

## RESEARCH ARTICLE

# Electrocommunication in pulse Gymnotiformes: the role of electric organ discharge (EOD) time course in species identification

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**ABSTRACT**

Understanding how individuals detect and recognize signals emitted by conspecifics is fundamental to discussions of animal communication. The species pair *Gymnotus omarorum* and *Brachyhypopomus gauderio*, found in syntopy in Uruguay, emit species-specific electric organ discharge (EOD) that can be sensed by both species. The aim of this study was to unveil whether either of these species is able to identify a conspecific EOD, and to investigate distinctive recognition signal features. We designed a forced-choice experiment using a natural behavior (i.e. tracking electric field lines towards their source) in which each fish had to choose between a conspecific and a heterospecific electric field. We found a clear pattern of preference for a conspecific waveform even when pulses were played within 1 Hz of the same rate. By manipulating the time course of the explored signals, we found that the signal features for preference between conspecific and heterospecific waveforms were embedded in the time course of the signals. This study provides evidence that pulse Gymnotiformes can recognize a conspecific exclusively through species-specific electrosensory signals. It also suggests that the key signal features for species differentiation are probably encoded by burst coder electroreceptors. Given these results, and because receptors are sharply tuned to amplitude spectra and also tuned to phase spectra, we extend the electric color hypothesis used in the evaluation of objects to apply to communication signals.

**KEY WORDS:** Field lines tracking, Navigation, Phase encoding, Syntopic species, *Gymnotus omarorum*, *Brachyhypopomus gauderio*, Electroreception

**INTRODUCTION**

Electroreception is a sensory modality found in many vertebrates, but the dual use of the same electric field for exploring the environment (active electroreception) and sending messages to conspecifics (electrocommunication) is only found in the superfamily Mormyridae, native to Africa, and the order Gymnotiformes, found in Central and South America. Species in both groups emit either a continuous sinusoidal electric field (electric organ discharge, EOD) with a predominant frequency component (i.e. wave fish) or discrete pulsatile EODs separated by a silence of at least twice the pulse duration (i.e. pulse fish). The self-generated field acts as a carrier for signals received by electroreceptors, which are differentially tuned to the species-specific EOD.

Wave-emitting fish, including the mormyrid *Gymnarchus niloticus* and Gymnotiformes of the families Sternopygidae and Apterontidae, possess electroreceptors that are sharply tuned ('V shaped') to their species-specific frequency (Bastian, 1977; Hopkins, 1976; Viancour, 1979; Zakon and Meyer, 1983). As the summation of sine waves of similar frequencies results in an amplitude-modulated sine wave in the same range of frequencies, these fish evaluate the envelopes of the incoming signals (Metzen and Chacron, 2014). The responses of the whole electroreceptor population are used to separate the information on either an object or a conspecific signal (Clarke et al., 2015). Furthermore, social signals create phase shifts in the envelopes that act as very powerful stimuli to electroreceptors as a result of their adaptation properties (Benda et al., 2005; Metzen et al., 2016).

The mechanisms that underlie the separation of object and conspecific signals in pulse-discharging fish are distinct from those of wave fish. Pulse Mormyridae have a private electrosensory pathway for processing communication signals (Hopkins, 1974; Bell, 1989; Bell and Grant, 1989; Bell and Szabo, 1986). They possess a specific type of receptor (Knollenorgans) which are 'U shaped', tuned to the power spectral density of the species (Hopkins, 1981; Bell and Grant, 1989; Lyons-Warren et al., 2012). Knollenorgan population evaluation of frequency components, pulse duration and the time course of allo-generated waveforms plays a role in species and sex identification (Lyons-Warren et al., 2012; Vélez and Carlson, 2016). Demonstrating this from a cognitive point of view, when Mormyridae of different species were placed in the same recording arena, all fish were optimally attracted to their own species, but also selectively responded to the presence of the other species with behavior that ranged from attraction to avoidance (Moller and Serrier, 1986).

Pulse-emitting Gymnotiformes, the focus of this study, possess two main types of electroreceptors, referred to as pulse markers and burst coders (Bastian, 1976, 1977; Caputi and Aguilera, 2019; McKibben et al., 1993; Rodríguez-Cattáneo et al., 2017; Watson and Bastian, 1979; Yager and Hopkins, 1993). Both types are innervated by a single primary afferent and respond to active electrolocation and communication signals. Pulse markers fire a single latency-modulated spike (Rodríguez-Cattáneo et al., 2017), and exhibit V-shaped tuning with a diversity of best frequencies located at the center of the power spectrum (Watson and Bastian, 1979). When stimulus signals are manipulated, they show better tuning to the amplitude than to the phase spectrum of the stimulus (Caputi and Aguilera, 2019). Burst coders, in contrast, respond to the EOD with a burst of spikes. They show tuning curves of different shapes (U and V) covering different frequency ranges of the amplitude spectrum of the self-generated discharge (Bastian, 1976, 1977; McKibben et al., 1993; Watson and Bastian, 1979; Yager and Hopkins, 1993). Burst coders are extremely sensitive to changes in the time course of the signal (Caputi and Aguilera, 2019, 2020).

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The dual sensitivity of electroreceptors to the amplitude and time course of a stimulus signal led to the concept of ‘electric color’, defined as an emergent feature that arises when different aspects of the stimulus electric field, separately encoded by distinct types of electroreceptors, converge at some still unknown point in the central nervous system. This neural signal appears to be relatively robust to changes in object location and indicates a qualitative feature of a stimulus object (Budelli and Caputi, 2000). Recognition and discrimination of the electric color of objects has been shown using behavioral assays in both Mormyridae (Gottwald et al., 2018; von der Emde, 1990; von der Emde and Ronacher, 1994) and pulse-discharging Gymnotiformes (Aguilera and Caputi, 2003; Rodríguez-Cattáneo et al., 2017).

