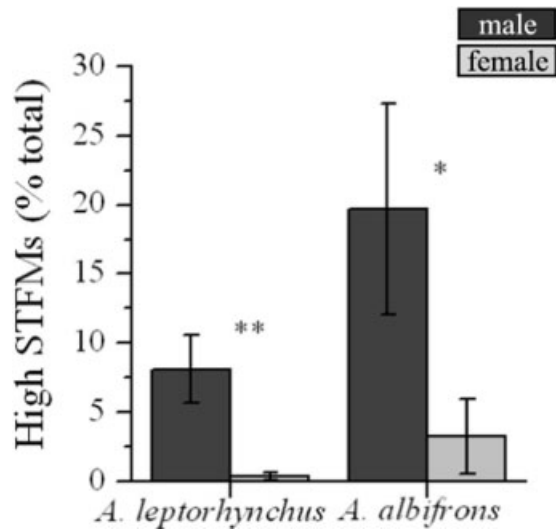


Figure 5 Sex differences in proportion of high STFMs produced. Males (dark bars) produced a higher proportion of high frequency STFMs than females (light bars) in *A. leptorhynchus* ($n = 9$ males, 11 females) and in *A. albifrons* ($n = 12$ males, 12 females). Bars represent mean \pm SEM. * $p < 0.05$, ** $p < 0.01$.

albifrons electrocommunication behavior (Dunlap and Larkins-Ford, 2003). *A. albifrons* high STFMs tended to have greater frequency modulation in males than in females, but the fact that only three females produced high STFMs limited the statistical power of this comparison, and the difference was therefore not statistically significant [$F(1, 8) = 5.15$; $p = 0.053$; Fig. 6(D)].

Substance P Immunofluorescence

In agreement with previous studies (Weld and Maler, 1992; Dulka et al., 1995), small fibers throughout the CP/PPn of *A. leptorhynchus* stained heavily for substance P in males, but not females [Fig. 7(A,B)]. *A. leptorhynchus* males had significantly more SPLir in

Figure 6 Sex differences in STFMs structure within types. (A) Low STFMs in *A. leptorhynchus* males ($n = 9$, dark bars) had more positive frequency modulation (+FM) than those of females ($n = 11$, light bars). (B,C) Male *A. albifrons* produced longer duration low STFMs ($n = 12$ males, 12 females) and high STFMs ($n = 7$ males, 3 females) than females. (D) High STFMs of male *A. albifrons* ($n = 7$) tended to have more frequency modulation than those of females ($n = 3$), but this difference was not significant, possibly due to a low sample size and thus low statistical power (see Results). Bars are means \pm SEM. * $p < 0.05$, ** $p < 0.01$.

