

# Stimulus frequency differentially affects chirping in two species of weakly electric fish: implications for the evolution of signal structure and function

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## Summary

During social interactions, apteronotid electric fish modulate their electric organ discharges (EODs) to produce transient communication signals known as chirps. Chirps vary widely across species and sex in both number and structure. In *Apteronotus leptorhynchus*, males chirp far more than females and their chirps have greater frequency modulation than those of females. High-frequency chirps are produced by males most often in response to female-like electric signals. As such, they have been hypothesized to function in courtship. The more common low-frequency chirps, produced by both males and females in response to same-sex signals, are hypothesized to function as aggressive signals. To determine whether the two chirp types in the closely related *Apteronotus albifrons* have similar functions, we stimulated chirping in male and female *A. leptorhynchus* and *A. albifrons* with playbacks simulating the EODs of same-sex versus opposite-sex conspecifics. As in *A.*

*leptorhynchus*, male and female *A. albifrons* produced low-frequency chirps most often to same-sex signals. Unlike *A. leptorhynchus*, however, *A. albifrons* also produced more high-frequency chirps to same-sex stimuli than to opposite-sex stimuli. This suggests that high-frequency chirps in *A. albifrons*, unlike those in *A. leptorhynchus*, may not function as courtship signals and that the function of similar chirp types has diversified in *Apteronotus*. Examples such as this, in which the function of a communication signal has changed in closely related species, are rare. The electrocommunication signals of apteronotids may thus provide a remarkable opportunity to investigate the evolutionary interactions of signal structure and function.

Key words: communication, chirping, sexual dimorphism, electric fish, signal evolution, *Apteronotus*, difference frequency.

## Introduction

Weakly electric fish in the genus *Apteronotus* provide a unique vertebrate model for studying the evolutionary interplay between signal structure, production, and function in complex communication systems. This diverse group of fish produces quasisinusoidal electric organ discharges (EODs) for electrolocation and communication. EODs are relatively simple and are easy to record, analyze and manipulate. These signals can vary in frequency, amplitude and waveform, and therefore reliably convey information about species, sex and individual identity (Hopkins, 1988; Smith, 1999; Zakon and Smith, 2002).

In many species, sex differences in EOD frequency are stable enough to unambiguously distinguish males from females. In the brown ghost knifefish *Apteronotus leptorhynchus*, males produce EODs between 800 and 1000 Hz whereas females produce lower-frequency EODs between 600 and 750 Hz (Hagedorn and Heiligenberg, 1985; Kirschbaum, 1983; Meyer et al., 1987). In the closely related black ghost knifefish *Apteronotus albifrons*, EOD frequency is also sexually dimorphic, but in the opposite direction. In this species, females discharge at higher frequencies than males (Dunlap et al., 1998; Kolodziejski et al., 2005).

EODs in both *A. leptorhynchus* and *A. albifrons* can be modulated to produce discrete signals that are often sexually dimorphic and species-specific. EOD modulations known as chirps and rises are transient changes in the frequency and/or amplitude of an otherwise constant-frequency EOD. Chirps are produced most often in response to social stimulation and vary in structure both within and between species. Several studies have proposed that chirps, and to a lesser extent rises, function as intraspecific communication signals (Bastian et al., 2001; Dunlap and Larkins-Ford, 2003; Engler et al., 2000; Hagedorn and Heiligenberg, 1985; Kolodziejski et al., 2005; Tallarovic and Zakon, 2002; Triefenbach and Zakon, 2003; Zupanc and Maler, 1993).

Chirps can be classified into categories based on frequency excursion and duration. Although the classification of chirp types differs somewhat across studies, two broad classes of chirps, low-frequency and high-frequency chirps, are consistently described in *A. leptorhynchus* (Bastian et al., 2001; Engler et al., 2000; Engler and Zupanc, 2001; Hagedorn and Heiligenberg, 1985; Kolodziejski et al., 2005; Tallarovic and Zakon, 2002; Triefenbach and Zakon, 2003; Zupanc et al., 2006). Low-frequency chirps (also called type II chirps) are by

far the most common chirp type (Engler and Zupanc, 2001; Hagedorn and Heiligenberg, 1985). Although male *A. leptorhynchus* produce more low-frequency chirps than females (Zupanc and Maler, 1993), both sexes produce low-frequency chirps most often in response to stimuli close in frequency to their own EOD. These stimuli simulate the presence of a same-sex conspecific, and therefore a likely rival for mates or resources. Because low-frequency chirps are produced during these simulated competitive encounters, they have been hypothesized to function as aggressive signals (Bastian et al., 2001; Dunlap et al., 1998; Engler and Zupanc, 2001; Hagedorn and Heiligenberg, 1985).

High-frequency chirps (also called type I chirps) are distinct from low-frequency chirps in abundance, structure and putative function, and are produced almost exclusively by males in *A. leptorhynchus* (Bastian et al., 2001). Although females can produce this type of chirp, they rarely do (Kolodziejski et al., 2005). High-frequency chirps are most often produced during mating attempts rather than during same-sex agonistic encounters (Hagedorn and Heiligenberg, 1985). Furthermore, male *A. leptorhynchus* produce more high-frequency chirps in response to playback stimuli simulating female EOD frequencies than to male frequencies (Bastian et al., 2001). High-frequency chirps have therefore been hypothesized to play a role in intersexual communication, possibly conveying information necessary for courtship and successful spawning (Bastian et al., 2001; Engler and Zupanc, 2001; Hagedorn and Heiligenberg, 1985).

