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RESEARCH ARTICLE

Electrocommunication signals and aggressive behavior vary among male morphs in an apteronotid fish, *Compsaraia samueli*

Megan K. Freiler[1,2,*,‡], Melissa R. Proffitt[1,2,*] and G. Troy Smith[1,2]

ABSTRACT

Within-species variation in male morphology is common among vertebrates and is often characterized by dramatic differences in behavior and hormonal profiles. Males with divergent morphs also often use communication signals in a status-dependent way. Weakly electric knifefish are an excellent system for studying variation in male morphology and communication and its hormonal control. Knifefish transiently modulate the frequency of their electric organ discharge (EOD) during social encounters to produce chirps and rises. In the knifefish *Compsaraia samueli*, males vary extensively in jaw length. EODs and their modulations (chirps and rises) have never been investigated in this species, so it is unclear whether jaw length is related to the function of these signals. We used three behavioral assays to analyze EOD modulations in male *C. samueli*: (1) artificial playbacks, (2) relatively brief, live agonistic dyadic encounters, and (3) long-term overnight recordings. We also measured circulating levels of two androgens, 11-ketotestosterone and testosterone. Chirp structure varied within and across individuals in response to artificial playback, but was unrelated to jaw length. Males with longer jaws were more often dominant in dyadic interactions. Chirps and rises were correlated with and preceded attacks regardless of status, suggesting these signals function in aggression. In longer-term interactions, chirp rate declined after 1 week of pairing, but was unrelated to male morphology. Levels of circulating androgens were low and not predictive of jaw length or EOD signal parameters. These results suggest that communication signals and variation in male morphology are linked to outcomes of non-breeding agonistic contests.

KEY WORDS: Androgens, Chirping, Electric fish, Jaw morphology

INTRODUCTION

Males often vary dramatically in morphology, aggression and reproductive behavior. Male phenotypic variation may be continuous or discrete. In alternative reproductive strategies (ARS), discrete male types differ in morphology and behavior (Sinervo and Lively, 1996; Smallegange and Johansson, 2014). Territorial males usually have larger weapons and/or body size to compete for mates (Emlen, 1997; Husak et al., 2009; Preston et al., 2003). 'Sneaker' males often resemble females to steal copulations, but have large testes for sperm competition (Apostólico and Marian, 2018; Simmons et al., 1999; Simmons and Emlen, 2006). While some species have discrete male strategies, males in other species vary continuously in territoriality, body size or weapon size (Del Sol et al., 2021; Hill et al., 1999; Holberton et al., 1989; Paterson and Blouin-Demers, 2017). Continuous male morphological variation is also linked to behavioral variation, which can influence outcomes of male–male competition and female choice.

Weapons or ornaments used in male–male contests or in courtship often vary substantially. Male weaponry is common in species with intense competition for resources, including access to females (Emlen, 2008; Miller, 2013). Weapon size may indicate fighting ability, and large disparities in weapon size between competing males often results in reduced physical combat (Barki et al., 1997; Iwata et al., 2005; Jennions and Backwell, 1996). Unlike weapons, ornaments are usually not used directly in combat, but may signal competitive ability or male quality (Husak and Swallow, 2011). For example, dark-eyed junco (*Junco hyemalis*) males with more tail white are more attractive to females and more often win contests (Hill et al., 1999; Holberton et al., 1989). Weapons can also sometimes function as ornaments (Emlen, 2008; Oliveira and Custodio, 1998). For example, enlarged claws of fiddler crabs can act as an ornament or an armament (Callander et al., 2013; Oliveira and Custodio, 1998; Swanson et al., 2013). Traits used as ornaments versus as weapons may carry trade-offs. Fiddler crabs with larger claws that are highly effective ornaments are less able to wield these claws as weapons (Swanson et al., 2013). Male variation in ornaments or weapons is often related to dominance (Bywater et al., 2008; Sneddon et al., 1997).

Intrasexual morphological variation may also be associated with differences in communication. For example, in midshipman fish, large, territorial males call to attract females, whereas small, sneaker males do not call (Brantley and Bass, 1994; Lee and Bass, 2004). Differences in communication signals are common in ARS (Brantley and Bass, 1994; Malavasi et al., 2003; Rotenberry et al., 2015), but communication also varies across males in species with continuous morphological variation. For example, in many frog species, larger males produce lower-pitched vocalizations (Hoskin et al., 2009).

Organizational and activational effects of hormones often regulate intrasexual variation in morphology and communication. In *Onthophagus* beetles, horn growth depends on sensitivity to juvenile hormone during development (Emlen and Nijhout, 1999; Moczek and Nijhout, 2002). In some frog species, males with higher testosterone levels call more in response to playbacks (Solís and Penna, 1997). Pleiotropic androgen effects can also mediate trade-offs associated with intrasexual morphological variation. For example, male juncos with more tail white can increase circulating testosterone levels more (McGlothlin et al., 2008). In fish, territorial, courting males often produce more 11-ketotestosterone (11-KT) (Brantley et al., 1993).

Weakly electric ghost knifefish (apteronotids) are an excellent model for examining relationships between hormones and male

[1]Department of Biology, Indiana University, 1001 E. 3rd St., Bloomington, IN 47405, USA. [2]Center for the Integrative Study of Animal Behavior, Indiana University, 409 N. Park Ave., Bloomington, IN 47405, USA.
*These authors contributed equally to this work

‡Author for correspondence (mkfreile@indiana.edu)