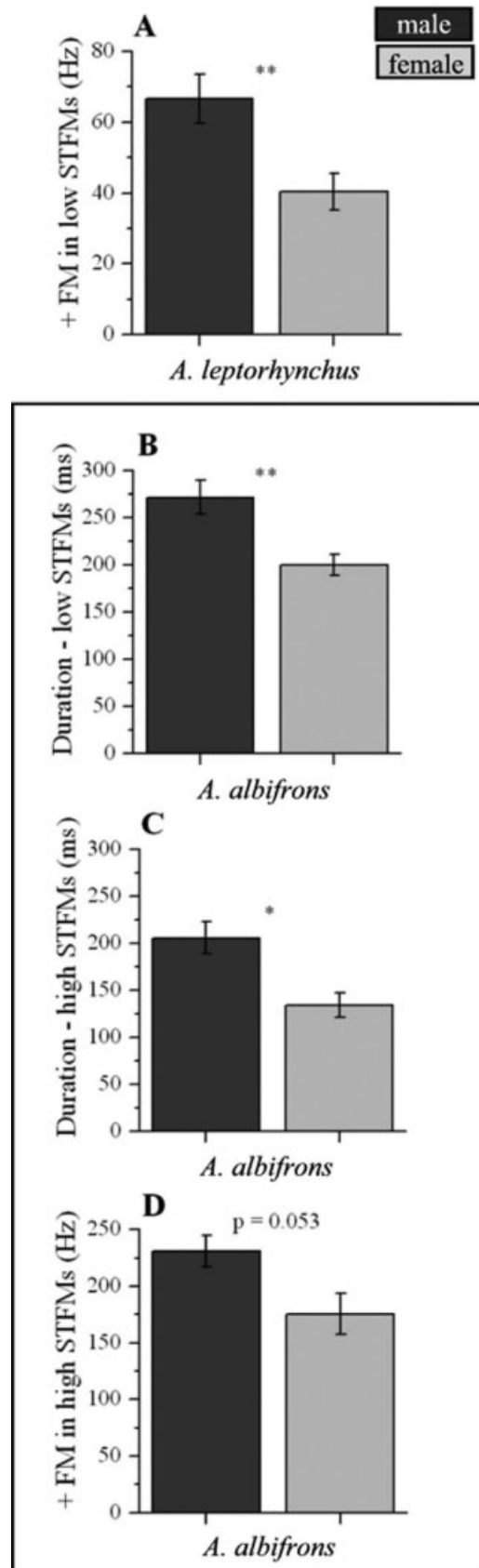


Figure 6

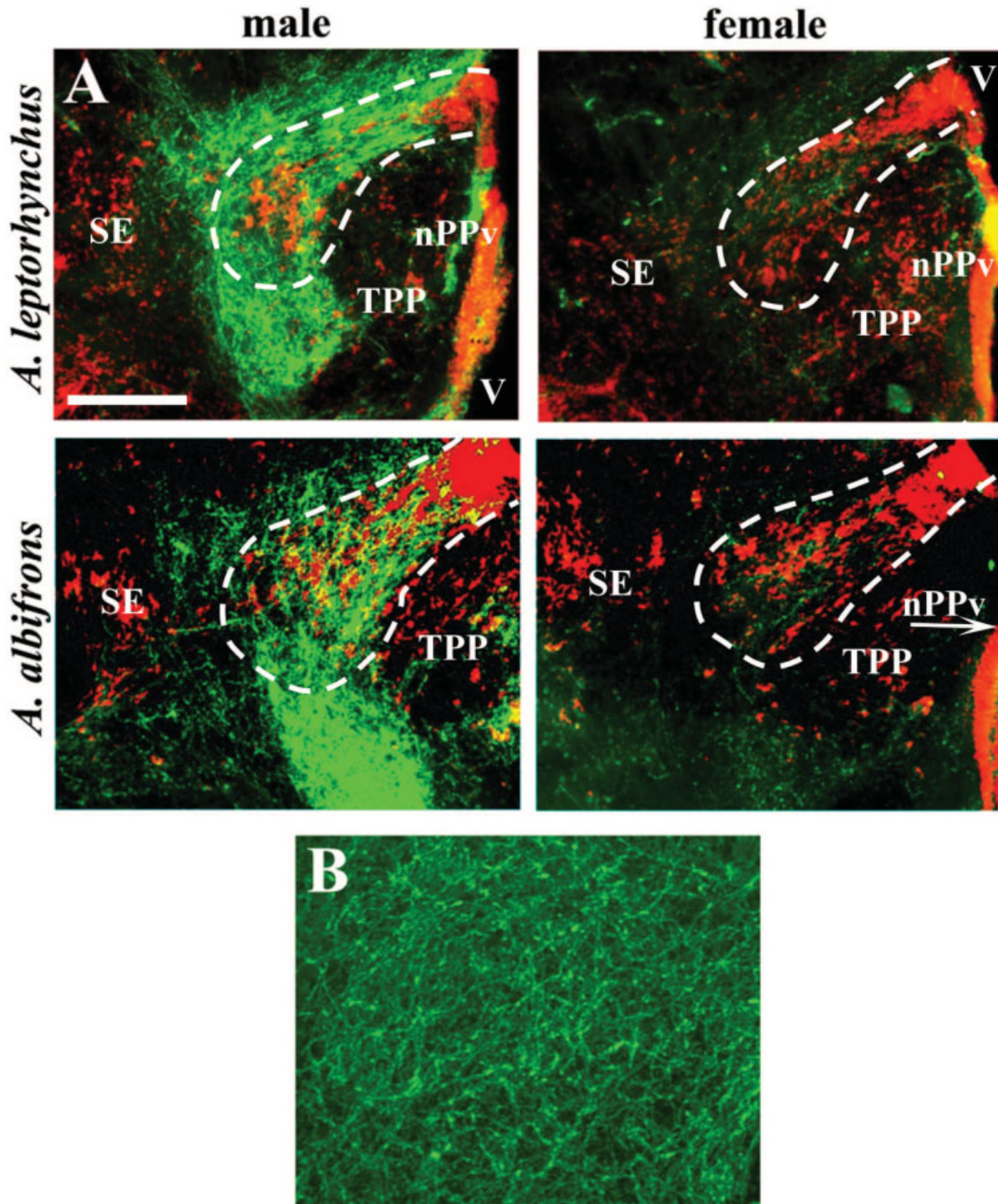


Figure 7 Substance P-like immunofluorescence in CP/PPn of male and female *A. leptorhynchus* and *A. albifrons*. (A) Males of both species had more SPlir fibers (green) in the CP/PPn than females. The CP/PPn was identified by comparing propidium iodide staining (red) to the *A. leptorhynchus* brain atlas (Maler et al., 1991); the approximate boundaries of the CP/PPn are indicated by the dashed lines in each panel. Dendrites of CP/PPn neurons may extend beyond these borders (Kawasaki et al., 1988). Scale bar (200 μm) in first panel applies to all four panels. Brain regions near the CP/PPn are indicated with the following labels: (V) ventricle, (SE) nucleus subelectrosensorius, (nPPv) nucleus posterioris periventricularis, (TPP) periventricular nucleus of the posterior tuberculum. (B) Higher magnification confocal image of typical CP/PPn fiber staining seen in males of both species.