*A. albifrons* also produce low- and high-frequency chirps. Unlike *A. leptorhynchus*, however, there is no sex difference in the number of low-frequency chirps produced by *A. albifrons* (Dunlap and Larkins-Ford, 2003; Kolodziejski et al., 2005). Furthermore, although male *A. albifrons* produce more high-frequency chirps than females, this sex difference is less robust than that in *A. leptorhynchus* (Kolodziejski et al., 2005). Thus, despite similarities in the types of chirps produced, it is not known whether low-frequency chirps in *A. albifrons* function as intrasexual signals, and high-frequency chirps as intersexual signals, as has been hypothesized in *A. leptorhynchus*.

To test the hypothesized functions of these two chirp types, we examined chirps produced by *A. albifrons* and *A. leptorhynchus* in response to male-like and female-like stimuli. Because *A. leptorhynchus* and *A. albifrons* produce both types of chirps during social interactions, we hypothesized that the function of each chirp type would be similar in the two species. Thus, if low-frequency chirps are used in agonistic encounters, we predicted that male and female *A. albifrons* would produce more low-frequency chirps in response to same-sex stimuli as seen in *A. leptorhynchus*. Similarly, if high-frequency chirps function as courtship signals across *Apteronotus* species, we predicted that *A. albifrons* would produce more high-frequency chirps in response to opposite-sex stimuli.

## Materials and methods

### Subjects

Twenty reproductively mature *Apteronotus leptorhynchus* Ellis 1912 (9 male, 11 female) and 31 reproductively mature *Apteronotus albifrons* L. (16 male, 15 female) were purchased from commercial suppliers. Fish were housed in individual 36 l

or 64 l tanks within two 2000 l recirculating aquarium systems maintained on a 12:12 light:dark cycle at 26.0–26.4°C, pH 4.5–6.0, conductivity 100–300  $\mu\text{S cm}^{-1}$ . The sex of each fish was initially determined by EOD frequency and later confirmed by laparotomy or *post-mortem* 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).

### Stimulus presentation and electrical recordings

We recorded electrocommunication signals of seven *A. albifrons* during the summer of 2005 following a protocol described previously (Kolodziejski et al., 2005); recordings from 44 additional fish (24 *A. albifrons* and 20 *A. leptorhynchus*) were collected in 2002 as part of a previous study (Kolodziejski et al., 2005). Briefly, fish were placed in PVC tubes covered with plastic mesh at both ends and over a window half way down the length of the tube. The recording tubes were secured to the bottom of a 38 l 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 to deliver stimuli. The signal from the recording electrodes was amplified and band-pass filtered (Model P-55, Grass Instruments; W. Warwick, RI, USA; gain 100 $\times$ , 0.1 Hz–10 kHz). All recordings were collected in the dark; the recording tank was maintained at 25.8–26.1°C and 100–200  $\mu\text{S cm}^{-1}$ .

EOD frequency was monitored with a Fluke multimeter (model 187; Everett, WA, USA) and was also measured in Cool Edit Pro (Syntrillium; Phoenix, AZ, USA). These frequency readings were used to calculate the frequency of each playback stimulus (see below). Sinusoidal electrical stimuli were produced with a function generator (Instek model GFG 8216A or 8219A; Chino, CA, USA) and attenuated to a field intensity of 1.5–2.0 mV  $\text{cm}^{-1}$  (measured parallel to, and midway between, the stimulus electrodes). This signal intensity approximates that of a medium to large conspecific. The amplified signal from the recording electrodes was digitized on the left channel of a sound card (44.1 kHz; SoundBlaster Live; Creative Technologies; Milpitas, CA, USA) and a copy of the stimulus signal was recorded on the right channel.

Before each recording session, fish were allowed 30 min to acclimate to the test tank. After acclimation, five stimuli were presented to the fish in random order. The stimuli varied in their difference frequency (Df) relative to the fish's own EOD frequency. Stimuli 20 Hz above or below the fish's own EOD frequency (+ or –20 Hz Df) simulated same-sex conspecifics; the +150 and –150 Hz Df stimuli simulated a conspecific of the opposite sex or a fish of another species; and the –5 Hz Df stimulus simulated a same-sex conspecific with a similar EOD frequency. Each recording session began with a baseline recording (no stimulus) followed by the five stimulus trials with an intertrial interval of 10 min. Each trial consisted of a 1 min pre-stimulus period (stimulus off), a 2 min stimulus playback, and a 1 min post-stimulus period (stimulus off).

### Analysis of EOD modulations

Chirps and rises, previously referred to as 'short term

frequency modulations' (Kolodziejski et al., 2005), did not exceed 45 s in this study, distinguishing them from longer-term EOD modulations such as the jamming avoidance response (Bullock et al., 1972) and long-term frequency elevations (Oestreich and Zakon, 2002). EOD recordings were analyzed offline with customized procedures similar to those described elsewhere (Kolodziejski et al., 2005; Nelson, 2004) and running in Igor Pro 4.0 (WaveMetrics; Portland, OR, USA). Stimulus contamination was reduced by subtracting an appropriately scaled and phase-shifted copy of the stimulus from the recording. The fundamental EOD frequency was then measured with an autocorrelation algorithm using a 6 ms Hanning window, shifted 2 ms per iteration, resulting in a temporal resolution of 2 ms and a frequency resolution of less than 1 Hz (Nelson, 2004).

The customized Igor procedure also identified and counted chirps and rises. Baseline frequency was measured as the mode of EOD frequency in 2 s windows; chirps and rises were defined as events in which EOD frequency deviated from baseline frequency according to user-defined parameters. For most recordings, chirps and rises were defined as events in which EOD frequency deviated from baseline by 3 Hz or more (frequency excursion) and remained elevated for more than 5 ms, but less than 45 s. Adjacent modulations were analyzed as independent events if separated by at least 100 ms. In rare instances, the threshold for modulations was increased from 3 Hz to 5 Hz above/below baseline to compensate for decreased signal-to-noise ratios. For each chirp or rise identified, the procedure then defined the beginning and end of the modulation as the time at which EOD frequency deviated by 1 Hz above/below baseline EOD frequency. The duration and frequency excursion of each modulation was then calculated with these defined boundaries. Chirps and rises were binned according to which of the 5 stimuli elicited them and when they occurred during each trial: before (pre-stimulus/spontaneous), during (evoked) or after (post-stimulus) the stimulus presentation. Visual inspection of the frequency trace of each modulation confirmed that the automated procedure correctly identified and measured the event.

