Title: Why the brown ghost chirps at night.

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Abstract

Since the pioneering work by Moeller, Szabo and Bullock, weakly electric fish have been widely used as a model to approach the study of both spatial and social cognitive abilities in a vertebrate taxon typically less approachable than mammals or other terrestrial vertebrates. Through their electric organ, weakly electric fish generate low-intensity electric fields around their bodies with which they scan the environment to orient themselves and interact with conspecifics even in complete darkness. The brown ghost knifefish is probably the most intensely studied species due to the large repertoire of individually variable and sex-specific electric signals it produces. Their rich electric vocabulary is composed of brief frequency modulations - or *chirps* - of the oscillating dipole moment emitted continuously by their electric organ. Chirps of different types are thought to convey very specific and behaviorally salient information, and are thus used as references to specific internal states during recordings - of either the brain or the electric organ - or during behavioral observations. However, while this notion undoubtedly contributed to the success of this model in neuroethology during the past seven decades, the logic underlying this electric communication still remains unclear.
This study re-evaluates this view in the attempt to provide an alternative, and perhaps complementary, explanation for why these freshwater bottom dwellers emit electric chirps. By revealing correlations between chirping, electric field geometry and detectability in enriched environments, we provide evidence for a previously unexplored role of chirps as specialized self-directed signals used to expand conspecific electrolocation ranges during social encounters.

Introduction

Homeoactive sensing - i.e. self-directed signal production (Zweifel and Hartmann, 2020) - is often used as a strategy to enhance spatial navigation in heterogeneous and unpredictable environments. By emitting high-pitch calls (8-200 kHz; Fenton et al., 1998), bats are able to localize stationary and moving objects while flying at high speeds and in complete darkness (Jones and Holderied, 2007). Similarly, odontocete cetaceans are capable of echolocating objects by emitting very short (a few ms long) clicks covering a broad frequency spectrum (5 Hz-100 kHz). A few other mammals (shrews and tenrecs), but also bird species (oilbirds and swiftlets) and even blind humans (Griffin, 1944; Thaler et al., 2011), are known to be capable of echolocating target objects through the emission of brief high-frequency echolocation calls (Konishi and Knudsen, 1979; Brinkløv et al., 2013; Gould, 1965). In general, all biosonar systems represent a remarkable adaptation to low-light or turbid environments in which other sensory modalities would offer only insufficient information required to optimize fast spatial navigation during foraging or social interactions.
But navigating in the dark is not only about detecting sounds. The electrosensory systems of weakly electric fish offer analogous advantages, yet at the same time presenting fundamental differences. Brown ghost knifefish are one of the most studied species. They belong to the gymnotiform group (as does the electric eel) and occupy benthic habitats in the Amazon river basin (Crampton, 2019). Brown ghosts generate quasi-sinusoidal electric fields at relatively high frequencies (600-1000 Hz; Zakon et al., 2002) by means of modified motor projection neurons forming a so-called electric organ, located along the fish body or in the caudal region.

For this reason, the electric fields generated by these species are also referred to as electric organ discharges (EODs).
Together with a few other species (Rose, 2004), brown ghostspossess the unique capability to localize bioelectric sources (such as muscle activity in prey or other organisms) using electrosensory information emanating from biotic sources (i.e. passive electrolocation; Rose, 2004). Compared to echolocation, passive electrolocation is possible only at shorter ranges (0-5 cm; Lissmann and Machin, 1958; Von der Emde, 1999; Knudsen, 1974) and, although it is so fast to be considered almost instantaneous, it is more sensitive to signal degradation (Benda, 2020; Nelson and MacIver, 2006).
Active electrolocation (i.e. the assessment of the location of an object based on the detection of its interference with actively generated electric fields) allows to extend the *active space* of electrolocation (Hopkins and Westby, 1986; Brenowitz, 1982) slightly beyond such short range: since EODs spread roughly equally in all directions, all affected surrounding objects (preys, substrate, vegetation) are simultaneously detected as they cast their electric image on the fish skin (up to 10-12 cm away from the skin; von der Emde, 1999; Snyder et al., 2007). But the real advantage of active electrolocation can be appreciated in the context of social interaction: when two fish approach each other, the alternating constructive and destructive interaction of their EODs results in an amplitude- and phase-modulated signal (also referred to as *beat*) detectable by each of them within a 30-40 cm radius (see appendix 1: *Detecting beats at a distance*; Knudsen, 1975; Fotowat et al., 2013; Henninger et al., 2018; Benda, 2020). The frequency of such beat modulations is equal to the frequency difference (DF) of the two sinewave signals. Because each brown ghost has a different EOD frequency (at 27°C female range 600-800 Hz, male range 800-1000 Hz, Bastian et al., 2001) the beat frequency can be used not only to sense an approaching conspecific but also to determine its identity and sex (low DFs = same sex, high DFs = opposite sex).
In summary, weakly electric fish are capable of passively detecting bioelectric sources in close proximity to their body (0-5 cm), but also inanimate objects, by actively sensing the instantaneous perturbations they cause to their EODs (6-12 cm; Chen et al., 2005; Snyder et al., 2007; Fotowat et al., 2013). In addition, by detecting beatmodulations of their EOD induced by other EOD emitting sources, they can instantaneously sense the presence of other conspecifics at distances of up to 30-40 cm (Fotowat et al., 2013; Henninger et al., 2018; Silva A., personal communication).
Importantly, since the strength of electric fields decays with the squared distance (see appendix 1), compared to sound waves generated by bioacoustic sources, EODs are more prone to anisotropic degradation even at short ranges. This is mainly due to the presence of noisy interferences encountered during locomotion through electrically heterogeneous underwater materials (Gómez-Sena et al., 2014; Pedraja et al., 2016; Milam et al., 2019; Yu et al., 2019).
The picture is further complicated by the presence of another critical source of beat interference: communication signals. These consist in brief EOD frequency modulations - also called *chirps* - which can be broadly classified in at least 4 different types, based on their duration and the amplitude of the chirp frequency modulation (Engler et al., 2000; Zupanc et al., 2006). Different types of chirps are thought to carry different semantic content based on their occurrence during either affiliative or agonistic encounters (Larimer and MacDonald 1968; Bullock 1969; Hopkins 1974; Hagedorn and Heiligenberg 1985; Zupanc and Maler 1993; Engler et al. 2000; Engler and Zupanc 2001; Bastian et al., 2001).
Since chirps can only be detected as beat perturbations (Petzold et al., 2016), this signaling is likely to interfere with electrolocation and with any other information detected through the beat (i.e. the location of other EOD emitting conspecifics). This is likely to occur most of the times during social interactions (Yu et al., 2012; Fotowat et al., 2013), due to the highest occurrence of chirps within beat detection ranges (< 50 cm; Zupanc et al., 2006; Hupé and Lewis 2008; Henninger et al., 2018; see appendix 1). How brown ghosts succeed in the complicated task of assessing conspecific location through blurred EOD amplitude modulations, while communicating through chirping signals highly distorting them, is not known.

In this study, we propose a solution to this problem by providing evidence for a previously unexplored function of chirping in weakly electric fish. We have gathered a large dataset of more than 54.000 chirps, obtained from staged social pairings, playback experiments and behavioral assessments of fish locomotory activity. We first show that the choice of different chirp types does not significantly correlate with any particular behavioral or social context. It is instead correlated to the biophysical properties of underwater electric fields and to the spatial arrangement of interacting EODs. By using high-speed infrared video recordings synchronized with multi-channel voltage data acquisition, we then analyzed chirps considering both their effects on beat processing and their spatial attributes, i.e. the spatial location of the interacting fish. Analyses of cross-correlation of chirp time-series, chirp-type transition sequences and of behavioral responses to playback chirps were used to assess the presence of meaningful correspondences. Finally, by recording fish in different conditions of electrical "visibility", we provide validating evidence in support of a new functional hypothesis to explain why brown ghosts chirp at night: homeoactive sensing.

Results

Experience is the main factor affecting chirp variability

During social interactions, communication signals are often related to immediate aspects of behavioral physiology (i.e. internal states, Owren et al., 2010). As a consequence, correlations between signals and behavioral contexts are often indicative of signal meaning (Flack and De Waal, 2007). We thus predicted that, if behavioral meaning can be attributed to different types of chirps, as posed by the prevailing view (e.g., Hagedorn and Heiligenberg, 1985; Larimer and MacDonald, 1968; Rose, 2004), one should be able to identify clear correlations between behavioral contexts and chirp types. To this aim, we recorded fish pairs in different contexts broadly divided in three groups based on the experience with the recording environment (*tank experience*): *residents* (experienced), *intruders* (naïve) and *equal* (pairs in which both fish were naïve). Additional sub-groups were identified based on other factors underlying a pair social relationship (*context*): social experience, simulated breeding conditions, dominance status and physical accessibility (N = 130 fish pairs, see methods for details).

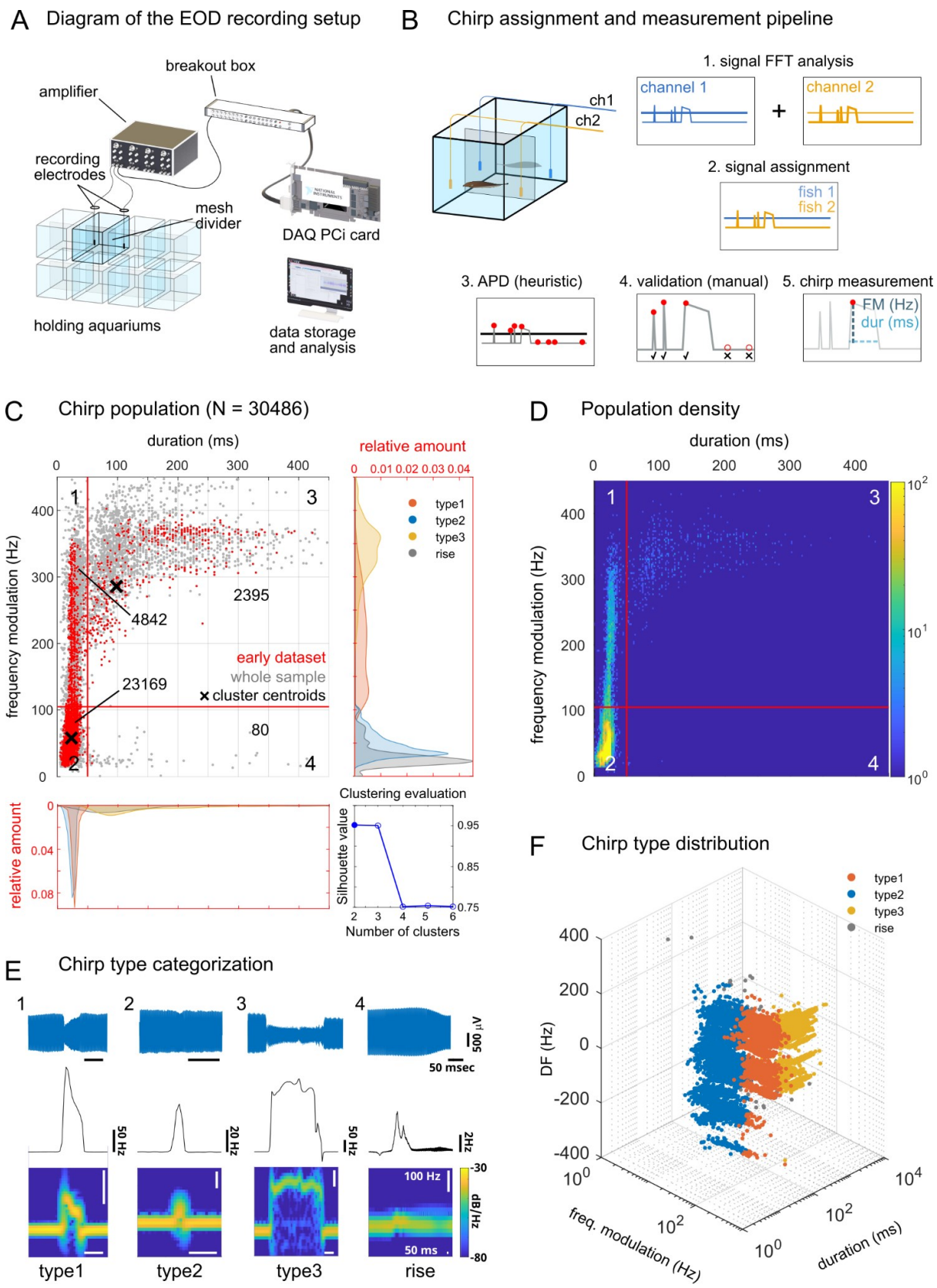

Figure 1. Chirp type categorization based on FM parameters. *A: Schematic diagram of the recording setup: holding aquariums (N = 8) were divided by a plastic mesh to prevent physical contact between the interacting fish (N = 2 per aquarium) while allowing electric interactions. Brown ghost EODs were detected by electrodes placed in each tank*

compartment (2 input channels per tank), amplified, digitized (20 kHz sampling rate) and recorded using custom-written MATLAB scripts. B: Electric signals and chirps were analyzed using their fast-fourier transforms (FFT, 1) and assigned to different fish based on the signal intensity (2). Frequency modulations were detected using a MATLAB-based heuristic method for automatic peak detection (APD, 3). False-positives, false-negatives or wrongly assigned chirps were revised manually (4) and chirp FM and duration were then measured (5). C: Chirp distribution by frequency modulation (Hz) and duration (ms). K-means chirp clustering indicated an optimal value of 2 for clustering chirps based on these two parameters (cluster centroids are indicated with black crosses). The red lines indicate the cut-off values used to classify the different chirp types (type 1 = 1, type 2 = 2, type 3 = 3, rises = 4): duration 50 ms and modulation 105 Hz. These values are based on a dataset acquired at the beginning of the study (red, in the scatter plot; N chirps = 11342; N chirping fish = 16, N fish pairs = 8) and on the distribution density (D) of the whole chirp population (gray, in the scatter plot; N chirps = 30486; N chirping fish = 130, N fish pairs = 78). E: Representative examples of the 4 different chirp categories (voltage data on the first row, instantaneous frequency on the second and spectrogram on the third). F: Scatter plot showing the distribution of different chirp types by DF (frequency difference between sender and receiver fish): note the gradual change in chirp type composition (color coded) at different DF values (specially visible for type 1-3). Due to the sex difference in the brown ghost EOD frequency, negative DF values correspond mainly to females, positive values to males.