M.K.F., 0000-0002-4872-1849; M.R.P., 0000-0003-2902-8149; G.T.S., 0000-0001-6930-7105

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variation in morphology and communication. In some species, including *Parapteronotus hasemani*, *Apteronotus leptorhynchus* and *Sternarchogiton nattereri*, males vary markedly in body size, jaw morphology and/or dentition (Cox-Fernandes et al., 2002, 2010). Male morphological variation often accompanies differences in electrocommunication signals, social status and androgens (Cox-Fernandes et al., 2010; Petzold and Smith, 2016). Electric fish produce continuous electric organ discharges (EODs) for electrolocation and electrocommunication. They also modulate their EODs to produce chirps [large, abrupt increases in EOD frequency (EODf)] and rises (smaller, slower increases in EODf). Chirps and rises coordinate agonistic and reproductive interactions (Dunlap et al., 2002; Henninger et al., 2018). Chirping is often sexually dimorphic and sensitive to hormones (Smith, 2013). Androgens increase EODf and chirp rate in *A. leptorhynchus*, a species in which males have a higher EODf and chirp more (Dulka and Maler, 1994; Schaefer and Zakon, 1996). Electrocommunication signals may also correlate with social rank (Fugère et al., 2011; Hagedorn and Zelick, 1989). 11-KT increases in males with high EODfs when socially housed, suggesting that androgens and EODf are related to status in *A. leptorhynchus* (Cuddy et al., 2012). Male variation in jaw morphology is often related to variation in electrocommunication and androgens. *Sternarchogiton nattereri* males with exaggerated teeth have higher EODfs and 11-KT levels than toothless males (Cox-Fernandes et al., 2010). In contrast, *P. hasemani* males with elongated jaws have similar EODs and androgen levels as short-jawed males (Petzold and Smith, 2016). Thus, although male variation in morphology, signals and androgens are common in electric fish, these relationships vary across species.

Compsaraia samueli is one of several apteronotid species with sexually dimorphic jaws (Albert and Crampton, 2009). Some males have substantially longer jaws than females, and jaw length varies continuously across males (Keeffe et al., 2019). Apteronotid males often compete by biting and/or locking jaws (Triefenbach and Zakon, 2008). Because the long jaws of *C. samueli* males are slender, poorly ossified and easily warped, they are poorly suited for gripping, biting and combat (Evans et al., 2019; Keeffe et al., 2019). These elongated jaws might also hinder feeding, as wild-caught, long-jawed males lack food in their stomachs (Keeffe et al., 2019). Long jaws in *C. samueli* may instead function as ornaments in ritualized combat or mate attraction (Evans et al., 2019; Keeffe et al., 2019). Older males might have longer jaws, but average-sized adult males can still sometimes have very long jaws, suggesting that jaw length does not depend strongly on age (Keeffe et al., 2019). The life history and ecology of *C. samueli* are poorly understood, however, so conclusions about functions of elongated jaws in the wild are somewhat speculative.

The pronounced variation in jaw length in *C. samueli* provides an opportunity to investigate relationships between male variability in morphology, aggression, communication and hormones. This study examines relationships between male jaw morphology, social status, and signal structure and function. We recorded EOD modulations in three contexts: in response to artificial playbacks, during short-term dyadic encounters and over 1 week of paired housing. We also measured androgen levels to examine relationships between morphology, electrocommunication signals and hormones.

MATERIALS AND METHODS

Animals

Adult male *Compsaraia samueli* (Albert and Crampton 2009) (N=38) were collected in Peru by a commercial supplier (Riverland, Iquitos) and were transported to Indiana University in three groups (March 2017, January 2018 and December 2018). Fish were housed individually in 35 liter tanks within a 2000 liter recirculating aquarium on a 12 h:12 h light:dark cycle. Water was maintained at a temperature of 25–27°C, at a pH of 5.5–6.5, and at a conductivity of 300–500 µS cm^{-1}. Animal care and experimental protocols were approved by the Indiana University Bloomington Institutional Animal Care and Use Committee.

Species determination

Fin clips were obtained from four individuals to determine which *Compsaria* species was used in this study. Genomic DNA was extracted from fin clips using the Qiagen DNeasy Blood and Tissue Kit, following the manufacturer's instructions (Qiagen, Valencia, CA, USA). PCR products for cytochrome oxidase (COI) were amplified using Fish-BCL: TCAACYAATCAYAAAGATATY-GGCAC, Fish-BCH: ACTTCYGGGTGRCCRAARAATCA (Baldwin et al., 2009; Becker et al., 2011) with a GoTaq polymerase kit (Promega, Madison, WI, USA). Samples were then purified and sequenced by Eurofins Genomics (Eurofins Genomics, Louisville, KY, USA). Poor quality areas (at the ends of the reads) of the raw sequencing results were identified and trimmed using Chromas (Technelysium, South Brisbane, QLD, Australia). The trimmed sequence fragments (from the forward and reverse primer sequences) were aligned to a reference sequence for COI using a combination of MUSCLE to align the fragments to the reference sequence and Jalview (Waterhouse et al., 2009) to view, trim and export the consensus sequence for each individual. The consensus sequences were created from the forward and reverse read fragments.

We obtained COI sequences from *C. samueli* and *C. compsa* from the NCBI database and aligned the NCBI sequences with the ones we obtained using the MUSCLE algorithm. We viewed the sequences with Jalview (Waterhouse et al., 2009) to ensure proper alignment and to compare COI sequences across species. We then assembled a phylogenetic tree of COI sequences from this study and from NCBI by using a maximum likelihood approach with 1000 bootstrap replicates, with RAxML v. 7.2.4 (Stamatakis, 2006). We used the GTR+gamma substitution model for this tree.

EODf measurements

EODf was measured at least two times – within 2 weeks of arrival into the lab, and after the fish had been in the laboratory for at least a month. Most fish had their final EODf measured within 2 weeks of blood sampling and dissection at the end of the experiment. The voltage signal of the EOD was detected by two wires placed next to the fish and amplified (gain 100X, Grass-Telefactor, West Warwick, RI, USA). EODf was measured with a multimeter (Fluke 187 True RMS multimeter, Fluke, Everett, WA, USA) connected to the output of the amplifier. Reported EODfs were temperature-adjusted to that expected at 26°C using a Q_{10} of 1.63 (Dunlap et al., 2000).