#### Statistical analysis

We previously used a k-means cluster analysis to categorize modulations into three relatively robust categories: high-frequency chirps, low-frequency chirps, and long-duration rises (Kolodziejski et al., 2005). We used the FM and duration ranges from this previous study to categorize modulations in the present study (Table 1). The production of chirps and/or rises in response to each of the five stimulus frequencies was analyzed with repeated-measures two-way

analysis of variance (ANOVA) in Statistica (StatSoft Inc.; Tulsa, OK, USA) with sex as an independent variable, difference frequency as a repeated measure, and the number of each category of modulation as dependent variables. We also used repeated-measures ANOVA to test for an effect of stimulus presence (i.e. pre-stimulus, evoked and post-stimulus) on the production of different types of modulations. For variables that were significantly affected by difference frequency or stimulus condition, we used Tukey's Honest Significant Difference (HSD) *post-hoc* test to determine which stimuli differentially affected the response. Significance was defined by  $P < 0.05$ .

## Results

### *Sex and species differences in electrocommunication signals*

All fish in both species were in reproductive condition. This was determined by sex differences in EOD frequency and measurements of gonadosomatic index (GSI) reported previously (Kolodziejski et al., 2005). The gonads of the additional fish laparotomized for this study were in similar reproductive condition (yolked follicles and large testes). Sex and species differences in EOD frequency and the production of EOD modulations for most of the animals in this study were reported previously (Kolodziejski et al., 2005); data from the additional seven *A. albifrons* included in this study did not change the previously reported differences. EOD frequency was sexually dimorphic in both *A. leptorhynchus* (males > females) and *A. albifrons* (females > males). The production of chirps and rises also differed across sex and species. Briefly, in *A. leptorhynchus*, males chirped more often than females and produced more high-frequency chirps. In *A. albifrons*, there was no sex difference in the number of chirps produced, although males produced a greater proportion of high-frequency chirps than females. The production of rises was not sexually dimorphic in either species (Kolodziejski et al., 2005).

### *Effect of difference frequency (Df) on chirp and rise production*

Stimulus frequency (as measured by difference frequency, Df, between the stimulus and the fish's own EOD) strongly affected chirping in both *A. leptorhynchus* (main effect of Df:  $F_{(4,72)} = 13.40$ ,  $P < 0.0001$ ) and *A. albifrons* ( $F_{(4,116)} = 13.31$ ,  $P < 0.0001$ ; Fig. 1A). Male and female *A. leptorhynchus* chirped more in response to stimuli close in frequency to their own EOD (5 Hz lower, 20 Hz higher/lower) than to stimuli farther away (150 Hz higher/lower; Tukey's HSD,  $P < 0.05$ ; Fig. 1A). Similarly male and female *A. albifrons* chirped more to the stimuli within 20 Hz of their own EOD than to stimuli 150 Hz higher or lower (Tukey's HSD,  $P < 0.05$ ; Fig. 1A). Stimulus frequency did not affect the number of long duration rises

Table 1. Frequency and duration ranges for EOD modulations\*

	Low-frequency chirps		High-frequency chirps		Long-duration rises	
	Frequency (Hz)	Duration (s)	Frequency (Hz)	Duration (s)	Frequency (Hz)	Duration (s)
<i>A. leptorhynchus</i>	3–167	0.007–0.091	176–433	0.011–0.11	3–270	0.092–37
<i>A. albifrons</i>	2–132	0.021–0.673	142–411	0.086–0.34	2–60	0.71–20.7

\*Based on Kolodziejski et al. (Kolodziejski et al., 2005).

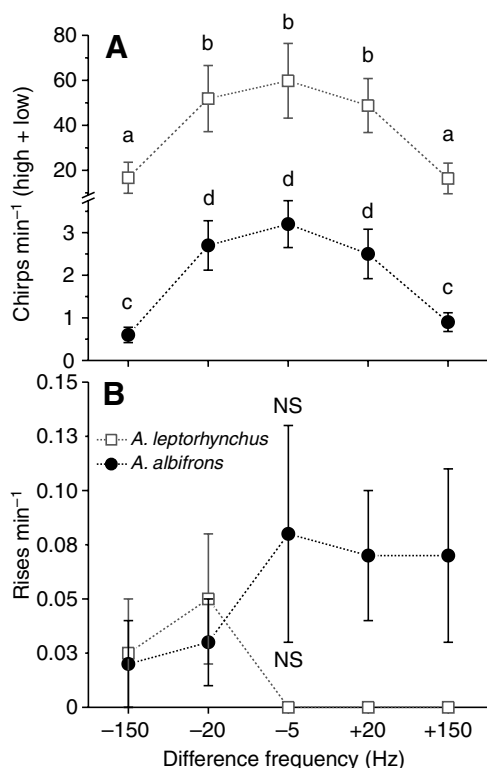


Fig. 1. Number of chirps (A) and rises (B) (mean  $\pm$  s.e.m.) produced during playback stimulation as a function of difference frequency (Df) in *A. leptorhynchus* ( $N=20$ , open squares) and *A. albifrons* ( $N=31$ , closed circles). (A) Both species produced significantly more chirps (low- and high-frequency chirps combined) in response to stimuli close in frequency to their own EOD (within 20 Hz) than to distant frequencies ( $\pm 150$  Hz). (B) There was no effect of Df on the number of rises (long-duration modulations) produced by either species. Data points with different letters differ significantly from each other; Tukey's HSD,  $P < 0.05$ . NS, no significant effect of Df (repeated-measures ANOVA,  $P > 0.05$ ).

produced by either *A. leptorhynchus* (main effect of Df:  $F_{(4,76)}=1.38$ ,  $P=0.26$ ) or *A. albifrons* (main effect of Df:  $F_{(4,120)}=0.70$ ,  $P=0.59$ ; Fig. 1B).