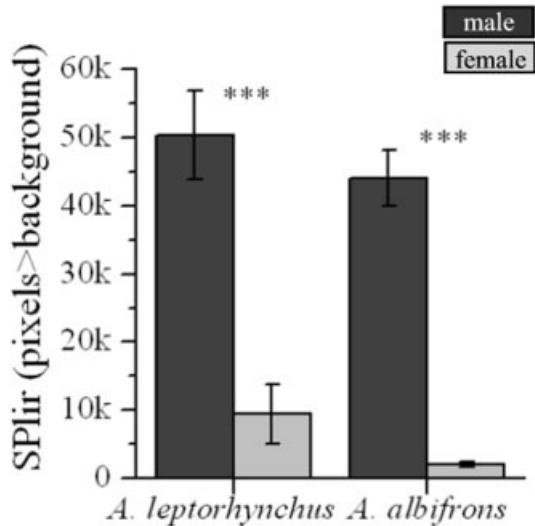


Figure 8 Sex differences in substance P-like immunoreactivity (SPlir) in the CP/PPn. SPlir (number of pixels above the nonspecific background threshold) was greater in the CP/PPn of males (dark bars) than females (light bars) in both *A. leptorhynchus* ($n = 5$ males, 8 females) and *A. albifrons* ($n = 7$ males, 8 females).*** $p < 0.001$.

the CP/PPn than females [$F(1, 11) = 30.17$; $p < 0.001$; Fig. 8]. This sex difference in SPlir within the CP/PPn of *A. leptorhynchus* was accompanied by sex differences in both the number and structure of STFMs.

SPlir in the CP/PPn of *A. albifrons*, like that in *A. leptorhynchus*, was also highly sexually dimorphic [Fig. 7(A)]. Male *A. albifrons* had significantly more SPlir in the CP/PPn than female *A. albifrons* [$F(1, 13) = 124.5$; $p < 0.0001$; Fig. 8]. SPlir was evident in numerous small fibers throughout the CP/PPn of males extending ventrally to the hypothalamus in a staining pattern similar to that seen in *A. leptorhynchus* males. Such fibers, although present, were clearly sparse in females of both species. This sex difference in SPlir within the CP/PPn of *A. albifrons* was accompanied by a sex difference in structure of STFMs, but not number.

No staining was evident in the CP/PPn in negative control sections of either species, which were not incubated in the substance P antibody.

DISCUSSION

Consistent with a previous report (Weld and Maler, 1992), this study found SPlir in fine fibers within the CP/PPn in *A. leptorhynchus*, and found that males of this species had significantly more SPlir in the CP/

PPn than females. In addition, the distribution of SPlir in the CP/PPn of *A. albifrons* was similar to that in *A. leptorhynchus* and was also sexually dimorphic; male *A. albifrons* had far more SPlir in the CP/PPn than females. Despite the fact that substance P expression was sexually dimorphic in both species, only *A. leptorhynchus* showed an associated sex difference in the number of STFMs produced; male *A. leptorhynchus* produced far more STFMs than female *A. leptorhynchus* whereas male and female *A. albifrons* produced similar numbers of STFMs. These results suggest that sex differences in substance P afferent input to the CP/PPn are not sufficient to produce sex differences in the number of STFMs produced, as previously hypothesized for *A. leptorhynchus* (Zupanc and Maler, 1993; Dulka et al., 1995). In contrast, sexually dimorphic SPlir was consistently associated with sex differences in STFM structure in both *A. leptorhynchus* and *A. albifrons*. This suggests that sexually dimorphic substance P expression may regulate sex differences in qualitative aspects of STFM structure (frequency excursion and/or duration) that are common to both *Apteronotus* species.

It is unlikely that the lack of sex differences in the number of STFMs in *A. albifrons* was due to the reproductive condition of animals used in this study. Fish were exposed to low water conductivity ($100\text{--}300 \mu\text{S} \cdot \text{cm}^{-1}$), which is the primary environmental cue for gonadal recrudescence in gymnotiform fish (Kirschbaum, 1975, 1979; Hagedorn and Heiligenberg, 1985). Furthermore, the high GSI, strong sexual dimorphism in EOD frequency, and presence of yolk-ing follicles in females were all typical of fish in breeding condition (Table 1). These criteria of reproductive condition indicated that fish in this study were at least as reproductively mature as those used in other studies of sexually dimorphic electrocommunication behavior (Dunlap et al., 1998; Bastian et al., 2001; Dunlap, 2002; Dunlap and Larkins-Ford, 2003).

