Variations in the electrocommunication behaviour of the weakly electric fish *Apteronotus Leptorhynchus*

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Abstract
*Apteronotus lepto rhynchus* (Gymnotiformes) is a weakly electric fish which produces wave-like electric organ discharges as well as brief modulations in the frequency of these discharges, termed chirps. Thought to be used for communicating, this study explores variations in previously described chirp types by investigating fish chirping behaviour through recordings of external electric field modulations, and several novel phenomena were characterized. An alternative form of the type 1 chirp was discovered that is preceded by a gradual rise in frequency lasting 8-10 ms. Two sub-categories of type 2 chirps emerged, distinguished by frequency excursions of 23-86 Hz and 86-183 Hz respectively, and individual fish chirp almost exclusively within a single subgroup. Type 3 chirps occupied a much lower range of durations (10-60 ms) than those previously described, and no type 4, 5 or 6 chirps were observed. Finally, a single unusual chirp characterized by an extreme frequency and amplitude modulation raises interesting questions about chirp production mechanisms. While we cannot exclude that the differences observed across chirping studies are a consequence of subtle differences in methodology, we propose that geographical variation in electrocommunication behaviour should be investigated as an alternative explanation with possible implications for speciation.

Keywords
Electrocommunication: communication through weak electric fields generated by the specialized cells of an animal which can be perceived by special receptors in the skin; chirps: a short and sharp rise in the frequency of electrical discharges which create the animal’s external electric field; *Apteronotus lepto rhynchus*: the electric fish used in this study.

Introduction
Weakly electric fish generate weak electric fields around themselves which they use to monitor their environment and interact with conspecifics. This field is generated by means of electrical discharges produced by a group of specialized cells called electrocytes that make up the electric organ. The brown ghost knifefish (*Apteronotus lepto rhynchus*, Figure 1) used in this study is a native to freshwater habitats of Central and South America and is a wave-type electric fish whose electric organ discharges (EODs) are quasi-sinusoidal and extremely regular (Møller 1995). The frequency of EODs in this fish are set by the pacemaker nucleus in the brain and can be driven up by excitatory input from a prepacemaker nucleus to produce short and sharp rises in EOD frequency, called “chirps”, which are frequently studied in this and other species of wave-type fish (Dunlap et al. 1998; Engler & Zupanc 2001; Zakan et al. 2002). Chirps are produced primarily by male fish in the presence of conspecifics and behavioural evidence suggests that they play an important role in aggressive and courtship encounters (Hagedorn & Heiligenberg 1985; Dunlap & Larkins-Ford 2003).

In laboratory studies of electric communication behaviour, an artificial electrical stimulus is typically used to mimic the EOD of a conspecific and elicit chirping (Larimer & MacDonald 1968; Dye 1987; Maler & Ellis 1987; Zupanc & Maler 1993; Dulka & Maler 1994; Dunlap et al. 1998; Engler et al. 2000). At least four main chirp types (named types 1 through 4) have been identified to date based on their duration, frequency excursion, and amplitude reduction characteristics (Engler et al. 2000; Zupanc et al. 2006). However, fish tested by different experimental groups have not performed completely consistently. For example, great variation in the range of frequency excursions is often reported for the same chirp type. Zupanc et al. (2006) describes type 2 chirps as having frequency excursions up to 156 Hz, but in an earlier report Zupanc (2002) placed them in a much lower range around 50 Hz, and Kolodziejski et al. (2005) describe an even lower range around 20 - 40 Hz. Furthermore, different studies often report completely different and sometimes unique chirp types. Beyond the typical chirp types, Kolodziejski et al. (2005) describes a long modulation with a frequency excursion of only 10 Hz lasting for up to 500 ms, while Zupanc et al. (2006) describes novel modulations (types 5 and 6) characterized by frequency depressions rather than increases.