By analogy, this led us to propose here that the allo-generated electrosensory field may carry a species-specific electric color when evaluated at a certain distance. This has broad implications for the study of electrocommunication, specifically with regards to sexual selection and speciation. This study contributes to the evaluation of the ‘species-color’ hypothesis. Because mechanisms involved in these processes are evidently taxa dependent, and should have broad ecological and evolutionary implications, we elected to focus on a syntopic pair of pulse gymnotiforms, *Brachyhypopomus gauderio* and *Gymnotus omarorum* (which live in syntopy in much of Uruguay; see Silva et al., 2003).

To fully validate the hypothesis that electric color may act as a feature for species identification, one has to show that electric color signals are detected and discriminated from other similar stimuli, and are also preferred and recognized at the cognitive level. At the sensory level, Hopkins and Westby (1986) proposed three possible non-exclusive mechanisms for conspecific waveform recognition based on receptor sensitivity to amplitude and phase spectra and also to scan sampling. At present, it is known that both pulse markers and burst coders both respond to conspecific signals (McKibben et al., 1993; Yager and Hopkins, 1993) and that they show differential sensitivity to the amplitude and phase spectra of the stimulus (Caputi and Aguilera, 2019). Behaviorally, pulse-emitting electric gymnotiforms detect the presence of a phase-locked signal and discriminate between the waveforms of two distinct allo-generated signals with the same power spectra (Heiligenberg and Altes, 1978). In addition, *B. gauderio* and *G. omarorum* exhibit sustained pacemaker rate accelerations in the presence of conspecific or allospecific EOD phase locked at 25 ms following their own discharge. They also show transient acceleration of the pacemaker rate triggered by switching between the phase-locked waveforms (Waddell and Caputi, 2020). This suggests that both species are able to detect and discriminate between signals in the presence of another fish solely through use of the EOD. However, as novelty responses are triggered even in a decerebrated animal (A.A.C., unpublished observations), decision-making experiments exploring the cognitive level of behavior are required to confirm that these species use the EOD waveform as a marker for identification of species, sex or even individual. In this sense, the tendency toward aggression found in *Gymnotus* has been used to show that these fish can learn to identify individuals (though the subjects were possibly different species; McGregor and Westby, 1992, 1993). In that study, fish were placed in individual side-to-side partitions of a common tank, and after 6 months the fish at the center showed no aggressive behavior toward either neighbor. However, when the EOD of each of the neighbor fish was played back on the same and opposite sides, differential aggressive displays were observed, indicating that fish learn to associate the territory held by another fish with the waveform of its EOD (McGregor and Westby, 1992).

Here, we used another natural behavior – the tendency to track the electric field generated by external electrogenic sources (Davis and Hopkins, 1988; Hopkins et al., 1997; Westby, 1974) – to test the hypothesis that the EOD waveform emitted by the same species has a preference value over that of the other species. Using a single electrogenic source, we implemented a forced-choice study by offering the fish pairs of signals – either artificially generated, or emitted by another two fish, one of each species – with very similar results for the two methods. To further explore the differential effects of amplitude and phase spectra, we employed a spectra translocation protocol (Caputi and Aguilera, 2019).