The effect of stimulus frequency on the production of low-frequency chirps mirrored its effect on the production of all chirp types combined. Both *A. leptorhynchus* (main effect of Df:  $F_{(4,72)}=14.1$ ,  $P < 0.0001$ ) and *A. albifrons* (main effect of Df:  $F_{(4,116)}=6.38$ ,  $P < 0.001$ ) produced more low-frequency chirps in response to stimuli close in frequency to their own EOD (Fig. 2A). In *A. leptorhynchus*, the effect of Df was different in males and females (sex  $\times$  Df interaction:  $F_{(4,72)}=8.32$ ,  $P < 0.0001$ ). Males produced more low-frequency chirps in response to stimuli 5 Hz lower and 20 Hz higher or lower than to stimuli 150 Hz higher or lower than the fish's own EOD (Tukey's HSD,  $P < 0.05$ ; Fig. 2A). Although females also tended to produce more low-frequency chirps to stimuli close in frequency to their own EODs, the production of low-frequency chirps in females was not significantly affected by stimulus frequency ( $F_{(4,40)}=1.98$ ,  $P=0.12$ ; Fig. 2B). In *A. albifrons*, the effect of Df was similar to that in *A. leptorhynchus*, but did not differ between males and females (main effect of Df:

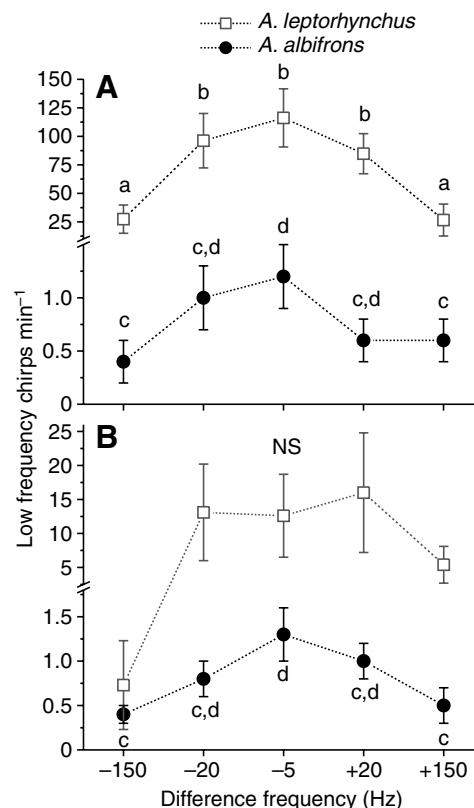


Fig. 2. Number of low-frequency chirps (mean  $\pm$  s.e.m.) produced during playback stimulation as a function of Df in male (A) and female (B) *A. leptorhynchus* (open squares) and *A. albifrons* (closed circles). (A) Male *A. leptorhynchus* ( $N=11$ , open squares) produced more low-frequency chirps to stimuli close in frequency to their own EOD (within 20 Hz) than to distant frequencies ( $\pm 150$  Hz). Similarly, *A. albifrons* males ( $N=16$ , closed circles) produced more low-frequency chirps to stimuli 5 Hz lower than their own EOD compared to stimuli 150 Hz higher or lower. (B) There was no effect of Df on the production of low-frequency chirps in *A. leptorhynchus* females ( $N=9$ , open squares), although the trend was similar to the overall effect of Df seen in males. Female *A. albifrons* ( $N=15$ , closed circles) produced more low-frequency chirps to stimuli within 5 Hz of their own EOD than stimuli 150 Hz lower or higher. Data points with different letters differ significantly from each other; Tukey's HSD,  $P < 0.05$ . NS, no significant effect of Df (repeated-measures ANOVA,  $P > 0.05$ ).

$F_{(4,116)}=6.38$ ,  $P < 0.001$ ; sex  $\times$  Df interaction:  $F_{(1,29)}=0.07$ ,  $P=0.80$ ). Male and female *A. albifrons* produced more low-frequency chirps in response to stimuli 5 Hz lower than to stimuli 150 Hz higher or lower than their own EOD (Tukey's HSD,  $P < 0.05$ ; Fig. 2A,B).

Stimulus frequency also affected the number of high-frequency chirps, but the effect was in opposite directions in the two species. The production of high-frequency chirps in *A. leptorhynchus* was significantly affected by Df (main effect of Df:  $F_{(4,72)}=2.90$ ,  $P=0.028$ ), but the effect differed between males and females (sex  $\times$  Df interaction  $F_{(4,72)}=2.93$ ,  $P=0.026$ ). *A. leptorhynchus* males produced more high-frequency chirps to the 150 Hz lower, opposite-sex, stimulus than to the 5 Hz lower, same-sex, stimulus (Tukey's HSD,  $P < 0.05$ ; Fig. 3A). Female *A. leptorhynchus* rarely produced high-frequency chirps, and the