Most of our behavioral results agree with those of previous studies of *Apteronotus* electrocommunication behavior. Previous work has demonstrated that male *A. leptorhynchus* produce more STFMs than females (Hagedorn and Heiligenberg, 1985; Dye, 1987; Zupanc and Maler, 1993; Dulka and Maler, 1994; Dulka et al., 1995; Dunlap et al., 1998; Bastian et al., 2001; Dunlap and Larkins-Ford, 2003), and that male *A. leptorhynchus* produce a greater proportion of high frequency STFMs than females (Hagedorn and Heiligenberg, 1985; Dulka and Maler, 1994; Dulka et al., 1995; Bastian et al., 2001; Dunlap and Larkins-Ford, 2003). In *A. albifrons*, previous studies found that males and females did not differ in the number of modulations produced, but that male *A. albifrons* pro-

duced longer duration modulations than females (Dunlap et al., 1998; Dunlap and Larkins-Ford, 2003). The results of the present study are consistent with all of these previous findings and suggest that both *A. albifrons* and *A. leptorhynchus* express sex differences in STFMs structure (expressed as the proportion of different categories of STFMs and/or parameters of STFMs within categories), but that only *A. leptorhynchus* expresses sex differences in STFMs number.

Some of the behavioral results of this study, however, differed from those reported in similar studies of sexually dimorphic communication behavior in *Apteronotus*. For example, although Bastian et al. (2001) and the present study both reported that males produced a higher proportion of type 1 chirps (high STFMs) than females, the two studies differed in the presence of sex differences in STFMs structure within types. Bastian et al. (2001) did not find a sex difference in the frequency excursion of type 2 chirps, whereas we found that low STFMs (similar to type 2 chirps) produced by male *A. leptorhynchus* had greater frequency excursion than those of females. One possible explanation for the difference between the studies might be differences in the reproductive condition of the fish used in each study. Although Bastian et al. (2001) did not report GSI or EOD frequency, fish in that study were housed in water with relatively high conductivity ($500\text{--}1000\ \mu\text{S} \cdot \text{cm}^{-1}$), which is typically experienced by the fish during the nonbreeding season. The fish in the present study were maintained in water with significantly lower conductivity levels ($100\text{--}300\ \mu\text{S} \cdot \text{cm}^{-1}$), typically experienced by fish during the breeding season. Therefore, it is possible that the fish examined in Bastian et al. (2001) were not in reproductive condition at the time of testing. Because STFMs are elicited during agonistic and reproductive encounters, it is possible that STFMs structure varies with reproductive condition and breeding season. Thus, differences in the degree of sexual dimorphism in STFMs structure between studies might be explained by differences in the reproductive condition of the fish used in those studies.

Similarly, Dunlap and Larkins-Ford (2003) did not report any sex differences in the frequency excursion of STFMs in *A. albifrons*, whereas the present study found evidence for sexually dimorphic frequency excursion. Male *A. albifrons* produced a greater proportion of high frequency STFMs than females, and high frequency STFMs produced by males tended to have greater frequency modulation than those produced by females. Again, this discrepancy might be due to reproductive condition. Unlike the present study, no sex difference in EOD frequency was reported in Dunlap and Larkins-Ford (2003), and the lack of

sexual dimorphism in EOD frequency might be attributable to relatively high conductivity levels ($400\text{--}800\ \mu\text{S} \cdot \text{cm}^{-1}$). Alternatively, differences between studies in sexually dimorphic frequency excursions of STFMs might be due to differences in playback stimuli. Dunlap and Larkins-Ford (2003) used only a stimulus 10 Hz below the fish's EOD frequency to elicit STFMs whereas the present study used five different stimulus frequencies that spanned the range of conspecific EOD frequencies. Because the structure of STFMs varies with stimulus frequency (Bastian et al., 2001), it is possible that this difference between studies might be explained by the different stimuli used to elicit STFMs.

Relationship between Sex Differences in Substance P and STFMs

The lack of sexual dimorphism in the number of STFMs produced in *A. albifrons*, despite a pronounced sex difference in SPLir, demonstrates that sexually dimorphic substance P in the CP/PPn is not sufficient for quantitative sex differences in STFMs production. This raises the question of what neural mechanisms regulate sex differences in the number of STFMs produced in *A. leptorhynchus*. Other possible neural correlates of sex differences in STFMs production rate include, but are not limited to, sex differences in other neuromodulators in the CP/PPn, differences in input from electrosensory regions, and differences in the intrinsic circuitry and/or excitability of the CP/PPn. Further comparative studies of sex differences in the chemical neuroanatomy, circuitry, and physiology of the CP/PPn are needed to address this question.

An additional issue is whether sex differences in SPLir are accompanied by differences in the distribution of substance P receptors. A previous study of tachykinin binding sites in *A. leptorhynchus* found no sex difference in the density of substance P binding in the CP/PPn despite the pronounced sex difference in SPLir fibers (Weld et al., 1994). It is not known, however, whether tachykinin receptors are present in the CP/PPn of *A. albifrons* or whether their distribution is sexually dimorphic. Future work examining the distribution of substance P receptors in *A. albifrons* may provide additional insight into the role of substance P in modulating sexual dimorphism in the production and structure of STFMs.