This investigation began as an exploratory study intended to search for further variants in chirping behaviour in order to compare them to the results of previous investigations, and several novel chirp characteristics emerged, serving as a further testament to the variability of this system. While some researchers have attributed these discrepancies to differences in stimulation procedures, there is no obvious difference in methodology which explains finding different chirp structures. Instead, we propose that this emergent variability between studies might be a product of local geographical variations in chirping behaviour emerging between studies using fish collected at different geographical locations.

Materials & Methods

**Animals**
Eight brown ghost knifefish (*Apteronotus lepto rhynchus; Gymnotiformes, Teleostei*) originating from the Peruvian Amazon were used in this study (DAP Aquatic Haven,
Etobicoke, ON. Fish ranged in length from 12.1 – 18.5 cm, and EOD frequencies at 28 ± 1 °C ranged from 889 – 1025 Hz (Table 1). Because female fish do not typically respond to external electrical stimulation with chirps (Zupanc 2002), only males were admitted to the experiment by excluding fish that did not respond to a trial stimulus and whose baseline EOD was below the threshold of male EOD frequencies (850Hz).

Between experiments, fish were kept in communal tanks, under a 12 h light / dark cycle, with other males as well as females. These tanks were maintained at approximately 28 °C, pH 7.0 - 7.5, conductivity 100 - 150 μS cm⁻¹, and aquarium water was continuously filtered and aerated.

**EOD Recording**

Recordings of chirping behaviour were performed in a test tank (31 x 32 x 61 cm) whose water was conditioned to match that of the respective home tank. To obtain recordings, fish were housed in a cylindrical chirp chamber (19.0 x 5.6 cm) similar to those used in previous studies (e.g. Dye 1987).

EOD fluctuations were recorded using paired carbon-rod electrodes, 6 mm in diameter, placed at the ends of the chirp chamber (Figure 2), and were simultaneously monitored on an oscilloscope. The signal was amplified 500x (bandpass-filter 300 - 3000 Hz) on a differential amplifier (Model 3000, A-M Systems, Sequim, WA) before being digitized at a sampling rate of 20 kHz via a Digidata 1320-A, 16-bit data acquisition device and accompanying Axoscope (v9.0) software (Axon Instruments, Sunnyvale, CA). Recordings were analyzed in MATLAB v7.0 (MathWorks, Natick, MA) using custom-written programs.

Baseline EOD was determined in MATLAB, by calculating the median value of the instantaneous frequency measured during a 2 second sample recording taken prior to each trial.

**Electrical Stimulation**

The stimuli were sine waves generated by MATLAB and output through the computer's 16-bit soundcard. The signal was fed through an attenuator (SmartStep 8310-1-2-R; Attenuator; Aeroflex/Weinschel, Frederick, MD) and an analog stimulus isolator (Model 2200 A-M Systems, Sequim, WA) and was delivered by paired carbon rod electrodes (6 cm long and 6 cm apart) situated orthogonally on either side of the fish (Figure 2). Stimulus amplitude was calibrated with a pair of silver wire electrodes at the location usually occupied by the fish and was set to 1 mV/cm by adjusting the attenuator.

**Results**

**Chirp Types Observed**

A total of 3338 chirps produced by 8 fish in 112 individual trials were recorded and examined in the course of this study (Figure 4a). Qualitative analysis revealed at least three chirp types produced in response to electric stimulation, each characterized mainly by frequency excursion from the fish's baseline and by duration. Many chirps observed resemble those described previously as types 1 through 3 (Engler et al. 2000; Zupanc et al. 2006), and so the same nomenclature shall be employed here to facilitate comparisons. Nevertheless, several novel and interesting variations of established chirp types were observed throughout this study and will be described presently.

No chirps were found which corresponded to the "classical" type 1 chirp, characterized by a steep EOD frequency increase to a peak of 338 - 537 Hz above baseline followed by an equally steep decline leading to an undershoot of the baseline EOD and having a total duration of 18 - 31 ms (Zupanc et al. 2006). Instead, what appears to be a variant of this type consisted of a gradual and approximately linear rise of the EOD followed by a sudden steep rise to the peak frequency and an equally steep decline and undershoot typical of a type 1 chirp (n = 5, Figure 3a). While the total chirp duration of 15 - 25 ms is similar to that for type 1 chirps, the frequency excursions observed were 400 - 700 Hz, a higher range than previously observed for chirps of this type. Because of their distinctive gradual rise and their resemblance to type 1 chirps, artificial EOD, whose frequency was defined as the fish's baseline EOD frequency immediately prior to each trial, plus the difference frequency (Δf) being tested (Δf = 300, 200, 100, 50, 20, -10, -4, 0, 4, 10, 20, 50, 100, 200, 300 Hz).