## MATERIALS AND METHODS

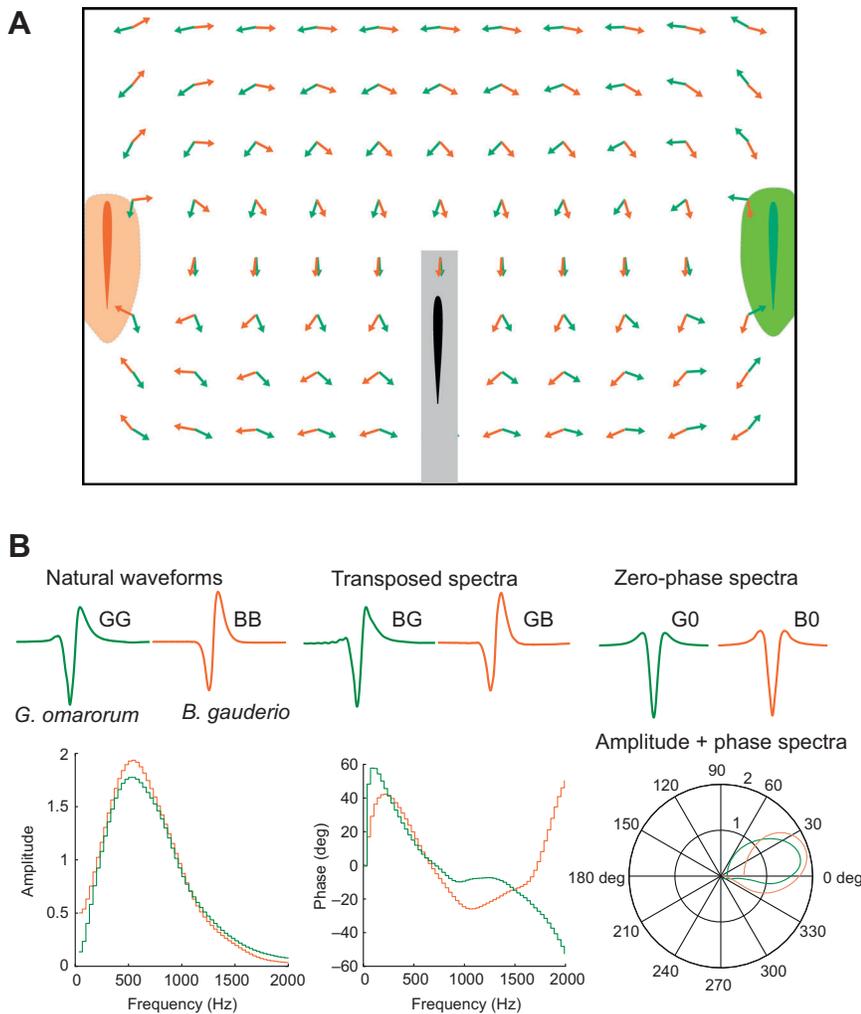
### Animals and ethical considerations

Two species were used in this study: *Gymnotus omarorum* Richerde-Forges, Crampton and Albert 2009 and *Brachyhypopomus gauderio* Giora and Malabarba 2009. Fish were captured using dip nets in Laguna del Sauce (Maldonado) y Rio Negro (on the border between Durazno and Tacuarembó) Uruguay, under permission of the Ministerio de Agricultura y Pesca. All experiments were performed under the regulations of animal care and use committees of the Instituto de Investigaciones Biológicas Clemente Estable (protocol no. 001/03/2011). None of the procedures caused lasting harm to the specimens, nor did they involve surgical procedures or chemical agents, and all specimens were returned to captivity after completion of trials. Thirty-six fish were used in this study. They ranged in size from 13 to 23 cm (mean 17.6 cm, median 17 cm) for *G. omarorum*, and from 12 to 19 cm (mean 16.3 cm, median 16 cm) for *B. gauderio*. During the captivity period, fish were kept in individual tanks with a water conductivity of 100  $\mu\text{S cm}^{-1}$  and 18°C water temperature, and were fed *ad libitum* with insect larvae. The light cycle was natural, provided by large windows, and matched light levels found in wild conditions.

### General experimental design

Experiments were carried out in a tank (150 cm×100 cm and 10 cm depth, 18°C and 100  $\mu\text{S cm}^{-1}$ ) in a closed laboratory with low ambient noise. We implemented a forced-choice experiment by taking advantage of the natural attraction electric fish have to electric sources, which led to the discovery of electroreception (Shackelford, 1941) and was later characterized as a ‘field line-tracking’ behavior (Davis and Hopkins, 1988; Hopkins et al., 1997; Westby, 1974). The experiments were carried out during the summer months, after fish had adapted to the animal care facility conditions for 2–6 weeks.

Each fish was exposed to two trains of pulsatile stimulus signals either emitted by two fish (one of each of the two species) placed in closed pens made of bamboo sticks and pantyhose fabric, or through two pairs of electrodes, with each pair placed at the center of one of the shorter walls of the arena (Fig. 1A). In the latter case, each train of signals was emitted through a pair of bare nichrome wires tightly coiled on the ends of a thin bamboo stick (15 cm spacing). Each of these pairs of electrodes was connected to an analog stimulus isolation unit (A-M Systems Model 2200) which was driven by the audio port of a personal computer emitting a signal in a range of  $\pm 1$  V, 16 bits at 44,100 kilosamples  $\text{s}^{-1}$ . Each computer was connected to an isolation unit through a potentiometer, allowing for the fine adjustment of signal amplitude. Every trial was video monitored and recorded with a cellphone camera (Motorola G4, 13-MP CMOS, f/2.0) placed 1 m above the bottom of the tank, using the freely available DroidCam software (<https://www.dev47apps.com/>). The root mean squared (rms) value of both



**Fig. 1. Forced-choice arena and stimulus waveforms.** (A) Aquarium used for trials, showing field line vectors. Green and orange fish silhouettes represent *Gymnotus omarorum* and *Brachyhyppopomus gauderio* emitters (mimicking dipole pairs were placed at the same positions), arrows of the same colors indicate the direction of the field, and the black fish silhouette in the rectangle represents a receiver fish studied in each trial. (B) Natural and synthetic waveforms used in trials [normalized to their root mean squared (rms) value]. Natural received signals are shown for *G. omarorum* in green and *B. gauderio* in orange. We maintain this color scheme in the synthetic waveforms of transposed spectra (color indicates phase spectra source) and in zero-phase spectra (color indicates amplitude spectra source). Fast Fourier transforms of natural signals for both species are shown below with the same color code. Amplitude (as a percentage of the total energy, frequency bin 33 Hz) and phase spectra (deg) are plotted separately (left and middle, respectively) and together (right) in a polar plot.

stimulus electric fields, measured at the door of the pen, was maintained ( $1200 \mu\text{V cm}^{-1}$  for a waveform centered on a trace of 10 ms length) and corresponded to the mean between the stimulus electric fields received from four *B. gauderio* and four *G. omarorum* of  $15 \pm 0.5$  cm length placed in parallel at 10 cm (following Waddell and Caputi, 2020). As a reference, the potential and field lines emitted by each emitter fish, or by each pair of dipoles, were calculated using a finite element model (Caputi et al., 1998).

In each trial, the exploring fish was placed in a pantyhose fabric pen placed perpendicular to and at the center of one of the longer walls, with the door facing the center of the tank. In every trial, just after the door was opened, both stimuli were started, in a different order each time. After a variable period (between seconds and a few minutes), the fish left the pen and chose one of the two signal emitters.

Stimuli were played back at  $30 \pm 0.5$  Hz. We chose this stimulus rate because this is a typical active discharge for *G. omarorum*, and while *B. gauderio* typically exhibit a slower discharge rate, they commonly reach these rates during swimming and exploration maneuvers (Waddell et al., 2016). We have no reason to believe that the higher discharge rate would in any way negatively affect the reception of the stimulus for *B. gauderio*. Playback signal repetition was phase offset differently for each trial to avoid synchrony in time, and also had a small frequency difference to avoid a systematic effect of phase lock between the two stimuli. Additional studies are

currently in progress to explore the extent to which information may be encoded in repetition rate, which may indeed provide some information regarding identity (Waddell et al., 2016).

Stimulus pairs were randomly assigned to the left and right sides of the tank, to avoid systematic bias due to potential preference for movement in a left or right direction, and to avoid any accidental ‘training’ of fish to prefer one side versus the other. Individual fish underwent a maximum of one trial per day, and fish were returned to their aquariums between trials – both measures to minimize any potential for stress and also to minimize their time spent in the experimental tank. Fish were fed *ad libitum* both before and after trials.