EOD recordings took place in chambered aquariums using a multi-channel acquisition setup (**Figure 1A**). Chirp detection was carried out using a semi-automatic working pipeline (**Figure 1B**, see methods section for details). Chirps have been previously categorized in different types based on the extent and the duration of their frequency modulation (in Hz and ms, respectively; Engler et al., 2000). Initially, we searched for clusters in our chirp dataset in an unbiased fashion using the k-means method and FM and duration as input parameters (**Figure 1C**). This algorithm identified the presence of 2 clusters - evaluated using the silhouette method (Rouseeuw, 1987) - and indicated a roughly bimodal distribution of chirps, at least relative to these basic features. Nonetheless, for consistency with the existing literature, we categorized chirps using rough cut-off values based on the distribution density of an early subset of chirps (N = 11342 chirps, see methods for details). These values roughly matched the broad parameter ranges previously reported for each type of chirp (Engler et al., 2000; Zupanc et al., 2006; Zakon et al., 2002; Bastian et al., 2001). Based on these cut-off values (duration = 50 ms, FM = 105 Hz), chirps were categorized in short (with small - type 2 - or large - type 1 - frequency modulation) and long (with small - rises - or large - type 3 - frequency modulation; **Figure 1C**). Slower events, such as gradual frequency changes (or *gradual frequency rises*; Engler et al., 2000), were more sporadic and were not considered in this analysis.

Overall, the majority of chirps were produced by male subjects, in comparable amounts regardless of environmental experience (resident, intruder or equal; **Figure S1A**,**C)**, social status (dominant vs subordinate; **Figure S1B**) or social experience (novel vs experienced; **Figure S1D**). Interestingly, this sex difference was reversed when fish were allowed to interact freely, as opposed to fish whose interaction was limited due to the presence of a plastic mesh divider (divided vs free; **Figure S1E**,**F**). Sex differences were found also in the types of chirp produced, mainly in the case of male-to-female exchanges (MF; **Figure S2C**,**D**), confirming previous accounts of marked sex dimorphisms in both the number and the type of chirps produced by brown ghosts (Dye, 1987; Zupanc and Maler,1993; Dulka and Maler, 1994).

To better define the main factors underlying chirp variability (i.e. types, frequency of beat and carrier signals, behavioral context, etc.), we used a 3D factor analysis for mixed categorical and numerical data (FAMD) to evaluate for each recorded chirp several parameters relative to the interacting fish (both *sender* and *receiver*) and their respective EODs (see methods for

details; **Figure 2**). The model obtained was adjusted in order to include the most significant variables (those explaining above 20% of the total variance). The least number of variables required to explain the highest percentage of variance (N = 3: *tank experience*, *DF* and *context*) was obtained by further simplifying the model through subsequent iterations (percent of variance explained 52.3%). Overall, this analysis indicated that environmental and social experience, together with beat frequency (DF) are the most important factors explaining chirp variability (**Figure 2B**). Analysis of the distribution of individual chirps revealed some cluster segregations in correspondence of resident-intruder or novel-experienced fish pairs, while less evident clustering can be seen for other variables (e.g., social status). Notably, no obvious cluster segregation was found between sender and receiver fish of either sex, as cluster centroids overlap in both cases. Type 2 chirps (see marginal chirp type distributions in **Figure 2C**) are mainly localized around resident fish and novel encounters (**Figure 2C**); type 1 and type 3 chirps seem to be equally distributed between naive and experienced pairs (see kernel distribution plots in **Figure 2C**); rises seem to be clustered more around female subjects, regardless of status and experience (**Figure 2C**). Of particular interest is the segregation of chirps produced by fish pairs subject to water changes to simulate the onset of the breeding season, with (breeding-naive, experienced-divided) or without tank dividers (breeding-experienced-free). The proximity of the latter condition to chirps produced by naïve and freely swimming mixed-sex pairs (no breeding-naïve-free) suggests that either locomotion and/or social contact might explain the segregation of this data from the parent groups (i.e. breeding-related) much better than breeding status. Collectively, these results confirm the strong effect of EOD frequency difference (DF) on chirp type distribution (**Figure 1**; Bastian et al., 2001; Petzold et al., 2016) and reveal that experience is a significant factor determining chirp heterogeneity. In addition, they suggest chirp production might be affected by locomotory activity and behavioral variables linked to direct close-range social interaction.

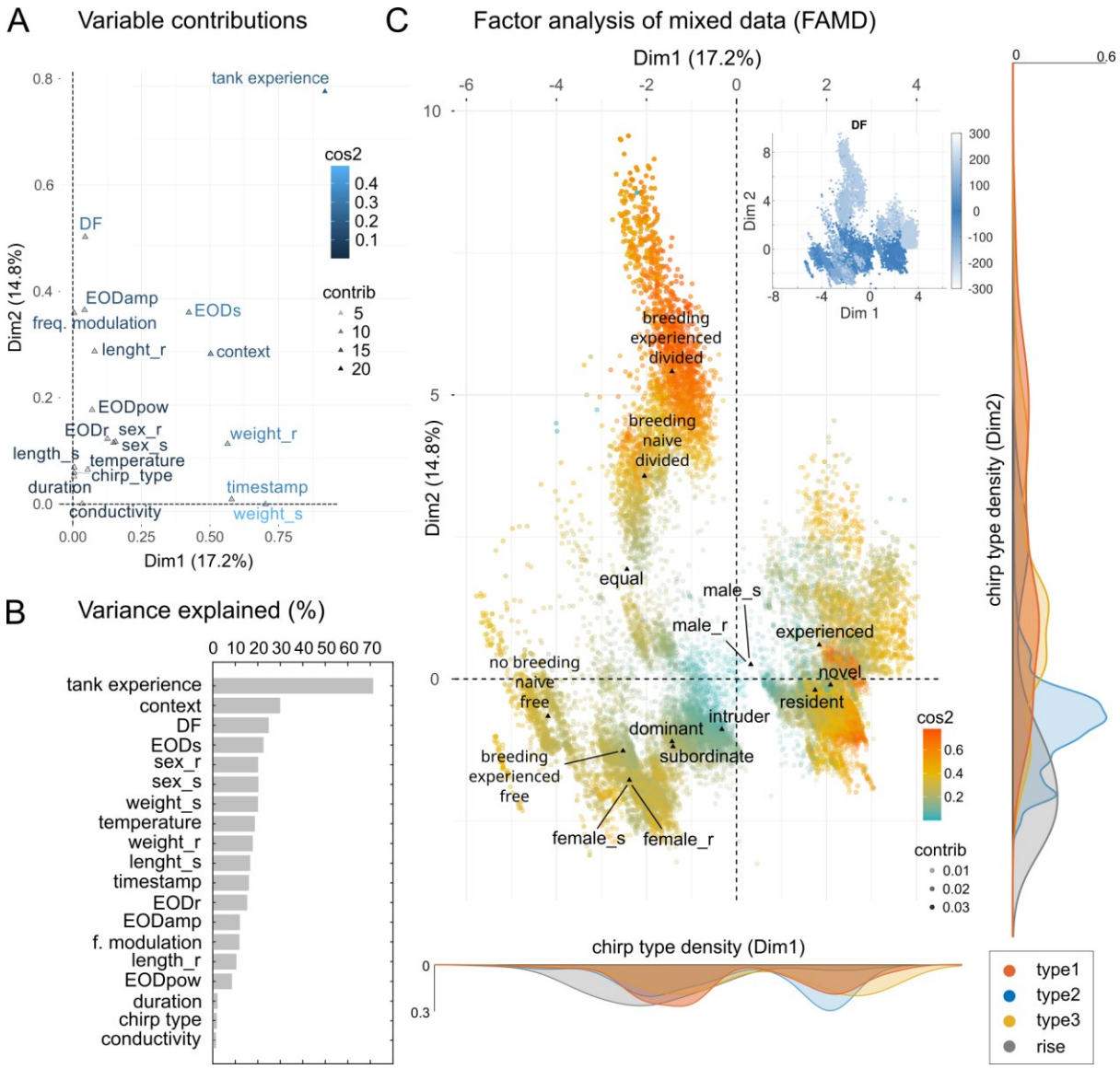

Figure 2. Factor analysis of mixed data (FAMD) – social contexts and tank experience. *A: Scatter plot showing the contribution of all chirp-related variables to the overall variance of the whole chirp dataset: among these, EOD parameters such as amplitude (EODamp), frequency (EODs or EODr, based on sender or receiver identity), spectral power density (pow) were considered together with variables related to chirps, such as frequency modulation (f. modulation), duration, sex of sender or receiver fish (sex_s, sex_r), the time of occurrence within a 1 hour trial (timestamp), the type (1 to 4), the DF. Variables related to the fish experience with either the tank environment (tank experience: resident = 1-week tank experience, intruder = new to the tank, equal = both new) and experience with the paired conspecific (context). This latter category refers to the reciprocal experience of each fish pair (novel or experienced), their hierarchical status (dominant or subordinate), the type of interaction (divided = behind a plastic mesh barrier, free = freely swimming) and the simulated breeding season (based on water conductivity levels: high conductivity = ca. 400 µS, no breeding; low conductivity = ca. 100 µS, breeding) at which the interaction takes place (see methods for details). Triangles indicate the coordinates of the variable centroids, their contribution ("contrib") is coded by color intensity, whereas the quality of their representation on the transformed coordinates is coded by color hue ("cos2"). Note how "tank experience", "context" and "DF" stand out of the variable group. B: Estimates of the total variance explained*

indicate that tank experience, together with DF and context, are the most important factors explaining chirp variance. C: Representation of chirps in the transformed coordinates. The clustering is based on qualitative coordinates (tank experience, context and chirp type). Cluster distance represents the correlation among variables. The marginal plots show the kernel distribution of the chirp population color-coded according to chirp type (legend on the bottom right). Labeling chirps by DF shows how chirps can be meaningfully clustered based on this parameter (inset, top right).

Invariant chirp responses to chirping playback EOD mimics

A complementary approach to uncover correspondences between communication signals and behavioral states implies the use of playback experiments. For this purpose, we adopted a variation of the classic "chirp chamber" approach (e.g. Dye, 1987; Bastian, 2001; Dunlap and Larkins-Ford 2003) and assessed fish responses to chirping signals in freely moving conditions (**Figure 3A**). In these experiments, both the matching of emitted signals to playbacks as well as the locomotor activity displayed during close-range interactions with EOD sources (i.e. decisions to approach or avoid playback signals), can be used as a possible measure of signal value (Fugere and Krahe, 2010; King and McGregor, 2016).
Fish were allowed to swim in a 30 x 80 cm aquarium at whose opposite ends two 13 x 30 cm playback compartments were created using plastic mesh barriers. Playback electrodes were positioned parallel to the longest tank axis and ca 10 cm apart. Electric stimuli were EOD mimics at different frequency steps (DFs) of both positive and negative sign, relative to the fish's own EOD frequency (values in Hz: ±240, ±160, ±80, ±40, ±20, ±10, ±5, 0). The EOD playback intensity was set at 1mV/cm at ca. 5 cm distance (which is in the range of natural field strengths of this species). Playback sessions were organized in 4 different *modes*, based on the pattern of frequency modulations contained (mode 0 = no chirps, mode 1 = type 3 chirps, mode 2 = type 2 chirps, mode 3 = rises). Chirp parameters were set to represent rises and the 2 main chirp categories defined by the k-means clustering without the constraints of our cut-off values (*short* - type 1 and type 2 - and *long* - type 3 - chirps; **Figure 1C**,**D**). Each playback trial lasted ca. 60 sec with 180-sec inter-trial intervals (see methods for details).
Fish responses to plain sinewave EODs confirmed previously reported findings: fish respond with large chirps (type 1 and type 3) to high DFs and small chirps (type 2) to low DF signals (**Figure S3)**, with males producing more chirps than females (**Figure 3B**; Bastian et al., 2001; Engler and Zupanc, 2001). Notably, a transient decrease in chirping was observed in the immediate temporal surround of playback chirps (modes 1-3, **Figure S4**). This effect was similar for all chirp types and seemed to be limited to the short time around chirp occurrence. Accordingly, a more pronounced decrease in chirping was observed during 3 Hz trains of type 2 chirps, possibly due to the higher frequency at which these events were repeated (**Figure S4**). Apart from this, fish responses to chirping EODs were hardly distinguishable from non-chirping EOD mimics in terms of number of chirps produced (**Figure 3C**,**D**).
Analysis of chirp time course further revealed two interesting details: first, while type 1 and type 3 chirps seem to be randomly scattered throughout the whole trial (60 s), the number of type 2 chirps seems to increase over time (**Figure S5**). This trend was independent of the type of playback and is therefore intrinsically related to the use of this particular type of chirp. All chirp types are subject to a brief decrease only in concurrence with 2 Hz trains of type 2-chirp playback (**Figure S5**). Second, the vast majority of rises was produced right at the end of the playback signal, regardless of whether or not playback chirps were occurring (**Figure S5**). Overall, these results agree with previous reports showing an increase of type 2 chirps over time during paired interactions (Hupé and Lewis 2008) and the presence of a short delay between consecutive chirps produced by different subjects (Hupé and Lewis 2008; Henninger et al., 2018). The consistent occurrence of rises at the end of the playback signal has also been reported previously (Kolodziejski et al., 2007). The strong symmetrical dependency of

chirp production on positive and negative beat frequencies (DFs; **Figure 3C, S3**; Bastian et al., 2001; Engler and Zupanc, 2001; Triefenbach and Zakon, 2007; Kolodziejski et al., 2007) and the lack of specific matching in the response of brown ghosts to playback chirps suggests that, in this species, chirping is primarily conditioned by beat parameters. In addition, these findings indicate that, irrespective of the meaning or valence chirps may have, the detection and the production of chirps may be two independent processes.