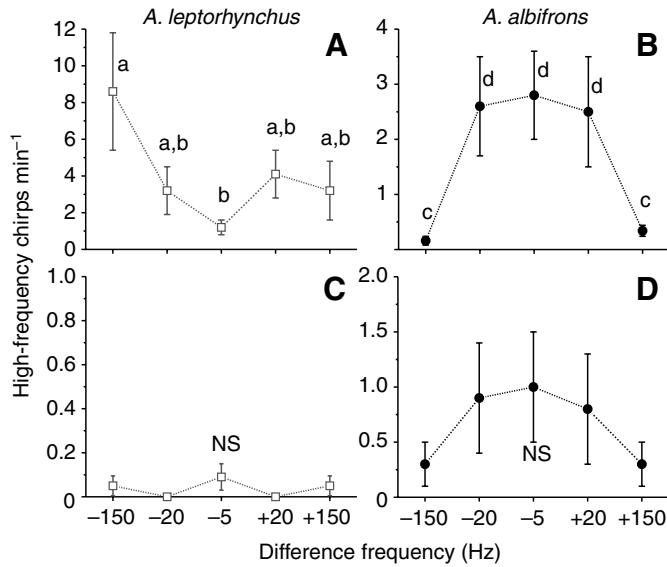


Fig. 3. Number of high-frequency chirps (mean  $\pm$  s.e.m.) produced during playback stimulation as a function of Df in male (A,B) and female (C,D) *A. leptorhynchus* (A,C) and *A. albifrons* (B,D). (A) Male *A. leptorhynchus* ( $N=11$ ) produced significantly more high-frequency chirps to the  $-150$  Hz Df than to the  $-5$  Hz Df. (B) In a pattern opposite to that seen in *A. leptorhynchus*, male *A. albifrons* ( $N=16$ ) produced significantly more high-frequency chirps to same-sex stimuli (within  $20$  Hz) than to opposite sex stimuli ( $\pm 150$  Hz). (C) Female *A. leptorhynchus* ( $N=9$ ) produced almost no high-frequency chirps, and Df did not affect their production. (D) There was no effect of Df on the number of high-frequency chirps produced by female *A. albifrons* ( $N=15$ ), although the trend was similar to the effect of Df seen in males. Data points with different letters differ significantly from each other Tukey's HSD,  $P<0.05$ . NS, no significant effect of Df (repeated-measures ANOVA,  $P>0.05$ ).

number was not affected by stimulus frequency ( $F_{(4,40)}=1.46$ ,  $P=0.23$ ; Fig. 3C). Some previous studies have subdivided high-frequency chirps in *A. leptorhynchus* based on their duration (Engler et al., 2000; Zupanc et al., 2006). We were unable to define a boundary that clearly delineated distinct categories of high-frequency chirps based on duration; because very few males produced longer duration high-frequency chirps, we could not conduct a separate statistical analysis. However, a qualitative analysis indicated that high-frequency chirps in *A. leptorhynchus*, regardless of duration, were more often produced to opposite-sex than same-sex stimuli.

As in *A. leptorhynchus*, stimulus frequency significantly affected the production of high-frequency chirps in *A. albifrons* ( $F_{(4,116)}=7.04$ ,  $P<0.0001$ ), and this effect differed between males and females (sex  $\times$  Df interaction:  $F_{(1,116)}=2.47$ ,  $P<0.05$ ). Surprisingly, the direction of the effect of stimulus frequency on high-frequency chirps in *A. albifrons* was the opposite of that in *A. leptorhynchus*. *A. albifrons* produced more high-frequency chirps to same-sex stimuli than to opposite-sex stimuli (main effect of Df:  $F=7.04$ ,  $P<0.0001$ ). Males produced more high-frequency chirps to stimuli  $5$  Hz lower,  $20$  Hz lower, and  $20$  Hz higher than to stimuli  $150$  Hz lower or higher than their own EOD frequency (Tukey's HSD,  $P<0.05$ ; Fig. 3B). Although there was a trend for a similar

pattern, the effect of Df on the production of high-frequency chirps by female *A. albifrons* was not significant (Tukey's HSD,  $P>0.05$ ; Fig. 3D).

The production of high-frequency chirps in both *A. albifrons* and *A. leptorhynchus* was affected by stimulus frequency, which indicates that both species respond selectively to conspecifics depending on the frequency of their EOD. This effect, however, was opposite in direction in the two species. Whereas *A. leptorhynchus* males increased the production of high-frequency chirps during encounters with female-like stimuli, *A. albifrons* produced fewer high-frequency chirps in the presence of opposite-sex stimuli.

#### Stimulus onset, offset, and the production of EOD modulations

Playback stimuli increased chirping in both *A. leptorhynchus* and *A. albifrons*. *A. leptorhynchus* showed a marked increase in chirping at the start of the stimulus, and a decrease in chirping at stimulus offset (main effect of stimulus condition: ( $F_{(2,36)}=31.91$ ,  $P<0.0001$ ). This effect was much stronger in males than in females (sex  $\times$  stimulus condition interaction:  $F_{(2,36)}=19.13$ ,  $P<0.0001$ ). Despite the strong sexual dimorphism in chirp rate, however, both males and females chirped more when presented with a stimulus than before or after the stimulus (Tukey's HSD,  $P<0.05$ ; Fig. 4A). Similarly in *A. albifrons*, chirping increased in both males and females during stimulus presentation ( $F_{(2,58)}=13.71$ ,  $P<0.0001$ ) and decreased upon stimulus offset (Tukey's HSD,  $P<0.05$ ; Fig. 4A). This effect was not sexually dimorphic in *A. albifrons* (sex  $\times$  Df interaction:  $F_{(2,58)}=1.14$ ,  $P=0.33$ ).

The production of rises was also stimulus dependent, but in a different pattern than that seen with chirps. Unlike chirps, rises were most often produced after the stimulus was turned off in both *A. leptorhynchus* (main effect of condition:  $F_{(2,36)}=21.93$ ,  $P<0.0001$ ) and *A. albifrons* ( $F_{(2,58)}=13.71$ ,  $P<0.0001$ ). In *A. leptorhynchus*, males and females produced the most rises after stimulus offset, significantly fewer rises prior to stimulus onset, and fewer still during electrical playbacks (Tukey's HSD,  $P<0.05$ ; Fig. 4B). Similarly in *A. albifrons*, more rises were produced after stimulus offset than before or during the stimulus (Tukey's HSD,  $P<0.05$ ; Fig. 4B).