#### Forced-choice experiments; natural conspecific versus allotropic stimuli

In the first set of experiments, we showed that fish have a significant preference for tracking the electric field lines that possess the temporal course characteristic of its own species. We used 20 *G. omarorum* and 16 *B. gauderio* that had never been exposed to the arena and stimulated them with artificial signals. These signals were built by averaging species-specific waveforms (one set for each emitting species) taken from a small pair of electrodes (3 mm spacing) aligned perpendicular to the skin 1 mm from the snout of a receiving fish, connected to a differential amplifier (A-M Systems Model 1800, band-pass filter 0.1 Hz to 10 kHz, gain  $\times 1000$ ). These

signals represent the allo-generated stimuli produced by emitter fish oriented in antiparallel on the electrosensory fovea of a receiver fish (i.e. the rostral region of the fish that exhibits a high concentration of electroreceptors; see Castello et al., 2000). To validate the veracity of the artificial signal playback, we compared the performance of 16 *G. omarorum* and 15 *B. gauderio* when live specimens were used in place of playback stimuli (as before, using one of each species).

In all cases, we counted a movement toward a waveform as a decisive approach when the fish rested stationary or show repetitive exploration movements within 15 cm of a stimulus source (either dipole or other fish). Fish generally exited the starting chamber and moved toward either the left or right, and swam in wide an arc moving along field lines calculated as previously (for method, see Caputi et al., 1998; Fig. 1; Movie 1), and finally rested along the dipole after the final approach. Sometimes the fish would sit with its head sticking out of the starting chamber, which is a typical behavior for Gymnotiformes (Pereira et al., 2005) when apparently evaluating signal sources. Slight side to side motions were often seen upon exit of the start chamber, and also while swimming towards its chosen target, which we interpret as the fish controlling its trajectory by partially aligning its body with the emitted field. In some rare cases, fish exhibited occasional exploratory approaches, and in others the fish swam backwards before snagging its trajectory to the field lines (see Fig. 2B; Movie 2), but in nearly every such case, fish only more closely approached the dipole they ultimately chose. Decisions were usually made in ca. 5–10 min, though some fish took only seconds (or longer than 10 min) to leave the starting chamber.

### Analysis of preference between conspecific and allospecific signals

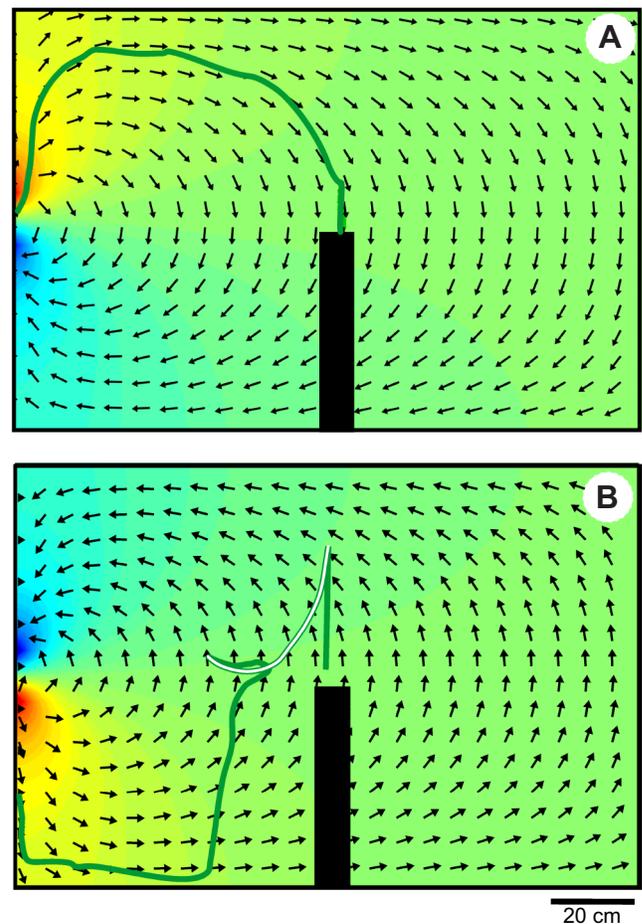
For each experiment type, we quantified the proportion of fish that decided to first explore the time course of the field of the same species versus the field of the other species. We tested the hypothesis that fish would prefer a conspecific discharge (and explore the source of the conspecific field) in the following ways: first, we performed a Fisher exact test to show association between fish preference and the species-specific EOD; and second, we calculated for each species whether the score of conspecific preference (i.e. fish preferring the same species EOD waveform, referred to as hits) exceeded that expected by the following equiprobability equation:

$$\sum_{i=0}^{\text{hits}} \frac{0.5^n \times n!}{i! \times (n-i)!} = 0.975, \quad (1)$$

where  $n$  is the number of studied fish (significance level 0.05 after Bonferroni correction).

### Forced-choice experiments; weighing signal features for species preference

Taking into account previous data that indicate that burst coders respond to changes in phase spectrum better than to changes in amplitude spectrum and that pulse markers show opposite preference (Caputi and Aguilera, 2019), we posed the hypothesis that both amplitude and phase spectra were relevant with regards to preference, but that because in this case amplitude spectra overlap, phase spectra would be the predominant factor. To test this hypothesis, a small library of six playback signals was built from the original signals (Caputi and Aguilera, 2019). As amplitude spectra largely overlap, one would expect that when a pair of stimuli having zero phase [i.e. signals having the amplitude spectrum of



**Fig. 2. Example of stimulus choice and field tracking behavior.** Field is shown only for one stimulus for simplicity, but both experiments shown were undertaken with both left- and right-side stimuli. Fish track is shown in green. The color map encodes the potential. (A) Simple track in which the fish follows the field line (corresponding to Movie 1). (B) Example in which the fish moved first backwards in an arcuate exploratory movement (white trace) before heading towards the emitter dipole (corresponding to Movie 2).

