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Phylogenetic comparative analysis of electric communication signals in ghost knifefishes (Gymnotiformes: Apteronotidae)

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Summary

Electrocommunication signals in electric fish are diverse, easily recorded and have well-characterized neural control. Two signal features, the frequency and waveform of the electric organ discharge (EOD), vary widely across species. Modulations of the EOD (i.e. chirps and gradual frequency rises) also function as active communication signals during social interactions, but they have been studied in relatively few species. We compared the electrocommunication signals of 13 species in the largest gymnotiform family, Apteronotidae. Playback stimuli were used to elicit chirps and rises. We analyzed EOD frequency and waveform and the production and structure of chirps and rises. Species diversity in these signals was characterized with discriminant function analyses, and correlations between signal parameters were tested with phylogenetic comparative methods. Signals varied markedly across species and even between congeners and populations of the same species. Chirps and EODs were particularly evolutionarily labile, whereas rises differed little across species. Although all chirp parameters contributed to species differences in these signals, chirp amplitude modulation, frequency modulation (FM) and duration were particularly diverse. Within this diversity, however, interspecific correlations between chirp parameters suggest that mechanistic trade-offs may shape some aspects of signal evolution. In particular, a consistent trade-off between FM and EOD amplitude during chirps is likely to have influenced the evolution of chirp structure. These patterns suggest that functional or mechanistic linkages between signal parameters (e.g. the inability of electromotor neurons increase their firing rates without a loss of synchrony or amplitude of action potentials) constrain the evolution of signal structure.

Supplementary material available online at
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Key words: communication, behavior, signal evolution, comparative approach, phylogenetic analysis, electric fish, chirping.

Introduction

The evolution of communication behavior, like that of all traits, is shaped both by external forces of natural and sexual selection and by internal forces of development and physiology. Complexity in both the function and production mechanisms of communication signals makes them a powerful model for understanding evolutionary interactions within and across levels of biological organization (Ryan, 2005).

Electrocommunication in weakly electric fish is an outstanding model for integrating mechanistic and historical biology (*sensu* Autumn et al., 2002) because these behaviors are diverse across species, are easily recorded and analyzed, and are controlled by a well-characterized neural circuit. The family Apteronotidae is particularly well-suited for a comparative approach because it has the highest species diversity among Neotropical electric fish (Crampton and Albert, 2006). Apteronotids continuously emit a quasi-sinusoidal voltage signal or electric organ discharge (EOD) that has two functions. Nearby objects locally distort the electric field generated by the EOD, and by detecting these distortions with their electroreceptors, fish can electrolocate. The fish can also use their EODs to communicate by detecting the interactions of their own EOD with those of other fish. Individual apteronotid fish maintain an extremely stable EOD frequency (EODf) (Bullock, 1970; Moortgat et al., 1998) and waveform (Rasnow and Bower, 1996). In addition to using the baseline EOD frequency and waveform as communication signals, fish also modulate the frequency and amplitude of the EOD during social interactions

(Hagedorn and Heiligenberg, 1985; Larimer and MacDonald, 1968). Two categories of EOD modulations (EODMs) are typically produced: chirps (Bullock, 1969), which have short durations (~10–1000 ms) and relatively large increases in frequency (50–600+ Hz), and gradual frequency rises (GFRs), which usually have longer, more variable durations (~10 ms–60 s) and less frequency modulation (<100 Hz) (Engler et al., 2000).

Comparative studies (>2 species) (Garland and Adolph, 1994) of apteronotid electrocommunication have focused primarily on EOD frequency and waveform because the EOD is constantly emitted and its frequency and waveform are therefore easily measured (Crampton, 1998; Crampton and Albert, 2006; Heiligenberg and Bastian, 1980; Hopkins and Heiligenberg, 1978; Kramer et al., 1981; Steinbach, 1970). These signals vary across species and are sexually dimorphic in some species. They can therefore function as broadcast signals that continuously allow receivers to gain information about the sex and species of the signaler. EODf and waveform overlap between some sympatric species, however, which may limit their ability to unambiguously convey species identity (Crampton, 1998; Crampton and Albert, 2006; Kramer et al., 1981).

Unlike the EOD, chirps are evoked signals that are emitted primarily during agonistic encounters and courtship (Hagedorn and Heiligenberg, 1985). Because chirps are actively produced in response to social stimuli rather than being continuously emitted, they can convey immediate motivational and conditional information as well as information about species identity and sex (Hopkins, 1974b). The function of chirps in active agonistic and reproductive communication might have exposed them to strong sexual and natural selection. If so, the structure and production of chirps should have evolved to be at least as diverse across species as EOD frequency and waveform. The transience of chirps and GFRs, however, also makes them more difficult to record than EOD frequency and waveform, and these signals have been studied in few species. Of the 60+ apteronotid species, chirps and GFRs have been described in only three (*Apteronotus leptorhynchus*, *Apteronotus albifrons* and *Adontosternarchus devenanzii*) (Dunlap and Larkins-Ford, 2003; Engler et al., 2000; Kolodziejski et al., 2005; Zhou and Smith, 2006; Zupanc and Maler, 1993). The structure of chirps, and to a lesser extent GFRs, varies considerably across these three species, but broader patterns of EODM evolution remain unexplored.

To gain a more complete understanding of the evolution of communication in apteronotids, we described the structure of electrocommunication signals in 10 additional apteronotid species, focusing particularly on chirps and GFRs. We then used discriminant function analysis to characterize species diversity in key signal features. Finally, to look for phylogenetic evidence of mechanistic relationships shaping signal evolution we tested for correlations between several electrocommunication parameters.

Materials and methods

Animals

Subjects from five species [four *Parapteronotus hasemani* (Ellis 1913), five *Sternarchella terminalis* Eigenmann and Allen 1942, seven *Sternarchorhynchus* cf. *curvirostris* Boulenger 1887, two *Porotergus gimbeli* Ellis 1912, and one '*Apteronotus*' n. sp. B Crampton and Albert 2006] were collected from the Rio Solimões/Rio Negro confluence at Ilha do Catalhão near Manaus, Brazil in May 2005. These subjects were housed in an aquarium system at the Laboratory of Behavioral Physiology (LFC) in the Instituto Nacional de Pesquisas da Amazônia in Manaus. Table S1 in supplementary material summarizes sex and body size data for the subjects. Fish were housed individually (if aggression was observed) or in groups in 36-liter tanks and were recorded within one week of capture.

Subjects from seven species [10 *P. hasemani*, 10 '*Apteronotus*' *bonapartii* (Castelnau 1855), seven *P. gimbeli*, 10 *Adontosternarchus balaenops* (Cope 1878), nine *Sternarchogiton nattereri* (Steindachner 1868), one *Sternarchogiton porcinum* Eigenmann and Allen 1942, and eight *Sternarchorhynchus* cf. *roseni* Mago-Leccia 1994] were obtained through a commercial tropical fish dealer (Ornamental Amazon Fish Aquarium, Iquitos, Peru) in May 2006. The dealer collected the fish from the Amazonas River (*P. hasemani*, *A. bonapartii*, *P. gimbeli*, *S. nattereri*, *A. balaenops*) and the Itaya River (*S.* cf. *roseni*) near Iquitos. Fish were housed individually (if aggression was observed) or in groups in 36-liter, 64-liter, 210-liter or 340-liter tanks within a recirculating aquarium system at Indiana University. The tanks were maintained on a 12 h:12 h light:dark cycle at 26.0–26.7°C, pH 4.5–6.0, and conductivity of 100–300 μS cm^{-1}. Fish from Peru were recorded within 4 months of receipt from the fish dealer. This study was conducted within the guidelines outlined by the National Institute of 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) at Indiana University.

To compare the production of communication signals across species, we re-analyzed previously published data (Kolodziejski et al., 2005; Zhou and Smith, 2006) from three additional species [*Apteronotus leptorhynchus* Ellis 1912, *Apteronotus albifrons* Linnaeus 1766, and *Adontosternarchus devenanzii* Mago-Leccia, Lundberg and Baskin 1985].

Behavioral recordings

Electrocommunication signals were recorded with a stimulus playback design following the methods of Kolodziejski et al. (Kolodziejski et al., 2005). Experiments occurred at various times of day and night and were performed in a completely darkened recording chamber. Although more EODMs are produced in the dark phase of the light:dark cycle, experiments with constant light or dark conditions demonstrate that the production of EODMs is directly responsive to light condition rather than being under an endogenous circadian rhythm (Zupanc et al., 2001) (C.R.T., unpublished observations). Subjects were placed inside a custom-built PVC tube with plastic mesh covering both ends and a mesh-covered aperture at the tube's midpoint. The size of the tube was adjusted for each subject to minimize movement while allowing normal body position. The tube was placed in the center of a 37-liter recording aquarium maintained at 25.8–27.0°C and containing water from the fish's home tank. The behavioral chamber was then closed and the fish was allowed to acclimate to the recording tank for 30 min. A pair of carbon electrodes placed at the fish's head and tail recorded its EOD, and a second pair of

electrodes on either side of the recording tube was used to present playback stimuli. The signal from the recording electrodes was band-pass filtered (0.1 Hz–10 kHz), amplified [100–1000×; model P-55 (Grass Instruments, West Warwick, RI, USA) or model 3000 (A-M Systems, Sequim, WA, USA)] and digitized at 44.1 kHz on the left channel of a sound card in a computer running Cool Edit Pro (Syntrillium, Phoenix, AZ, USA). Playback stimuli were sinusoidal voltage signals generated by a function generator (Model AFG320; Sony/Tektronix, Tokyo, Japan) or by a computer using Cool Edit Pro and were calibrated to a species-specific root-mean-square (RMS) field amplitude (0.3–1.5 mV cm^{-1}) measured parallel to the stimulating electrodes and midway between them. The amplitude was kept the same within each species and was chosen to mimic the EOD amplitude of that species. A copy of the stimulus was digitized on the right channel of the sound card. A 4 min baseline recording was made from each fish without stimulation, and five recordings were made with different playback stimuli. Each recording consisted of a 1 min baseline period with no stimulation, two minutes of playback stimulation and 1 min post-stimulus. The frequencies of the playback stimuli were set relative to each subject's own baseline EOD frequency: 150 Hz above and below the EOD frequency (±150 Hz), 20 Hz above and below the EOD frequency (±20 Hz) and 5 Hz below the EOD frequency (–5 Hz). The playback frequencies spanned the species-typical range of EOD frequencies and were meant to simulate the presence of a conspecific fish in the recording tank. The –5 Hz stimulus was expected to evoke a jamming avoidance response (Bullock et al., 1972). The stimuli used were the same as those in previous studies of EODMs in *A. leptorhynchus*, *A. albifrons* and *A. devenanzii*, which allowed us to compare our results directly with those studies (Kolodziejski et al., 2005; Zhou and Smith, 2006). Stimuli were presented in random order and were separated by 10-min intervals without stimulation. We pooled measurements of EODMs across all of the playback stimuli, and our measurements therefore represent the overall signal production to playbacks across a species-typical range of EOD frequencies. Because this study focused primarily on species differences in the structure of signals, cross-species comparisons of chirp and GFR production as a function of stimulus frequency are beyond the scope of the present study and will be presented as part of a subsequent study.