Although sex differences in SPLir in the CP/PPn were not consistently associated with sex differences in the number of STFMs, sex differences in substance P distribution were consistently associated with sex differences in the structure of STFMs. Males and

females of both species differed both in the proportion of different categories of STFMs produced and in the structure of STFMs within categories. In *A. leptorhynchus*, high frequency STFMs were produced by only 2 of the 11 females, compared to all 9 of the males. In *A. albifrons*, only 3 of the 12 females (compared to 7 of 12 males) produced high frequency STFMs. Furthermore, males of both species produced a greater proportion of high STFMs than females. Of those *A. albifrons* that produced high STFMs, male modulations were 50% longer and had over 30% more frequency modulation than those of females. In addition, low frequency STFMs were sexually dimorphic in frequency modulation in *A. leptorhynchus* and duration in *A. albifrons*. Thus, males in both species were more likely than females to produce STFMs with greater frequency modulation and/or longer duration.

Several lines of evidence suggest that sex differences in substance P distribution may be related to sex differences in the structure of STFMs. First, substance P is associated with sexually dimorphic behavior in other taxa. Substance P facilitates male copulatory behavior in rats (Dornan and Malsbury, 1989), is sexually dimorphic in brain areas involved in reproductive behavior in birds (Aste et al., 1995), and regulates male-specific agonistic behavior in reptiles (Gobbetti et al., 1994). Second, androgen treatment of *A. leptorhynchus* females up regulated SPLir in the CP/PPn and masculinized not only STFM number, but also STFM structure (Dulka et al., 1995). Third, sex differences in SPLir in the CP/PPn were associated with sex differences in STFM structure in both *A. leptorhynchus* and *A. albifrons*. Finally, direct injection of substance P into the CP/PPn of *A. leptorhynchus* elicits chirps in the absence of electrical stimulation, and chirps evoked by substance P injections into the CP/PPn of *A. leptorhynchus* differed in structure from those evoked by glutamate (Weld et al., 1991). Future studies of how substance P affects chirping behavior and CP/PPn physiology in *A. leptorhynchus* and *A. albifrons* will help confirm the behavioral consequences of sexually dimorphic substance P distribution and identify the specific mechanisms of action.

Species Diversity in Electrocommunication Behavior

EOD frequency and waveform are remarkably diverse among the gymnotiform fish (Hopkins, 1974a; Kramer et al., 1980). Sex differences in EOD frequency and EOD modulations are also diverse, although sex differences in electrocommunication behavior have been studied in relatively few species

(Fig. 9). EOD frequency is sexually dimorphic in almost every gymnotiform species studied. The direction of this sexual dimorphism, however, has reversed at least once. EOD frequency is lower in males than in females in most gymnotiform species, but is higher in males than in females in *A. leptorhynchus* (Hopkins, 1974b; Kirschbaum, 1983; Hagedorn and Heiligenberg, 1985; Dunlap et al., 1998). The structure, rate, and sexual dimorphism of EOD modulations also differ between species (Table 2; Fig. 9). In *A. leptorhynchus* and *Eigenmannia virescens*, males produce more STFMs than females (Hopkins, 1974c; Zupanc and Maler, 1993; Dulka and Maler, 1994). In *A. albifrons*, however, the number of STFMs is sexually monomorphic (Dunlap et al., 1998). In addition, sexual dimorphism in the structure of STFMs (as measured by proportion of high STFMs produced and/or structural differences within STFM categories) is present in both *Apteronotus* species studied and suggested in two gymnotiform fish from other families, *E. virescens* and *Sternopygus* spp. (Hopkins, 1974b,c).

By comparatively studying the neural and hormonal control of sexually dimorphic EOD modulations, we can gain insight into the evolution of sex differences in neural circuits and behavior. The present study showed that sex differences in SPLir in the CP/PPn were not limited to *A. leptorhynchus*, but were also present in *A. albifrons*. In addition, we found that sex differences in SPLir were not consistently associated with STFM production rate, but were instead associated with sex differences in STFM structure common to both species. It is possible that sex differences in substance P afferents to premotor electrocommunication circuits may exist in other gymnotiform fish. If so, this study provides testable hypotheses for examining how sex differences in substance P are related to sexually dimorphic electrocommunication behavior in other electric fish species and how the neural and hormonal control of sexually dimorphic communication evolved in gymnotiforms.