*G. omarorum* (G0) or of *B. gauderio* (B0) but in both cases zero-phase synchronous phase spectra) are compared, no preference would be found. In addition, when phase spectra of natural stimuli were transposed between species [i.e. the amplitude of *G. omarorum* but the phase spectra of *B. gauderio* (GB) and the amplitude of *B. gauderio* but the phase spectra of *G. omarorum* (BG)], fish would prefer the signal with the phase spectrum of its own species. As a control comparison, we used the original pair of signals described above. We refer to these as GG and BB for *G. omarorum* and *B. gauderio*, respectively, as each of them has amplitude and phase spectra of the same species (see Fig. 1B for the time course and spectra of the six stimuli).

For the first series, we compared the effect of the power spectral densities of the signals by exploring fish decisions between B0 and G0. In the second series, we tested whether phase or amplitude spectra carried the predominant feature used in decision making by comparing fish decisions made between BG versus GB. Finally, we again compared the original signals (BB versus GG). In this part of the study, 15 individuals of each species, previously tested for preference for fish emitted signals, were explored using dipole playback in place of live fish. Because we made the assumption that preference may not be wholly decisive in single trials either in

transposed stimuli or, especially, in zero-synchronous phase spectra stimuli experiments, we elected to perform three trials for each fish when evaluating preference using any artificial stimuli and judge each fish preference according to the majority of the choices in the three trials.

### Analysis of the weight of signal features in species preference

To represent the preference of each fish with a single parameter, we implemented a simple scoring system whereby a single point was given to either waveform A or waveform B over the course of the three trials, resulting in a final score for each fish, for each trial. For example, in a trial where the fish swam three times to A, we would give 3 points to A, 0 points to B, and then subtract the points for B from A, resulting in a score of 3 ( $3-0=3$ ). Therefore, in each comparison (i.e. BB versus GG, B0 versus G0 and BG versus GB), four possible outcomes of the experiment were considered for each fish, referring to the compared waveforms as A and B: absolute preference for A ( $3-0=3$ ), absolute preference for B ( $0-3=-3$ ), relative preference for A (score:  $2-1=1$ ), relative preference for B (score:  $1-2=-1$ ).

As fish prefer their own species' waveform when using fish as stimulus generators, we reasoned that if the two components of the waveform were equipotent features, fish would choose the transposed stimuli in a random manner, and therefore the summed score would tend to cancel out, which would result in a median score of 0. In contrast, if the fish population were to show a homogeneous preference for one signal spectrum over the other, the distribution would be skewed – which would result in a large absolute value of the summed score, and the median value would be significantly different from zero. Following this line of reasoning, a small summed score would also be expected if the population expressed heterogeneous preferences. In each experiment, a zero median of score values would indicate symmetrical distribution of scores having either flat, bimodal or centered, monomodal distribution corresponding either to a heterogeneous decision criteria in individuals of the same species or to a lack of preference. In contrast, a non-zero median of the score values would indicate a strongly skewed distribution and a significant preference for one of the two waveforms. Thus, we tested (using a sign test) whether the median value of the scores in each experiment was significantly different from zero. As we performed 6 tests, Holm–Bonferroni correction was applied to circumvent underestimation of type I error using an ultimate  $P$ -value of 0.05. In addition, we performed a two-way ANOVA Friedman test across the three comparisons and the two species.

## RESULTS

All studied fish exhibited a preference for one of two natural signals. Fish behavior consisted of a period of variable length in which the fish remained quiet inside the pen (or just out of the pen, in the middle of the tank), and a second period in which the fish navigated at a relatively fast speed following the field lines, where it ended up exploring the 'head' electrode of the chosen stimulus pair. Fig. 2 shows two typical tracks. Calculation of fish tracks from raw video data for each of these trials is shown in Movie 1.

### Each species prefers tracking signals with its own waveform

Fish chose to navigate to their conspecific in 15 out of 16 cases for *G. omarorum* ( $P=2.59\times 10^{-4}$ ) and in 12 out of 15 for *B. gauderio* ( $P=0.0176$ ). In addition, 16 out of 20 *G. omarorum* ( $P=0.006$ ) and 13 out of 16 *B. gauderio* ( $P=0.011$ ), none of which had previously

been exposed to the experimental arena, preferred their own species-specific EOD waveform to the waveform of the other. The strong association between the species-specific waveform and the species of the tested fish (Fisher exact test,  $P=3.53\times 10^{-5}$ ) clearly indicates that both species prefer to track the waveform emitted by conspecifics (Fig. 3A,B).