#### Discussion

Stimulus frequency differentially affected chirping in both *A. leptorhynchus* and *A. albifrons*. Both species produced more low-frequency chirps in response to stimuli close in frequency to their own EOD, suggesting that low-frequency chirps are universally directed towards individuals of the same sex. The production of high-frequency chirps was also affected by stimulus frequency, but in opposite directions in the two species. In *A. leptorhynchus*, high-frequency chirps were more often produced in response to opposite-sex stimuli. In contrast, high-frequency chirps were more often produced in response to same-sex stimuli in *A. albifrons*.

The presence of the playback stimulus affected the production of chirps and rises differently. Both species produced more chirps during stimulus presentation than before or after the stimulus. Rises, on the other hand, were produced most often after stimulus offset and were rarely produced spontaneously (before stimulus onset) or during stimulation.

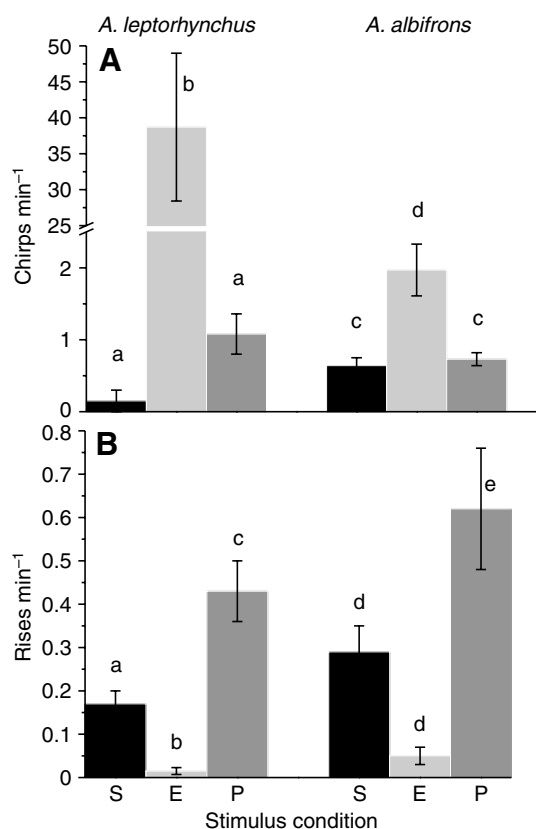


Fig. 4. Number of chirps (A) and rises (B) (mean  $\pm$  s.e.m.) produced before (S, spontaneous), during (E, evoked), and after (P, post-stimulus) stimulus presentation. (A) Both *A. leptorhynchus* and *A. albifrons* produced more chirps during stimulation (E) than before stimulus onset (S) or after stimulus offset (P). (B) Both species produced more rises after stimulus offset (P) than before stimulus onset (S) or during stimulus presentation (E). Data points within each species that have different letters differ significantly from each other; Tukey's HSD,  $P < 0.05$ .

#### Function of chirps in communication

The high- and low-frequency chirps in this study are similar to the type I and type II chirps reported by others (Engler et al., 2000; Bastian et al., 2001; Engler and Zupanc, 2001). Because high-frequency chirps in *A. leptorhynchus* are produced almost exclusively by males and are preferentially elicited by female-like stimuli, they have been hypothesized to function as courtship signals (Bastian et al., 2001; Engler and Zupanc, 2001; Hagedorn and Heiligenberg, 1985). Similarly, because both male and female *A. leptorhynchus* produce more low-frequency chirps in response to same-sex stimuli, low-frequency chirps have been hypothesized to function in an agonistic context (Bastian et al., 2001; Dunlap et al., 1998; Engler and Zupanc, 2001; Hagedorn and Heiligenberg, 1985). Our results replicate these findings in *A. leptorhynchus*.

Although our data support the hypothesis that low-frequency chirps are used during same-sex aggressive interactions in both species, our data suggest that, unlike in *A. leptorhynchus*, high-frequency chirps are not likely to be courtship signals in *A. albifrons*. The fact that high-frequency chirps are produced by both male and female *A. albifrons* and are best elicited by same-sex stimuli suggest that high-

frequency chirps may function as intrasexual agonistic signals in this species.

Why might same-sex stimuli elicit low-frequency chirps in *A. leptorhynchus* but both low- and high-frequency chirps in *A. albifrons*? One possibility is that low- and high-frequency chirps represent a single graded signal in *A. albifrons* that varies in frequency excursion with changes in motivation; high-frequency chirps might simply be structurally exaggerated forms of low-frequency chirps that convey similar information. A second possibility is that both low- and high-frequency chirps are directed towards same-sex receivers, but carry different or even opposite information during intrasexual communication in *A. albifrons*. More studies examining the context in which each chirp type is produced and the responses of receivers to each chirp type are needed to address this question. In either case, high-frequency chirps appear to have been co-opted for courtship in *A. leptorhynchus*.