Although this study begins to examine the relationship between sex differences in neuromodulation and electrocommunication behavior across species, several questions remain to be comparatively addressed. First, is the association between sexually dimorphic substance P and STFM structure widespread in gymnotiform species, or limited to apteronotid fish? This question could be addressed by examining the distribution of sex differences in substance P and STFM structure in other gymnotiform species (e.g., other apteronotids, *Eigenmannia*, or *Sternopygus*). Second, how are sex differences in substance P and in STFM number and structure regulated by gonadal steroids

	<i>Sternopygus</i> spp.	<i>Eigenmannia</i> <i>virescens</i>	<i>Apteronotus</i> <i>albifrons</i>	<i>Apteronotus</i> <i>leptorhynchus</i>
EOD frequency	F >> M ¹	F > M ^{2,3}	F > M ⁴	M >> F ³
androgen effects	↓ ⁵	↓ ⁶	↓ ⁴	↑ ⁷
# of EOD modulations (interruptions, STFM's)	?	M > F ²	M = F ⁴	M > F ^{8,9,10}
androgen effects	?	?	No effect ⁴	↑ ^{10,11}
EOD modulation structure	?	?	M ≠ F ¹⁴	M ≠ F ^{3,10,11,12,14}
androgen effects	?	?	?	masculinize ^{10,11}
SPlir in CP/PPn	?	?	M >> F ¹⁴	M >> F ¹³
androgen effects	?	?	?	↑ ¹¹

¹Hopkins (1972); ²Hopkins (1974c); ³Hagedorn and Heiligenberg (1985); ⁴Dunlap et al (1998); ⁵Meyer (1983); ⁶Dunlap and Zakon (1998); ⁷Schaefer and Zakon (1996); ⁸Dye (1987); ⁹Zupanc and Maler (1993); ¹⁰Dulka and Maler (1994); ¹¹Dulka et al (1995); ¹²Bastian et al (2001); ¹³Weld and Maler (1992); ¹⁴see Results

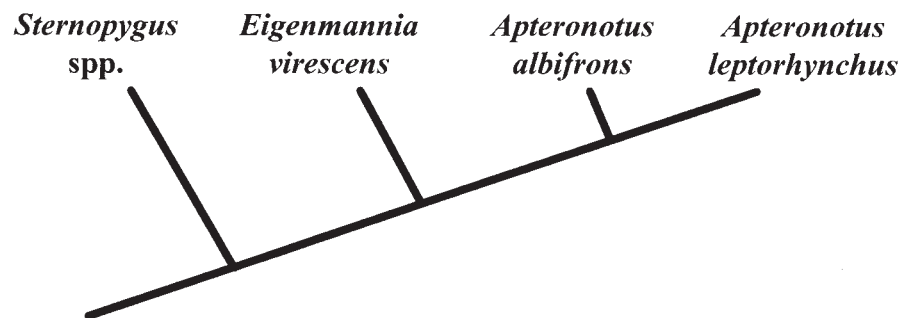


Figure 9 Species differences in sexual dimorphism and androgen regulation of electrocommunication behavior in four gymnotiform species. Cladogram is based on consensus phylogeny from Alves-Gomes et al. (1995) using molecular, behavioral, and morphological data. ↑ androgens increase parameter, ↓ androgens decrease parameter, ? sex difference and/or androgen effect not known.

across taxa? In *A. leptorhynchus*, androgens masculinize EOD frequency, chirp rate, and structure, and SPlir in the CP/PPn (Dulka and Maler, 1994; Dulka et al., 1995). Although androgens have no effect on chirp rate in *A. albifrons* (Dunlap et al., 1998), whether they affect STFM structure or SPlir is unknown. Similarly, the effects of androgens on STFM's and SPlir in non-apteronotid gymnotiform fish remain to be examined.

Comparative studies of neural circuits that underlie diverse behaviors in closely related taxa can elucidate how evolutionary changes in neural circuitry can produce species diversity in behavior. Similar comparative studies in rodents and birds have examined the neural correlates of social behavior in closely related

species that exhibit different degrees of sexually dimorphic behavior and different life history traits (Brenowitz and Arnold, 1986; Insel et al., 1991, 1994; Insel and Shapiro, 1992). Many vertebrate models of sexually dimorphic behavior, however, involve multiple interconnected brain regions with complex intrinsic circuitry. The study of sex differences in electrocommunication behavior is aided by the fact that the underlying electromotor circuitry is well-defined and relatively simple, in some cases spanning only two to four synapses from the premotor nucleus to electric organ (Heiligenberg et al., 1996; Smith, 1999). In addition, there is a straightforward relationship between excitability of neurons in this circuit and resultant electrocommunication behavior. It is there-

fore easier to understand how evolutionary changes in circuitry (or sex differences in circuitry) are related to behavioral diversity. Future studies that take advantage of the relative simplicity of the electromotor circuit and the diversity of electrocommunication behaviors will shed light on general mechanisms by which evolutionary changes in motor and premotor circuits produce behavioral diversity both within and between species.