EOD modulations in an artificial playback paradigm

One to four months after arrival in the lab (group 1: May 2017; group 2: April–May 2018; group 3: January 2019), chirps and rises were recorded from 31 fish by using a modified chirp chamber paradigm that has been described previously (Kolodziejski et al., 2005; Smith et al., 2016; Zhou and Smith, 2006). The fish was placed into a mesh hammock inside a darkened tank. A pair of carbon electrodes were placed at the ends of the tank opposite the head and tail of the fish to record signals produced by the fish. Playback stimuli were produced with audio software (Cool Edit Pro, Syntrillium, Phoenix, AZ, USA) running on a Windows-based computer and were presented to the fish via a pair of carbon

electrodes in the middle of the tank on the left and right sides of the fish. The orthogonal orientation of the stimulus electrodes relative to the recording electrodes minimized the contamination of the recording by the playback. The stimulus was calibrated, midway between the playback electrodes, to be 0.6 mV cm^{-1} root-mean-square amplitude. This intensity was chosen to be similar to the intensity of the electric field generated near the body by the EODs of an adult *C. samueli*. After a 50-min acclimation period and a 4-min baseline recording, we presented each fish with five stimuli in a randomly assigned order for each fish. Each stimulus was a sinusoidal stimulus mimicking the EOD of another fish, +5, −20, +20, −150 or +150 Hz relative to the fish's own EODf. For each stimulus recording session, we recorded 1 min before stimulus presentation, followed by 2 min with the stimulus and 1 min after stimulus offset. Playbacks were separated by 10 min to reduce habituation.

Chirps and rises were analyzed using custom procedures (Brian Nelson, University of Oregon, Eugene, OR, USA) in Igor Pro (WaveMetrics, Portland, OR, USA), as described previously (Kolodziejski et al., 2005; Ho et al., 2010). This program eliminated contamination from the playback stimulus by subtracting a copy of the playback (appropriately scaled and phase shifted) from the recording. The code identified EOD modulations (chirps and rises) when EODf increased by 3 Hz or more above its baseline for at least 10 ms. To be counted, chirps or rises had to be separated from each other by at least 100 ms. The beginning and end of the EOD modulation were defined by when the EODf was within 1 Hz of the baseline EODf. Frequency traces were visually inspected to ensure accurate identification and quantification of each EOD modulation. A few chirps had more than one distinct frequency peak. When more than one frequency peak was present, the highest peak was used to determine chirp frequency modulation (FM). Chirps were distinguished from rises with the same criteria used by Turner et al. (2007) to classify EOD modulations in other apteronotid species; i.e. chirps were defined as EOD modulations in which the FM (Hz) >21×duration (s)+25.

Live dyadic social encounters

Experimental design

Fish (N=34) were recorded during live, free-swimming dyadic encounters in November–December 2017 (group 1, N=8), July–August 2018 (group 2, N=12) and January–February 2019 (group 3, N=14). Each fish was paired with a novel conspecific between one and three times for a total of 45 trials. Fish were also paired to ensure a range of jaw length differences. All but one of the fish in this study were male (determined at the end of the experiment by gonadal inspection). Data from the trials including the one female were removed from all statistical analyses. If fish were paired more than once, trials were separated by at least 3 days. Fish were removed from their home tanks and acclimated to a 52×60×20 cm experimental tank in a dark room with infrared LED lighting for 30 min before the start of a trial. Two pairs of carbon electrodes were placed on opposite sides of the tank to capture a global stimulus of the fish's electric signals. Signals were amplified ×1000 with a p55 A.C. pre-amplifier (Grass-Telefactor) and were captured with a Sound Blaster Audigy sound card (Creative Labs, Milpitas, CA, USA). Video was captured with an infrared-sensitive video camera [eZ HD EQ900F (EverFocus, Duarte, CA, USA) or Exwave HAD SSC-M383 (Sony, New York, NY, USA)] and was collected with a MyGica Capit USB video card (MyGica, Xili, Nanshan, Shenzhen, China). Each fish was kept behind a plexiglass divider on opposite sides of the tank during acclimation to prevent the perception of the other fish's electric signals. A PVC shelter tube was placed in the center of the tank to serve as a resource for the fish to defend. After 25 min, electrical recordings were started to obtain a baseline measure of EOD activity prior to interaction. After a further 5 min, the dividers were raised, and fish were allowed to interact for 7 min. The overhead room lights were then turned on, and fish were returned to their home tanks.

Behavioral analysis

Electrical recordings from live dyads were analyzed in Adobe Audition (Adobe Systems, San Jose, CA, USA), where chirps and rises were counted by visual inspection on a spectrogram (sampled 32 bit at 48,000 Hz with an FFT size of 16,384). Wave-type weakly electric fish have a unique and relatively stable EODf, so the EODs of individual fish can be tracked by frequency. Chirps and rises can then be easily attributed to the fish whose EODf shows an abrupt increase in frequency. Small EOD modulations (<100 Hz FM) at the fundamental EODf were conservatively classified as rises. Except for two individuals, all fish increased their EODf immediately after the dividers were lifted. The magnitude of the EODf increase was calculated by comparing the average EODf of each fish over 1 min starting 2 min before the trail began and 1 min after the trial began (after most fish had stabilized their EOD at a higher frequency).

Video files were analyzed by a single observer using BORIS v. 7.5.3 (Friard et al., 2016), an open-source event logging software. All attacks were time-stamped and counted for each fish. An attack was defined as any time a fish made aggressive movement toward or contact with another fish by nipping, lunging, charging or performing open jaw gape displays (Table S1). Many of these behaviors have been reported in other apteronotids, and biting (nipping) and jaw gapes have been seen previously in *C. samueli* (Albert and Crampton, 2009; Evans et al., 2019; Hupé and Lewis, 2008; Triefenbach and Zakon, 2008). Attack latency was defined as the time to first attack after dividers were raised, and was set at the length of the trial (7 min) if the fish never attacked. The time spent parallel and antiparallel swimming and in the PVC shelter tube was also quantified (Table S1). Dominance was established if the difference in the number of attacks was greater than 5. If there were no attacks or if the difference between the fish in the number of attacks was less than 5 between the two fish, no clear hierarchy was considered to have been established (N=4 trials).

Long-term social housing

Experimental design

To collect data on chirping dynamics over a longer-term social experience, fish (N=28) were housed together for 1 week and recorded on the first and seventh nights of pairing in March–May 2017 (group 1, N=10), May–June 2018 (group 2, N=12) and April 2019 (group 3, N=6). Fish were paired to ensure a range of jaw length differences, but never with a fish they had previously interacted with. A plastic mesh divider was placed in the middle of a 56×30×37 cm tank, which allowed electrocommunication, but prevented aggression and physical interaction. At least 1 h before lights off, a pair of carbon electrodes was placed on each side of the tank. Electrodes were connected to an H6 Handy Recorder (Zoom, Hauppauge, NY, USA), which was left on overnight to record signals throughout the night. Signals were sampled 16 bit at 48,000 Hz, and the pre-amplifier on the recorder was placed at a setting between 6 and 8. After 1 week of social housing, fish were returned to their original tank. All but one fish was also recorded alone in their home tanks to collect a baseline recording of chirping during isolation.