Signal parameters

Baseline EOD

The baseline EODf (Fig. 1A) of each fish was measured during its recording session by using the frequency analysis function in Cool Edit Pro [fast Fourier transform (FFT) size=65536]. Water temperature was recorded to the nearest 0.1°C, and a $Q_{10°C}$ of 1.6 was used to correct each EODf measurement to that expected at 25.0°C (Dunlap et al., 2000). To quantify one parameter of EOD waveform, we also measured 'waveform complexity' (Fig. 1B) as the powers of the second and third FFT harmonics relative to that of the fundamental (F2–F1 and F3–F1, in dB). More positive values of F2–F1 and F3–F1 indicated more complex (i.e. polyphasic) waveforms. We used a customized procedure written by Brian Nelson (BSound version 1; available at http://homepage.mac.com/bsnelson/Igor/BSound.html) and running in Igor Pro (Wavemetrics, Lake Oswego, OR, USA) to generate a power spectrum (8192 points, Hanning window) of the baseline

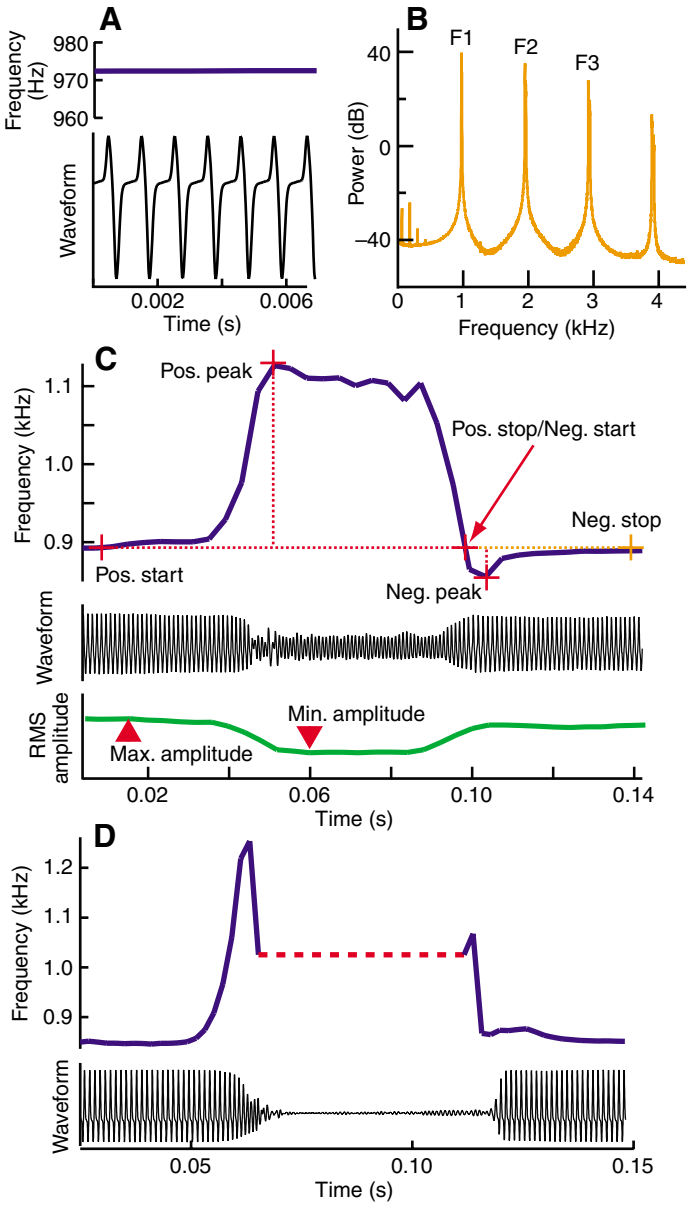

Fig. 1. Signal parameter measurements. (A) EOD frequency trace (top) and head-to-tail EOD waveform (voltage) trace (bottom). (B) Power spectrum (8192 points, Hanning window) of an EOD recording showing fundamental (F1), second (F2) and third (F3) harmonic frequencies. (C) EOD frequency trace (top), head-to-tail EOD waveform (voltage) trace (middle), and root-mean-square (RMS) EOD amplitude trace (bottom) for a single chirp. Points used to measure signal parameters are indicated with crosses and/or arrows. See Table 1 for details on how duration and FM parameters were calculated from these points. (D) EOD frequency trace (top) and head-to-tail EOD waveform (voltage) trace (bottom) for a chirp with extreme and prolonged reduction of EOD amplitude. Broken red line indicates where EOD frequency was not measurable (i.e. when RMS EOD amplitude dropped below 15% of baseline). Estimates of EODf were fixed at this frequency until RMS EOD amplitude returned to at least 15% of its baseline.

recording of each fish and to measure the power of the first three harmonics. The power of the fundamental (in dB) was then subtracted from the power of the second and third harmonics to quantify the relative power of each harmonic.

EOD modulations

We used a customized procedure written by Brian Nelson (eFish version 23e; available at http://homepage.mac.com/bsnelson/eFish/efish.html) and running in Igor Pro to count and measure the parameters of EODMs (see Kolodziejski et al., 2005). Briefly, any playback-induced contamination of the recording was removed by subtracting an appropriately scaled and phase-shifted copy of the playback signal. The fundamental frequency of the EOD was calculated by using an autocorrelation algorithm on 6 ms Hanning windows, advanced 2 ms per iteration. This process resulted in a temporal resolution of 2 ms and a frequency resolution of 0.5–3 Hz, depending on the signal–noise ratio of the recording. The RMS amplitude of the EOD was calculated on the same 6 ms windows.

eFish used the mode of EODf in sliding 2 s windows as a baseline frequency from which to detect EODMs. The procedure counted EODMs as any event in which EODf exceeded this baseline frequency by more than 3 Hz for more than 10 ms and less than 60 s. The beginning and end of each EODM was then defined as the time at which EODf crossed a threshold of 1 Hz above or below the baseline frequency. eFish then recorded the time and EODf at five points on each EODM: positive start, positive peak, positive stop, negative peak, and negative stop (Fig. 1C). For EODMs without undershoots (negative phases), only the first three points were recorded. The RMS amplitude data from each recording were imported into Microsoft Excel (Microsoft Corp., Bellevue, WA, USA), and we measured the maximum and minimum RMS amplitude during the positive phase of each chirp (Fig. 1C). Each EOD modulation was also examined by the experimenter to confirm that the automated procedure accurately identified the EODM and measured its parameters. Using the measurements of time, EODf and EOD amplitude, we calculated the following signal parameters for each EODM: duration, frequency modulation (FM), relative amplitude modulation (%AM), undershoot FM, positive FM slope, and negative FM slope (Table 1).

We differentiated GFRs from chirps in each species by visually examining the positive FM *versus* duration of all EODMs on a scatter plot (see Results). In all species except *S.* cf. *roseni*, *A. leptorhynchus*, *A. albifrons* and *A. devenanzii*, the two categories of EODMs formed discrete clusters distinguishable by the amount of FM. In *S.* cf. *roseni*, *A. leptorhynchus*, *A. albifrons* and *A. devenanzii*, the FM and duration of low-FM chirps and GFRs overlapped somewhat, but they could be distinguished based on a combination of FM and duration. Because the FM and the duration of GFRs are positively correlated within species, we used lines based on this FM–duration relationship to distinguish chirps from GFRs. For *S.* cf. *roseni*, *A. leptorhynchus* and *A. albifrons*, we classified all EODMs having FM>21×(duration)+10 as chirps. In *A. devenanzii*, GFRs and chirps formed discrete clusters distinguishable by the line FM=21×(duration)+25 (see Results).

In two species (*P. hasemani* and *P. gimbeli*), some chirps displayed extreme and prolonged reduction of EOD amplitude (Fig. 1D) (see Results). When EOD amplitude dropped below 15% of its baseline, the ability to resolve EODf became erratic. Therefore, we fixed the estimate of EODf at its last measurable value between the first sampled window in which RMS amplitude dropped below the 15% threshold and the first window in which the RMS amplitude returned to at least 15% of baseline (Fig. 1D). We used the mode of RMS amplitude in sliding 1-s windows as a baseline amplitude from which to detect levels below 15%. Once RMS amplitude returned to at least 15% of baseline, the EODf was calculated as before in eFish.

Values for signal parameters were averaged for each fish, and all statistical and phylogenetic analyses were performed on the individual means.

Discriminant function analysis

We used discriminant function analyses (DFAs) to assess signal diversity and to quantify the ability of different signals (EODs, chirps and GFRs) to carry species-identifying information. One of the assumptions of DFA is low multicolinearity of independent variables [i.e. lack of strong correlations between variables (Spicer, 2004)]. To ensure that the signal parameters used in the DFA were independent of each

Table 1. *Calculation of signal parameters*

Signal parameter	Abbreviation	Definition
EOD frequency	EODf	Baseline frequency of the electric organ discharge
Waveform complexity (F2–F1)	(F2–F1)	Power (dB) of second harmonic relative to fundamental
Waveform complexity (F3–F1)	(F3–F1)	Power (dB) of third harmonic relative to fundamental
Chirp/GFR rate	Rate	Total number of chirps or GFRs divided by the total recording duration
Chirp/GFR duration	DUR	Length of time between the positive start time and the positive stop time
Chirp/GFR frequency modulation	FM	Frequency difference between the positive start frequency and the positive peak frequency
Chirp % amplitude modulation	%AM	Amplitude difference between maximum amplitude and minimum amplitude, divided by maximum amplitude
Chirp undershoot FM	Undershoot FM	Frequency difference between the negative start frequency and the negative peak frequency
Chirp/GFR positive FM slope	+FM slope	Frequency modulation (see above) divided by the length of time between the positive start time and the positive peak time
Chirp/GFR negative FM slope	–FM slope	Frequency modulation (see above) divided by the length of time between the positive peak time and the positive stop time

other, we first performed separate principal components analyses (PCAs) on parameters of EODs, chirps and GFRs. The PCA factors were then used as independent variables in the DFAs, with species as a grouping variable. Four separate DFAs were performed: one with all signal variables, and separate DFAs with only EOD, only chirp and only GFR variables. The contribution of different signal parameters to species diversity and discrimination was estimated by the loadings of the signal variables on the DFA. To assess the ability of different signal types (EODs, chirps and GFRs) to identify species, we also used a cross-validated classification using the canonical roots created by the DFAs (Spicer, 2004). We excluded one individual of each species from the analysis and predicted the species of the excluded individuals based on the canonical roots from DFAs generated from the remaining individuals. This process was repeated with different individuals excluded until each individual was classified at least once. The percentage of individuals whose species was correctly classified provided an index of the ability of the signals in the DFA to characterize species identity.

Phylogenetic comparative analysis and relationships between signal parameters

To detect phylogenetically independent relationships between signal parameters, we used a method based on Felsenstein's independent contrasts [FIC (Felsenstein, 1985)]. The phylogenetic generalized least squares method [PGLS (Martins and Hansen, 1997)], as implemented in the program COMPARE (Martins, 2004), was used to test *a priori* hypotheses about the relationships between these parameters (see Martins et al., 2004; Martins and Lamont, 1998; Ord and Martins, 2006). We chose PGLS because it performs well with small interspecific sample sizes, uses a range of microevolutionary models, and scales branch lengths by using the comparative data (see below) (Martins, 1999; Martins et al., 2002; Martins and Hansen, 1997). PGLS has been used previously to investigate the evolution of communication signals in other vertebrate species (Martins et al., 2004; Ord and Martins, 2006; Ord and Stuart-Fox, 2006).