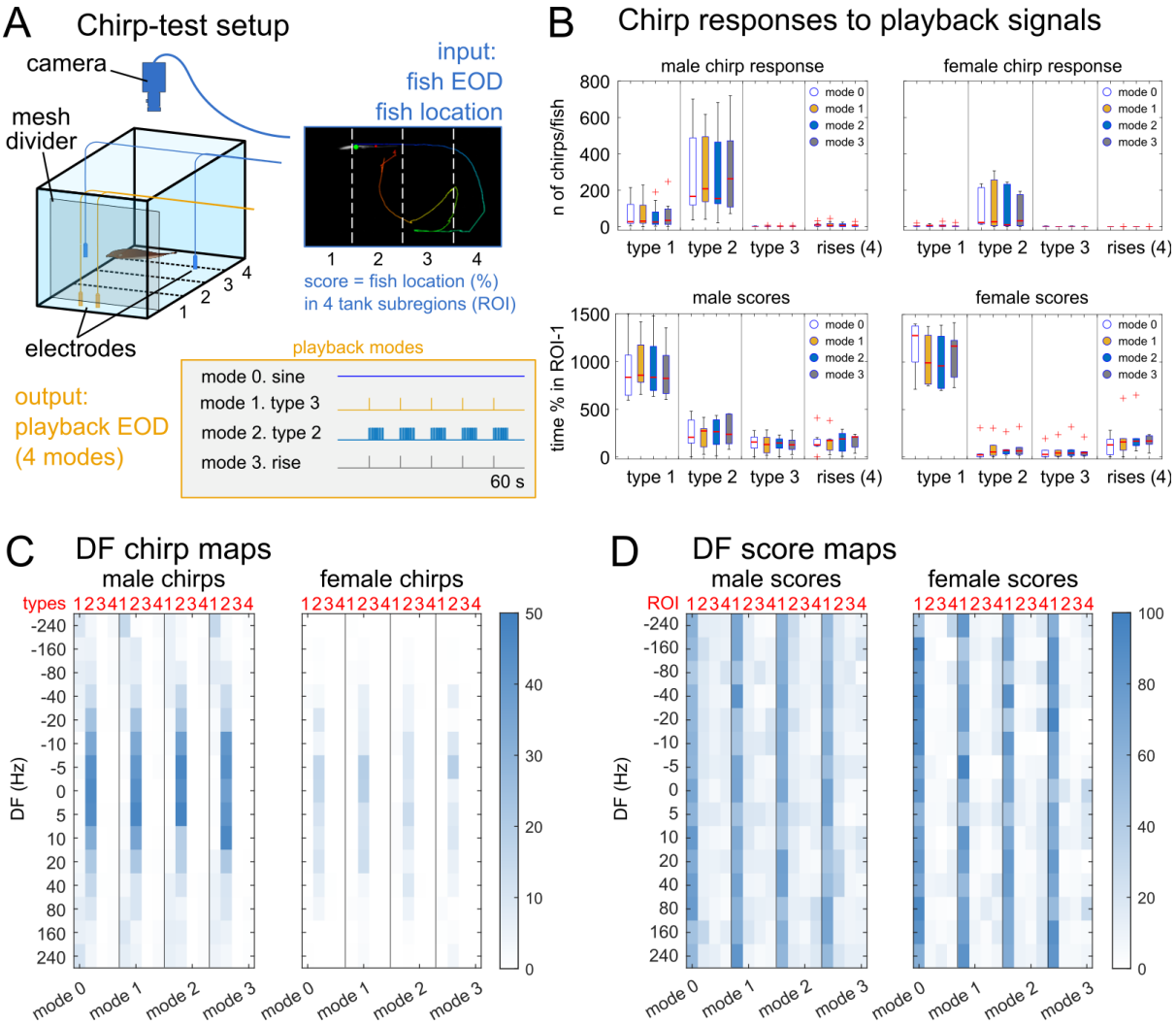

Figure 3. Invariant chirping responses to playback chirps in freely swimming fish. *A: Schematic diagram of the setup used for the playback experiments: both the fish EOD and the fish swimming behavior are recorded during 60 s long playback trials. During each trial the fish locomotion is scored based on the % coverage of tank space (60 x 30 cm) in 4 regions of interest (ROI) at increasing distance from the playback electrodes (1 = close, 2-3 = intermediate, 4 = far). Playback trials are organized in 4 different modes (0-3), each including 15 DF levels, all shuffled and randomized. B: The box plots on the top row show the total number of chirps produced by either male or female brown ghosts produced in response to plain EOD mimics (mode 0), sinewaves containing type 3 chirps (red, mode 1), sine waves containing trains of type 2 chirps (blue, mode 2) or rises (grey, mode 3). Chirp counts relative to each individual fish are summed across different DFs. The boxplots on the bottom row show the trial scores relative to the same subjects and summed across different DFs. C: Heatmaps showing the DF-dependent but mode-invariant distribution of mean chirp types produced by male and female subjects in response to different playback regimes. D: Score heatmaps*

showing that fish of both sexes approach playback sources (i.e. higher scores in ROI 1) with equal probability regardless of playback types or DFs. Female fish may be more stationary in proximity of the electrodes, resulting in slightly higher ROI-1 scores.

Chirping is mainly a self-referenced behavior

As shown in other communication systems (bats, Bohn et al., 2008; cetaceans, Dunlop, 2017; primates, Slocombe and Zuberbüler, 2005a,b; Notman and Rendall, 2005), valence and behavioral meaning could reside in conserved motifs that can serve as unequivocal signifiers for the specific context in which they are produced (Berwick et al., 2011; Nieder and Mooney, 2020). Similarly, in brown ghosts, while single chirps may neither be behaviorally salient nor informative, meaning could be attributed to chirp patterns or chirp transitions produced by the senders of the signals, the receivers or both. According to this, in weakly electric fish, specific chirp sequences have been proposed to be relevant to specific behavioral contexts (Henninger et al., 2018; Dye, 1987), and even emotional states (Triefenbach and Zakon, 2008; Smith et al., 2013; Silva et al., 2013).

To validate this idea and assess whether specific chirp patterns could be considered signatures for different types of behavioral interactions, we evaluated all possible chirp sequences in the chirp time-series recorded. This was done first independently (each subject considered separately; **Figure S2B,D**) and then interactively (considering sender-receiver transitions; **FIgure 4**). Although this analysis was conducted in similar ways for different chirp sequence lengths (2, 4, 6 and 8 chirps), since very similar results were found (not shown), for simplicity we focus here on 2-chirp sequences (i.e. chirp *transitions*).

Considering all recordings independently, type 2 chirps are overall the most frequently produced chirp type, both in females as well as males (**Figure 4A**, **Figure S2A**,**C**). As a direct consequence, the most common chirp type transition found is “type 2”-”type 2”, except for the case in which males are chirping to females (MF; **Figure 4B**, **Figure S2B**). In this case, repeats of type 1 chirps become more prominent in experienced pairs, possibly due to the higher amount of type 1 chirps produced by males at high frequency beats (i.e. high DF; **Figure 1D, Figure 3C**) and to the concurrent time-dependent decrease in the number of chirps produced (**Figure 2A, Figure S1D**). In any other case, neither chirp transitions nor chirp type ratios were differently distributed. Rather, the proportion of different chirp types and the transition frequencies from each type to the others were remarkably similar, regardless of the context and the sex of the interacting fish (**Figure S2**). Overall, these results show that, at an individual level, the most common chirp sequence is a repeat of type 2 chirps in both males and females, with type composition being only slightly more heterogeneous for male senders. Thus, at least when chirp sequences are considered individually, chirp-type patterning is highly conservative and not connected to any of the behavioral contexts considered.

Yet, it is possible that meaningful chirp transitions (i.e. correlated to a specific behavioral context) are to be identified only when considering time series interactively (Chow et al., 2015; Fröhlich, 2017). Recordings from brown ghosts, conducted both in the field and in captivity, suggested the presence of correlations between male chirp rate (type 2 and type 3) and female larger chirps (type 3; Henninger et al., 2018). Meaningful correlations have been also suggested to consist more broadly in temporal correlations between time-series, with chirps produced with a preferred latency by two interacting subjects. However, different authors reported different latency values (Zupanc et al., 2006, latency 500-1000 ms; Hupé and Lewis 2008, latency 200-600 ms). These discrepancies - either due to breed differences or differences in the recording environments - do not suggest the presence of stereotyped temporal patterning.

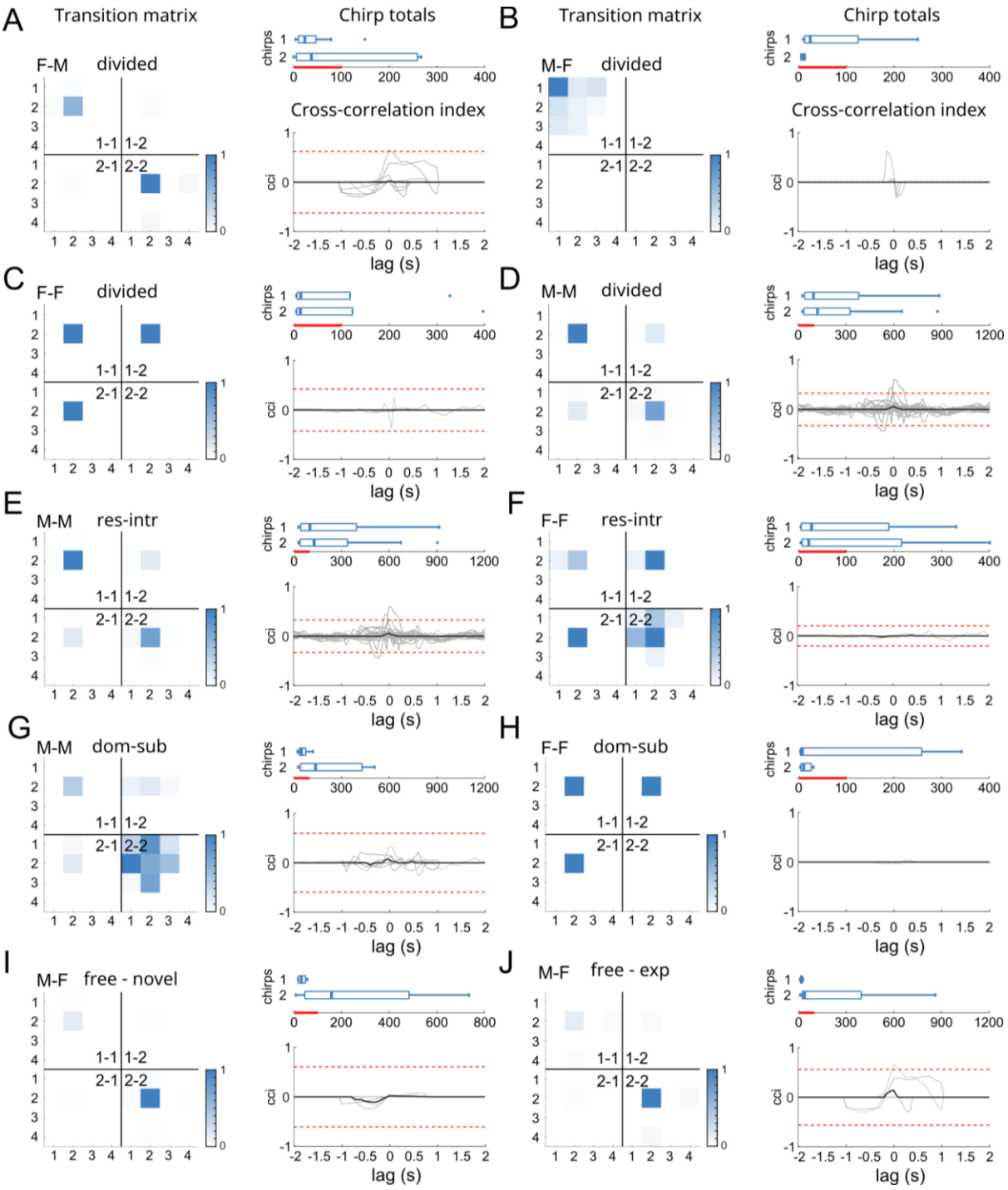

Figure 4. Chirp time-series correlations during social interactions. *A,B: Chirp transition maps representing the median transition probability (normalized) of all possible chirp type pairs calculated for female and male fish, considered together. For each type of social pairing, the identity of the two fish is indicated by different ID numbers in the different map quadrants: 1-1 = fish 1 to fish 1, 1-2 = fish 1 to fish 2, 2-1 = fish 2 to fish 1, 2-2 = fish 2 to fish 2. In mixed pairs, the order given in the sex tag of each plot follows the same order of the fish ID. The presence of higher chirp-transition frequencies in the 2nd and 4th quadrants of the matrices (labeled with 1-1 and 2-2) indicates a substantial independence between chirp time-series (i.e. lack of temporal correlation). Transitions from female to male fish (A) or male-to-female fish (B) are considered separately. For each case, chirps were selected based on the sex of the sender fish (1 = sender, 2 = receiver). On the right side of each matrix, chirp totals are*

displayed in boxplots for sender and receiver fish (outliers are represented as dots laying beyond the boxplot whiskers; red bars = 100) and cross-correlation indexes (cci, 50 ms binning) are provided for chirp time series relative to the same fish pairs (red dotted lines = confidence intervals corresponding to 3 cci standard deviations). C,D: Chirp transitions relative to same sex pairings. A higher level of interaction for F-F pairs (visible in the first and third quadrants in C) is probably due to the extremely low chirp rates in these pairs. Notably, higher chirp rates (as in M-M pairs, D,E) do not result in higher level of cross-correlation. E,F: Chirp transitions for resident-intruder pairs (M-M and F-F). Since most chirps are produced by M-M resident-intruder pairs in divided aquariums, plots in E resemble those in D, as they are relative to overlapping datasets. G,H: Chirp transitions for dominant-subordinate pairs (M-M and F-F). I,J: Chirp transitions for freely swimming (naive, I or experienced, J) opposite-sex pairs. Note the reversed sexual dimorphism in chirp rates, in both cases.