Chirp analysis

Overnight recordings were subsampled in four 1-h sections for chirp counting: an hour before lights off, an hour after lights off, an hour in the middle of the night, and an hour before lights on. Chirps were counted using custom MATLAB (MathWorks, Natick, MA, USA) scripts written by G.T.S. (available upon request). The algorithm used by these scripts generated spectrograms (FFT window size=2048 samples; 95.3% window overlap) from recordings of each electrode pair in the tank. The center frequency of the spectrogram frequency bins with the highest power was used to estimate the EODfs more precisely. Putative chirps and rises were identified and counted in the third or fourth harmonic of the EOD, which provided greater separation of the EODfs of the two fish than the lower harmonics. The script first compared the spectrograms on the two recording channels and blanked the frequency bins corresponding to the EOD of the fish on the other side of the tank, so that each channel represented the EOD of the fish on the same side of the tank as the recording electrodes for that channel.

Putative chirps and rises were identified in the recordings with three criteria: EOD FM, rate of EODf change, and spectral ratio. To identify putative chirps based on FM, EODf was Gaussian filtered at 0.3 s, and the baseline EODf was calculated as the running mode of EODf (rounded to the nearest Hz) in 30 s windows. Putative chirps and rises were defined as events in which the filtered EODf (in the third or fourth harmonic) exceeded the baseline EODf by at least 15 Hz for at least 8 ms. Chirps and rises also had to be at least 0.25 s apart. To identify chirps based on the rate of EODf change, the first derivative of EODf was rectified and Gaussian filtered (0.3 s). Putative chirps were identified as events in which EODf changed at a rate greater than 125 Hz s^{-1} and in which the mean EODf rate change was greater than 90 Hz s^{-1} for at least 8 ms. Chirps were also identified based on spectral ratios. During chirps, the power at the baseline EOD frequency declines, while the power in frequencies above the baseline EOD frequency increases. To calculate spectral ratios, the power in the frequency bin corresponding to the baseline EODf was divided by the mean power in the six frequency bins above the baseline EODf for each time point in the spectrogram. The spectral ratio was $\log_{10}$ transformed and Gaussian filtered (0.3 s). Putative chirps were identified as events during which the log-transformed spectral ratio fell below 0.5 for at least 8 ms.

To confirm chirps and distinguish them from noise in the recordings, we used two discriminate function analyses (DFAs) on putative chirp parameters. One DFA was used to distinguish noise from chirps or rises, and a second DFA was used to distinguish chirps from rises. The discriminate functions were developed with 6342 putative chirps that were manually categorized as chirps, rises or noise. In the training dataset, the discriminate functions correctly categorized putative chirps as chirps, rises or noise with 97.3% accuracy. In addition to the automated chirp counting, all files were manually checked to ensure that any chirps or rises missed or misclassified by the algorithm were counted or corrected.

Blood sampling, dissection and hormone assays

After all behavioral experiments were completed, blood samples were collected to measure hormone levels, and fish were dissected on the same day to confirm sex and reproductive condition (group 1: December 2017; group 2: September 2018; group 3: May 2019). We measured the EODf within 2 weeks of dissections (using the same method as baseline EODfs as above) and then lightly anesthetized each fish with 0.1% 2-phenoxyethanol (Sigma-Aldrich, St Louis, MO, USA) in tank water. Body mass, body length and jaw length (from the tip of the upper jaw to the rictus) were measured. Blood (~20–40 µl) was collected from the caudal vein using a 1 ml syringe and a heparinized 25 G×5/8″ needle. Fish were placed back in anesthetic for several minutes until fish were deeply anesthetized and unresponsive. Brains were removed for potential use in a later study. Sex was confirmed by inspection of the gonads, which were removed and weighed to assess reproductive condition. Gonadosomatic index (GSI) was calculated as 100×gonad mass/body mass. Blood samples were placed in microhematocrit tubes and were centrifuged for 8 min at 4400 ***g*** to extract plasma. Plasma samples were stored at −80°C. Testoterone and 11-KT concentrations were determined by ELISA (Cayman Chemical, Ann Arbor, MI, USA). Plasma samples were diluted in assay buffer (testosterone 1:30, 11-KT dilutions ranged from 1:40 to 1:50). Each sample was assayed in duplicate. Intra-assay variation was calculated using the coefficient of variation of the six replicate wells distributed across the plate containing the 62.5 pg ml^{-1} standard (testosterone) or the 12.5 pg ml^{-1} standard (11-KT). Intra-assay variation was 12.9% for the testosterone assay and 13.1% and 5.4% for each of the 11-KT assays. The inter-assay variation between the two 11-KT assays was 9.0%. The minimal detection limit of the 11-KT assay kit was 0.78 pg ml^{-1}, and the minimal detection limit of the testosterone assay was 3.9 pg ml^{-1}. Because samples were diluted (1:40–1:50 for 11-KT; 1:30 for testosterone), the minimum detectable concentration in the samples was 31.2–39 pg ml^{-1} for 11-KT and 117 pg ml^{-1} for testosterone.

Statistical analysis

Results were analyzed in R (https://www.r-project.org/, v 3.6.1) and most figures were plotted using the ggplot2 package (Wickham, 2016). Some multi-panel figures also required the use of the cowplot package in R (https://CRAN.R-project.org/package=cowplot). Effect sizes for paired Wilcoxon tests were calculated using the rstatix package (https://CRAN.R-project.org/package=rstatix), in which values between 0 and 0.3 were considered small effects, values between 0.3 and 0.5 were considered moderate effects, and values over 0.5 were considered large effects.