To account for potential phylogenetic error, we used two alternative apteronotid phylogenies for this analysis. The first is a preliminary phylogeny (C.D.d.S., unpublished data) (Fig. 2A) created using maximum parsimony analysis of 103 morphological characters (majority consensus of 597 most parsimonious trees). The second – Crampton and Albert (Crampton and Albert, 2006) (Fig. 2B) – is based on a maximum parsimony analysis of 249 morphological, physiological and behavioral characters (Albert, 2001) with minor additions from two single-genus studies (Campos-da-Paz, 2000; Mago-Leccia et al., 1985). Note that although Crampton and Albert (Crampton and Albert, 2006) used '*Apteronotus*' *porcinum*, here we use the older name, *Sternarchogiton porcinum*. Because branch-length estimates were not available for either phylogeny, we set the total length of each phylogeny equal to one such that tip species were aligned at the top of the tree (Fig. 2). Importantly, the PGLS method in COMPARE uses the comparative data themselves to estimate best-fit branch lengths and is thus robust to inaccuracy in the initially specified branch lengths (Martins et al., 2002).

PGLS uses a single parameter, alpha (α), which can be interpreted as the fit of the comparative data with a specific evolutionary model. When α=0, phenotypic change (i.e. change in signal parameters) and phylogenetic distance are linearly related and PGLS behaves identically to FIC. In this linear model, evolutionary change happens *via* random genetic drift or

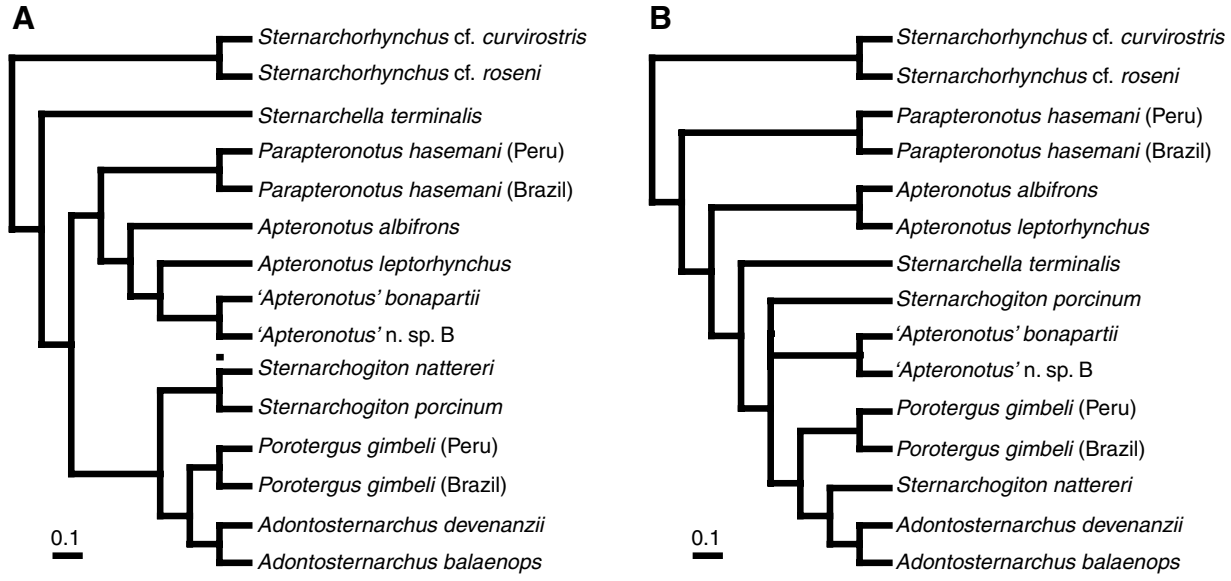

Fig. 2. Phylogenies used for comparative analyses. (A) Modified from the preliminary phylogeny of de Santana (C.D.d.S., unpublished data). de Santana's phylogeny is a majority consensus from 597 most parsimonious trees in a maximum parsimony analysis of 103 morphological characters. (B) Modified from Crampton and Albert (Crampton and Albert, 2006). Scale bar applies to branch lengths. Note that we use the older name *Sternarchogiton porcinum* whereas this species is referred to as '*Apteronotus*' *porcinum* in Crampton and Albert (Crampton and Albert, 2006). Note differences between the two phylogenies in the placement of *S. terminalis*, *S. nattereri* and *S. porcinum* and in the relationship between *A. albifrons* and *A. leptorhynchus*.

fluctuating directional selection (Brownian motion). When α is greater than zero, phenotypic change and phylogenetic distance are exponentially related. In this exponential model, evolutionary change happens *via* stabilizing selection, and the magnitude of α represents the strength of constraint around a fixed optimum. Thus, when α is extremely large (e.g. 15.5, the maximum value used in COMPARE), the constraint is extremely large, and phylogenetic effects on trait evolution are unimportant. PGLS trait regressions using the large α are identical to standard, non-phylogenetic regressions (TIPS).

We used a contrasts approach to examine relationships between different signal parameters across apteronotid species. Although all possible relationships between signal parameters could have been evaluated, we limited our significance tests to those for which we had *a priori* hypotheses (see Results). This approach avoids the problems associated with large numbers of statistical comparisons. For each regression, the PGLS-Relationships module of COMPARE provided separate results using the TIPS model (α=15.5), the FIC model (α=0) and a maximum-likelihood (ML) estimate of α. Using these three different PGLS models allowed us to assess the robustness of particular results to assumed models of phenotypic evolution. Significance tests were performed on each of the three results for all regressions, and thus correlation coefficients are reported as ranges. Following the method outlined in Martins (Martins, 1996) we tested the significance of correlation coefficients by developing a 95% confidence interval around the mean regression slope estimated using the two alternative phylogenies separately. This procedure addresses potential error due to phylogenetic uncertainty. A correlation coefficient was considered to be significantly different from zero if the confidence interval of the mean regression slope did not include zero (P<0.05).

Results

Electrocommunication signals in the family Apteronotidae were diverse across species. The parameters of EODs and EODMs in each species are summarized in Tables 2–4, and Fig. 3 illustrates representative EOD waveforms and chirps for each species. Variation in EODf and waveform was consistent with that found in previous studies (Crampton and Albert, 2006; Kramer et al., 1981). EODfs ranged from 644 to 1433 Hz across species, with intraspecific ranges typically spanning 100–300 Hz. Modified biphasic EOD waveforms were common, but some species (e.g. *Sternarchorhynchus* spp., *S. terminalis* and '*A.*' n. sp. B) had more complex, triphasic waveforms. Each taxon produced EODMs that fit into the two existing general categories of chirps and GFRs, but production rate and structure differed across species. Below, we highlight particularly novel or interesting aspects of the electrocommunication signals in each genus. EODMs in *A. leptorhynchus*, *A. albifrons* and *A. devenanzii* have been described previously (Dunlap and Larkins-Ford, 2003; Engler et al., 2000; Kolodziejski et al., 2005; Zhou and Smith, 2006; Zupanc and Maler, 1993) and are therefore not described in detail here.

Sternarchorhynchus spp.

Chirps in both *Sternarchorhynchus* species were produced at extremely low rates and had FM that only slightly exceeded that of GFRs. The seven recorded *S.* cf. *curvirostris* produced a total of 16 chirps and 65 GFRs. The eight recorded *S.* cf. *roseni* produced a total of 5 chirps and 26 GFRs. Although both *Sternarchorhynchus* species had EODs with triphasic waveforms, this feature was more pronounced in *S.* cf. *roseni*, as evidenced by the greater relative power of the second and third harmonics in this species (Table 2, Fig. 3B). EODf was higher in *S.* cf. *roseni* than in its congener, and EODf ranges did not overlap between the two species.

Parapteronotus hasemani

P. hasemani produced chirps with extraordinarily high frequency modulation (>500 Hz above baseline) and amplitude modulation (>90%AM). Chirps with the most pronounced amplitude reduction resulted in a brief, complete cessation of

Table 2. *Species means (±s.e.m.) for EOD parameters*

Species	*N*	EOD frequency range (Hz)	EOD frequency (Hz)	Waveform complexity (F2–F1) (dB)	Waveform complexity (F3–F1) (dB)
Sternarchorhynchus sp.	7	776–1056	870.24±33.02	2.45±0.97	–0.043±1.08
Sternarchorhynchus cf. *roseni*	8	1125–1344	1255.79±23.27	4.58±2.21	2.29±3.56
Parapteronotus hasemani (Peru)	10	755–935	825.57±14.71	–6.05±0.49	–18.68±0.70
Parapteronotus hasemani (Brazil)	4	736–807	773.37±14.8	–5.48±0.92	–21.26±2.41
Apteronotus albifrons[1]	42	824–1110	949.24±13.42	–15.1±1.73[†]	–16.13±0.62[†]
Apteronotus leptorhynchus[1]	20	644–883	742.55±20.14	–12.73±0.70*	–15.3±0.44*
'*Apteronotus*' *bonapartii*	10	1164–1433	1314.77±29.57	–3.33±0.81	–16.71±2.08
'*Apteronotus*' n. sp. B	1	n/a	1034.2	6.74	4.62
Sternarchogiton nattereri	9	867–1271	1071.17±42.39	–1.51±0.68	–12.56±2.79
Sternarchogiton porcinum	1	n/a	899.02	–2.25	–17
Porotergus gimbeli (Peru)	7	1147–1377	1225.04±37.61	–13.41±2.37	–25.54±5.09
Porotergus gimbeli (Brazil)	2	1094–1104	1099.18±4.71	–9.06±0.40	–15.06±0.04
Sternarchella terminalis	5	1180 –1262	1234.09±14.76	7.6±0.92	5.62±1.6
Adontosternarchus balaenops	10	765–971	880.11±21.92	0.107±0.418	–4.14±0.98
Adontosternarchus devenanzii[2]	21	931–1186	1093.92±14.59	–4.96±0.84*	–19.39±1.99*

*Measured on a subset of 10 individuals; [†]measured on a subset of six individuals; [1]data based on Kolodziejski et al. (Kolodziejski et al., 2005); [2]data based on Zhou and Smith (Zhou and Smith, 2006).

the EOD. This type of EODM has been commonly observed in the non-apteronotid genera *Sternopygus* (Hopkins, 1974a) and *Eigenmannia* (Hagedorn and Heiligenberg, 1985), where it was termed an 'interruption'. Many chirps also had extremely long durations (>1 s), and the range of chirp durations was the largest of all species (Table 3). Low-FM chirps (<200 Hz above baseline) were rare.

The 10 recorded *P. hasemani* from Peru produced a total of 1158 chirps and 1404 GFRs in two recording sessions. 711 of the GFRs were a distinct and novel type of EODM that we have named 'rasps' because of their sound when transduced into audio form (Fig. 4). Rasps were characterized by a variable-duration sequence of small (<25 Hz) peaks in EODf. The EODf peaks were generally highest in the middle of a rasp. Rasps were produced both spontaneously and during playback stimulus. The four recorded *P. hasemani* from Brazil produced a total of 363 chirps and 94 GFRs. Chirps from the Brazilian population were almost exclusively interruptions (90% of chirps had >90%AM), and none of the GFRs resembled rasps. These differences suggest that the structure of electrocommunication signals may vary not only across species but also across populations of the same species.

'Apteronotus' spp.

Both species in this genus commonly produced chirps with two distinct frequency peaks (Fig. 3C), and nearly every chirp ended with a small frequency undershoot. Although many elements of chirp structure are similar between these congeners, the complexity of their EOD waveforms differed dramatically. '*A*.' *bonapartii* produced a modified biphasic EOD with relatively little power in the second and third harmonics whereas '*A*.' n. sp. B emitted triphasic EODs with more power in the second and third harmonics than in the fundamental (Table 2, Fig. 3B). The 10 recorded '*A*.' *bonapartii* produced a total of 224 chirps and 117 GFRs in two recording sessions. '*A*.' *bonapartii* chirps had a narrow range of duration (0.01–0.04 s), but the range of chirp FM (100–600 Hz) was the largest of all species (Fig. 3E). The one recorded '*A*.' n. sp. B produced 40 chirps and 49 GFRs.