Different stimulation frequencies were ordered randomly and served primarily to mimic the presence of male and female conspecifics to elicit the production of different chirp types.

One fish was tested per day. Each fish was measured and photographed before being placed inside the chirp chamber, and allowed to acclimate for 10 minutes prior to testing. The stimulus regime consisted of 15 trials corresponding to the randomly ordered Δf, each lasting 60 seconds, separated by 2 minutes of rest to avoid habituation. Each trial recording was saved to file, and once all trials were completed, fish were returned to a separate compartment of the communal tank.

**Data Analysis**

Data were analyzed primarily using MATLAB. Peak-to-peak amplitude was calculated using Hilbert Transform, and instantaneous EOD frequency was calculated by determining the periods between successive zero-crossings of the EOD signal. Chirps were detected automatically when the instantaneous frequency rose to ≥ 10 Hz above baseline frequency and did not return below that value for at least 5 ms. When chirps were found, their duration, frequency excursion (defined as the maximum frequency reached minus the baseline EOD), and time of occurrence relative to the start of stimulation were calculated and automatically recorded in a text file upon completion of analysis. In-depth analysis of chirp structure was also performed in MATLAB by expanding and smoothing chirps within a given time interval using a 3-point gliding average function. Chirp frequency excursions, which typically increase with increasing temperature, were normalized to a temperature of 28 °C by using the slope of a linear fit to a correlation of frequency excursion and temperature that indicated a small rise in frequency excursions of 2.9 Hz per degree (data not shown).
these chirps will henceforth be referred to as gradual rise type 1 chirps (GR-1).

Chirps corresponding to type 2 were most common (n = 3313, Figure 3b), and conformed well to the classical definition of a chirp lasting 10 – 27 ms and having a relatively small frequency excursion of 50 - 200 Hz without a terminal undershoot (Zupanc et al. 2006). Interestingly, two previously undescribed sub-categories of type 2 chirps emerged consisting of a group concentrated in the 23 - 86 Hz and 86 - 183 Hz ranges respectively, with few chirps occurring at the boundary as defined by the minimum of this clearly bimodal distribution (Figure 4b). Each fish produced chirps almost exclusively within one subgroup. This bias was not influenced by variations in test temperatures, as the chirp distributions were essentially identical after corrected to a standard temperature of 28 °C (Table 1, Figure 4c).

A third group of chirps correspond to those of type 3, as distinguished having a sharp rise in frequency of several hundred Hz sustained with some jitter over 40 – 100 ms followed by a sharp return to baseline accompanied by a simultaneous amplitude collapse nearly to zero (Figure 3d). It was the sole chirp produced by fish 4 in response to a stimulation frequency of -300 Hz. While the chirp’s duration (41 ms) resembles that of type 3 chirps, its frequency excursion of 1057 Hz exceeds that of commonly produced chirps of any type and puts the fish’s EOD at slightly over 2000 Hz at the peak of the chirp. This chirp resembles a type 3 chirp in shape, but the usual plateau is more variable and is punctuated by one brief but dramatic frequency collapse to well below baseline EOD rate accompanied by a simultaneous amplitude collapse nearly to zero.

Effect of Stimulation Frequency Difference (Df)
Stimulation of fish with signals of varying frequency differences yielded responses of varying degrees. The magnitude of the response was gauged by the number of chirps produced during each stimulation period at a particular Df (Figure 5).