Morphology, EODf and artificial playback recordings

Relationships between jaw length, hormones and signal parameters (EODf, chirp rate, duration, FM) in the artificial playback paradigm were analyzed with linear regressions. A paired Wilcoxon signed-rank test was used to examine the change between arrival and final EODf.

Live behavioral data

A principal components analysis (PCA) was performed on all aggressive behaviors to assess the relationships between nips, lunges, charges and jaw gapes. Most analyses did not produce normally distributed residuals owing to zero-inflated count data. Paired Wilcoxon signed-rank tests were used to analyze paired differences between dominants and subordinates and the change in EODf after social interaction in the live dyads. *P*-values were adjusted for multiple comparisons using Benjamini–Hochberg correction. The variation in the change in EODf across status was assessed using a Kruskal–Wallis rank sum test. Generalized linear models (GLMs) were used to analyze the relationship between chirping and attacks in live-dyadic trials and the relationship between chirping and the absolute difference in EODf in overnight recordings. A negative binomial distribution was used because zero-inflated chirp count data led to overdispersion. Gaussian linear regression was used to assess relationships between the difference in

the number of attacks, chirps and jaw length between fish in live dyads, because GLM error distributions require non-negative values. For the same reason, the relationship between the change in EODf during live dyads between fish and the number of attacks was assessed with a linear regression. Linear regressions were also used to assess the relationship between the difference in jaw length and attack total in the dyads and the difference in jaw length and the number of chirps or the change in the number of chirps across weeks in the overnight recordings. General linear mixed models (GLMMs) fitted with a negative binomial distribution were used to analyze how chirp/rise rate changed overnight across a week of social housing with fish ID used as a random effect. GLMs were run using the MASS package (Venables and Ripley, 2002), GLMMs were run using the package glmmTMB (Brooks et al., 2017) and pairwise contrasts were analyzed with emmeans (https://CRAN.R-project.org/package=emmeans).

Temporal analyses

The temporal relationships between EOD modulations and attacks both within and across fish were analyzed by generating probability distributions of the times of chirps or rises relative to the times of reference events (i.e. chirps, rises or attacks of the same fish or of the other fish in the dyad). The following temporal relationships were analyzed: chirps of the subordinate fish relative to chirps of the dominant fish; rises of the subordinate fish relative to rises of the dominant fish; chirps relative to attacks of the same fish; rises relative to attacks of the same fish; chirps relative to attacks of the other fish; rises relative to the attacks of the other fish. We also conducted these analyses separately for different attack types (nips, lunges, charges, jaw gapes). Charges and jaw gapes were rare, however, and the patterns of temporal relationships for the nips and lunges were similar to each other. Consequently, we report only the temporal relationships for the pooled attack types. These relationships were analyzed using custom MATLAB (MathWorks) scripts written by G.T.S. (available upon request). For each temporal analysis, a matrix of time differences between each chirp or rise and the reference event was generated. All time differences in the range of ±12 s were convolved with a Gaussian filter with a standard deviation of 0.5 s and were summed across all reference events. The filtered and summed time differences were normalized by dividing by the number of reference events to generate a temporal probability distribution of the chirps or rises relative to the time of the reference event. The null hypothesis was that the chirps or rises that occurred within ±12 s of the reference event would be randomly distributed within that time window. To generate null distributions, the times of all chirps or rises that occurred within ±12 s of a reference event were randomly shuffled within ±12 s of the reference event. The randomly shuffled time differences were then Gaussian filtered, summed and normalized as above. This process was repeated 10,000 times to generate a 95% confidence interval for the null probability distribution. A similar process was used to analyze temporal relationships of the chirps of the two fish in the overnight recordings, except that a window of ±32 s relative to the reference event was used because the peak of the temporal probability distribution was broader for the overnight recordings than for the dyadic interactions.

Chirps and rises compared between artificial playback experiments and live interaction

Chirp rates and magnitude of rises were not normally distributed, so a paired Wilcoxon signed-rank test was used to determine whether chirp rates or magnitude of rises [or jamming avoidance response (JAR)-like EODf modulations] differed between the live interactions and artificial playback experiments.

RESULTS

Morphology and EODf

Species identification, jaw length and body size

COI sequences of fish in this study clustered phylogenetically with those of 13 *C. samueli* from the NCBI database and were distinct from those of *C. compsa* (Fig. S1). Males varied in size, morphology and reproductive condition. Body mass was 12.4±0.7 g (mean±s.e.m., range 5.2–19.7 g). Most fish were not in reproductive condition (GSI=0.22±0.05%, range 0.05–1.46%). Jaw length varied substantially across males (2.3±0.2 cm, range 1.1–5.0 cm), but was not correlated with reproductive condition [linear regression model (LRM): β=0.49, R^2=0.002, P=0.31]. Notably, the only fish that had a GSI indicative of robust reproductive condition (1.46%) had intermediate jaw length (2.1 cm). Total body length was 20.1±0.4 cm (range 13.4–25.5 cm) and explained 30% of the variance in jaw length (LRM: β=0.202, R^2=0.298, P<0.001). With jaw length subtracted from total body length, however, the rest of body length was unrelated to jaw length (LRM: β=0.102, R^2=0.031, P=0.153). Because jaw length itself explained variation in body length, we used jaw length in further analyses.

EODf and jaw length

EODf varied across males. Within the first 2 weeks of arriving in the laboratory, jaw length explained 22% of the variance in EODf (Fig. 1A). This relationship between EODf and jaw length disappeared after a month or more in the laboratory (Fig. 1B). Moreover, EODf of most fish declined after the fish had been in the laboratory (Fig. 1C).

EOD modulations in response to artificial playback

EOD modulations varied within and between individuals

Like many other apteronotid species, *C. samueli* produced two types of EOD modulations: chirps (Fig. 2A,B) and rises. Chirps had large FMs (26.5–807.8 Hz) and short durations (0.01–0.19 s), whereas rises had modest EODf increases (1.6–57.3 Hz) and variable durations (0.005–26.5 s; Fig. 2C). Variation in chirp FM and duration was mostly continuous (Fig. 2C), but very few chirps had FMs between 480 and 520 Hz, and chirps with more than 480 Hz of FM always resulted in an extreme reduction in EOD amplitude that led to an EOD interruption (Fig. 2B). Only five fish produced chirps with FM greater than 480 Hz. The jaw length of fish that produced these chirps did not differ from that of fish that produced only smaller chirps (Mann–Whitney: P=0.13). Chirp structure varied both within and between individuals (Fig. 2D). Chirp rate did not differ significantly in response to any of five playback frequencies. Chirp parameters (duration, FM, rate) were not correlated with jaw length (LRM: R^2=–0.03 to 0.08, P=0.27–0.79).