Sternarchogiton spp.

The nine recorded *S. nattereri* produced a total of 1107 chirps and 112 GFRs. Although a few chirps had FM as high as 500 Hz, most fell between 50 and 300 Hz. Of all the species measured, *S. nattereri* had the broadest range of EODf (404 Hz) (Table 2). The one recorded *S. porcinum* produced 217 chirps and eight GFRs. The chirps produced by this individual were similar in structure to the lower-FM chirps produced by *S. nattereri*.

Porotergus gimbeli

The seven recorded *P. gimbeli* from Peru produced a total of 27 chirps and 26 GFRs. The two recorded *P. gimbeli* from Brazil produced a total of 20 chirps and 22 GFRs. Chirps from the Brazilian population exhibited greater AM than those from the Peruvian population. Also, the Brazilian *P. gimbeli* chirps, unlike those from the Peruvian population, nearly always ended with a small frequency undershoot (Table 3). *P. gimbeli* from Peru produced only two chirps with more than 70%AM,

Table 3. *Species means (±s.e.m.) for chirp parameters*

Species	*N*	Chirp rate (chirps min^{-1})	Chirp duration (s)	Chirp FM (Hz)	Chirp AM (%)	Undershoot FM (Hz)	Chirp +FM slope (Hz ms^{-1})	Chirp –FM slope (Hz ms^{-1})
Sternarchorhynchus sp.	7	0.095±0.039	0.126±0.050	102.7±32.4	24.5±14	0	5.96±1.92	–5.2±3.63
Sternarchorhynchus cf. *roseni*	8	0.022±0.007	0.155±0.045	68.0±20.1	13.6±2.1	0	2.7±1.15	–1.14±0.3
Parapteronotus hasemani (Peru)	10	2.41±0.71	0.627±0.103	479.3±14.6	83.2±3.7	0	27.69±2.64	–1.65±0.33
Parapteronotus hasemani (Brazil)	4	3.78±1.49	0.395±0.133	349.3±37.1	94.7±1.9	–0.05±0.05	25.04±2.89	–2.39±0.86
Apteronotus albifrons[1]	42	0.77±0.17	0.215±0.017	102.6±11.8	11.5±4[†]	–0.09±0.04	4.42±0.85	–0.84±0.09
Apteronotus leptorhynchus[1]	20	13.37±3.49	0.021±0.001	64.6±6.4	6.6±1.2*	–4.00±1.03	5.67±0.65	–9.39±1.02
'*Apteronotus*' *bonapartii*	10	0.47±0.27	0.03±0.005	252.8±26.9	31.3±5.9	–2.14±0.44	22.46±3.62	–19.84±2.57
'*Apteronotus*' n. sp. B	1	1.67	0.021	118.0	10.4	–2.46	13.87	–10.02
Sternarchogiton nattereri	9	5.13±1.82	0.04±0.003	106.1±13.4	11.1±2.4	0	12.88±2.14	–3.85±0.53
Sternarchogiton porcinum	1	9.13	0.144	74.4	3.5	–0.28	8.18	–6.7
Porotergus gimbeli (Peru)	7	0.16±0.065	0.063±0.005	178.0±21.4	42.6±5.7	0	20.1±2.84	–3.74±0.75
Porotergus gimbeli (Brazil)	2	0.42±0.38	0.061±0.016	217.3±12.2	62±27.4	–1.05±1.05	23.51±2.57	–4.62±1.27
Sternarchella terminalis	5	19.23±8.42	0.021±0.002	151.5±16.4	20.7±6.1	–0.07±0.05	27.82±3.5	–12.39±0.98
Adontosternarchus balaenops	10	0.17±0.12	0.036±0.008	232.6±37.8	41.6±10.2	0	19.05±1.93	–12.23±1.31
Adontosternarchus devenanzii[2]	21	0.49±0.10	0.078±0.01	169.1±7.5	13.7±1.4*	–1.74±0.28	9.67±1.14	–7.25±0.97

*Measured on a subset of 10 individuals; [†]measured on a subset of six individuals; [1]data based on Kolodziejski et al. (Kolodziejski et al., 2005); [2]data based on Zhou and Smith (Zhou and Smith, 2006).

Table 4. *Species means (±s.e.m.) for GFR parameters*

Species	*N*	GFR rate (GFRs min^{-1})	GFR duration (s)	GFR FM (Hz)	GFR +FM slope (Hz ms^{-1})	GFR –FM slope (Hz ms^{-1})
Sternarchorhynchus sp.	7	0.39±0.19	5.70±2.03	8.61±1.19	0.081±0.022	–0.0108±0.0056
Sternarchorhynchus cf. *roseni*	8	0.12±0.03	20.51±8.7	18.88±8.92	0.043±0.012	–0.0278±0.0133
Parapteronotus hasemani (Peru)	10	2.93±0.37	2.00±0.17	6.22±0.24	0.119±0.021	–0.0695±0.0120
Parapteronotus hasemani (Brazil)	4	0.98±0.11	2.97±1.24	7.43±1.71	0.099±0.034	–0.0364±0.0110
Apteronotus albifrons[1]	42	0.63±0.06	1.56±0.2	8.94±0.58	0.103±0.007	–0.0473±0.0045
Apteronotus leptorhynchus[1]	20	0.23±0.03	1.40±0.93	10±1.06	0.260±0.036	–0.292±0.074
''*Apteronotus*'' *bonapartii*	10	0.24±0.05	4.35±0.86	5.37±0.52	0.042±0.008	–0.0103±0.0037
''*Apteronotus*'' n. sp. B	1	2.04	1.07	5.40	0.048	–0.0122
Sternarchogiton nattereri	9	0.52±0.11	2.87±0.86	4.93±0.59	0.030±0.005	–0.0122±0.0049
Sternarchogiton porcinum	1	0.33	7.02	4.23	0.015	–0.0014
Porotergus gimbeli (Peru)	7	0.16±0.047	6.52±1.84	6.86±1.22	0.078±0.024	–0.0076±0.0024
Porotergus gimbeli (Brazil)	2	0.46±0.21	7.15±0.59	7.86±0.34	0.022±0.001	–0.0022±0.0003
Sternarchella terminalis	5	1.36±0.58	2.14±0.88	5.89±1.38	0.213±0.105	–0.0839±0.0409
Adontosternarchus balaenops	10	0.16±0.07	5.92±1.99	11.89±1.52	0.267±0.167	–0.119±0.081
Adontosternarchus devenanzii[2]	21	0.26±0.04	1.22±0.48	11.64±1.77	0.392±0.037	–0.236±0.021

[1]Data based on Kolodziejski et al. (Kolodziejski et al., 2005); [2]data based on Zhou and Smith (Zhou and Smith, 2006).

whereas most of the chirps produced by the Brazilian population were interruptions (>90%AM). EOD waveform was more complex in *P. gimbeli* from Brazil, and fish from this population also had lower baseline EOD frequencies than all of the *P. gimbeli* from Peru (Table 2, Fig. 3B). As with *P. hasemani*, these differences suggest population-level divergence in electrocommunication signals.

Sternarchella terminalis

The five recorded *S. terminalis* produced a total of 2308 chirps and 163 GFRs. *S. terminalis* produced chirps at a very high rate and in a novel 'burst-like' fashion, in which trains of very short duration chirps occurred on top of a slightly elevated baseline EODf (Fig. 5). Occasionally, these short-duration chirps occurred in extremely rapid succession to form long-duration, multi-peaked chirps (Fig. 3D). Most *S. terminalis* chirps fell within a comparatively narrow range of duration (0.007–0.04 s) but a broad FM range (50–300 Hz above baseline).

Adontosternarchus balaenops

The 10 recorded *A. balaenops* produced a total of 40 chirps and 43 GFRs. As in *A. devenanzii*, this species produced chirps infrequently. The two species differed, however, in the relative variability of chirp FM and duration (Fig. 3E). Chirps in *A. balaenops* spanned a larger range of FM (50–450 Hz) than in *A. devenanzii* (90–350 Hz), whereas the range of chirp duration was much narrower in *A. balaenops* (0.02–0.2 s) than in *A. devenanzii* (0.02–2.0 s). *A. balaenops* produced chirps with much more AM and steeper +FM slopes than those of its congener, and the multi-peaked chirps of *A. devenanzii* (Fig. 3D) (Zhou and Smith, 2006) were never seen in *A. balaenops*. The EOD waveform of *A. balaenops* was more complex than that of *A. devenanzii* (Table 2) due to a prolonged 'shoulder' where voltage transitioned from the negative to positive phase (Fig. 3B). *A. balaenops* also had a lower baseline EODf than *A. devenanzii*. Thus, the two species of *Adontosternarchus* provide another example in which the electrocommunication signals of closely related species have diverged significantly.

Table 5. *Principal components analysis on EOD, chirp and GFR variables*

EOD variables	EOD factor 1	EOD factor 2	EOD factor 3
Factor loadings			
EOD frequency	–0.27	**0.96**	0.09
Waveform complexity (F2–F1)	**–0.95**	0.002	–0.30
Waveform complexity (F3–F1)	**–0.91**	–0.28	0.29
Variance explained	60.4%	33.4%	6.2%
Chirp variables	**Chirp factor 1**	**Chirp factor 2**	**Chirp factor 3**
Factor loadings			
Chirp duration	**–0.72**	–0.38	0.46
Chirp FM	**–0.93**	0.22	0.11
Chirp %AM	**–0.93**	0.09	0.04
Undershoot FM	0.39	0.57	**0.70**
+FM slope	**–0.71**	0.57	–0.31
–FM slope	0.22	**0.90**	–0.08
Variance explained	49.5%	27.7%	13.4%
GFR variables	**GFR factor 1**	**GFR factor 2**	**GFR factor 3**
Factor loadings			
GFR duration	**0.69**	–0.65	0.33
GFR FM	0.42	**–0.85**	–0.31
+FM slope	**–0.83**	–0.48	0.06
–FM slope	**–0.81**	–0.50	0.05
Variance explained	50.0%	40.7%	5.2%

Bold values indicate significance at $P<0.0001$.