#### Evolution of electrocommunication signals: does form predict function?

The function of chirps as communication signals in gymnotiforms has been studied mostly in *A. leptorhynchus*. Electrocommunication signals in *A. leptorhynchus*, however, differ in several respects from those in most other species: EOD frequency is higher in males than females, sexual dimorphism in chirping is extremely pronounced, and certain aspects of chirp structure (short duration, frequency undershoot) are shared with few other species (Dunlap and Larkins-Ford, 2003; Kolodziejcki et al., 2005; Turner et al., 2005; Zhou and Smith, 2006). *A. leptorhynchus* and *A. albifrons* are closely related taxa, and recently published gymnotiform phylogenies suggest that they belong to the same monophyletic genus (de Santana, 2002; Crampton and Albert, 2006). Differences between *A. leptorhynchus* and *A. albifrons* in signal structure and production, therefore, suggest broader species diversity in electrocommunication signals. In fact, species diversity in EOD frequency and waveform is well-documented (Crampton and Albert, 2006; Hopkins, 1988; Kramer et al., 1980), and the structure of chirps also differs across apteronotid species (Turner et al., 2005; Zhou and Smith, 2006). Sympatry could also influence the rapid evolution of chirp structure, as has been suggested for EOD frequency and waveform (Hopkins and Heiligenberg, 1978). Indeed, *A. leptorhynchus* and *A. albifrons* are sympatric over part of their range (de Santana, 2003; de Santana et al., 2007); and it is possible that differences between these species in chirping might have resulted from character displacement.

Chirping is a common social trait shared by many wave-type electric fish species, both within and outside the Apteronotidae, and is most likely ancestral to the lineage leading to this family (Dunlap et al., 1998; Hopkins, 1974a; Hopkins, 1974b; Zhou and Smith, 2006). However, chirp structure has evolved rapidly in apteronotids. For example, chirps produced by *Adontosternarchus devenanzii*, another apteronotid, differ from those produced by *A. leptorhynchus* and *A. albifrons*. Although all three species produce chirps and rises, *A. devenanzii* does not produce any low-frequency chirps analogous to those of *Apteronotus*. Instead, this species only produces high-frequency chirps and long-duration rises. Furthermore, many chirps in *A.*

*devenanzii* have complex spectro-temporal structure and multiple frequency peaks (Zhou and Smith, 2006).

The evolutionary plasticity in chirp structure across different apteronotid species might correlate with, or at least provide the opportunity for, plasticity in chirp function. Our results not only demonstrate sex and species differences in chirp structure (frequency modulation, duration) and chirp production within *Apteronotus*, but also suggest that the functions of different chirp types might be just as labile. Because the function of chirping has been studied in few apteronotids, more comparative studies are needed to assess structural and functional variation across species.

#### *Functional specificity and sexual dimorphism in signal production*

Well-documented examples of signals evolving different functions in closely related species are rare. Signal function has, however, evolved differently in males and females of single species (Berglund et al., 1996). For example, claw-waving in male fiddler crabs, *Uca pugilator*, is context-dependent and primarily directed at females. Although other males may be present during signaling, potential recipient males are not attentive to the displays and therefore do not elicit claw-waving in the absence of females (Pope, 2000a; Pope, 2000b). Dual-function signals such as birdsong used for mate attraction, territoriality and defense are also relatively common (Berglund et al., 1996). Still other examples exist in which the context-dependence of signal production has evolved differently in males and females. In the sex role-reversed deep-snouted pipefish, both males and females engage in mate competition and courtship and use color ornaments in signaling. The function and context of the ornament display, however, differs between the sexes. Females display their sexual ornament for both courtship and competition. Males, on the other hand, display their ornament only during courtship, but not during intrasexual competition (Berglund et al., 2005).

These intraspecific examples, however, do not necessarily indicate how interspecific differences in signal function might evolve. The difference in the context-dependence of high-frequency chirps in *A. leptorhynchus* and *A. albifrons* represents a rare example in which a communication signal has evolved distinct functions in two closely related species. Our findings that both signal structure and function may have recently and rapidly diversified across species in *Apteronotus* thus provide a unique opportunity to investigate the evolutionary mechanisms linking signal structure and function.

#### *Sexual dimorphism in EOD frequency, electrosensory processing and chirp function*

The proposed differences in chirp function in *A. leptorhynchus* and *A. albifrons* necessarily require that EOD frequency be a reliable indicator of sex in both species. This dimorphism is well established in *A. leptorhynchus*, but is less clear in *A. albifrons*. EOD frequency has been repeatedly shown to be sexually dimorphic in *A. albifrons* when fish are in reproductive condition (Dunlap et al., 1998; Kolodziejewski et al., 2005). However, the sex difference in EOD frequency is not as robust as that seen in *A. leptorhynchus* and is therefore often not reported (Dunlap and Larkins-Ford, 2003). Variation in the

magnitude of sex differences in EOD frequency could lower the reliability of the EOD as an indicator of sex. As such, it is possible that chirp production, structure and function could change dramatically as populations diverge in their ability to decipher sex and individual information from EOD frequency alone. Thus, the evolution of sex differences in the function of chirps might be linked to the evolution of sex differences in EOD frequency.

Interactions between the electromotor and electrosensory systems could also influence the evolution of electrocommunication signals. Chirp structure and function are likely linked to the sensory mechanisms that allow fish to detect the EODs and modulations of conspecifics. For example, P-type electroreceptors (P-units) in *A. leptorhynchus* encode low-frequency and high-frequency chirps differently, depending on the social context in which they are produced (Benda et al., 2006). The synchrony of P-unit firing depends on the beat frequency created by the interference of two EODs (Benda et al., 2005; Benda et al., 2006; Chacron et al., 2005). P-units fire asynchronously in response to small difference frequencies (same-sex EODs), but fire synchronously to large Dfs (opposite-sex EODs). Low-frequency and high-frequency chirps alter the synchrony of P-units in opposite directions: low-frequency chirps transiently increase synchrony of P-units, whereas high-frequency chirps desynchronize P-unit activity (Benda et al., 2006). The sensory mechanisms by which beat frequency and chirping are encoded might provide a reception-based framework for sex differences in the production and function of different chirp types. Low-frequency chirps produced in response to same-sex stimuli dramatically synchronize the relatively asynchronous P-units. Similarly, high-frequency chirps produced by male *A. leptorhynchus* in response to opposite-sex stimuli, desynchronize otherwise strongly synchronous P-units. The structure and function of different chirp types in *A. leptorhynchus* thus capitalizes on these sensory mechanisms such that each type of chirp is produced in the context in which it is most detectable.