Steady-state increases in EODf and rasps

Steady-state increases in EODf, which were like JARs in other electric fish (Dye, 1987; Metzner, 1993), were elicited by artificial playbacks (Fig. S2A). Unlike JARs in other species, these JAR-like responses were elicited not only by playbacks with frequencies close to the fish's own EODf, but also by frequencies distant from the fish's EODf (Fig. S2B). Fish also occasionally produced rasps, trains of rapid, small (5–30 Hz) oscillations of EODf (Turner et al., 2007) (Fig. S2C,D).

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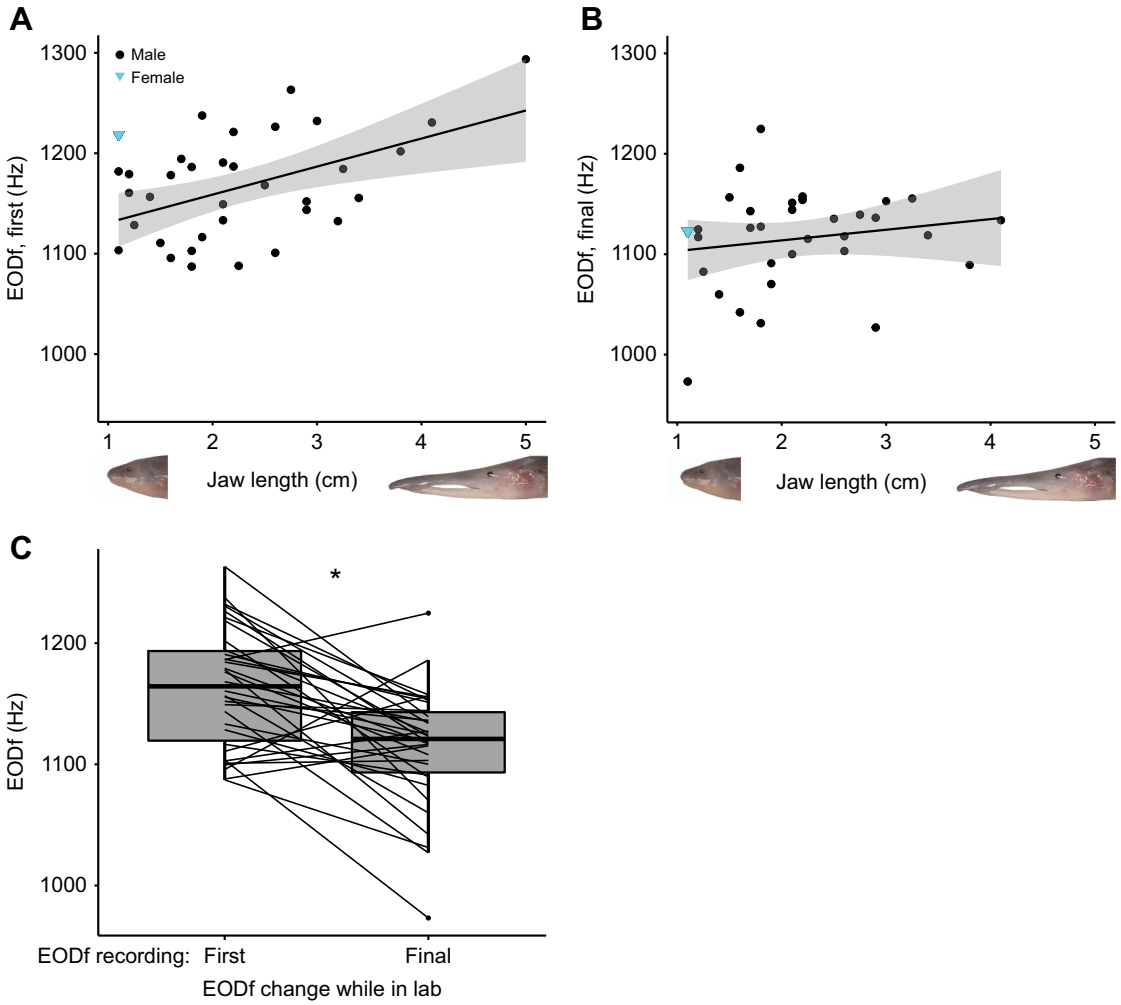

Fig. 1. Jaw length and electric organ discharge frequency (EODf). (A) EODf (N=36, mean±s.e.m.: 1165±9 Hz) within 2 weeks of arrival into the laboratory correlated with jaw length (LRM: β=27.93, R^2=0.24, P<0.01). Female (teal triangle) was excluded from the regression. Light gray shading indicates the 95% confidence intervals (CI). (B) Last measured EODf (N=34, 1115±8 Hz), after at least 1 month in the laboratory, was not correlated with jaw length (LRM: β=11.71, R^2=0.03, P=0.3). Initial EODf was not measured in one fish, and final EODf was not measured in three fish. (C) First versus final EODf, including the female (N=34). Black line and boxplots indicate median and interquartile range (IQR). Whiskers extend to smallest and largest values, at a maximum of 1.5×IQR. Points outside whiskers are outliers. Lines connect first versus final EODf of individual fish. Wilcoxon: *P<0.001, effect size=0.42.

Androgens, communication signals and jaw length

Plasma 11-KT concentrations were 252±48 pg ml^{-1} (range: 8–1203 pg ml^{-1}), and plasma testosterone concentrations were 119±8 pg ml^{-1} (range: 67–187 pg ml^{-1}). Other than a weak correlation between testosterone and chirp rate in the dyadic encounters (LRM: β=5.2, R^2=0.19, P=0.04), 11-KT and testosterone were not correlated with jaw length, EODf or chirping (chirp rate, duration or FM during artificial playbacks or chirp rates during live encounters) (LRMs: R^2=–0.01 to 0.06, P=0.2–0.95).