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REFERENCES

- Alves-Gomes JA, Ortí G, Haygood M, Heiligenberg W, Meyer A. 1995. Phylogenetic analysis of the South American electric fishes (Order Gymnotiformes) and the evolution of their electrogenic system: A synthesis based on morphology, electrophysiology, and mitochondrial sequence data. *Mol Biol Evol* 12:298–318.
- Aste N, Viglietti-Panzica C, Fasolo A, Panzica G. 1995. Mapping of neurochemical markers in quail central nervous system: VIP- and SP-like immunoreactivity. *J Chem Neuroanat* 8:87–102.
- Bastian J, Schniederjan S, Nguyenkim J. 2001. Arginine vasotocin modulates a sexually dimorphic communication behavior in the weakly electric fish, *Apteronotus leptorhynchus*. *J Exp Biol* 204:1909–1923.
- Brenowitz E, Arnold A. 1986. Interspecific comparisons of the size of neural song control regions and song complexity in duetting birds: Evolutionary implications. *J Neurosci* 6:2875–2879.
- Bullock T, Hamstra R, Scheich H. 1972. The jamming avoidance response of high-frequency electric fish, I & II. *J Comp Physiol A* 77:1–48.
- Dornan W, Malsbury C. 1989. Peptidergic control of male-rat sexual-behavior: The effects of intracerebral injections of substance P and cholecystokinin. *Physiol Behav* 46:547–556.
- Dulka J, Maler L, Ellis W. 1995. Androgen-induced changes in electrocommunicatory behavior are correlated with changes in substance P-like immunoreactivity in the brain of the electric fish *Apteronotus leptorhynchus*. *J Neurosci* 15:1879–1890.
- Dulka JG, Maler L. 1994. Testosterone modulates female chirping behavior in the weakly electric fish *Apteronotus leptorhynchus*. *J Comp Physiol A* 174:331–343.
- Dunlap K. 2002. Hormonal and body size correlates of electrocommunication behavior during dyadic interactions in a weakly electric fish, *Apteronotus leptorhynchus*. *Horm Behav* 41:187–194.
- Dunlap K, Larkins-Ford J. 2003. Diversity in the structure of electrocommunication signals within a genus of electric fish, *Apteronotus*. *J Comp Physiol A* 189:153–161.
- Dunlap KD, Thomas P, Zakon HH. 1998. Diversity of sexual dimorphism in electrocommunication signals and its androgen regulation in a genus of electric fish, *Apteronotus*. *J Comp Physiol A* 183:77–86.
- Dunlap K, Zakon HH. 1998. Behavioral actions of androgens and androgen receptor expression in the electrocommunication system of an electric fish, *Eigenmannia virescens*. *Horm Behav* 34:30–38.
- Dye J. 1987. Dynamics and stimulus-dependence of pacemaker control during behavioral modulations in the weakly electric fish, *Apteronotus*. *J Comp Physiol A* 163:445–458.
- Engler G, Fogarty C, Banks J, Zupanc G. 2000. Spontaneous modulations of the electric organ discharge in the weakly electric fish, *Apteronotus leptorhynchus*: A biophysical and behavioral analysis. *J Comp Physiol A* 186:645–660.
- Engler G, Zupanc G. 2001. Differential production of chirping behavior evoked by electrical stimulation of the weakly electric fish, *Apteronotus leptorhynchus*. *J Comp Physiol A* 187:747–756.
- Gobbetti A, Zerani M, Fiore MD, Botte V. 1994. Relationships among GnRH, substance P, prostoglandins, sex steroids, and aromatase activity in the brain of the male lizard *Podarcis sicula sicula* during reproduction. *J Reprod Fert* 101:523–529.
- Hagedorn M, Heiligenberg W. 1985. Court and spark: Electric signals in the courtship and mating of gymnotoid fish. *Anim Behav* 33:254–265.
- Heiligenberg W, Metzner W, Wong CJH, Keller CH. 1996. Motor control of the jamming avoidance response of *Apteronotus leptorhynchus*: Evolutionary changes of a behavior and its neural substrate. *J Comp Physiol A* 179:653–674.
- Hopkins CD. 1972. Sex differences in electric signaling in an electric fish. *Science* 176:1035–1037.
- Hopkins CD. 1974a. Electric communication in fish. *Am Scientist* 62:426–437.
- Hopkins CD. 1974b. Electric communication in the reproductive behavior of *Sternopygus macrurus* Gymnopteroidea. *Z Tierpsychol* 35:518–535.
- Hopkins CD. 1974c. Electric communication: Functions in the social behavior of *Eigenmannia virescens*. *Behav* 50:270–305.
- Hopkins CD. 1988. Neuroethology of electric communication. *Annu Rev Neurosci* 11:497–535.
- Insel T, Gilhard R, Shapiro L. 1991. The comparative distribution of forebrain receptors for neurohypophyseal peptides in monogamous and polygamous mice. *Neuroscience* 43:623–630.
- Insel T, Shapiro L. 1992. Oxytocin receptor distribution reflects social organization in monogamous and polygamous voles. *Proc Natl Acad Sci USA* 89:5981–5985.
- Insel T, Wang Z, Ferris C. 1994. Patterns of brain vasopressin receptor distribution associated with social organization. *J Neurosci* 14:5381–5392.