Although highly variable among fish pairs (**Figure 4**), the temporal correlation of chirp series seems to depend consistently on the number of chirps produced (Zupanc et al., 2006, estimated maximum chirp rate 0.4/sec; Hupé and Lewis 2008, estimated maximum chirp rate 0.53/sec; Henninger et al., 2018, estimated maximum chirp rate 3/sec) raising the question of whether the previously reported instances of higher temporal correlations could be just a direct consequence of higher chirp rates occurring when fish get closer to each other. Chirp transitions were assessed considering both senders and receivers interactively for different sex pairings (M-M, M-F, F-M, F-F). Transition frequencies were mapped onto four quadrants defined by fish identity (1 or 2) and temporal order: 1-1, 1-2, 2-1 and 2-2.
What stood out most consistently from this analysis was the much lower (or sometimes completely absent) extent of sender-receiver chirp transitions, as shown by the higher transition probabilities along the 1-1 and 2-2 quadrants of the probability maps. According to this, cross-correlation indices are very low or near zero levels in all cases (**Figure 4**). Although slightly different interaction rates were observed in different contexts, in all cases the most frequent chirp transitions were self-referenced (1-1 or 2-2). Although within FF pairs 2-1 and 1-2 interactions seem to be more frequent (**Figure 4C**,**F**,**H**), this is probably due to the much lower number of chirps produced by this sex (see boxplots showing the total number of chirps in each group).
Eventually, the absence of conserved temporal correlations could also depend on the type of behavioral interactions and the proximity at which they take place (Dunlap, 2002). Social distance in particular could explain the high chirp-rates previously observed during courtship (Hagedorn and Heiligenberg 1985; Henninger et al., 2018) and short-range agonistic interactions between fish confined in a relatively small aquarium (Hupé and Lewis, 2008) as opposed to the more sporadic chirping observed during encounters over wider spatial ranges (Henninger et al., 2018). Alternatively, the higher chirp temporal cross-correlation previously observed by other authors could be due to neuro-endocrine effects of courtship or other mating-related factors.
To test whether cross-correlation would be significantly affected during courtship, we simulated the onset of the reproductive season by gradually lowering water conductivity over the course of a 40-day period (from 400 µS to 100 µS, Kirschbaum, 1979). As a result of this treatment, at least 75% of the females used in this experiment (N = 8) showed visible signs of egg maturation. Mixed-sex pairs (N = 8 pairs) held in such conditions were then allowed to interact freely, in absence of any tank divider. As a control group we paired males and females in absence of any water conductivity change ("novel" vs "experienced", **Figure 4I**,**J**). Although female fish produced a surprisingly higher number of chirps in these conditions, only minimal signs of cross-correlation could be detected, without reaching on average the threshold for statistical significance (**Figure 4J**). Collectively, these results indicate the use of a very limited variety of chirp type transitions in different behavioral contexts and a lack of any correspondence between contexts and specific chirp type repertoires. More importantly, they

show that - at least in the behavioral conditions analyzed here - chirp time series produced by different fish during paired interactions are consistently independent of each other. This further confirms the notion of chirp production and detection as independent processes.

Chirp type choice can be predicted by chirp interference with beat periodicity

Since brown ghost electroreceptors are highly tuned to the fish's own EOD (Scheich and Bullock, 1974; Zakon, 1986), they are directly affected by the EOD amplitude modulation and their firing rates are entrained by the beat periodicity (Benda et al., 2005). It follows that any frequency modulation will result in alterations of the periodic electroreceptor firing and therefore will be detected as an abrupt change in electroreceptor activity (Benda et al., 2006). This perturbation of the beat is scaled by mainly 3 parameters: the extent of the chirp frequency modulation, its duration and the underlying DF (or beat frequency) at which such modulation is produced (Benda, 2006). It follows that chirps of different types will have different effects on electroreceptor firing based on the frequency of the interacting EODs (i.e. the beat frequency; Walz et al., 2013).

Although this differential effect of chirps has been proposed to be behaviorally salient (Benda et al., 2006; Walz et al., 2013), given the invariance of chirp patterns across different social contexts (**Figure S2**) and the more significant impact of beat frequency (compared to any other physical factor considered) on chirp variance (**Figure 2B**, **Figure S3**), we posit that chirp function is likely to be mainly related to the physiological aspects of beat processing by the chirping individual.

To further validate this idea, we have computed an estimate of the interference caused by a chirp on the beat periodicity (*beat interference*). This was done by calculating the ratio between the duration of the beat cycles affected by the chirp (beat interpeak intervals) and the total duration of the beat cycles detected within a fixed time window (roughly double the size of the maximum chirp duration, 700 ms). Such an estimate of beat interference was made for a wide range of Gaussian chirps (duration 10-400 ms, modulation range 0-400 Hz) on a wide range of beat frequencies (±300 Hz; **Figure 5A**). For each chirp, the interference values obtained for 4 different phases (90° steps) were averaged. As a result, we obtained a three-dimensional space on which recorded chirps (N = 30486 chirps) could be localized and matched to their putative interference (**Figure 5B**).

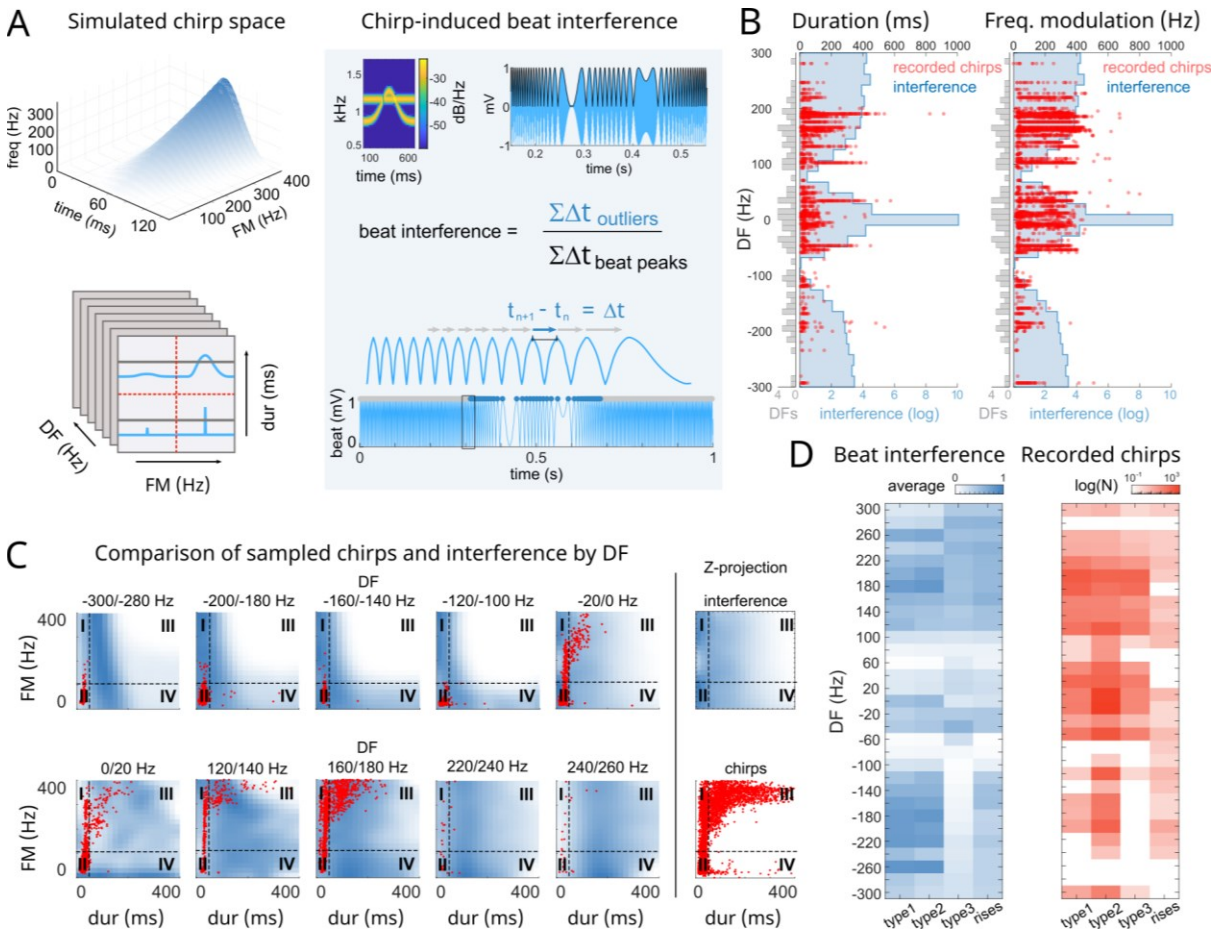

Figure 5. Chirp interference with beat regularity. *A: Artificial chirps were generated on an 862 Hz EOD baseline covering a 0-400 ms duration range and a 0-400 Hz peak amplitude. An additional EOD signal was added to the chirping signal (-300-300 Hz DF range) to simulate a wide range of beat frequencies. The sign of the DF is referred to the baseline reference EOD. The beat interference induced by chirps was calculated as the ratio of the cumulative duration of beat interpeak intervals (IPI) affected by a chirp (i.e. outliers in the IPI population relative to each EOD pair) on the total cumulative beat IPI duration (including outliers) within a 700 ms time window (see methods for details). B: Duration and frequency modulation (FM) histograms of recorded chirps (red, N = 30486) sorted by DF and matched to their estimated beat interference (blue). The gray histograms on the Y-axis represent the beat frequencies sampled. C: Normalized heatmaps showing examples of chirp-induced beat interferences (color coded in blue) calculated at different DF values. Real chirps produced at the same DF are overlaid in red. Only overlapping FM and duration ranges are shown. In the plots on the extreme right: beat interference values are summed over all DFs (top) and are shown next to the corresponding chirps (bottom). D: Comparison of average chirp-induced beat interferences sorted by type (left map, blue) and the actual observed chirps (log N, right map in red).*

What we found is that chirps were indeed often localized in correspondence of the areas of high interference, at different beat frequencies: at negative DFs chirps were localized mainly in the type 2 range, whereas at positive DFs recorded chirps overlapped more often with the type 1 and 3 ranges (**Figure 5C**). Notably, by calculating the normalized cumulative interference for each chirp type, a high degree of matching was found between the DF values at which the interference is higher and those at which chirps were actually recorded (**Figure 5B**).

In general, chirp-induced interference is variable across different DF values as chirp types are, supporting the idea that chirps are probably chosen based on beat parameters in order to exert a tangible effect on the electrosensory system (Benda et al., 2006; Walz et al., 2014). A comparison of chirp interference across different chirp types (**Figure 5D**) further revealed that, relative to other chirp types, type 2 chirps at low or even negative DFs may induce similar interference levels as those induced by larger chirps at positive beat frequencies. This could perhaps explain the lower occurrence of larger chirps in low frequency fish (being more often in negative DF encounters). In addition, areas of lower interference seem to be symmetrically localized around 60 Hz, the frequency level to which electroreceptors are most sensitive (Bastian, 1981) and at which chirp occurrence was lower (**Figure 5B-D**). Overall, these results corroborate the idea that chirp production may be constrained mainly by the effects chirps have on the perceived beat.

Chirping is significantly affected by locomotion and social distance

To further test this idea, we decided to assess the presence of correlations between chirping and locomotion during the exploration of a beat source. This is in fact an ideal context in which beats exert highly variable effects on the electrosensory system due to fish locomotory activity, thus allowing for an independent testing of beat intensity and frequency (Fotowat et al., 2013). This experiment was set in an enriched environment to evaluate other aspects of locomotor and social behavior, which were neglected in our previous experiments and which could possibly explain the high interindividual chirping variability (e.g., thigmotaxis, novelty- or shelter-seeking, sociability).

Fish were allowed to swim freely (for 30 min) in a 160 x 80 cm arena containing shelters (plastic tubes and plants). The social stimulus - and also the “beat source” - consisted of a conspecific caged in a mesh tube located on one side of the arena. A “novel object” (a 1 x 5 cm rod of graphite) was hidden behind a barrier and used to test novelty seeking. Shelters consisted of transparent PVC tubes and plastic plants. Different regions of interest (ROIs) were used to score the occurrence of fish swimming behavior around the shelter area, the tank walls (within 5 cm distance), the novel object and the conspecific (**Figure 6A**). The time spent swimming in the arena’s open space (5 cm away from the walls) was also evaluated. Behavioral observations were conducted taking into account the subject’s sex, as it is the behavioral variable most directly correlated to DF and beat frequency.