EOD modulations varied with jaw length, aggression and status during dyadic trials

Aggressive behaviors

A PCA assessed whether aggressive behaviors (nips, lunges, charges and jaw gapes) were correlated with each other (N=84 fish). Two components explained 79.2% of the total variance. PC1 explained 53.8% of the variance and loaded primarily with nips, lunges and charges. PC2 explained 25.4% of the variance and loaded primarily with jaw gapes. Dominant individuals varied more in aggressive behaviors, whereas the principal components of agonistic behaviors from subordinate fish and fish with no established status were clustered. Because different types of aggressive behaviors were correlated with each other, all aggressive behaviors were pooled for remaining analyses. Shelter tube entries (9/45 trials) and parallel and antiparallel swimming (11/45 trials) were infrequent and were not analyzed further. Although fish occasionally displayed jaw gapes, jaw locking, which has been observed previously in *C. samueli* (Evans et al., 2019), was not seen in the dyadic trials. This might be because most pairings in this study were between long-jawed and short-jawed fish, and jaw displays and jaw locking are most often produced in encounters between long-jawed males (Evans et al., 2019; Triefenbach and Zakon, 2008).

Jaw length, EOD modulations and attacks

Fish that attacked more chirped more (Fig. 3A) and produced more rises (Fig. 3C). Between fish in a dyad, the difference in attack rate explained 27% of the variance in the difference in chirp rate (Fig. 3B) and 13% of the variance in the difference in rise rate (Fig. 3D). However, the difference in jaw length between the two fish in the dyad did not predict the total number of attacks (LRM: β=4.13, R^2=–0.016, P=0.56) nor the difference in the number of chirps, rises or attacks (LRMs: β<5.4, R^2<–0.016, P>0.55; Fig. S3).

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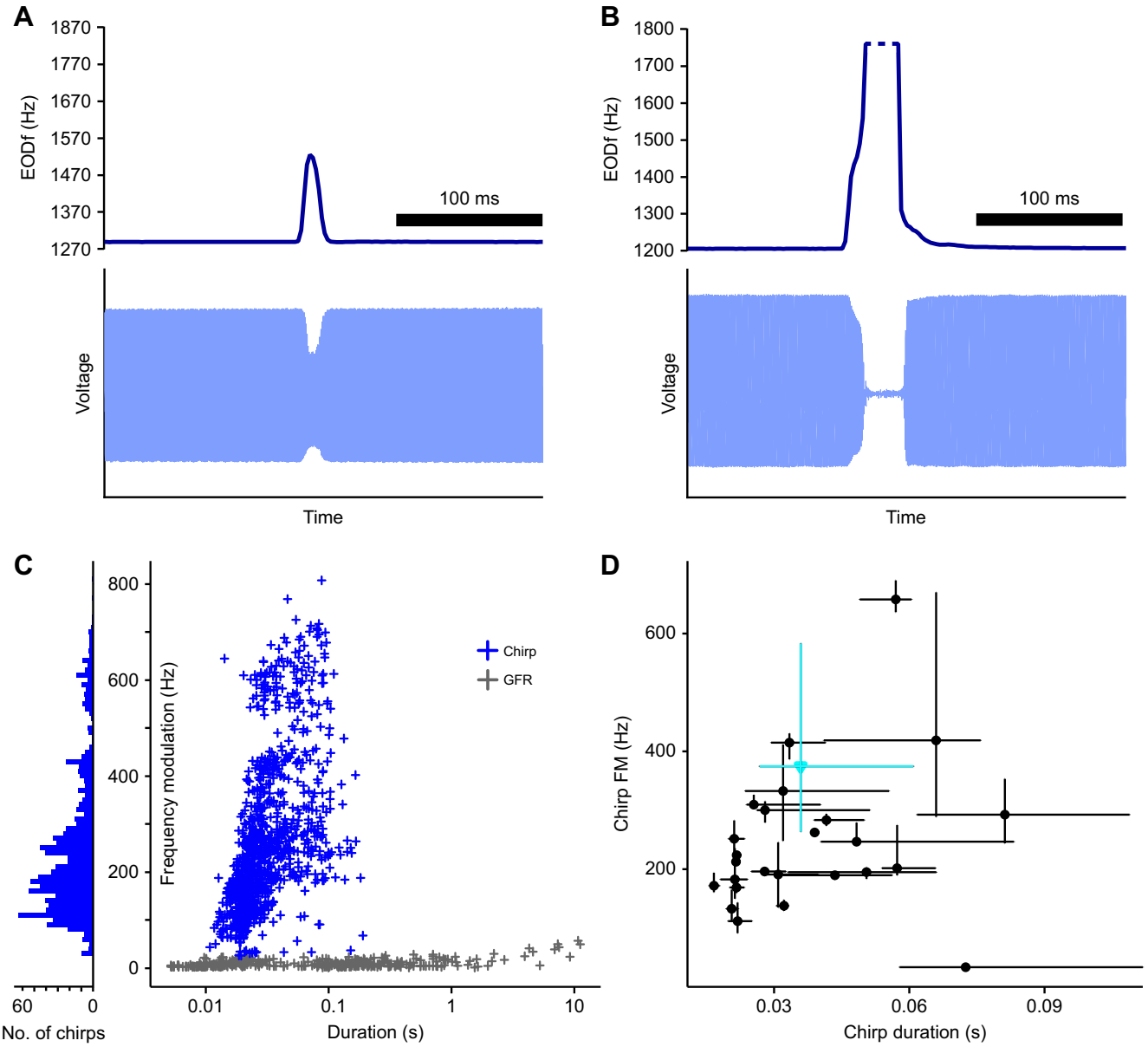

Fig. 2. EOD modulations varied within and across individuals. (A) EODf (top) and head–tail voltage (bottom) during a small chirp. Note the frequency increase concurrent with the decrease in EOD amplitude. (B) Large chirp. Note the extreme reduction in EOD amplitude that interrupted the EOD. Dashed line in frequency trace represents time during which EOD amplitude was too low to measure EODf. (C) Histogram of chirp frequency modulation (FM, left of *y*-axis) and scatter plot of FM and duration (right of *y*-axis) for all chirps (blue crosses) and rises (gray crosses). Classification of chirps versus rises follows Turner et al. (2007). When FM exceeded 480 Hz, all chirps resulted in EOD interruptions. (D) Chirp FM and duration (median±IQR) of individual fish (N=27). Note the variation in chirp FM and duration both across and within individuals. Teal point indicates the female. Point in lower right is from a male that produced a few EOD modulations whose classification as chirps versus rises was somewhat ambiguous, but which met our operational definition of chirps.