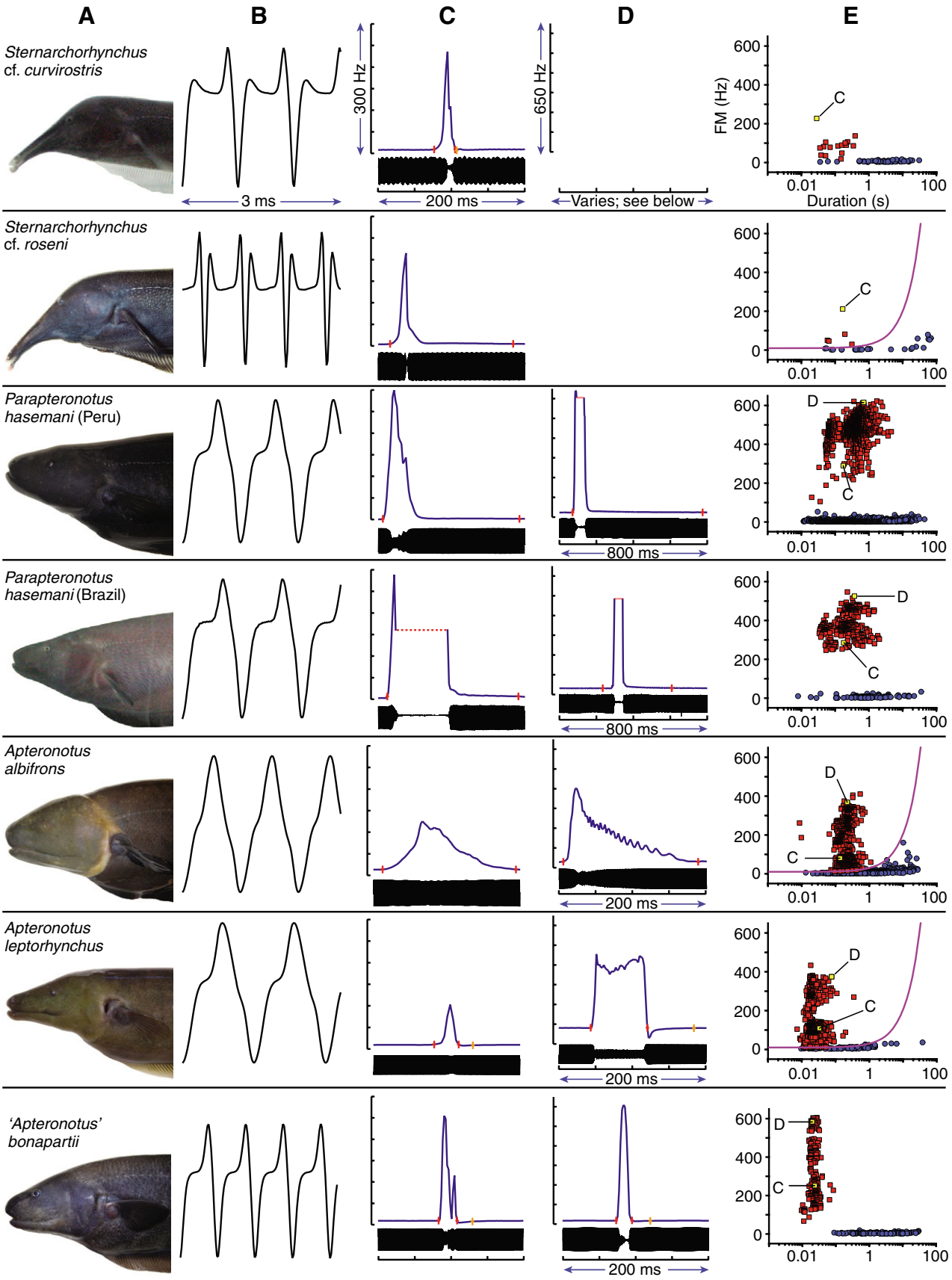

Fig. 3 continued on next page.

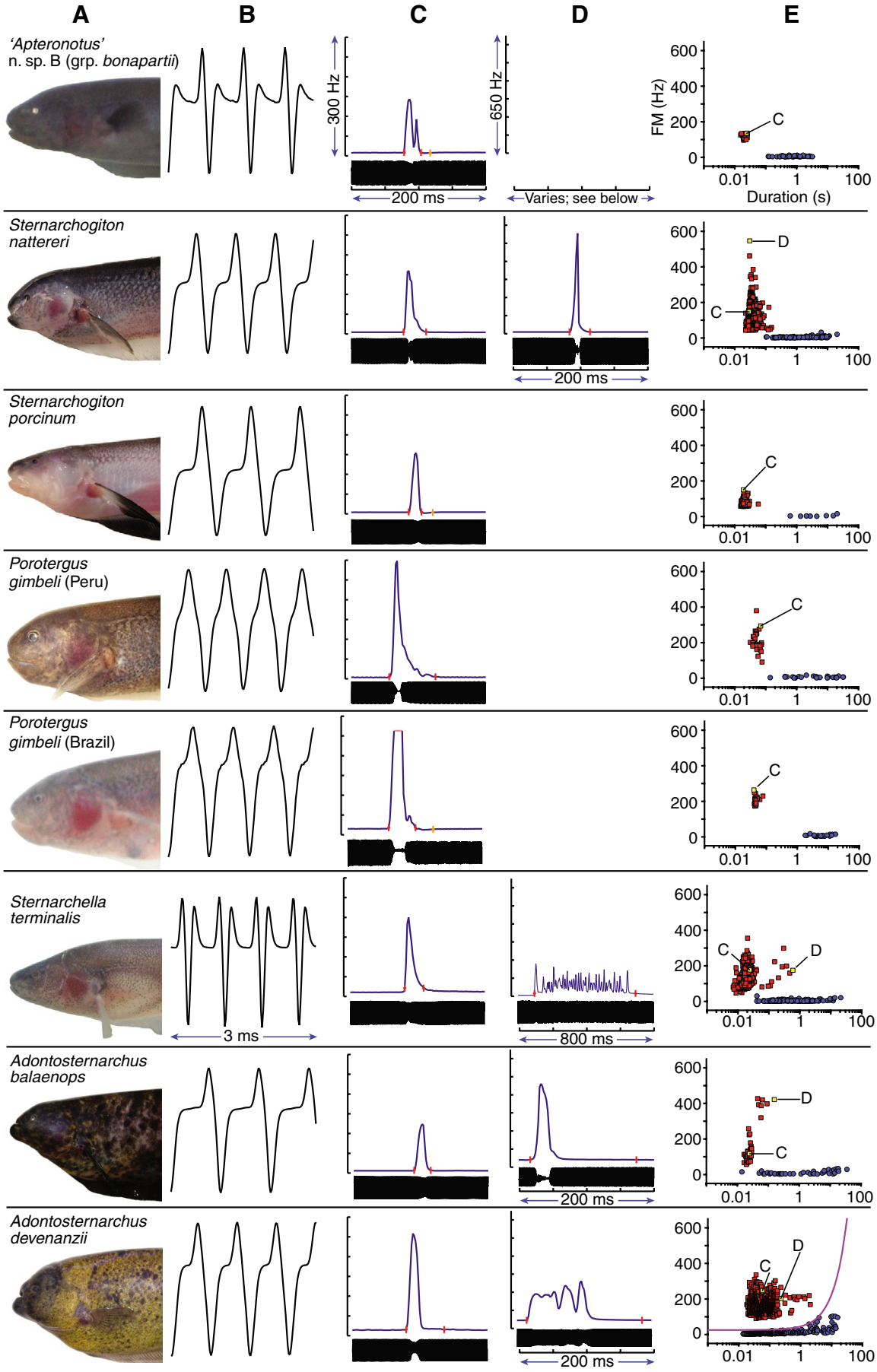
A
B
C
D
E
'Apteronotus'
n. sp. B (grp. bonapartii)
300 Hz
650 Hz
200 ms
Varies; see below
FM (Hz)
Duration (s)
Sternarchogiton nattereri
Sternarchogiton porcinum
Porotergus gimbeli (Peru)
Porotergus gimbeli (Brazil)
Sternarchella terminalis
3 ms
800 ms
Adontosternarchus balaenops
Adontosternarchus devenanzii

Fig. 3. Photographs and species-typical electrocommunication signals of 15 apteronotid taxa. (A) Photograph of the head, not shown to scale. (B) A 3 ms section of head-to-tail EOD waveform with peak-to-peak amplitude normalized for all taxa. (C) EOD frequency trace (top) and head-to-tail EOD waveform (voltage) trace (bottom) of characteristic chirps in each species. Amplitude reductions during chirps are apparent from the amplitude envelope of the waveform trace in some species. Axes are the same as in Fig. 1A. Axis scales are identical within column C across all panels to allow direct comparison of chirp FM and duration. Red bars show positive start and stop times, respectively. Orange bars show the negative stop time. (D) Additional examples of chirps for each species shown as in C. Note the different frequency scale in D compared with C. The frequency axis scales are identical across panels within column D, but time scales vary as indicated. (E) Scatter plot of positive FM and duration showing all EODMs recorded in each taxon. Chirps are shown as red squares, GFRs as blue circles. The chirps identified in columns C and D are colored yellow and labeled. The purple line illustrates the linear function used to distinguish chirps from GFRs when necessary (see Materials and methods for details). Note that the duration axis is logarithmic, and thus the linear functions appear curved. Axis scales are identical across all panels within column E.

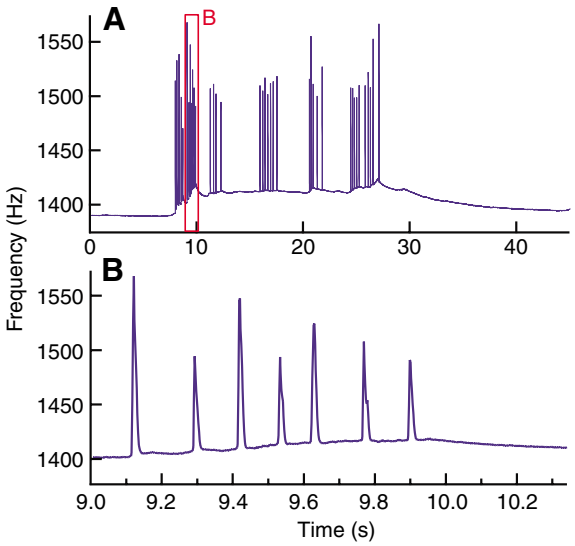

Fig. 4. EOD frequency trace of chirp 'bursts' from *S. terminalis*. (A) Entire burst. (B) The red-boxed portion of the burst in A is shown on an expanded time scale. Note the plateau-like elevation of the baseline EODf and the clustering of chirps within the burst.

Discriminant function analyses

Separate principal component analyses on EOD, chirp and GFR parameters were used to generate independent variables for DFA. The first factor of the PCA on EOD parameters was loaded primarily by EOD waveform (F2–F1 and F3–F1) and accounted for over 60% of the variance (Table 5). EOD frequency loaded robustly on the second factor, which accounted for most of the remaining variance. The chirp PCA was more complex, with chirp %AM, FM, duration and + FM slope all loading heavily on the first factor, –FM slope on the second factor, and undershoot FM on the third factor. Together, these three factors explained more than 90% of the variance. The PCA on GFR parameters was dominated by two factors that were strongly influenced by all four structural parameters (duration, FM, and + and –FM slopes).

The DFA using all of the factors from the EOD, chirp and GFR PCAs revealed strong influences of EOD and chirp parameters, but not GFR parameters, on interspecific signal variation. The first two chirp factors and the first two EOD factors were by far the strongest contributors to the discriminant model, whereas the GFR parameters contributed the least to the model (Table S2 in supplementary material). Both EOD and chirp variables were highly correlated with the first three canonical roots of the DFA, which explained over 80% of the variance in the model, whereas GFR variables were poorly correlated with these roots (Table 6). The DFA model based on the combined EOD, chirp and GFR parameters was largely successful at segregating species based on these signals, although there was still overlap between some species (Fig. 6A,B). This was also revealed by the fact that the DFA using all signal parameters correctly classified species identity 78.3% of the time in leave-one-out cross-validations, which is far greater than the 9.1% expected based on chance alone but still less than 100% (Fig. 6C).

Separate DFAs on EOD parameters only, chirp parameters only, and GFR parameters only confirmed that EOD and chirp parameters varied more across species and are much stronger predictors of species identity than GFR parameters. Cross-validated classifications based on EOD- or chirp-based DFAs correctly identified the species of 63.5% and 67.5% of individuals, respectively, whereas DFAs based on GFR parameters correctly identified the species of only 28.9% of individuals (Fig. 6C).