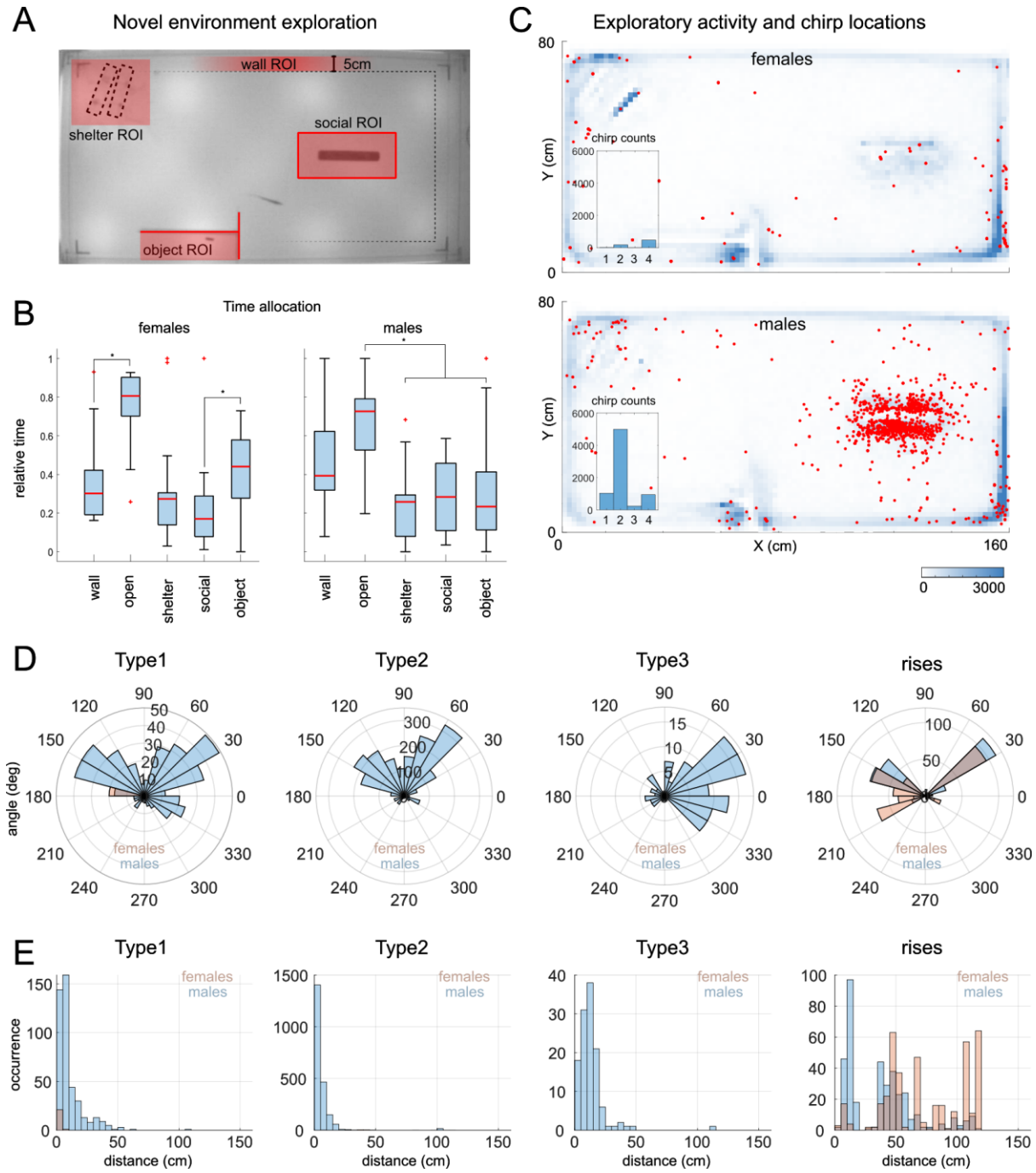

Figure 6. Chirping during novel environment exploration. *A: Diagram of the recording arena showing the criteria used to define the different regions of interest (ROIs): 1) the presence of shelters (PVC tubes), 2) the proximity to the tank walls (distance < 5 cm), 3) the presence of a fish (caged in a mesh tube), 4) the presence of an "unknown/novel" conductive object (a 3 cm piece of graphite). B: Proportion of time spent in the different ROIs (N = 14 females, Friedman X^2 22,9 $p < 0.001$ wall vs open $p < 0.001$, social vs object $p = 0.02$, open vs shelter/social/object < 0.01; N = 15 males, Friedman X^2 19,8 $p < 0.001$ wall vs open $p = 0.049$, wall vs shelter/object $p < 0.05$, open vs shelter/social/object $p < 0.001$). C: Chirp locations (red) overlaid to the heatmaps showing the average swimming activity of females and males. D: Polar histograms showing the angles between the two fish during chirping. Angles are referred to the X-axis and are sorted based on chirp type and sex (male = blue, female = red). E: Histograms of the chirping distances in males (blue) and females (red) relative to different types of chirps.*

We found that female brown ghosts spent more time swimming in the open space areas of the arena, as opposed to swimming closer to the tank walls (thigmotaxis; **Figure 6B**). Female subjects also spent more time investigating the novel object than the conspecific (**Figure 6B**). Male behavior showed a different trend as males spent comparatively more time swimming close to the tank walls and more time in contact with conspecifics (**Figure 6B**). Possibly as a direct consequence of this, they also produced a higher number of chirps, compared to their female counterparts. Notably, most of the chirps were produced around the caged conspecific (**Figure 6C**). Analysis of the angles and distances of fish during chirps revealed that first, chirps were mostly produced when fish were located at 30°, 150° or 330° angles relative to the axis defined by the mesh tube (**Figure 6D**), and second, most chirps were produced at a distance of less than 25-50 cm (**Figure 6E**). While the angle bias could be due to the circular loops described by fish swimming around the conspecific, the rather limited range at which chirps are produced could be explained by the range of the EOD field (beat amplitude reaches 1% at around 30 cm; Fotowat et al., 2013; Henninger et al. 2018; Benda, 2020; see appendix 1) and confirms previous observations (Hupe and Lewis, 2008; Zupanc et al., 2006; Henninger et al., 2018).

Interestingly, chirps of different types were not all produced at similar angles: while type 1 and type 2 chirps seem to be more often produced at 30-150 angles, type 3 chirps - produced in this experiment only by males - seem to be used in the ±30° range (i.e. on the right side of both the arena and the plane representing the angles). Rises - in both males and females - seemed more often produced at multiples of 30° located on the left side of the cartesian plane (30°, 150° and 210°). All chirp types, except for rises, were produced within a 50 cm radius from the conspecific fish (**Figure 6E**). Rises were produced almost ubiquitously, by both sexes, and most of the time at locations far away from the caged fish (**Figure 6C**, **Figure 6E**). Factor analysis (FAMD) confirmed the already known sex-dimorphisms in chirp distribution (males chirp more than females and more often towards other males, **Figure S6**). Collectively, caged fish produced more chirps than freely swimming subjects (4410 vs. 3483 chirps). In addition, variables associated to beat frequency (such as DF, EOD frequency, sex, chirp type) explain a good percentage of chirp variance (73,7% of variance explained as opposed to the 55% explained including all other variables 55%; see also **Figure 2B**). A relatively high loading on principal components was also found for variables associated with locomotion, such as distance and time spent exploring different tank areas (**Figure S6B**). Accordingly, chirp clustering is affected by the EOD frequency difference of the emitting fish (DF) and by distance (**Figure S6C**, insets).

Modelling of field current and iso-potential lines using dipole models of our fish allows to visualize more intuitively how both chirp angles and distances can be explained by the electric field geometry (**Figure 7A**). Most chirps (90%) in fact are produced within distances corresponding to the 1% range of sinewave field intensity (roughly 30 cm; **Figure 7B**) and are possibly aligned with the current lines (compare **Figure 6D** with **7A**), which could represent meaningful cues tracked by electric fish during target-reaching tasks (Hopkins, 2009). The correlation between beat range and chirping is even more evident when chirp distances are compared to the values of field intensity measured at different distances (**Figure 7C**, **D**). To assess whether, within the same range of distances, chirps have a detectable effect on randomly generated beats, we have estimated the interference caused by different chirp types on representative sinewave pairs using the reference values obtained from our test recordings (**Figure 7D**). Not surprisingly, we found a linear and direct correlation of chirp-induced beat interference with beat decay over distance (**Figure 7E-H**). This clearly confirms that chirps affect the beat within beat-detection ranges but also that our beat-interference estimates are meaningfully related to beat detection.

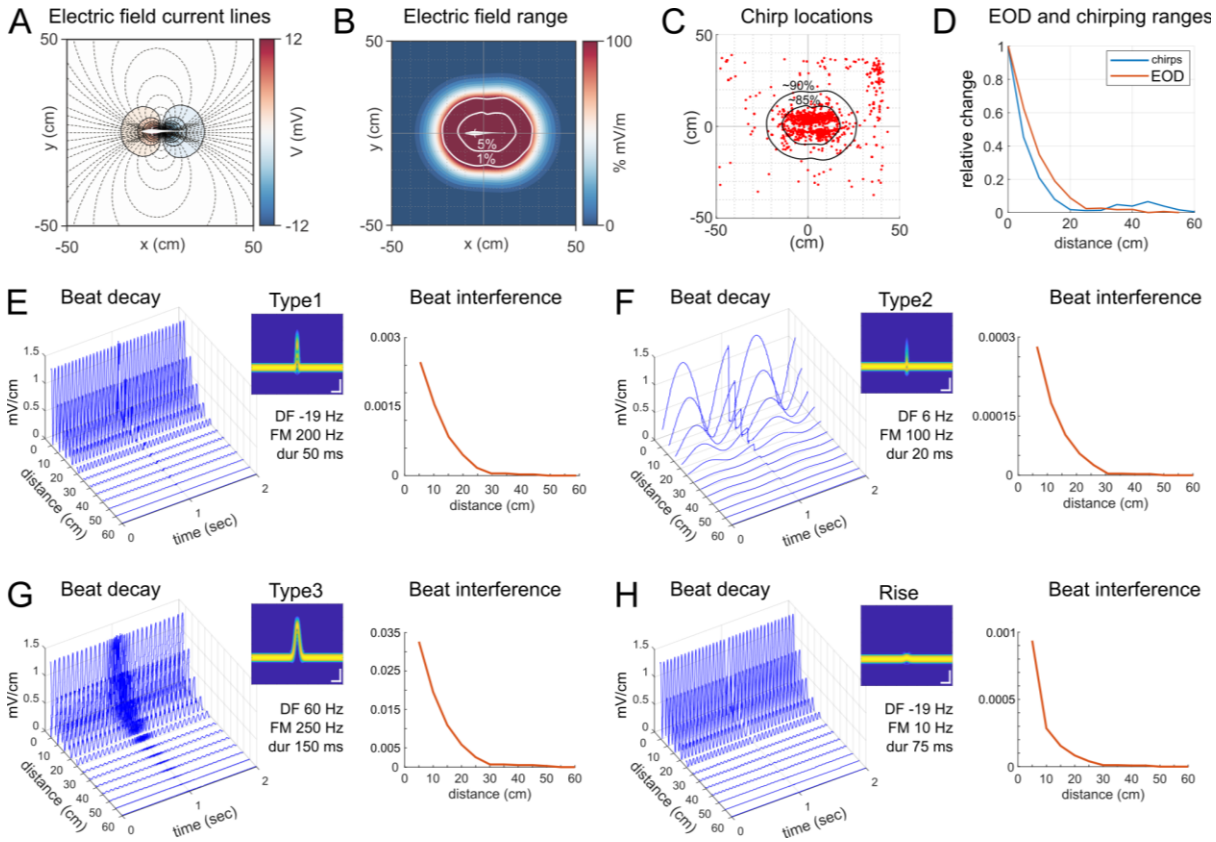

Figure 7. Chirps interfere with the beat within the electric field range. *A: Electric field generated by a 16 cm 3D dipole modeling an electric fish of the same size using BEM (boundary element methods). Iso-potential lines are shown for the near-field range in different colors, based on field polarity. Current is represented by the dashed lines, perpendicular to them. B: Electric field intensity mapped around the same modeled fish. The level lines represent the 1% and 5% intensity of the electric field generated by the ideal fish. C: Scatter plot of chirp locations. The overlay is centered at the origin and corresponds to 90% and 85% of all chirps produced, respectively. D: Plot showing the intensity range of an EOD mimic calculated at 22-25 °C and 200 µS (red) and the distribution range of chirps emitted by real fish (blue), for comparison. E-H: 3D plots showing the ideal beats calculated for different sinewave pairs during different chirp types and plotted over distance. The 2D plots on the side represent the beat interference (calculated using a threshold of 1% of maximum beat amplitude) caused by each chirp type over distance. Scale bars in the spectrograms are 100 ms and 100 Hz.*

Differences in the beat interference elicited by chirps of different types were found: the interference induced by type 1 chirps is almost one order of magnitude higher than the estimates of the same parameter for type 2 chirps, but one order of magnitude lower than the beat interference induced by type 3 chirps. Rises induce interferences in the range of type 1 chirps. Overall these results indicate that during social encounters, all types of chirps, but not rises, are produced within a short distance. This possibly may explain why female subjects, apparently less motivated in exploring social stimuli, also produce considerably fewer chirps. More broadly, these findings indicate that chirping, besides being strongly correlated to the frequency of the beat generated by the interacting fish, is also significantly affected by locomotion and by spatial relationships between subjects within beat detection ranges. This further corroborates the hypothesized role of chirps in beat processing.