The coding of same-sex *versus* opposite-sex beat frequencies and of low- *versus* high-frequency chirps by P-units has not been studied in *A. albifrons*. The fact that high-frequency chirps are produced most often to same-sex stimuli in this species, however, raises several interesting questions. For example, if P-units in *A. albifrons*, like those in *A. leptorhynchus*, encode conspecific EODs and chirps *via* changes in firing synchrony, are high-frequency chirps less detectable when produced in response to same-sex (low Df) stimuli? Alternatively, do *A. leptorhynchus* and *A. albifrons* use different sensory mechanisms to encode high- *versus* low-frequency chirps? These differences might specifically facilitate the detection of different chirp types in the different social contexts in which they are normally produced in each species. Species differences in chirp duration could also influence electrosensory mechanisms for chirp detection. Chirps are significantly longer in *A. albifrons* than in *A. leptorhynchus* (Dunlap and Larkins-Ford, 2003; Kolodziejewski et al., 2005). It is possible that the longer duration chirps in *A. albifrons* are easier to detect over the slow beats created by same-sex EODs. Thus, the evolution of chirp duration could be linked *via* sensory mechanisms to the social contexts in which they are produced. Additional studies



on how the electrosensory system processes beat frequencies and chirping in different species will increase our understanding of how the evolution of signal structure and function could both influence, and be influenced by, constraints on sensory processing.

#### *Stimulus condition and rise production*

Unlike chirps, long-duration rises were most frequently produced before and after stimulus presentation and were rarely produced during playbacks in either *A. leptorhynchus* or *A. albifrons*. Additionally, both *A. leptorhynchus* and *A. albifrons* produced more rises after stimulus offset than before stimulus onset. These results are similar to the pattern with which extremely long-duration rises (also called yodels) are produced in *A. leptorhynchus* (Dye, 1987). This indicates that the removal of a stimulus can elicit rises in both species. Although several studies have reported rises and hypothesized on the information that they convey, no clear consensus on their function has emerged (Dye, 1987; Hopkins, 1974b; Serrano-Fernandez, 2003; Tallarovic and Zakon, 2002; Zupanc et al., 2001). Our results demonstrate that playback stimuli actually suppress rise production. One possible explanation for this result is that rises might not function as communication signals at all. Rises might simply be startle responses to novel or unusual stimuli, in this case the abrupt removal of a stimulus. Rises might also aid in electrolocation and might be produced as search responses when a previously detectable EOD disappears.

If rises are, on the other hand, intraspecific communication signals similar to chirps, it is not entirely clear why stimulus removal might increase rise production. One hypothesis suggests that rises represent a dominant 'victory signal' at the apparent retreat of a potential rival (Dye, 1987), as suggested in other taxa (Grafe and Bitz, 2004a; Grafe and Bitz, 2004b). In female *A. leptorhynchus*, rises have been suggested to signal dominance to other females and to advertise reproductive condition to males (Tallarovic and Zakon, 2002). If rises do indicate dominance, this hypothesis would predict that rises would be produced more often by winners of physical agonistic encounters, rather than by losers. It might also predict that rises would be produced more often when two sparring individuals are in the presence of an audience of conspecifics similar to dominance signals produced in other fish species (Doutrelant et al., 2001; Matos and McGregor, 2002). Although our finding that rises are produced when the stimulus is removed is consistent with this hypothesis, further work is needed to assess the adaptive value of signaling dominance upon the retreat of a rival. In order to specifically test the hypothesis that rises are post-conflict victory signals, experiments would have to explicitly assess whether they are effective deterrents of future territorial disputes.

Another hypothesis suggests that rises are submissive signals, produced by less dominant individuals within an established social hierarchy (Hopkins, 1974b; Serrano-Fernandez, 2003). If, however, the removal of a stimulus indicates the retreat of a presumed competitor, then our data do not fully support this hypothesis as fish in both species produced more rises after stimulus removal. Another possibility is that rises are advertisement signals produced only by solitary individuals and

enable the signaler to indicate territorial claims, reproductive readiness, or other socially relevant behavioral states. These hypotheses can be tested by further examining the context in which rises are produced, whether they are produced in response to specific stimuli, and whether other fish respond to their production.

#### *Conclusions*

Common behavioral, environmental and physiological constraints faced by different taxa can provide insight into the evolution of signal complexity. Chirping in weakly electric fish is a useful model system for studying communication systems because of the sheer array of species diversity in the structure and sexual dimorphism of these signals. This diversity, as well as the ease with which electrocommunication signals can be recorded and analyzed, facilitates further comparative studies. Additionally, the relative simplicity of the underlying neural circuitry allows for simultaneous study of the evolution of communication behavior and its underlying physiological correlates. The finding that *A. leptorhynchus* and *A. albifrons* use high-frequency chirps in different social contexts suggests that chirp function may vary substantially across different species. To better understand this potentially complex relationship between chirp production, function, and social context, more studies correlating electrical and physical behaviors, similar to earlier works (Hagedorn and Heiligenberg, 1985; Hopkins, 1974a; Hopkins, 1974b), are needed. Similarly, ethograms of other species in naturalistic conditions, linking physical courtship and/or aggression with electrical displays, may help us to better define the ecological correlates of chirp structure and function. A better understanding of how the physical environment, ecology and social contexts influence both the structure and information content of electrocommunication signals across difference species of electric fish may lead to broader insights in the evolution of complex communication signals.

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