### The role of power spectra and time course of EOD waveform for signal preference

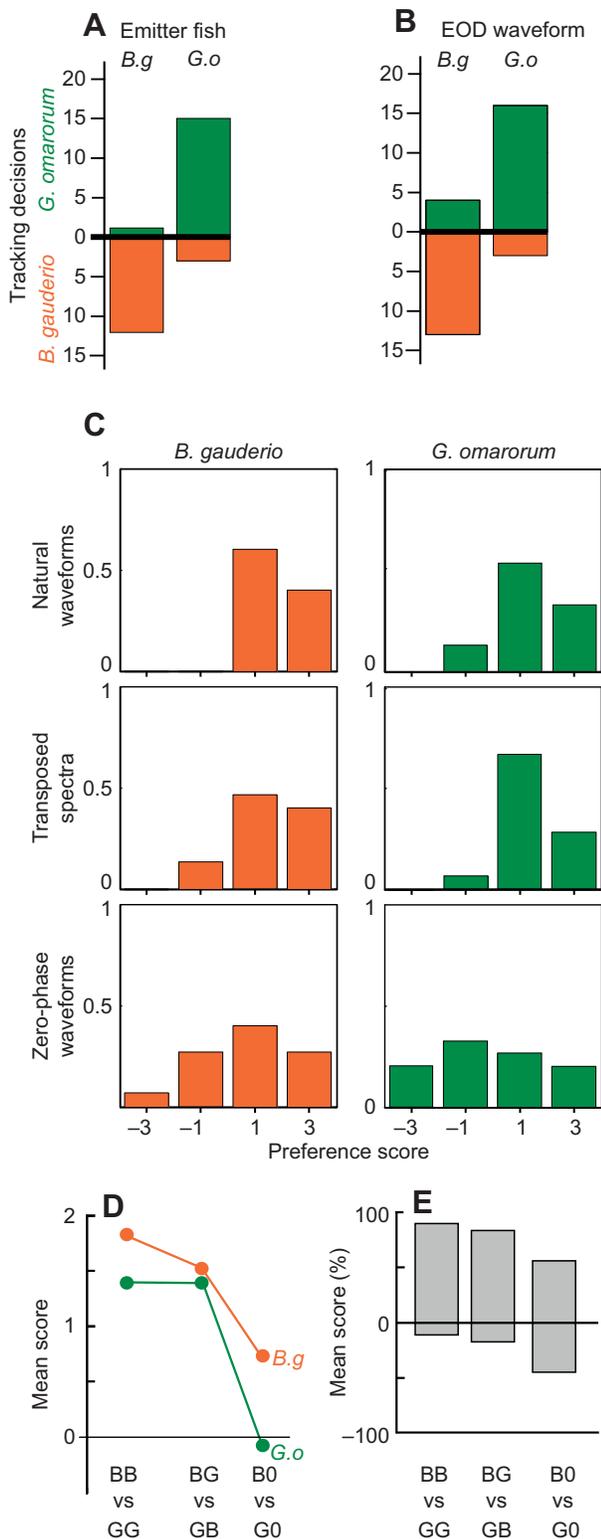
To test the preference for a waveform, we tested the role of amplitude spectrum and the time course of the waveform in 15 specimens of each species. Two-choice experiments, repeated 3 times per fish, yielded four possible score values per fish: absolute preference for the same species (3), absolute preference for the other species (–3), relative preference for the same species (1), relative preference for the other species (–1). Fig. 3C shows a comparison of these scores with those obtained on decisions with natural stimulus waveforms (i.e. BB versus GG) by the same fish in the same conditions (mean values: 1.40 and 1.8, sign test  $P$ -values of 0.0074 and 0.0001 for *G. omarorum* and *B. gauderio*, respectively;  $n=15$  in both cases; Fig. 3C, top row; decisions made by each fish are reported in Table S1).

Decisions between synthetic signals with different power spectral densities and the same, synchronic, zero-phase spectrum (i.e. B0 versus G0) yielded scores not significantly different from zero (mean values: –0.067 and 0.73 corresponding to  $P$ -values of 1 and 0.30 for *G. omarorum* and *B. gauderio*, respectively, sign test,  $n=15$  in each case; Fig. 3C, bottom row). In contrast, scores corresponding to decisions on transposed signals (i.e. BG versus GB) showed phase preference for the signals when combining the phase spectrum of the same species and the amplitude spectrum of the other species (mean values: 1.40 and 1.53 corresponding to  $P$ -values of 0.00098 and 0.0074 for *G. omarorum* and *B. gauderio*, respectively; sign test,  $n=15$  in each case; Fig. 3C, middle row).

Two-way ANOVA Friedman tests, with repetition, indicated a significant main effect of the pair of compared waveforms ( $\chi^2=7.47$ ,  $P=0.024$ , d.f.=2) on the scores, but did not show evidence of different behavior between species ( $\chi^2=1.53$ ,  $P=0.22$ , d.f.=1; Fig. 3D). When we pooled data for the two species, and binarized individual results (e.g. relative and absolute preference for a given signal were grouped together as a singular choice), we found that 93% of individuals chose their own waveform, 87% chose their own phase and only 57% chose their own amplitude. This showed that preference scores obtained when comparing zero-phase signals (i.e. B0 versus G0) were significantly smaller than those obtained with either natural or transposed signals, but there was no significant difference between the scores obtained when selection was between signals with different phase spectra (sign tests among scores obtained in experiments contrasting zero-phase with natural or transposed signals yielded  $P$ -values of 0.0034 and 0.0129, respectively, while the test between the scores obtained in these last two experiments yielded a  $P$ -value of 1). These data suggest that the phase spectrum was the main signal feature driving decisions between natural and transposed signals, and these syntopic species have no apparent preference for either amplitude spectra.

## DISCUSSION

The presence of species-specific communication channels is central to many discussions of social interaction, including mate choice and its consequences on speciation. *Gymnotus omarorum* and *Brachyhyppopomus gauderio* live in syntopy, and are often found in close proximity with one another – well within the working range



**Fig. 3. Summary of behavioral trials.** (A) Decision outcomes for trials using live fish as stimulus emitters (*G. omarorum*,  $n=16$ ,  $P=2.59 \times 10^{-4}$ ; *B. gauderio*,  $n=15$ ,  $P=0.0176$ ). (B) Counts of decision outcomes for artificially generated conspecific and allospecific signals, i.e. 'natural' waveforms (*G. omarorum*,  $n=20$ ,  $P=0.006$ ; *B. gauderio*,  $n=16$ ,  $P=0.011$ ). (C) Distribution of decision scores for natural, transposed and zero-phase waveforms (*G. omarorum*,  $n=15$ ; *B. gauderio*,  $n=15$ ). Scores are summarized for three trials per fish such that positive and negative scores indicate which waveform is preferred (positive being conspecific, negative being allospecific), with absolute values of 3 and 1 indicating absolute (3 choices in the 3 trials) and relative preference (2 choices in the 3 trials), respectively. (D) Comparison of the mean score obtained for the three comparisons in the artificial stimulus experiments: natural (BB versus GG), phase transposed (BG versus GB) and zero phase (B0 versus G0). The same order of preference indicates a strong concordance between the mechanisms used by the two species. Decisions taken by *B. gauderio* are in orange and those by *G. omarorum* are in green. Results of a two-way ANOVA are given in Results, and a full description of the statistical tests is provided in Materials and Methods. (E) Mean of scores for each of the three artificial stimulus experiments, for the two species pooled together, and with decisions binarized (described in full in Results). Positive values represent the percentage of individuals that chose their own signal (or given signal feature), negative values represent the percentage of individuals that chose the signal of the other species. Statistical analyses are described in Results. Individual decisions are reported in Table S1.