- Kawasaki M, Maler L, Rose G, Heiligenberg W. 1988. Anatomical and functional organization of the prepacemaker nucleus in gymnotiform electric fish: The accommodation of two behaviors in one nucleus. *J Comp Neurol* 276:113–131.
- Kelley DB. 1988. Sexually dimorphic behaviors. *Annu Rev Neurosci* 11:225–251.
- Kirschbaum F. 1975. Environmental factors control the periodical reproduction of tropical electric fish. *Experientia* 31:1159–1160.
- Kirschbaum F. 1979. Reproduction of the weakly electric fish *Eigenmannia virescens* (Rhamphichthyidae, Teleostei) in captivity. *Behav Ecol Sociobiol* 4:331–355.
- Kirschbaum F. 1983. Myogenic electric organ precedes the neurogenic organ in Apterodontid fish. *Naturwissenschaften* 70:205.
- Kramer B, Kirschbaum F, Markl H. 1980. Species specificity of electric organ discharges in a sympatric group of gymnotoid fish from Manaus (Amazonas). In: Szabó T, Czéh G, editors. *Sensory Physiology of Aquatic Lower Vertebrates*. Budapest: Akadémiai Kiadó, p 195–219.
- Larimer J, MacDonald J. 1968. Sensory feedback from electroreceptors to electromotor pacemaker centers in gymnotids. *Am J Physiol* 214:1253–1261.
- Maler L, Ellis W. 1987. Inter-male aggressive signals in weakly electric fish are modulated by monoamines. *Behav Br Res* 25:75–81.
- Maler L, Sas E, Johnston S, Ellis W. 1991. An atlas of the brain of the electric fish *Apterontus leptorhynchus*. *J Chem Neuroanat* 4:1–38.
- Metzner W. 1999. Neural circuitry for communication and jamming avoidance in gymnotiform electric fish. *J Exp Biol* 202:1365–1375.
- Meyer JH. 1983. Steroid influences upon the discharge frequencies of a weakly electric fish. *J Comp Physiol A* 153:29–37.
- Nelson B. 2004. Dynamics of frequency and amplitude modulations in vocalizations produced by eastern towhees, *Pipilo erythrophthalmus*. *J Acoust Soc Am* 115:1333–1344.
- Oestreich J, Zakon HH. 2002. The long-term resetting of a brainstem pacemaker nucleus by synaptic input: A model for sensorimotor adaptation. *J Neurosci* 22:8287–8296.
- Schaefer JE, Zakon HH. 1996. Opposing actions of androgen and estrogen on in vitro firing frequency of neuronal oscillators in the electromotor system. *J Neurosci* 16:2860–2868.
- Smith GT. 1999. Ionic currents that contribute to a sexually dimorphic communication signal in weakly electric fish. *J Comp Physiol A* 185:379–387.
- Tallarovic S, Zakon H. 2002. Electrocommunication signals in female brown ghost electric knifefish, *Apterontus leptorhynchus*. *J Comp Physiol A* 188:649–657.
- Triefenbach F, Zakon H. 2003. Effects of sex, sensitivity, and status on cue recognition in the weakly electric fish *Apterontus leptorhynchus*. *Anim Behav* 65:19–28.
- Weld M, Kar S, Maler L, Quirion R. 1994. The distribution of tachykinin binding sites in the brain of an electric fish (*Apterontus leptorhynchus*). *J Chem Neuroanat* 7:123–139.
- Weld M, Maler L. 1992. Substance P-like immunoreactivity in the brain of the gymnotiform fish, *Apterontus leptorhynchus*: Presence of sex differences. *J Chem Neuroanat* 5:107–129.
- Weld M, Maler L, Quirion R, Kar S. 1991. Sexually dimorphic distribution of substance P and its role in the regulation of communication in an electric fish. *Soc Neurosci Abstr* 17:1407.
- Zakon HH. 1993. Weakly electric fish as model systems for studying long-term steroid action on neural circuits. *Brain Behav Evol* 42:242–251.
- Zupanc G, Heiligenberg W. 1992. The structure of the diencephalic prepacemaker nucleus revisited: Light microscopic and ultrastructural studies. *J Comp Neurol* 323:558–569.
- Zupanc G, Maler L. 1993. Evoked chirping in the weakly electric fish *Apterontus leptorhynchus*: A quantitative biophysical analysis. *Can J Zool* 71:2301–2310.
- Zupanc G, Maler L. 1997. Neuronal control of behavioral plasticity: The prepacemaker nucleus of weakly electric gymnotiform fish. *J Comp Physiol A* 180:99–111.