Effects of environmental complexity on chirping

Based on these results we hypothesized chirps could be used as probes to fine-tune the detection of EOD sources (i.e. conspecifics) during social interactions. According to this hypothesis, one would expect that increasing levels of environmental complexity will result in higher probing rates (Siemers et al., 2009). To test this idea, we used both playback experiments and recordings of interacting fish pairs. These experiments aimed at assessing the responses of brown ghosts to EODs in presence of environmental clutter which would distort electric fields in tangible but unpredictable ways (von der Emde, 2006).
We first recorded social interactions using the same divided tank configuration as in our previous recordings. Fish pairs (N = 6) were recorded in 3 different conditions: 1) with lights turned ON and no object in the tank other than the mesh divider placed between the 2 fish (lights ON - clear), 2) with lights switched OFF (lights OFF - clear) and 3) with lights OFF and the addition of shelter PVC tubes and plastic plants filling almost entirely the two tank compartments (lights OFF – cluttered; **Figure 8A**).

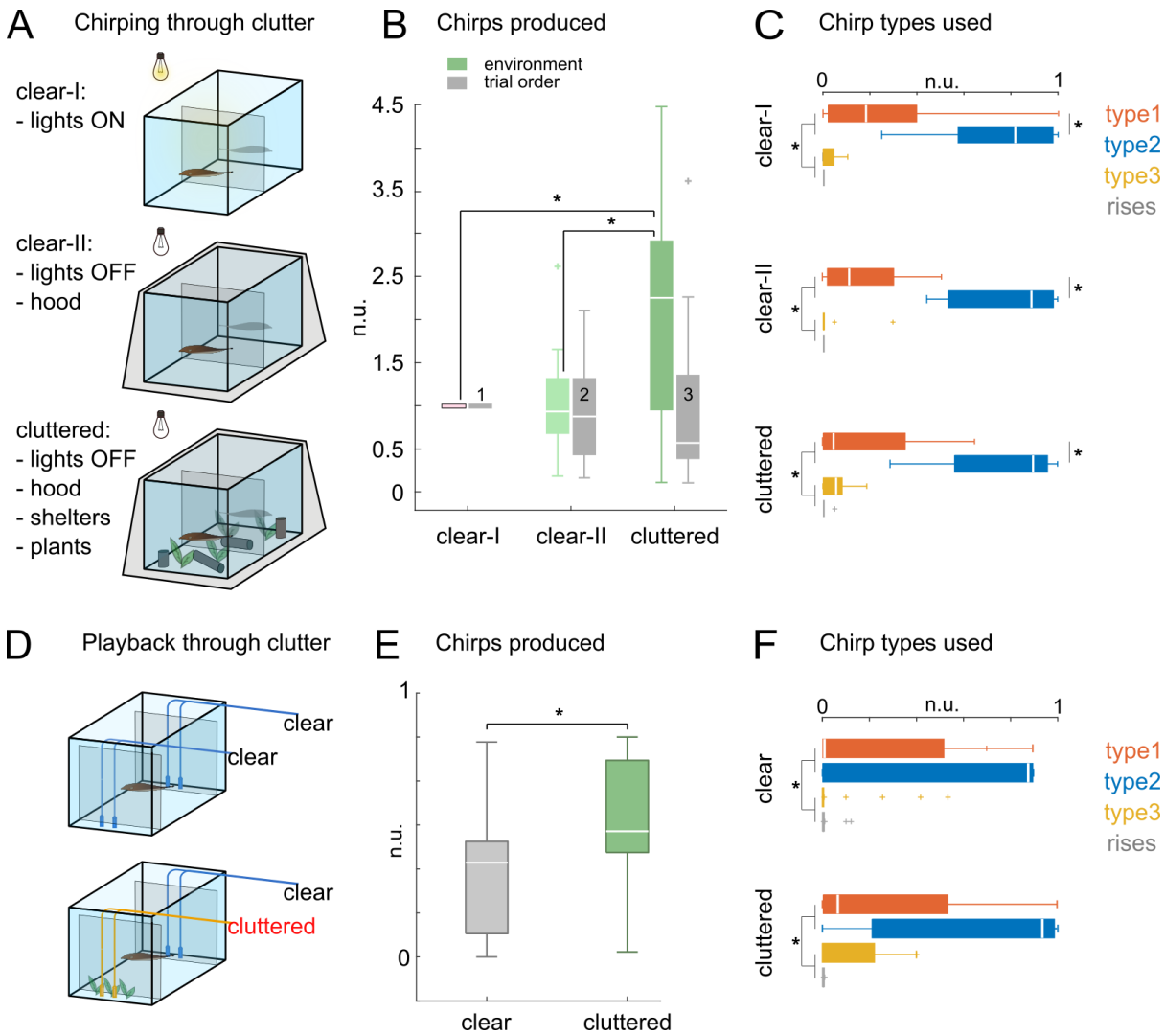

Figure 8. Effect of environmental clutter on interacting fish pairs. *A: Recording of fish pairs (N = 6) in environments of different sensory complexity: lights ON = clear tank environment and direct illumination, lights OFF = clear tank environment, no illumination, lights OFF + clutter = no illumination and cluttered environment. B: Total amount of chirps produced in each condition and normalized on the lights ON session (green bars; Friedman's p = 0.053; lights ON vs lights OFF + clutter = 0.024, lights OFF vs lights OFF + clutter = 0.051). Chirp counts relative to trials sorted in chronological order (1-3) are shown in gray. C: The box plots*

*show the normalized chirp type counts relative to each session (lights ON, Friedman's X^2 = 21.9 p < 0.001, pairwise comparisons 1*2 p = 0.006, type 1 vs 3, type 1 vs 4, type 2 vs 3, type 2 vs 4 p < 0.001; lights OFF Friedman's X^2 = 21.8 p < 0.001 pairwise comparisons type 1 vs 2, type 1 vs 3, type 1 vs 4, type 2 vs 3, type 2 vs 4 p < 0.001; clutter Friedman's X^2 = 19.5 p < 0.001 pairwise comparisons type 1 vs 2, type 1 vs 4, type 2 vs 3, type 2 vs 4 p < 0.001). D: Results of playback experiments in which EOD mimics were either directly detectable through a fine mesh barrier (clear) or more indirectly due to a barrier of plastic plants interposed between the mesh and the EOD source (cluttered). Clear and cluttered trials were presented in random succession (N fish = 6, 10 trials each, 60 sec ITI). E: Total chirp counts in the 2 conditions are normalized on the total amount of chirps produced by each subject (Wilcoxon, p = 0.025). F: Boxplots showing the chirp type composition of each condition (clear Friedman's X^2 = 17.4 p < 0.001 pairwise comparisons type 1 vs 3 p = 0.048, type 1 vs 4, type 2 vs 3, type 2 vs 4 p < 0.001; cluttered Friedman's X^2 = 29.9 p < 0.001 pairwise comparisons type 1 vs 3, type 1 vs 4, type 2 vs 3, type 2 vs 4 p < 0.001; Wilcoxon type 1 clear*type 1 clutter p = 0.034).*

Fish pairs were recorded for 15 minutes with no previous knowledge of either the environment or their social partner. Trials were shuffled in a randomized order and were spaced by 5-minute inter-trial intervals. Chirp counts were evaluated at the end of the trials. As expected, the total chirp rate increased in absence of light and even further when clutter was present in the environment (**Figure 8B**). In all cases, type 2 chirps were the most often produced type (**Figure 8C**). A slight (non-significant) increase in type 2 and type 3 chirps could be observed under cluttered conditions. These results clearly indicate that chirping is affected by environmental complexity, since in these recordings aggression- or courtship-related behaviors were either absent or randomly sorted in all three conditions and both fish had equal environmental and social experience.
Nonetheless, even in conditions of low "electrical detectability", fish can sense each other using visual cues, olfactory and mechanical cues induced by water movements during swimming. Therefore, chirp production in these experiments could also be affected by a range of sensory inputs co-occurring in an unnaturally confined space and for the whole duration of the recordings (15 min). To rule out these possibilities, we conducted playback experiments in a larger enclosure (160 x 50 cm), allowing fish to detect playback EODs from a wider range of distances. Playback electrodes were not directly accessible but placed behind a mesh barrier located at either side of the enclosure (**Figure 8D**). Environmental clutter (plastic plants) was interposed between the electrodes and the mesh divider at a random location in each trial. We found that chirp rates increased in response to playback signals specifically when they were partially concealed by clutter **Figure 8E**). Notably, this effect seems to be more evident for larger chirps (type 1 and type 3; **Figure 8F**) although combined effects of chirp types and clutter have not been found. Overall, these results indicate that chirping is significantly affected by the presence of environmental clutter partially disrupting - or simply obstructing - beat detection during locomotion. They further confirm electric signals alone are sufficient to trigger the chirping increase in cluttered conditions. All chirp types seem to be equally affected, with type 2 chirps being the most common type produced also in cluttered conditions. The enhanced use of chirping during locomotion around partially concealed EOD sources fully supports the proposed idea of chirps as self-referenced probing signals to optimize the electrolocation of conspecifics and fits with the tuning of probing rates to environmental complexity (Obrist, 1995; Schnitzler and Kalko, 2001; Slabbekoorn and Peet, 2003; Siemers et al., 2009;).

Discussion

In this study, we have analyzed the factors implied in the production of EOD frequency modulations (chirps) by brown ghost knifefish. We have collected a large chirp dataset by recording EODs in several types of social interactions to assess the effect of territory ownership, social hierarchy, sex, reproductive state and social experience. We have used this data to identify the most significant factors explaining chirping and found that by far the

strongest predictors are DF (beat frequency) and experience (environmental and social). We found that chirp patterns are for the most part constituted by repeats of chirps of the same type, whose choice strongly relies on the beat frequency. Fish responses to playback chirps inserted into a sinewave stimulus of a particular DF are indistinguishable both in terms of electric signaling as well as in terms of exploratory behavior (approach/avoidance). Behavioral analysis of freely swimming fish indicated that chirping occurs always at relatively short ranges and most of the time in association with an active social approach. Based on these results, we propose that chirps are self-referenced signals (sensory probes) used to enhance beat-detection and thus the processing of beat-related spatial information.

Existing functional hypotheses

Since the earliest reports (Larimer and MacDonald, 1968), chirps have been assigned behavioral meaning as communication signals based on the following observations (mostly in brown ghosts): fish chirp mainly during social encounters; males chirp considerably more than females (Dye, 1987; Bastian et al. 2001, Tallarovic and Zakon 2002, Dunlap and Larkins-Ford, 2003; Kolodziejski et al. 2007) and are also considerably more aggressive; during male interactions (often agonistic) smaller chirps are produced (i.e. shorter duration and lower frequency excursion: type 2) whereas larger chirps (type 3) are used more often during female-male encounters (presumably courtship-oriented); high chirp rates are observed in mating pairs, too, with a relatively higher production of large chirps (i.e. type 1 and type 3; Hagedorn and Heiligenberg, 1985; Henninger et al., 2018). This led to the hypothesis of small chirps being implied in agonistic interactions while larger chirps were considered pertaining to different aspects of courtship and mating.

This idea raises the following concerns: first, social encounters do not only involve communication but also higher levels of locomotion and reciprocal exploration, which could also be linked to chirp activity, but have not been considered in the past. Second, several factors (sex and the magnitude of beat-interference) covary with the main corollary of chirping, i.e. beat frequency. These covariates are not easy to untangle, which makes it difficult to separate neuroendocrine or other behavioral factors from the biophysical features of EOD fields affecting detection and localization of conspecific fish. Third, observations in a few species are generalized to all other gymnotiforms without testing for species differences (Turner et al., 2007; Smith et al., 2013; Petzold et al., 2016). Last, and most importantly, no causal evidence in support of any of the hypothesized functions of the different types of chirps has ever been provided.

The idea of chirps being used as *probes*, as proposed here, is not entirely new: due to the tight correlation between chirp features and beat frequency (mainly chirp frequency modulation), Benda and coauthors previously hypothesized a role of chirps in determining the sign of the beat frequency (Walz et al., 2014). This indeed represents an ambiguous parameter as two opposite frequency differences result in the same beat frequency: e.g. consider a fish producing an EOD of 750 Hz (EOD1) and interacting with conspecifics with an EOD of either 700 Hz (EOD2) or 800 Hz (EOD3). The two beats could be indistinguishable (assuming that the mechanism underlying the discrimination of the sign of DF at low DFs, and thought to be the basis of the so called jamming avoidance response (JAR; Metzner, 1999), is not functional at higher DFs). However, since any frequency modulation of EOD1 will have different effects on the beat frequency relative to EOD2 and EOD3, chirps could indeed solve the problem of disambiguating the sign of DF (which in brown ghosts could mean no less than discriminating between male and female conspecifics). However, it is also possible to obtain beat frequency information more reliably with a more gradual and slow change in the fish's own EOD frequency (which knifefish are capable of producing, see for instance Rose and Canfield, 1991 for more details). In addition, the frequency at which chirps are produced does not seem to realistically reflect such an aim (at low-beat frequencies, it is not rare for a fish to chirp more than 100 times per minute).