The number of small chirps was greatest for small frequency differences between Df = ±10 Hz, and in most cases small chirps were rare or absent outside the range of -100 Hz ≤ Df ≤ +100 Hz. In contrast, large chirps (corresponding to type 1 and type 3 chirps) were usually absent at small absolute Df but became more common for larger absolute Df ≤ -100 Hz or ≥ 100 Hz.

Discussion
The present study examined several aspects of the chirping behaviour of A. leptocephalus in order to investigate variations in chirping behaviour in comparison to previous studies, which may indicate that these fish have a richer communicatory repertoire than was previously thought.

Chirp Types Observed
Chirps corresponding to those of type 2 were by far the most frequently observed. Although identical in structure to those previously described, two distinct subgroups emerged within this category that have been noted in passing by Zupanc & Maler (1993), but were eventually combined and left uncharacterized. The reappearance of a strong bimodal distribution during this study provides evidence for the existence of real subgroups, and although our sample size was small (8 fish), the larger number of chirps recorded (3338) mitigates this limitation. This phenomenon may even explain the discrepancies in type 2 frequency excursion ranges previously reported (Engler et al. 2000; Zupanc 2001; Zupanc 2002; Kolodziejski et al. 2005; Zupanc et al. 2006). Here, we demonstrate for the first time that these sub-categories occupy the frequency excursion ranges of 50 - 86 Hz and 86 - 120 Hz, where the boundary is defined as the minimum of the bimodal distribution. Interestingly, individual fish produce chirps almost exclusively in a single sub-category. The basis
for this selection is still unknown and we speculate that it might reflect social status or perhaps the difference between immature and mature males, in which case it would be interesting to see whether there is a shift from one chosen subgroup to the other as young males mature.

Chirps loosely corresponding to those of type 1 were observed, but much less frequently. The main difference from the traditional type 1 chirp is the presence of a previously undescribed gradual frequency rise preceding the frequency spike. Frequency excursions also ranged slightly higher than has been previously observed for type 1 chirps, most recently defined as 338 - 537 Hz (Zupanc 2002; Zupanc et al. 2006), instead easily reaching 700 Hz. These chirps could be construed as an entirely novel chirp type or as a type 1 chirp to which an additional motif has been added, and whether or not they fulfill the same function as type 1 chirps do in other members of this species remains to be seen.

Chirps corresponding to those of type 3 were observed least frequently of all. While these chirps adhere very well to the frequency modulation profile previously described, their 10 – 60 ms durations occupy a much lower range than the 48 – 128 ms chirps of this type that are typically observed (Zupanc 2002; Zupanc et al. 2006). Although this range creates some overlap with type 1 chirps, their distinct shapes distinguish them from one another. Further investigation is required to accumulate enough specimens of these rare chirps for quantitative analysis in order to determine the true bounds of their duration range in this group of fish.

No chirps corresponding to those of types 4, 5 or 6 were observed, which may be an intrinsic trait of this group of fish or may simply reflect their general rarity or even the absence of real specifics which might more easily provoke these chirps (Zupanc et al. 2006).

The central findings on the effect of stimulation frequency on the incidence probability of type 1, 2 and 3 chirps agree with those of previous studies, in which small chirps are produced predominantly at small Df (-10 ≤ Df ≤ 10) while large chirps are produced predominantly at large Df (-50 ≥ Df ≥ 50) (Bastian et al. 2001; Engler & Zupanc 2001). This is consistent with the interpretation of the earlier studies that small chirps play a role in intrasexual communication, whereas large chirps are involved in intersexual communication, in which the difference in EOD frequency is larger. Since the relationship between chirp type and stimulation frequency has been well described by these previous studies, and since our results agree with those studies, we did not analyze this interaction in further depth.

Chirps corresponding to those of type 2 were observed most frequently, but they were left unexamined and likely represent a similar type of modulation which will be considered more carefully here. Zupanc & Maler (1993) briefly mention rare chirps which “displayed very large frequency increases...coupled with a near collapse of the EOD amplitude”, but they were left unexamined and likely represent a similar type of modulation which most recently observed.