Relationships between signal parameters

Our *a priori* hypotheses were based on aspects of the neuroanatomy and neurophysiology of the electromotor circuit in *A. leptorhynchus* (reviewed in Smith, 1999), many aspects of which are likely to be conserved across apteronotids. EODf in

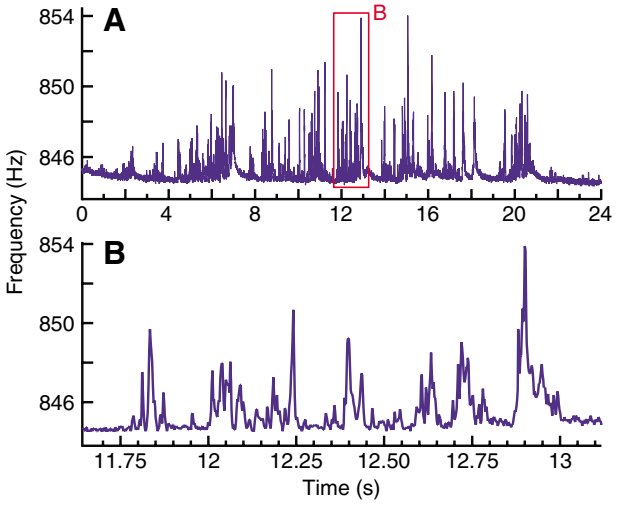

Fig. 5. EOD frequency trace of a 'rasp' from Brazilian *P. hasemani*. (A) Entire rasp. (B) The red-boxed portion of the rasp in A is shown on an expanded time scale.

Table 6. *Correlations of EOD, chirp and GFR parameters with canonical roots of discriminant function analysis*

	Root 1	Root 2	Root 3
EOD parameters			
EOD frequency	**–0.74**	0.42	–0.45
Waveform complexity (F2–F1)	**–0.54**	**0.52**	**0.59**
Waveform complexity (F3–F1)	**–0.51**	0.05	**0.59**
Chirp parameters			
Chirp duration	**0.66**	0.40	0.14
Chirp FM	**0.61**	**0.68**	–0.02
Chirp %AM	**0.67**	**0.61**	0.14
Chirp undershoot FM	–0.02	**–0.57**	–0.04
Chirp +FM slope	0.21	**0.64**	–0.08
Chirp –FM slope	–0.47	–0.07	–0.17
GFR parameters			
GFR duration	–0.23	0.08	0.04
GFR FM	–0.05	–0.27	0.15
GFR +FM slope	–0.03	–0.10	0.20
GFR –FM slope	0.02	–0.26	0.17
Percent variance explained	42.9%	25.8%	12.9%

Bold values indicate significance at $P<0.0001$.

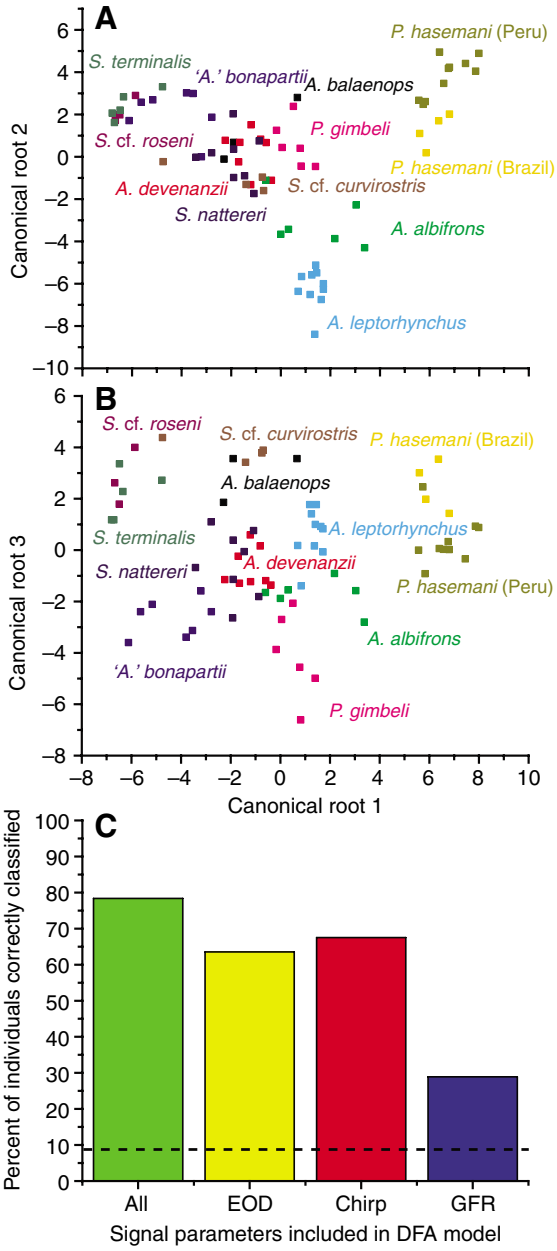

Fig. 6. Discriminant function analysis (DFA) of EOD, chirp and GFR parameters. Scatter plots of first *versus* second (A) and first *versus* third (B) canonical roots from a DFA based on all signal parameters reveal the ability of these roots to segregate apteronotid species. Each data point represents a single fish, and species are color-coded as indicated. Some species (e.g. *P. hasemani* and *A. leptorhynchus* in A, and '*A.*' *bonapartii* and *P. gimbeli* in B) were well-segregated. Other species (e.g. *S. terminalis* and *S.* cf. *roseni*) overlapped considerably. (C) Performance of DFAs based on all signal parameters, EOD parameters only, chirp parameters only, or GFR parameters only in correctly classifying the species of individuals in leave-one-of-each-species-out cross-validations (see Materials and methods). The broken black line represents performance predicted based on chance alone. All DFAs correctly classified individuals at rates exceeding chance, but DFAs based on EODs and chirps performed better than those based on GFRs.

this species is controlled by pacemaker neurons in the medullary pacemaker nucleus (Pn). Relay cells in the Pn convey this command signal to electromotor neurons in the spinal cord, whose axons form the electric organ. Firing rates of pacemaker, relay and electromotor neurons correspond directly to EODf. EODMs, including chirps and GFRs, are caused by glutamatergic excitatory inputs to the Pn from the thalamic prepacemaker nucleus (PPn) and midbrain sublemniscal prepacemaker nucleus (SPPn) (reviewed in Heiligenberg et al., 1996; Metzner, 1999; Zakon et al., 2002).

First, we asked whether EODf and waveform complexity (as assessed by the relative strength of the second and third harmonics) were positively correlated. In some gymnotiform species, waveform complexity is partly due to rostral and caudal portions of the electric organ firing slightly out of phase. This asynchrony results from small differences in the conduction time of the command signal from the Pn (reviewed in Caputi, 1999). If a similar mechanism contributes to EOD waveform in apteronotids, then species with higher EODf may have more complex waveforms. This might occur because fixed rostro-caudal delays in the conduction time would cause larger rostro-caudal phase differences in EODs with shorter periods. Alternatively, conduction delays could change with EODf or waveforms could be determined primarily by the pathway that electromotor axons follow in the electric organ (Bennett, 1970; Bennett, 1971). In this case, EOD waveform and EODf would be uncorrelated. EODf and waveform complexity were not significantly correlated across species (r=–0.33 to 0.26, P>0.05) (Table 7), which suggests that high-frequency EODs do not necessarily result in more complex EOD waveforms.

Second, we tested the hypothesis that species whose chirps had steeper returns to baseline EODf also produced chirps with larger frequency undershoots. Engler et al. proposed that undershoots in *A. leptorhynchus* resulted from rapid removal of the PPn-generated excitation of the Pn at the end of chirps (Engler et al., 2000). The frequency of the command signal for

Table 7. *Phylogenetic correlations between signal parameters*

Signal parameter relationship	Correlation coefficient: ML α α=(estimated)*	TIPS α=15.5	FIC α=0
EODf and waveform complexity (F2–F1)	0.26 (15.5)	0.26	–0.19
EODf and waveform complexity (F3–F1)	0.18 (15.5)	0.19	–0.33
–FM slope and undershoot FM	0.48† (15.5)	0.49‡	0.22
EODf and chirp FM	–0.19 (12.2)	–0.20	0.10
Chirp %AM and chirp FM	0.89‡ (14.4)	0.89‡	0.70‡
Chirp %AM and chirp positive peak frequency	0.24 (10.9)	0.26	0.17

*Average of the two αs estimated from the two alternative phylogenies; †P=0.0506; ‡statistically significant correlation, P<0.05.

the EOD is regulated by sodium and potassium currents in pacemaker and electromotor neurons (Dye, 1991; Smith and Zakon, 2000; Smith, 2006). Frequency undershoots might result if strong, PPn-mediated depolarization of the pacemaker network during chirps caused steady-state sodium channel inactivation that recovered more slowly than the removal of the excitatory input from the PPn. This hypothesis predicts that more rapid deactivation of this excitatory input (i.e. steeper –FM slope) should produce more pronounced undershoots. Consistent with this prediction, undershoots are absent in two species [*A. albifrons* and *A. devenanzii* (Dunlap and Larkins-Ford, 2003; Kolodziejski et al., 2005; Zhou and Smith, 2006)], that produce longer duration chirps with shallow –FM slopes. A significant positive correlation between –FM slope and undershoot FM of chirps was observed for the TIPS model, but this trend did not reach significance with the other two models (r=0.22–0.49, P<0.05 for TIPS; P=0.0505 for ML α) (Table 7, Fig. 7A). This result therefore only partly supports the hypothesis that, across apteronotid species, the rapid removal of excitation needed to produce short-duration chirps is mechanistically linked to the production of frequency undershoots.

Third, we hypothesized that species with higher baseline EOD frequencies would produce chirps with less FM. The electric organ and neurons in the pacemaker nucleus fire at rates unsurpassed by cells in any other organism (Moortgat et al., 1998; Smith, 1999). If these rapid firing rates approach an absolute physiological ceiling in species with the highest baseline EOD frequencies, they might constrain the magnitude of frequency increases during chirping. EODf and chirp FM were not significantly correlated (r=–0.2 to 0.1, P>0.05) (Table 7). This suggests that an absolute physiological ceiling on neuronal firing rates has not constrained the evolution of FM in apteronotid chirps, even in species in which EODf surpasses 2000 Hz during chirps.

Fourth, we asked whether chirp %AM was correlated with chirp FM or with chirp positive peak frequency. Chirps in all three of the apteronotid species studied previously have some AM (Dunlap and Larkins-Ford, 2003; Zhou and Smith, 2006; Zupanc and Maler, 1993). Although AM has only been quantified in *A. leptorhynchus* (Engler et al., 2000; Zupanc and Maler, 1993), high levels of AM are associated with high-FM chirps in this species and in *A. albifrons* (Kolodziejski et al.,

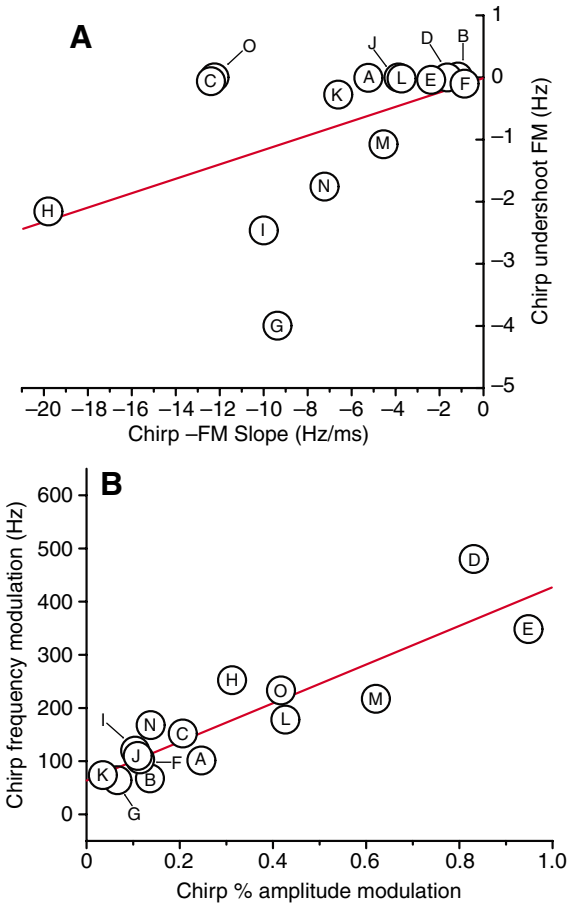

Fig. 7. Correlations between signal parameters. Raw species means are shown with the correlation calculated using TIPS model (large α; non-phylogenetic). (A) Correlation between chirp undershoot FM and chirp –FM slope. More negative values indicate larger undershoots (*y*-axis) and faster returns to baseline EODf (*x*-axis). (B) Correlation between chirp FM and chirp %AM. Larger values on the *x*-axis indicate greater reduction in EOD amplitude during chirps. Letters on each data point indicate species as follows: A, *S.* cf. *curvirostris*; B, *S.* cf. *roseni*; C, *S. terminalis*; D, *P. hasemani* (Peru); E, *P. hasemani* (Brazil); F, *A. albifrons*; G, *A. leptorhynchus*; H, '*A.*' *bonapartii*; I, '*A.*' n. sp. B; J, *S. nattereri*; K, *S. porcinum*; L, *P. gimbeli* (Peru); M, *P. gimbeli* (Brazil); N, *A. devenanzii*; O, *A. balaenops*.