More recent studies have further suggested that patterns of specific chirp types could be a salient signal used for communication purposes in the context of mating (Henninger et al., 2018). Results obtained from our recordings do not support the existence of a chirp-based syntax in the brown ghost's social communication, as fish pairs acclimated to breeding conditions (but not mating) used non-patterned chirp repertoires similar to fish pairs recorded in non-breeding conditions. One exception to the *monotony* of the chirping repertoires observed in this study is given by male-female pairs: in this case, type 1 and type 3 chirps are used more often, while the relative amount of type 2 chirps is lower (**Figure 3, Figure S2C,D**). However, this effect is likely to be a result of the combined effect of the pronounced dependency of chirp type on DF together with the higher chirp rates observed in males (i.e. if chirp variety increases with DFs and males chirp more, chirp type variety will be higher when males interact with high beat frequencies).

Inconsistencies between behavior and hypothesized signal meaning

In our playback experiments we observed no differences in the responses of fish of the two sexes to playback stimuli, regardless of whether they contained a particular chirp type or not: test subjects chirped at comparable rates in response to all types of EOD mimics (containing type 1 chirps, type 2 chirps or rises), confirming that the main factor determining type choice is DF and therefore self-referenced sensory requirements (as opposed to different semantic contents). This idea is further corroborated by the known symmetric distribution of chirp types within the ±400 Hz DF range (Bastian et al., 2001; Engler and Zupanc, 2001; Triefenbach and Zakon, 2008; Kolodziejski et al., 2007). Based on our beat interference estimates, we propose that the occurrence of the different types of chirps at more positive DFs (such as in male-to-female chirping) may be explained by their different effect on the beat (**Figure 5D**), an idea which seems congruent with published electrophysiological studies (Benda et al., 2006; Walz et al., 2013). Notably, rises reliably occurred at the end of the playback stimulations (as also reported by Kolodziejski et al., 2007), which could be explained by their use as probes to assess signal presence in case of abrupt interruptions or weakening of EOD mimics (either caused by objects occluding temporarily the EOD field - clutter - or by playback termination). Interestingly, rises were considered by other authors as "proximity signals", although most likely with a different connotation (Hupé & Lewis 2008).
Arguably, fish might display invariant responses to EOD mimics simply because these stimuli are only close - yet nonrealistic - copies of natural stimuli. However, EOD waveform and harmonic components – which make natural EODs different from playback mimics - do not seem to play a role in EOD discrimination as EOD frequency alone does (Dunlap and Larkins-Ford, 2003; Fugere and Krahe, 2010). Moreover, artificial EODs are widely used in electrophysiological studies as they elicit comparable responses to natural stimuli in the electrosensory system (e.g., Benda et al., 2006, Marsat et al., 2009). In line with our behavioral data, electrophysiological recordings conducted at all main nodes of the electrosensory pathway did not show consistent chirp-type specific responses in either peripheral or central brain areas (Metzen and Chacron, 2017; Allen and Marsat, 2018; Metzen et al., 2020). Instead, the temporal correlations between chirps and other events (locomotion-related) seem to be more salient factors, as recently proposed for midbrain circuits whose phasic responses to moving objects are used to timestamp their occurrence during spatial navigation (Wallach et al., 2018). Recordings from interacting fish pairs confirmed the absence of any significant correlation between chirp type choice and behavioral context (**Figure S2**) although the variance of chirp parameters is significantly affected by this factor (**Figure 2**). This may suggest that the effect of context is mainly detectable in the number of chirps produced (**Figure S1**), rather than the type. Previous experiments reported the use of long rises (larger events compared to those we have labeled as rises) during agonistic interactions (Hupé & Lewis, 2008; Raab et al., 2021). However, it is not clear whether these are aggressive signals (as proposed by Hupé and Lewis, 2008, Raab et al., 2021) or de-escalating submissive signals (as proposed by Hopkins, 1974 and Serrano-Fernández, 2003). In our recordings rises

are produced more or less with equal probability by female and male subjects (42.5% in males, 57.5% in females) and yet, compared to other chirp types, their relative probability is higher in females (1.2% in females vs 0.12% in males), although female brown ghosts are less frequently engaged in aggressive interactions.
Type 2 chirps are also considered to be significantly correlated with aggressive encounters, although it is not clear whether they represent purely aggressive, possibly deterring, signals (Zupanc, 2002; Triefenbach and Zakon, 2008) or attempts to de-escalate aggression and communicate submission (Hupé and Lewis, 2008). Our results do not support either of these hypotheses: type 2 chirps are produced at higher rates at low beat frequencies (a feature of any encounter in which fish with similar EOD frequency approach each other and which has nothing to do with their intentions), they are more often produced by newly introduced fish (which are not necessarily more aggressive but rather more explorative), less in experienced fish (which also move less; see **FigureS1A**,**D** but also **Figure S2**), more by males when separated from females (males are more motivated to interact socially; **Figure 6**) and more in females when freely swimming (females are more motivated to escape when exposed to males too directly; **Figure 5I**,**J**). Overall, these considerations collectively suggest that the main factors determining chirp rates are the activity levels and the need to localize the other fish through the beat. Accordingly, in our experiments chirp type choice seemed to be largely unaffected by the behavioral context in which chirps were recorded, whereas the extent of social interaction (evident, for example, when comparing novel vs experienced fish pairs) seemed to play a much larger role.

Probing with chirps

In this study we explore the possibility that chirps could improve conspecific localization by enhancing beat detection. This could be achieved in at least 3 different ways. First, chirping could temporarily adjust beat frequency to levels better detectable by the electrosensory system (Bastian et al., 2001). Second, chirps could be used to improve active electrolocation by briefly enhancing beat temporal resolution (all types of chirps will increase electroreceptor firing rates, acting on different input lines; Benda et al., 2006).
Third, as previously discussed within this study, chirps could be used to enhance electrosensory responses to the beat. This may be achieved by simply enhancing the output of the primary electrosensory afferents (through synchronization of different types of input; Benda et al., 2006) or by phase-shifting the carrier EOD in correspondence of the *destructive* components of the beat cycles (i.e. the beat minima). Although chirp production has been previously reported to be phase-invariant (Metzen and Chacron, 2017), this could be still a valid hypothesis if one considers the effects of beat phase adjustment only at specific body locations (such as the head, which can be considered the “electrosensory fovea”; Carr et al., 1982; Castelló et al., 2000).
In theory, chirps could also be used to improve electrolocation of objects as well (as opposed to beat detection, i.e. detection of EOD sources). Compared to other electric fish using pulse-type EODs, the frequency content of wave-type EODs is relatively poor and narrowly focused on limited components. The larger signal band-width (i.e. frequency content) of pulse EODs affords better object discrimination, when compared to wave-type EODs. However, given their lower production rates compared to the frequency of wave-type EOD cycles, EOD pulses grant a lower sampling rate and thus a poorer temporal resolution (Crampton, 2019; Bastian, 1976**;** Watson & Bastian, 1979). To compensate for this, EOD pulses are emitted at higher frequency during electrolocation bouts and locomotion in general (Jun et al., 2014). Conversely, for wave-type fish, chirping could represent a strategy to temporarily compensate for the lower frequency resolution while still being able to resolve EOD perturbations with a good temporal definition.
If this hypothesis finds further support, the question arises as to why not all gymnotiforms electrolocate using the same strategy. It may be that a trade-off exists between space- and

time-resolution in the evolution and maintenance of electrosensory systems (Crampton, 2019). While broadband pulse signals may be useful to capture highly complex environments rich in foliage, roots and vegetation featuring the more superficial habitats in which pulse-type fish live, wave-type EODs may be a better choice in the relatively simpler river-bed environments in which many wave-type fish live (e.g., the benthic zone of deep river channels; Crampton, 2019). In this case, achieving a good spatial resolution is critical during social encounters, especially considering the lower saliency of visual cues caused by the scarcer light penetration. In such habitats, social encounters would be less “abrupt” but spatially less “conspicuous” or blurred.

Conclusions

Although our results do not completely rule out a role of chirps in electrocommunication (probing cues could be used for instance as proximity signals to advertise a subject's presence, deter further approaches or coordinate synergistic behaviors as during spawning, Henninger et al., 2018), the high self-correlation of chirp time-series and the lack of any meaningful behavioral correlate of chirping, seems to suggest that this is an unlikely scenario for the majority of chirping observed. Moreover, the rate of chirp production (extremely variable) and the sparseness of the production of chirp types other than type 2 chirps, would make such a communication channel very unreliable and semantically quite limited to be compatible with the functions so far attributed to chirps. Our behavioral experiments - although they do not fill the gaps in causal descriptions of chirps and behavior - open the discussion to alternative functions of chirps beyond their presumed communication function.
In echolocating species, active sensing has been shown to clearly depend on environmental complexity and spatial features (Fenton and Bell, 1981; Siemers et al., 2009; Fouda et al., 2018). As probing cues, chirps of different types could be used by knifefish adaptively, based on the features of the foreign EOD source (frequency, position, location, orientation, distance, etc.). Here, we emphasize the role of chirps as homeoactive signals - as opposed to alloactive - to underline the fact that chirps represent active modulations of signal frequency possibly aimed at optimizing the otherwise passive beat detection occurring constantly and unintentionally (Zweifel and Hartmann, 2020). To our knowledge, this would be a unique biological example of how underwater object localization can be enhanced instantaneously by sinewave FM probes without the assessment of echo-delays. By proposing a novel and more parsimonious explanation to explain chirping behavior, this study revives the debate on the reasons why these nocturnal bony fish evolved this signaling system (electrolocation vs communication).

Materials and Methods

Animals

A total of 196 *Apteronotus leptorhynchus* of both sexes - age ranging between 2-3 years - were obtained from tropical fish importers and housed in individual 80 x 35 x 40 cm aquariums under a 12/12 light cycle. Fish were allocated to the different experiments as follows: 130 for the assessment of context-dependent effects on chirping, 16 fish were used for the playback experiments, 30 fish were used in the novel environment exploration assay, 12 fish were used to assess the environment effects on chirping and 8 to assess the impact of clutter on the fish responses to playback EODs. In all groups both sexes were equally represented. Throughout

the experiments, the water was continuously filtered, water conductivity was maintained between 150-300 µS cm^{-1}, temperature between 23-26 °C and pH between 6-8. The hometank environment consisted of PVC shelter tubes and plastic plants. The fish were fed 3 times a week with red mosquito larvae. Prior to each experiment, the fish's EOD frequency (EODf), body length and weight were measured. To identify their sex, the EODf was normalized to a water temperature of 25.0 °C using a Q_{10} of 2 (Dunlap and Ragazzi, 2015; Dunlap et al., 2000). The limitation of this approach is that females cannot be distinguished from immature males with absolute certainty, since no post-mortem gonadal inspection was carried out. Nevertheless, fish with a normalized EODf higher than 750 Hz are considered males and those with lower frequencies are considered females (Dunlap et al., 2000).

Recordings of fish pairs and playback experiments

Equipment

All technical equipment used in the playback experiments is listed in the table below.

Device	**Model**	**Manufacturer**
Amplifier	DPA-2FSL and DPA-2FS	npi electronic GmbH
Attenuator	839 attenuator	KAY elemetrics Corp.
Computer	OptiPlex 7050	Dell Technologies Inc.
Data acquisition system	NI 6211 USB	National instruments
Infrared illuminators	CM-IRP6-940 nm	CMVision
Stimulus isolator	ISO-STIM 01M	npi electronic GmbH
Infrared video camera	GS3-U3-41C6NIR-C	FLIR

Experimental setup and groups

Fish pairs were recorded in 2 adjacent chambers of tripartite 80 x 35 x 40 cm aquariums (**Figures 1**,**2**,**4**). Tank compartments were separated by a plastic mesh barrier to allow electrical but no physical interaction. EOD recordings were conducted using 2 graphite electrodes placed on opposite sides of the tank (1 pair per compartment). EOD recordings were assigned to the following categories based on the type of experience fish had with either the test aquarium or the paired subjects: *resident*, *intruder*, *dominant*, *subordinate*, *novel*, *experienced*, *courtship*, *no courtship*, *divided* and *free*. *Resident* fish were housed for 1 week alone in the same setup, before being paired with *intruder* fish. Dominance was assessed by means of shelter-competition tests prior to EOD recordings. These tests consisted of 30 min long trials in which fish pairs were allowed to interact freely and compete for the occupancy of a plastic shelter tube positioned in one of the 3 compartments of a tripartite aquarium (1 pair per aquarium). The fish spending more time in the shelter tube was considered to be the *dominant* one, the other the *subordinate*. Fish were selected such that one fish was always 3-5 grams heavier than the other, to ensure a predictable outcome of the competition and to limit aggressive displays to a minimum (Jennions and Backwell 1996; Umbers et al. 2012). In these and other recordings, fish were considered "*experienced*" after at least 1 week of pairing, as opposed to "*novel*" (just paired). This means that residents, intruders, dominant and subordinate fish were all assigned to the category "novel", whereas the category "experienced" included only resident and intruder fish, due to their longer pairing period (1 week). In a separate set of recordings, male and female pairs were subject to water conductivity changes to simulate the onset of the breeding season: during the course of a 4-week period water conductivity was lowered from 400 to 100 µS cm^{-1} (novel fish in high conductivity water = *no breeding*, experienced fish in low conductivity water = *breeding season*). Although, the term

“breeding” here refers to the context and not to the actual behavioral repertoire displayed by the fish, this treatment resulted in 6/8 female fish to show evident signs of developed eggs (no post-mortem exam was used to confirm egg presence in the other 2 fish). At the end of this period, fish were allowed to swim freely and interact without mesh barriers (experienced fish in low conductivity water = breeding + free). Recordings from such pairs were compared with naïve male-female pairs freely swimming in high conductivity (400 μS cm^{-1}) water (naïve fish in high conductivity water = no breeding + free). Although even in this case fish were paired with their tank partners for longer times, due to the different treatment they had been subject to, they were not included in the “experienced” category.