Although our experiments were not designed to evaluate whether waveform recognition is innate or learned, the fish used in these trials were caught in the wild and nearing adult size (though they were not yet sexually mature). This indicates that signal preference, if not innate, would have been learned in early life.

#### Proximate causes of waveform preference: why pulse fish employ 'waveform tuning'

The behavioral decision profile observed in the experiments reported here consists of a gradient of preference – beginning with the strongest preference for the conspecific waveform, significant preference of phase over amplitude spectrum of the same species when transposed signals are compared, and no preference between amplitude spectra when zero-phase signals are compared (we note only a slight, non-significant, trend toward the amplitude spectrum of the conspecific in *B. gauderio*; see Fig. 3).

To describe the time course of a signal with a broad amplitude spectrum, the relative phase differences between frequency components must be evaluated. Although here we used phase spectrum as a tool for manipulating the waveform received by the fish without affecting the relative power of each frequency component of the signal, we must stress that other signal features might be the basis of the signal evaluated by the fish (if, when combined, said features completely describe the stimulus space in an equivalent manner). When dealing with subtle features of a sensory system that humans cannot perceptually imagine, one must take into account that phase and amplitude representations of a signal are mathematical means to measure these signals, and do not necessarily reflect the way in which fish assess the same signals.

Wave fish emitting in a very narrow frequency band (Lissmann, 1958), to which their electroreceptors are fine tuned (Hopkins, 1976), do not have the option to use the relative phase between more than one frequency component, and can only use the phase of the signal when comparing between different parts of the body (Kawasaki and Heiligenberg, 1989), or when abrupt changes in the signals emitted by one of two or three socially interacting fish occur (Benda et al., 2005; Metzen and Chacron, 2014).

In contrast, pulse fish emit short stereotyped waveforms, and possess electroreceptors either broadly tuned to this amplitude spectrum (Mormyridae: Bell, 1990; *Gymnotus*: Watson and

of electroreception, which is the primary sensory modality in electric fish (Caputi et al., 2008; Lissmann, 1958; von der Emde, 2006). Here, we provide evidence for the identification of the electric field emitted by its own species and for a principal role of the whole time course of the signal rather than the power spectral density alone in species self-recognition. This study is in line with the finding that fish are able to learn the association between an EOD waveform and a territory (McGregor and Westby, 1992).

Bastian, 1979; *Brachyhypopomus*: Yager and Hopkins, 1993) or tuned to different frequencies within this range (Mormyridae: Lyons-Warren et al., 2012; Gymnotiformes: Bastian, 1976, 1977; Watson and Bastian, 1979). Some of these electroreceptors are also sensitive to changes in the time course of the signal (Mormyridae: Hopkins and Bass, 1981; Arnegard et al., 2006; Gymnotiformes: Caputi and Aguilera, 2019). This dual responsiveness opens the possibility of using the frequency range of the carrier spectrum to evaluate – with relative independence – the changes in amplitude and phase of the received pulse. As a consequence, the received signals may be mapped in a manifold of at least three different dimensions: the whole amplitude of the signal, the frequency distribution of its amplitude and the phase relationship between different frequency components. Thus, the hypothetical amount of information carried by potential changes in each of these dimensions is enormous, and may allow the central nervous system to develop an abstraction of some features that result from specific combinations of these parameters – all of which are represented in the time course of the received signal.

One of these abstractions is the so-called electric color hypothesis proposed (Budelli and Caputi, 2000) upon analysis of previous data (von der Emde, 1990; von der Emde and Ronacher, 1994), and confirmed both in *Gnathonemus petersii* (Mormyridae; Gottwald et al., 2017, 2018) and *Gymnotus omarorum* (pulse Gymnotiformes; Aguilera and Caputi, 2003; Rodríguez-Cattáneo et al., 2017) in the context of object recognition. Here, we provide evidence that the main parameter used for waveform differentiation in the pair of syntopic species *G. omarorum* and *B. gauderio* (when evaluating the EOD of these two species) is encoded in the time course of the signal. Our findings are consistent with the hypothesis that – just as many macroptic species use visual color for species, sex and individual recognition – pulse Gymnotiformes use electric color. Furthermore, taking into account that our results are consistent with what has been found in mormyrid fish, which exhibit EOD with species-specific phase and amplitude spectra (Hopkins, 1995; Moller, 1995), and a receptor type exclusive to communication (the Knollenorgans) that is sensitive to both phase and amplitude spectra (Arnegard et al., 2006), one can extend the electric color recognition hypothesis to all pulse-emitting electric fish.

### Ultimate causes of waveform preference and their implications

Pulse Gymnotiformes are often found in speciose, electrically noisy communities in which amplitude spectra of different species' discharges overlap (Crampton et al., 2013; Hopkins, 1981). *Gymnotus omarorum* and *B. gauderio* overlap along the Rio Negro, which crosses Uruguay from east to west, and in the drainage basin of the Rio Uruguay in the northern part of the country. The locality from which the individuals used in this study were collected is composed only of *B. gauderio* and *G. omarorum* (with occasional sightings of *Eigenmannia virescens*). Because the species almost entirely overlap in amplitude spectra, we found a predominant role for phase spectra in waveform recognition, which indicates that they are able to establish an intraspecific communication channel based mainly on the time course of their species-specific EOD. Further, even though these species are predominant in inland Uruguayan rivers and creeks, *Gymnotus* and *Brachyhypopomus* have fairly wide distributions, and are therefore expected to be able to differentiate among signals from a variety of species.