2005). Amplitude modulation may result from the inability of neurons in the electromotor circuit to fire synchronously and/or produce large amplitude action potentials (APs) when firing at high frequencies. However, a constraint on EOD amplitude at extremely high frequencies could be relative or absolute. If the constraint were relative, an upper limit to how fast the neurons could fire without a reduction in synchrony or AP amplitude would increase as a species' baseline EODf increased. Consequently, AM would increase as the amount of FM above the baseline EODf increased, and chirp AM would be positively correlated with chirp FM across species. By contrast, if the constraint were absolute, a fixed upper limit to how fast the neurons in the electromotor circuit could fire without a reduction in synchrony or AP amplitude would be independent of individual or species-specific baseline EODf. In this scenario, AM would increase as EODf approached the fixed upper limit rather than being determined by the amount of change in EODf. Consequently, chirp AM would be positively correlated across species with chirp positive peak frequency but not necessarily with chirp FM. We found that chirp AM and FM were positively correlated (r=0.7–0.89, P<0.05) (Table 7, Fig. 7B) but that chirp AM and positive peak frequency were not (r=0.17–0.26, P>0.05) (Table 7). Thus, chirps that had large increases in frequency above the baseline EODf were likely to result in more AM, independent of the baseline EODf itself. This result supports the hypothesis that a relative constraint on how fast neurons in the electromotor circuit can fire without a reduction in synchrony or AP amplitude shapes the evolution of chirp structure in apteronotids.

Discussion

We characterized the electrocommunication signals of 10 apteronotid species for the first time, compared signal parameters across species to understand patterns of signal diversification, and examined cross-species correlations between different signal parameters. Many aspects of the electrocommunication signals were similar across the different apteronotid species. For example, all species produced both chirps and GFRs. However, many signal parameters, particularly EOD frequency and waveform and the structure of chirps, varied substantially across species. Below, we highlight novel signal types found in this study, compare the species diversity of different signal parameters, propose hypotheses for how mechanistic relationships between signal parameters influence the evolution of communication signals, and discuss the implications of species diversity in signals for their production, perception and function.

Novel signal types

Several novel signal types were produced by some of the species in this study. One was the bursting of chirps on an elevated baseline EOD frequency produced by *S. terminalis*. The chirp bursts of *S. terminalis* contrast with the more uniform timing of chirps in response to playback stimulation in *A. leptorhynchus* (Engler et al., 2000; Zupanc and Maler, 1993). By producing chirps in bursts, *S. terminalis* may create an additional element of signal complexity that could open up new communication channels. For example, information about motivation or social status might be encoded in burst duration, interchirp intervals within bursts, or timing between bursts. Chirp bursts could also contribute to interactive chirping. *A. leptorhynchus* produces chirps in an 'echo response' pattern during interactions (Zupanc et al., 2006). Chirp bursts might similarly provide a mechanism for interacting *S. terminalis* to exchange 'packets' of chirps and prevent the overlap of chirps of different fish. To test these hypotheses, more information is needed about the social ecology and electrosensory physiology of *S. terminalis*.

The rasps produced by *P. hasemani* from Peru are another novel type of EODM. Rasps were relatively common and were produced by all fish from this population but were never produced by the Brazilian *P. hasemani*. Although the FM and duration of rasps were similar to those of GFRs, the structure of rasps was unlike that of chirps or GFRs in other species. It is possible that rasps have been recorded in other species but not identified above background recording noise because of their low and erratic FM. Indeed, changes in EODf that resemble rasps have been observed during playback stimulation in *A. leptorhynchus* and *A. albifrons* but could not be distinguished confidently from recording artifacts (J. A. Kolodziejski, personal communication). Our recordings and playback removal algorithm provided very low levels of background noise and thus allowed greater resolution of low-FM EODMs than in previous studies. Rasps did not coincide with fluctuations in EOD amplitude that occur during fish movement, and they were sometimes produced spontaneously without playback stimulation. These features allowed us to conclusively identify them and measure their parameters. Further studies are needed to determine the function of rasps, their evolutionary history and their mechanisms of production.

Finally, *P. hasemani* and the Brazilian *P. gimbeli* produced chirps that resulted in complete interruptions of the EOD. Although EOD interruptions are common in the non-apteronotid knifefish *Eigenmannia* and *Sternopygyus* and have similar functions as chirps (Hagedorn and Heiligenberg, 1985; Hopkins, 1974a; Hopkins, 1974b), they have not been reported previously in apteronotid species. The closest approximations are extremely rare chirps (e.g. two of 4116 spontaneous chirps) in *A. leptorhynchus* in which EOD amplitude was reduced by approximately 80–90% (Engler et al., 2000; Heiligenberg et al., 1996; Zupanc and Maler, 1993). However, amplitude reduction beyond 90%, as was common in the chirps of *P. hasemani*, has not been described in *A. leptorhynchus*. Thus, although it is possible that other apteronotids can produce complete interruptions, only *P. hasemani* and *P. gimbeli* use them extensively. Two possible mechanisms might cause these EOD interruptions. One possibility is that the excitatory input from the subdivision of the PPn that controls chirps, the PPn-C, to the Pn is particularly strong in *P. hasemani* and *P. gimbeli*. Such extreme excitation might cause both the large increase in EODf during the chirp and prolonged depolarization and inactivation of ion channels that leads to an EOD interruption. Alternatively, interruptions in these species might be controlled by an excitatory input to the Pn from the SPPn in the midbrain. In *A. leptorhynchus*, current injection into the SPPn caused large increases in EODf and reductions in EOD amplitude that resembled the rare chirps with extreme AM and FM in *A. leptorhynchus* and the interruptions we observed in *P. hasemani*

and *P. gimbeli* (Heiligenberg et al., 1996). The potential role of the SPPn in high-AM chirps in *A. leptorhynchus* has not been studied further, however, because the behavior is so rare. Because interruptions are common in *P. hasemani*, this species may provide a better opportunity to identify the premotor nucleus that controls these signals.

Species diversity and evolution of EOD signals

Discriminant function analysis indicated that EODs and chirps differed markedly across apteronotid species whereas the structure of GFRs was much less species-specific. EOD and chirp parameters were far stronger contributors to the DFA model than GFR parameters, and DFAs based on EODs or chirps alone more accurately classified the species of individuals than GFR-based DFAs. These results demonstrate that EODs and chirps, but not GFRs, can serve as species-identifying signals. They also suggest more interspecific variation and evolutionary lability in EODs and chirps than in GFRs. The evolutionary lability of chirps and EODs is also supported by the variability of these signals across closely related species and different populations of the same species (e.g. differences in EODf and chirp structure between *A. balaenops* and *A. devenanzii*, EOD waveform differences between '*A.*' *bonapartii* and '*A.*' n. sp. B, and the production of interruptions by Peruvian but not Brazilian *P. gimbeli*).

EOD frequency and waveform complexity were strongly correlated with the first canonical root of the DFA, demonstrating that they reliably vary across species. All of the chirp parameters also contributed strongly to the DFA, confirming that chirps, just like EODs, vary substantially across apteronotid species. Three chirp parameters in particular – duration, FM and %AM – strongly influenced the DFA. The most important variable contributing to the DFA model was the first chirp factor, which was loaded primarily by FM, %AM and duration; and these variables also correlated highly with the first two canonical roots of the DFA. This suggests that chirp duration, FM and %AM, like EOD frequency and waveform, are particularly capable of conveying species-identifying information and have undergone substantial evolutionary changes within the Apteronotidae.

Relationships between signal parameters

Testing relationships between signal parameters allowed us to look for phylogenetic evidence that conserved production mechanisms shape the evolution of electrocommunication signals. We hypothesized that as EODf increased, constraints on the conduction velocity of relay axons in the spinal cord might lead to increased rostro-caudal phase delays in the firing of the electric organ and increased waveform complexity. Both the independent loadings of EODf and waveform on the PCA (Table 5) and the lack of significant PGLS correlations (Table 7), however, revealed that waveform complexity was not related to EODf. Thus, either EOD waveform is influenced primarily by trajectory of the electromotor axons, rather than rostro-caudal phase delays, or conduction velocity is not constraining and can change to allow EODf and waveform to evolve independently.

Our results partially support the hypothesis that the rate at which excitation is removed from the Pn at the end of chirps (–FM slope) influences the evolution of chirp undershoots (Engler et al., 2000). The positive correlation between the –FM slope and undershoot FM of chirps was significant for the TIPS (large α, non-phylogenetic) model and closely approached significance with the ML α model but was not significant for the FIC model (Table 7). Thus, although the rapid deactivation of excitatory input that causes steep –FM slopes may contribute to the evolution of chirp undershoots, this linkage is not robust across the phylogeny. For example, chirps in *A. balaenops* have comparatively steep –FM slopes but no undershoot whatsoever (Table 3) (symbol O in Fig. 7A). In this species, adaptations of the electromotor circuit, such as differences in channel inactivation kinetics in the pacemaker neurons, might allow a smooth but rapid return to baseline EODf at the end of chirps.

We found no support for the hypothesis that the amount of FM in chirps was constrained by baseline EOD frequency. For example, the species with the highest EODf ('*A.*' *bonapartii*) still routinely produced chirps with up to 600 Hz of FM. This result suggests that as higher baseline EODfs evolved, the ability to transiently raise EODf to even higher levels during chirps was retained. This is particularly remarkable given that in species with EODfs that approach 2 kHz, the neurons that control the electric organ are producing action potentials at those frequencies. We were unable, however, to obtain individuals from the species with the highest reported baseline EODf (*Sternarchella schotti*; up to 2179 Hz) (Crampton and Albert, 2006), and it would be interesting to determine whether these 'extremists' are still able to increase EODf by hundreds of Hz during chirps.