Chirp type categorization

Overall, the coding of custom MATLAB scripts (for EOD recording, chirp detection and validation), the preparation and execution of the behavioral experiments and the manual analysis of chirps imposed very significant timing constraints for the execution of the experiments. For this reason, to come up with threshold values usable to categorize chirps over the course of the whole study, we have used a preliminary dataset of 11342 chirps obtained from our first set of recordings (8 male-male fish pairs). A more complete dataset (N = 30486) was obtained and validated only 2 years later.

The cut-off values used in our study (50 ms duration and 105 Hz frequency modulation amplitude) were chosen based on the distribution of our preliminary recorded chirps but also based on reference values previously published by other authors (see **Table 1**, for previously published chirp categories and **Table 3** for details on our own categorization).

Table 1. Chirp categories

chirp type	**FM (Hz)**	**duration (ms)**	**references**
type 1	200-400	25	Engler et al., 2000; Bastian et al., 2001, Zakon et al., 2002
type 2	50-100	15-20	Engler et al., 2000; Bastian et al., 2001; Hupé and Lewis, 2008
type 3, 4	200-300	75-200	Hagedorn and Heiligenberg 1985; Engler et al., 2000; Engler and Zupanc 2001
rises	10-20	50-100	Engler Zupanc 2001; Tallarovic Zakon 2002

Amplifier and recording settings

EOD recordings (3 channels) were amplified through DPA-2FSL and DPA-2FS amplifiers (*npi electronics*) with a gain of 200, low pass filtered at 10 kHz, high-pass filtered at 100 Hz. The acquisition sampling rate was kept at 20 kHz in all recordings.

Playback experiments

All playback experiments were conducted in 80 x 35 x 40 cm glass aquariums in a dark room (**Figure 3**). Water conductivity and temperature were similar to the fish housing conditions and ranged between 200-300 µS cm^{-1} and 23-26 °C, respectively. Water was pre-heated using commercially available heaters (EHEIM 3612 heating rod 50W). At one end of the tank, a mesh barrier separated the playback electrode from the fish. Electrodes were placed 10 cm apart and 1 cm away from the barrier. This layout ensured that playback stimuli consisted of naturalistic electric fields (Kelly et al., 2008). Electrodes were randomly placed at either side of the aquarium, in different experiments, to avoid playback location biases.
Three pairs of recording electrodes were placed in the fish compartment in accordance with an "IX" layout: 1 pair was parallel to the short tank side ("I") the other 2 pairs were oriented diagonally across the compartment ("X"). This layout ensured that the fish EOD was always detectable, regardless of fish movement and body orientation. Prior to commencing the study, the stimulus amplitude was calibrated to have a field intensity of approximately 1 mV/cm measured across the mesh divider at circa 5 cm distance from the playback dipole, and in line with its axis.
Playback sinewave stimuli were designed in MATLAB and their frequency based on the measured fish EOD frequency prior to trial onset. Stimuli were then delivered via a 6211-USB DAQ (National Instruments) to the aquarium through a stimulus isolator (ISO-STIM 01M, npi electronics). The stimuli played back were modified sinewaves mimicking conspecific EODs the frequencies of which were calculated as differences (DF) from the fish's own EOD frequency: -240, -160, -80, -40, -20, -10, -5, 0, 5, 10, 20, 40, 80, 160, 240 (Hz). Each of these playback stimuli was delivered in 4 different modes distinguished by their chirp content (modes, see **Table 2** below): mode0 contained no chirps, mode1 contained type 1 chirps, mode2 with type 2 chirps and mode3 contained small rises (abrupt frequency rises). Each playback trial (50 s with a 5 s time of fade-in and fade-out) had a 180 s inter-trial interval. Each playback session consisted in a randomized sequence of 15 x 4 playback trials (1 min + 3 min each) which lasted for 4 h.

Table 2. Playback chirp parameters.

mode	duration [ms]	freq. mod. [Hz]	Chirps
0	-	-	no chirps
1	approx. 120	500	0.1 Hz for 50 s (1 chirp every 10 s = 5/trial)
2	approx. 20	120	2 Hz for 5 s (n = 10), every 10 s (n=10x5)
3	approx. 60	30	0.1 Hz for 50 s (1 chirp every 10 s = 5/trial)

Video tracking

During playback experiments (**Figure 3**) but also novel environmental exploration assays (**Figure 6**), freely swimming fish were recorded at 40 FPS using an infrared USB camera (Grasshopper3, FLIR, model: GS3-U3-41C6NIR-C). The camera was set to acquire a frame every 500 samples of the EOD recording (1 sec = 20000 samples) through a TTL channel of the digital acquisition device (NI USB 6211 National Instruments). Swimming trajectories were extracted from the videos using the software BioTracker after converting the files from H264 to AVI (https://www.igb-berlin.de/en/biotracker). Playback experiments began around 1 p.m., after the onset of the animals lights OFF phase (12 AM lights ON, 12 PM lights OFF). Test subjects were acclimated for 30 min to the test aquarium, before trial onset.

Beat decay estimates

The beat dissipation range in our experimental conditions was estimated by recording the interaction of two EOD mimics (a static reference electrode pair and another identical electrode pair, placed parallel to it, as a moving source) at increasing distances and in the same water conditions as in our recordings (conductivity 200 µS/cm and temperature 25°C; **Figure 7**). Both the reference and the moving EOD mimic were scaled to natural fish signals (1mV/cm, measured at 2-3 cm from the playback dipoles). Recordings were made placing recording electrodes at opposite poles of the reference electrodes while shifting the moving source with 5 cm steps up to 60 cm away. The EOD field intensity generated by both reference and moving electrodes was recorded for 5 seconds at each distance step, always at the extremities of the reference pair.

Environmental manipulations

Fish pairs were exposed to changing environmental conditions in the experiments illustrated in **Figure 8**. Recordings in lights ON, OFF and cluttered conditions were carried out in 80x35x40 cm aquariums divided in 2 compartments by a plastic mesh divider. During *lights-ON*, even illumination of both tank compartments was provided via a Leica LED illuminator located on a shelf above the aquarium (Leica CLS 150 XE Microscope Cold Light Source, 150W). During *lights-OFF* and *cluttered* conditions, a drape obtained from a blackout curtain was used to fully cover the tank (room lights were switched OFF and daylight lowered by curtains). Cluttered conditions were exactly the same as *lights-OFF* with the addition of floating and submerged plastic shelter tubes and plants heterogeneously arranged in order to fill up each compartment.

Data analysis

Chirp detection

Chirps were detected in a two-step process consisting of a first automatic detection followed by a manual validation. The first step implied a measurement of the power density in the FFT of the recorded signal (FFT parameters: window size = 2^12; overlap = 90%) within the range set by the fundamental frequency and the first harmonic component (EODf - 5 Hz and 2 x EOD - 100 Hz). Chirps were detected as peaks in the power density. The manual validation consisted of a survey of the detected chirps to eliminate false positives. False negatives were estimated at a rate lower than 10%. Although the two electrode pairs used in each aquarium would detect EODs from both fish, chirps were properly assigned to the sender based on signal intensity and the baseline EOD frequency of the two fish, when possible (EODs generated by a fish will have higher intensity if recorded by electrodes placed in the same compartment). Notably, other authors reported the occurrence of more than 2 types of large chirps (namely: type3-6), we included all those in one single group based on the distribution of our data and taking into account the absence of any obvious clustering for large chirps (see **Figure 1**).

Table 3. Chirp categorization used for chirp detection

	Frequency excursion < 105 Hz	Frequency excursion > 105 Hz
Duration > 50 msec	rise	Type 3 chirp

Duration < 50 msec	Type 2 chirp	Type 1 chirp

Factor analysis mixed data - I

Factor analysis was conducted using the R package FactoMiner ("Analyse factorielle de donnees mixtes." Revue Statistique Appliquee. LII (4). pp. 93-111. Josse and Husson, 2008). Factor analysis of mixed data (FAMD, **Figure 2**) is equivalent to a principal component analysis adapted to analyze mixed datasets, containing both quantitative and qualitative variables (Pagès 2004) and explore their associations. The FAMD algorithm can be seen as a hybrid of principal component analysis (PCA) and multiple correspondence analysis (MCA): it works like PCA with quantitative variables and MCA for qualitative ones. Quantitative and qualitative variables are normalized in order to balance the influence of each set.
A total of 30486 sampled chirps were used. For each chirp, the following qualitative and quantitative variables were considered: freq_modulation (peak of EOD instant frequency during chirp), chirp duration (instant frequency peak duration), EOD frequency of the sender (EODs), EOD frequency of the receiver (EODr), EOD_amplitude (amplitude of the EOD sinewave during chirp), power (chirp triggered EOD intensity), water temperature, water conductivity, chirp timestamp, weight and length of the sender fish, weight and length of the receiver fish, sender and receiver sex, status of the sender and receiver fish (experience with the recording aquarium), fish ID (to account for interindividual variability), chirp type (categorized as above), state (based on the experience with the other fish of the pair).
A first model (3 dimensions, model#1, dim1 17.46%, dim2 15.76%, dim3 10.67%) was made considering all variables listed above. Subsequent models were calculated while progressively reducing the number of variables (down to 3, model#11, dim1 23.17%, dim2 20.4%, dim3 14.35%). At each iteration variables with eigenvalues lower than the mean average contribution were eliminated.

Factor analysis mixed data - II

To assess the correlations among swimming related variables and chirps, a total of 7893 chirps obtained from a separate experiment (novel environment exploration, Figure 6) were used. For each chirp, the following qualitative and quantitative variables were considered: the percentage of time spent near the tank wall ("wall"), the percentage of time spent in the tank open space ("open"), percentage of time spent in the shelter area ("shelter"), the percentage of time spent within a rectangular area 10 cm around the conspecific location ("social"), the percentage of time spent investigating the novel object ("object"), the time at which chirps were produced ("timestamp"), the chirp type, the fish ID, the EOD frequency of the freely swimming fish, the EOD frequency of the caged fish, the sex of the chirp sender, the sex of the chirp receiver, the DF (i.e. EOD frequency difference between sender and receiver), the distance between sender and receiver ("distance"), the average speed of the sender ("avg speed"), the angle between sender and receiver (referred to the a horizontal axis delineated by the tube, "angle"), the interference estimate weighed on the distance (i.e. calculated on a beat resulting from EODs attenuated as a function of distance, "actual interference") and the maximum beat interference possible for a given chirp (i.e. without distance attenuation, "interference").

Chirp transitions and time-series cross-correlation

The number of chirp transitions present in each recording (dataset used for **Figures 1**,**2**,**4**) was measured by searching in a string array containing the 4 chirp types, all their possible pairwise permutations. The number of each chirp-type transition was then used to create a 4x4 frequency matrix. Chirp transition diagrams were obtained by calculating the median of such frequency matrices calculated for different fish pairs. Chirp time series were extracted from each recording and binned (50 ms) to evaluate the cross-correlation index within a +-2 s lag window.

Chirp interference

Chirp interference was calculated as the cumulative duration of outlier beat inter-peak intervals (IPI) after calculating the beat envelope for each chirp/DF combination (**Figure 5**). Gaussian chirps were generated on top of a constant frequency carrier of 862 Hz (arbitrarily chosen) and the beat resulting from a paired sinewave with a frequency within ±300 Hz of 862 Hz. The frequency modulation and duration of gaussian chirps were determined by setting the sigma and the amplitude of the peak and by measuring the actual peak size of the instant frequency ("medfreq" MATLAB function). This was obtained from the FFT analysis of the chirping signal (window size 2^12, 90% overlap, sampling frequency 20kHz). For each chirp, beat peaks (i.e. the peaks of the amplitude modulation, AM) were detected searching for local maxima in the signal first order envelope. Adjacent beat peaks were used to calculate beat cycle durations for the whole segment. The different impact of different types of chirps on the beat would result in different populations of beat cycles (depending on the beat frequency, the duration and FM of the chirp). Outliers in the beat cycle durations resulting from each chirp were used as a measure of how a given chirp would perturbate the otherwise regular beat cycles. Beat cycle outliers were detected using a MATLAB built-in function - "isoutlier" - with the "quartile" setting to include peak durations on both tails of the peak distribution (below 25% and above the 75% quartiles). The interference value for each chirp was obtained by dividing the cumulative outlier beat cycle duration (i.e. the total duration of beat cycles significantly affected by the chirp) by the overall duration of the beat peaks. The duration of each beat cycle corresponded to the difference in time between consecutive beat peaks. In this way, the effect of a chirp could be weighted on the number of peaks (i.e. the beat frequency). The beat interference is calculated within a fixed time window (700 ms) which corresponds to the median inter-chirp interval (ICI; median = 0.6987) calculated for 30486 chirps (130 chirp time-series) and is centered around the chirp (chirp peak is always at 350 msec). This parameter is used to provide an estimate of the effect of a chirp in its immediate temporal surround.

Statistics on chirp counts

The numbers of chirps produced during the experiments assessing the effect of environmental factors on chirp rate production were compared using the Friedman's test (non-parametric ANOVA for repeated measures) as data were not following a Gaussian distribution.

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Author contributions

L.O. designed the experiments, collected and analysed the data and wrote the first draft of the manuscript. M.R, performed part of the resident-intruder experiments. M.L. recorded fish in simulated breeding conditions. L.K. performed the playback experiments. F.P. provided the electric field models, prepared the relative figure panels and provided feedback during the manuscript draft preparation. R.K. contributed with discussions throughout the course of the study and provided feedback on the manuscript drafts.

Conflict of interest statement

The authors declare no conflict of interest.

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