Although *G. omarorum* is mostly restricted to Uruguayan territory (Craig et al., 2018; Richer-de-Forges et al., 2009), it is found in sympatry with *Gymnotus paraguensis* (part of the

*Gymnotus tigre* clade within *Gymnotus*) in the provinces of Entre Ríos and Corrientes, in the Argentinian Mesopotamia between the Parana and Uruguay rivers (Craig et al., 2018). The two species are morphologically similar, and while their signals largely overlap in their power spectral density, the temporal course differs in the late negative wave component that originates in the tail region (Rodríguez-Cattáneo and Caputi, 2009). Further, *G. omarorum* is a relatively recent divergence from a species-rich clade with similar discharges, and likely originated in a much more species-rich region (Lehmberg et al., 2018).

Similarly, *B. gauderio* is known from the lower Paraná, Paraguay, Uruguay, Patos-Mirim and Tramandaí basins – which puts it in contact with tens of electric fish species. Notably, *B. gauderio* overlaps in range almost completely with the congener *Brachyhypopomus bombilla*, and to a lesser extent with *Brachyhypopomus draco*. In the furthest extent of its range, it overlaps with *Brachyhypopomus walteri* in the lower Parana and upper Paraguay, and in the upper Paraguay basin with *Brachyhypopomus brevirostris* (Crampton et al., 2016). Most of these species express a head to tail biphasic discharge typical of Hypopomidae in which a positive peak is followed by a negative peak (Giora et al., 2008; Loureiro and Silva, 2006; Sullivan et al., 2013; Waddell et al., 2016), both peaks being of variable duration and having mostly overlapping power spectral density.

Because both *G. omarorum* and *B. gauderio* are likely to encounter many congeners across their geographic range, we expect that conspecific recognition and discrimination of potentially confusing social and sexual signals through the use of multiple signal features is under significant selective pressure. While here we suggest the use of the time course of the emitted signal, which is able to carry more information than a smooth, relatively narrow (although wider than in wave fish) and monomodal power spectral density contains, and may be a simple and powerful evolutionary solution for efficiently solving this problem in this species pair, this important role of the time domain features of the EOD does not rule out the role of amplitude spectrum.

Here, we propose that the concept of electric color may be relevant to signal diversity in at least three cases. First, many *Brachyhypopomus* and some *Gymnotus* species exhibit sexually dimorphic EODs such that males have a longer discharge to the extent that the time course can be recognized by the naked eye, and thus tend to have a lower peak power frequency (Caputi et al., 1998; Crampton et al., 2011; Giora et al., 2008; Rodríguez-Cattáneo et al., 2013; Stoddard et al., 1999). In this case, changes in peak power frequency would result in different shades of the same electric color – a sort of fine-tuning mechanism utilized during the reproductive season. Second, some communities are composed of congeners which do vary significantly in amplitude spectra. For example, *Brachyhypopomus beebei* (with a peak power frequency of approximately 1 kHz; Waddell et al., 2016) is found in the wild with both *Brachyhypopomus sullivanii* (which has a peak power frequency of ca. 3 kHz) and *B. walteri* (which has a peak power frequency of ca. 0.2 kHz) – and some *Gymnotus* assemblages exhibit the same amount of variation (Crampton et al., 2011; Rodríguez-Cattáneo et al., 2013). In these communities, we predict stronger behavioral effects when large differences in amplitude spectra are presented. These differences in amplitude can be considered effectively as different electric colors entirely – and act as a sort of coarse tuning mechanism. Third, many weakly discharging pulse gymnotiforms are syntopic with species of the strongly discharging genus *Electrophorus* (de Santana et al., 2019), which exhibits a strong monophasic discharge. This large difference in amplitude spectra may be a coarse tuning mechanism to rapidly differentiate predators from

non-predators (Stoddard, 1999). Thus, the combination of amplitude and phase spectra, or another equivalent set of species-specific features, to define species electric color, may serve as way species can recognize and discriminate among stimuli, allowing for fast reactions in events such as predator–prey encounters, and for fine tuning during social interaction.

## Conclusions

Our results add a deeper understanding to important aspects of the main sensory channel of electric fish, which has broad implications for the study of animal communication. The dual sensitivity of electroreceptors to amplitude and phase spectra (Caputi and Aguilera, 2019) may allow a better discrimination among sensory stimuli, and specifically participate in the recognition of conspecific communication signals. This is necessary to avoid costly mismating events (Coyné and Orr, 2004; Endler, 1989), and recognition of signals from potential predators is similarly vital (Stoddard, 1999). In other species, color has an important role in social interaction, managing the identification of conspecifics and their sex (Albertson et al., 1999; Kocher, 2004), and here we suggest that electric color plays an analogous role. In the case of weakly electric pulse fish, species-specific signals exhibit a wide-amplitude spectrum that is able to broadly stimulate the mosaic of other species. Although the amplitude spectra largely overlap among many species, our data indicate that species specificity derives from a fine tuning of the effectiveness of the power distribution over the frequency range of a given signal by a species-specific sensitivity to the time course of conspecific signals, much like how color or timbre add dimensions to visual and auditory inputs. Broad similarities between electric color evaluation strategies found in pulse-discharging weakly electric fish of distant clades imply a convergence at anatomical, physiological and behavioral levels. We anticipate that further exploration of these similarities will be rewarding as we continue to tease apart the mechanisms that underlie detection, discrimination and recognition of electrocommunication signals in pulse-type electric fish.

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## Competing interests

The authors declare no competing or financial interests.

## Author contributions

Conceptualization: J.C.W., A.A.C.; Methodology: J.C.W., A.A.C.; Software: J.C.W., A.A.C.; Validation: J.C.W., A.A.C.; Formal analysis: J.C.W., A.A.C.; Investigation: J.C.W.; Resources: A.A.C.; Data curation: J.C.W.; Writing - original draft: A.A.C.; Writing - review & editing: J.C.W., A.A.C.; Visualization: J.C.W., A.A.C.; Supervision: A.A.C.; Project administration: A.A.C.; Funding acquisition: A.A.C.

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