In contrast to the lack of correlation between EODf and Chirp FM, we did find a strong relationship between chirp FM and EOD amplitude during chirps. Species whose chirps had greater FM also produced chirps in which amplitude decreased more (i.e. greater chirp %AM). Indeed, this was the strongest correlation between any of the measured signal parameters, suggesting that chirp AM and FM are linked across taxa by a relatively invariant physiological mechanism. Thus, the extremely high neuronal firing rates necessary to produce high EODfs have not limited the evolution of chirp FM. Instead, a transient drop in EOD amplitude is a necessary trade-off incurred by high-FM chirps. The interspecific trade-off between FM and EOD amplitude during chirps is paralleled by similar correlations within each species and within individuals (Engler et al., 2000) (C.R.T., unpublished observations). The consistency of this relationship at multiple levels (across species, individual fish and individual chirps) indicates that chirp FM and AM are physiologically linked rather than being generated independently and co-selected. The conserved mechanism producing the tradeoff is most likely an inability of pacemaker, relay and/or electromotor neurons to fire synchronously and/or produce large amplitude APs when firing at frequencies that greatly exceed their baseline firing rates.

Evolution of signal production mechanisms

Patterns in the species diversity and evolution of communication signals can direct research on the function and production mechanisms of these signals towards fertile ground. Signals or signal parameters with greater evolutionary lability may indicate which underlying mechanisms have evolved to

produce signal diversity across species (Emerson, 1996; Nishikawa, 1997).

Species diversity in chirp structure and EOD frequency and waveform suggests that mechanisms controlling these signals have evolved rapidly. The electromotor circuit is well-characterized in *A. leptorhynchus* (reviewed in Heiligenberg et al., 1996; Smith, 1999), but studies in other apteronotid species are needed to understand how the physiology of signal production co-evolves with signal structure. EOD waveform, which is one of the most evolutionarily labile signals, has been correlated across a few apteronotid species with the trajectory of axons in the electric organ. These axons have both rostral- and caudal-running segments in the electric organ of *A. albifrons*, which produces a biphasic EOD waveform, but they run only caudally in *Sternarchorhamphus*, which has a monophasic waveform (Bennett, 1970). Rostro-caudal asynchrony of electric organ firing occurs in some apteronotids and may also influence waveform as it does in some species that produce pulse-type EODs (Caputi, 1999; Rasnow et al., 1993). Studying electric organ morphology and physiology in species with complex EOD waveforms (e.g. *S.* cf. *roseni* and *S. terminalis*) (Fig. 3B) would provide a stronger test of these hypothesized mechanisms.

Comparative studies of the pacemaker and prepacemaker nuclei could reveal the mechanisms of species diversity in EOD frequency and modulations. EODf is controlled by spontaneous, high-frequency firing of pacemaker neurons in the Pn (Meyer, 1984). Species differences in EODf are likely to have evolved through changes in the physiology of these neurons, including properties of sodium and potassium currents (Smith, 1999). These properties, however, have been studied only in *A. leptorhynchus* (Dye, 1991; Smith and Zakon, 2000), and this hypothesis needs to be tested further by characterizing neuronal physiology in the Pn of other apteronotid species.

The only documented interspecific variation in the central electromotor system of apteronotids is a difference between *A. leptorhynchus* and *A. albifrons* in synaptic inputs to the Pn. In *A. leptorhynchus*, dendrites of pacemaker and relay cells receive extensive chemical synaptic input, whereas these dendrites are nearly absent in *A. albifrons* (Elekes and Szabo, 1985). Because the synapses on these dendrites are from the prepacemaker nuclei (PPn and SPPn), which control EODMs (Dye et al., 1989), differences in dendritic morphology and synaptic input may contribute to species diversity in the structure and production of EODMs, including chirps.

Species diversity in chirp structure, including the novel chirp types found in this study, warrant comparative studies of the PPn-C and its targets in the Pn. Of particular interest are mechanisms regulating chirp duration, AM and FM, which contributed strongly to the species-specificity of chirps. Dunlap and Larkins-Ford (Dunlap and Larkins-Ford, 2003) hypothesized that the nearly 10-fold difference in chirp duration between *A. albifrons* and *A. leptorhynchus* could result from species differences in whether glutamate from the PPn-C acted on NMDA or non-NMDA receptors in the Pn. Interspecific variation in chirp AM and FM (e.g. less than 100 Hz of FM and little AM in *Sternarchorhynchus* spp. *versus* more than 500 Hz of FM and extreme AM in *P. hasemani*) could result from species differences in the robustness of PPn-C to Pn projections, in the recruitment of PPn-C projection neurons, or in the strength of post-synaptic responses of Pn neurons. Similarly, species diversity in the spectro-temporal structure and timing of chirps, such as the dual-peaked chirps of '*Apteronotus*' spp., the multi-peaked chirps of *A. devenanzii* and the chirp bursts of *S. terminalis*, may result from differences in the excitability and/or coupling of PPn-C projection neurons. Specifically, PPn-C neurons may fire single, synchronous action potentials in species that produce single-peaked chirps, but doublets, multiple spikes or spike bursts in species that produce dual- or multi-peaked chirps or chirp bursts.

These hypotheses are testable with comparative studies because the electromotor circuit is relatively simple and accessible. A direct correspondence between the firing rates of neurons in the Pn and EODf and between the firing of PPn-C projection neurons and chirping means that diversity in the neuronal physiology can be readily related to behavioral diversity (Meyer, 1984; Schaefer and Zakon, 1996; Kawasaki et al., 1988). These neurons can also be recorded electrophysiologically both *in vivo* and *in vitro*, which allows studies of intrinsic excitability and synaptic connectivity (Dye, 1991; Kawasaki et al., 1998; Heiligenberg et al., 1996) (J. A. Kolodziejski and G.T.S., unpublished observations). The anatomy of the Pn and PPn, including cell types, synaptic inputs and the expression of neuromodulators, is also well-studied (Ellis and Szabo, 1980; Elekes and Szabo, 1985; Kawasaki et al., 1988; Zupanc and Maler, 1997; Heiligenberg et al., 1996; Smith et al., 2000; Kolodziejski et al., 2005). Thus, comparative studies of the Pn and PPn will be able to link the evolution of species diversity in the anatomy and physiology of the electromotor system to species differences in electrocommunication signals.

Function and perception of diverse signals

Just as species diversity in signal parameters can suggest how signal production mechanisms evolved, this diversity can also indicate which signal functions have been subjected to strong directional or disruptive selection (Cocroft and Ryan, 1995). One of the main findings of this study was that properties of EODs and chirps were much more species-specific than those of GFRs. This result raises the question of why EODs and chirps have evolved so much whereas GFRs have remained largely conserved. One possibility is that the signal functions of EODs and chirps have exposed them to strong natural or sexual selection. Both EOD frequency and chirping are sexually dimorphic in some species and function as signals used in courtship and/or intrasexual aggression (Dunlap et al., 1998; Hagedorn and Heiligenberg, 1985; Kolodziejski et al., 2005). By contrast, GFRs are not sexually dimorphic, and their function is more controversial. They have variously been postulated to be signals of dominance, signals of subordinance, 'victory cries' or not to be communication signals at all (Dye, 1987; Hopkins, 1974b; Kolodziejski et al., 2007; Serrano-Fernandez, 2003; Tallarovic and Zakon, 2002; Triefenbach and Zakon, 2003). If chirps and EODs, but not GFRs, are used to assess mates and same-sex rivals, particularly if that assessment includes species recognition, these signals may be subject to strong sexual selection and evolve more rapidly than GFRs. Similar examples have been reported in other taxa. Evolutionary

conservation of call structure in *Atelopus* frogs, for example, may result from the reduced importance of acoustic signals relative to visual signals in mate choice in this genus (Cocroft et al., 1990). Similarly, song components that are likely to be used in mate choice are evolutionarily labile in oropendolas, whereas other song components that are less likely to be mate assessment signals are conserved across species (Price and Lanyon, 2002).

EOD frequency and waveform varied substantially across apteronotid species, but were uncorrelated with each other. This suggests that these two signal parameters evolved independently. The independent evolution of EODf and waveform may effectively increase the signal space of the EOD and allow more species-distinctive EODs to evolve. Indeed, substantial overlap of EODf between sympatric and even syntopic apteronotid species suggests that EODf alone is not a particularly effective species recognition cue (Crampton and Albert, 2006; Kramer et al., 1981). Combinatorial variations of EODf and waveform may increase the utility of the EOD as a potential species identification signal, as is supported by the ability of a DFA based on EODf and waveform to classify species at rates that far exceed chance (Fig. 6C).

By contrast, the AM and FM of chirps were tightly linked to each other both within and across species. Although this linkage reflects constraints on the mechanisms of chirp production (see above), it also has important implications for the function and perception of chirps. The association between AM and FM creates redundancy in the signal value of these parameters and may thus constrain them to convey similar information. For example, in *A. leptorhynchus*, high-FM chirps that are used as courtship signals also have much AM, and chirps with less FM that are used in same-sex interactions have little AM (Bastian et al., 2001; Engler et al., 2000). That redundancy may extend to the electrosensory mechanisms used to detect chirp AM and FM. Chirps are encoded by P-type electroreceptors based on the perturbations they produce in the beat pattern of the interacting fishes' EODs (Benda et al., 2006). These beat perturbations are a product of the relative frequencies of the two EODs as well as the AM, FM and duration of the chirp. Additional studies could test the hypothesis that the potential redundancy of AM and FM in chirps may function in signal fidelity. For example, could correlated AM and FM in chirps help the electrosensory system decode them when they occur in complex social environments, such as when the EODs of multiple nearby fish interact to produce complex beat patterns?

Conclusions and future directions

The remarkable species diversity in apteronotid electrocommunication signals raises fascinating questions on their functions and mechanistic control. A more thorough understanding of the information conveyed by these signals, their function and social contexts, and the ability of receivers to detect them is needed to provide a clear picture of how this diversity evolved. For example, the evolution of EOD waveform in non-apteronotid gymnotiform fishes has been influenced by selection to avoid electroreceptive predators (Stoddard, 1999). Selective pressures contributing to diversity in EOD frequency, waveform and modulations in apteronotids are less well known. Although it is tempting to speculate that the evolution of 'extreme' chirps with extensive AM and FM, such as those that occur in *P. hasemani*, evolved through sexual selection on males to produce more conspicuous signals, comparative studies on chirp function are lacking. Indeed, the little comparative evidence to date suggests that chirp function may itself be evolutionarily labile. Chirp types with comparable structures in *A. leptorhynchus* and *A. albifrons* are produced in different social contexts and may have evolved distinct functions (Kolodziejski et al., 2007). Thus, to understand why some species produce chirps with more than 500 Hz of FM whereas others produce chirps with less than 100 Hz of FM, more comparative studies are needed on both the social contexts in which chirps are produced and how conspecific receivers respond to them. The evolution of signal structure and function is also likely to be linked to the evolution of electrosensory systems. Comparing the abilities of the electrosensory systems of different apteronotids to encode different types of electrocommunication signals will test the hypothesis that electrosensory systems are tuned to the complex structure of conspecific signals and provide a powerful model for examining the co-evolution of signal production and sensory systems.

List of abbreviations

%AM	relative amplitude modulation
AM	amplitude modulation
AP	action potential
DFA	discriminant function analysis
EOD	electric organ discharge
EODf	electric organ discharge frequency
EODM	electric organ discharge modulation
FIC	Felsenstein's independent contrasts
FM	frequency modulation
GFR	gradual frequency rise
ML	maximum-likelihood
PCA	principal components analysis
PGLS	phylogenetic generalized least squares
Pn	pacemaker nucleus
PPn	prepacemaker nucleus
PPn-C	subdivision of the PPn which controls chirps
PPn-G	subdivision of the PPn which controls GFRs
RMS	root-mean-square
s.e.m.	standard error of the mean
SPPn	sublemniscal prepacemaker nucleus
TIPS	standard, non-phylogenetic regression

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