### Table 1. Key physiological characteristics of each subject fish.

<table>
<thead>
<tr>
<th>Fish Number</th>
<th>Total Body Length (cm)</th>
<th>EOD at 28°C (Hz)</th>
<th>Temperature During Trials (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.5</td>
<td>889 - 920</td>
<td>28.0</td>
</tr>
<tr>
<td>2</td>
<td>15.5</td>
<td>1025 - 1035</td>
<td>25.5</td>
</tr>
<tr>
<td>3</td>
<td>13.8</td>
<td>926 - 935</td>
<td>29.0</td>
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<tr>
<td>4</td>
<td>14.7</td>
<td>950 - 957</td>
<td>28.0</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>989 - 1011</td>
<td>27.5</td>
</tr>
<tr>
<td>6</td>
<td>13.6</td>
<td>942 - 949</td>
<td>28.9</td>
</tr>
<tr>
<td>7</td>
<td>12.8</td>
<td>950 - 962</td>
<td>29.0</td>
</tr>
<tr>
<td>8</td>
<td>13.6</td>
<td>898 - 903</td>
<td>27.9</td>
</tr>
</tbody>
</table>

Extreme frequency and amplitude modulations

The occurrence of a novel and exceptional chirp type with extreme frequency and amplitude modulations beyond those observed in any previously described chirps, although it was singular among all the chirps observed, warrants discussion as to the possible underlying mechanisms because of the very fact that it is physically possible in an apparently healthy fish. While its duration (41 ms) and the presence of an undershoot make it similar to type 3 chirps, its maximal frequency excursion of 1057 Hz, as well as the dramatic frequency and amplitude collapse, distinguish it from any chirps previously described. Zupanc & Maler (1993) briefly mention rare chirps which “displayed very large frequency increases...coupled with a near collapse of the EOD amplitude”, but they were left unexamined and likely represent a similar type of modulation which will be considered more carefully here.
organ (EO) resulting in maximally destructive interference. This raises the interesting question of whether or not the A. leptorhynchus EO can fire asynchronously, a question which would be simple to address in future work by recording neural activity from different parts of the EO in an immobilized fish exposed to electrical stimuli. If the EO can indeed fire asynchronously, this would open a dimension for generating communication signals that has not been considered thus far. Alternatively, the collapse could be explained by the refractory period of the electrocytes. If the refractory period of the electrocytes were longer than the refractory period of the relay cells of the pacemaker nucleus driving them (Smith & Zakon 2000), then the electrocytes could miss EO Occurrences at the highest driving frequencies. This explanation might identify this chirp as a type 3 chirp which reached such an exceptionally high frequency excursion that just such a collapse took place, and further investigation on a cellular level is required to investigate this hypothesis.

Conclusions
The results of this study provide numerous examples of variations in all properties of chirping behaviour when compared to previous bodies of work on A. leptorhynchus. While it seems increasingly clear that previously defined chirp categories may not be as rigid as it was previously believed, these findings also raise interesting questions about the nature of chirp behaviour variability across studies.

As no obvious methodological reasons have arisen to explain the structural differences in chirps obtained during different studies, the possibility of natural geographical variations in chirping behaviour remains a possibility that warrants investigation. This tentative hypothesis is supported by the fact that the chirp behaviour of the fish used in this study investigation, which to the best of our knowledge originate from Peru, are disparate from the behaviour of fish used in another recent study of chirp behaviour by Zakon (2002) which originated from a geographically distinct river system in Columbia (Zakon personal communication, February 5, 2007).

The phenomenon of unique forms of communication in different populations is well documented in other species of fish, as well as in insects, birds, and even mammals, and may be an indicator of incipient speciation (Martens 1996; Pillay 2000; Boughman 2002; Yamada et al. 2002; Magurran & Ramnarine 2004; Patten et al. 2004). Consequently, the examination of chirping variation in geographically isolated populations of A. leptorhynchus could provide an opportunity to investigate real-time speciation as a result of sensory drive. Future work should aim to compare chirp characteristics between fish from different known geographical locations, and if systematic differences do exist, to determine whether they might form the basis for reproductive isolation between populations which could lead to speciation.

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References