Status varied with jaw length and behavior

Dominant fish attacked more than subordinate fish and had a shorter attack latency (Table 1). In dyads with clear hierarchies, the longer-jawed fish usually dominated the shorter-jawed fish (26/38 pairs), although sometimes shorter-jawed fish did win contests (Fig. 4). Dominant fish also produced more chirps and rises than subordinate fish (Table 1). Baseline EODf and total body length did not vary by status (Table 1).

Change in EODf

Except for two fish that decreased EODf, every fish rapidly increased EODf by 27.2±16.3 Hz after dividers were removed at the beginning of the trial (Fig. 5A). EODf 1–2 min into the dyadic trial (1110.0±40.3 Hz) was greater than EODf 1–2 min before dividers were removed (1082.8±36.8 Hz) (Fig. 5B). The EODf increase did not differ between dominant versus subordinate fish or fish in dyads with unresolved status (Kruskal–Wallis: P=0.137; Fig. 5B). However, 77% of the variance in one fish's EODf increase was explained by the other fish's EODf increase (Fig. 5C). Within fish, the EODf increase explained 7% of the variance in its attack rate (Fig. 5D).

Temporal relationships between EOD modulations and aggression

Distribution of attacks and EOD modulations

Attacks and EOD modulations were uncommon in the first minute of a trial. Fish chirped most in the second and third minutes of a trial.

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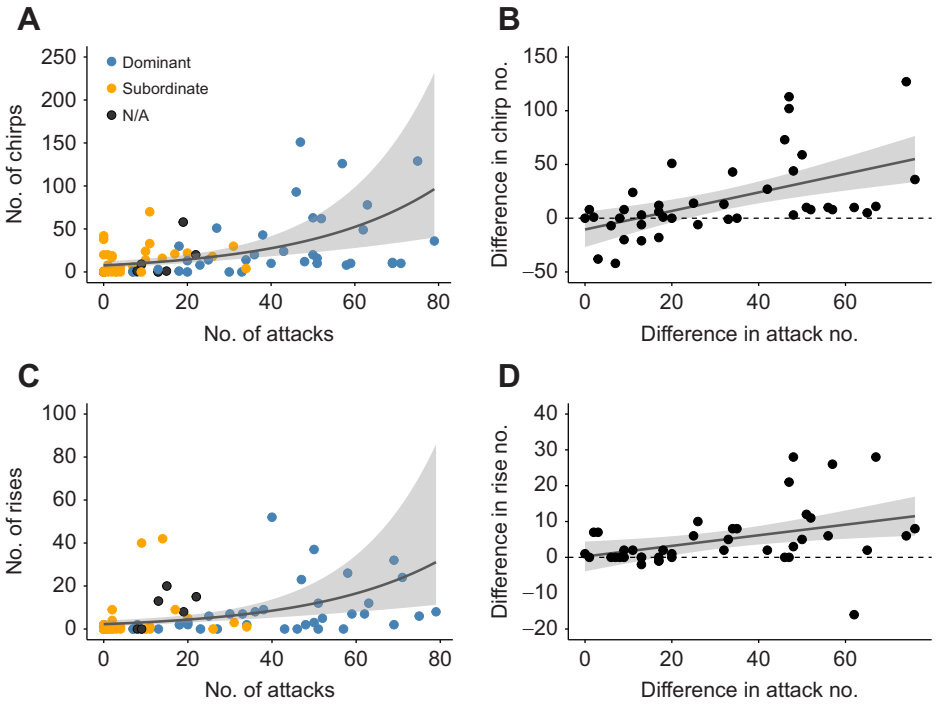

Fig. 3. Chirp and rise production were related to attack frequency. (A,C) Socially interacting males (N=82) chirped more (A: negative-binomial regression: P<0.001) and produced more rises (C: negative-binomial regression: P<0.001) when they attacked more. Each point represents the number of attacks and chirps/rises a fish produced during a trial. N/A refers to fish in trials with no established hierarchy. (B,D) Within a trial (N=41 trials), the fish that attacked more than the other fish chirped more (C: LRM: β=0.863, R^2=0.267, P<0.001) and produced more rises (D: LRM: β=0.148, R^2=0.132, P=0.011) than the other fish. Each point represents the difference between the two fish in the number of attacks and chirps for a single trial, with the fish that attacked more as the reference. Light gray shading indicates the 95% CI.

Dominant chirp rates remained high throughout the rest of the trial (Fig. S4B), whereas subordinate chirp rates declined steadily in the second half of the trial (Fig. S4E). Attacks by dominant males increased throughout the trial (Fig. S4A), but subordinate attacks increased only slightly after 1 min and stayed low throughout the trial (Fig. S4D). The temporal dynamics of rises mirrored that of attacks in both dominants and subordinates (Fig. S4). Like attacks, dominant rise production increased throughout the trial (Fig. S4C). In fish with no established hierarchy, attacks and EOD modulations did not usually occur until 3 min into a trial (Fig. S4G–I). Rates of attacks, chirps and rises peaked at 5–6 min in these trials, but declined by the end of the trials (Fig. S4G–I).

EOD modulations and attacks were temporally related within fish

Both dominants and subordinates were most likely to produce chirps and rises just before they attacked (Fig. 6). Dominants were significantly more likely to chirp 1.6–0.3 s before an attack (peak 1 s before attacking; Fig. 6A). Subordinates were more likely to chirp 1.9–0.2 s before attacking (peak of 1.1 s before attacking; Fig. 6B). Dominants were more likely to produce rises between 1.4 s before and 0.5 s after attacking (peak 0.6 s before attacking; Fig. 6C). Subordinates were more likely to produce rises 1.4–0.2 s before an attack (peak of 0.9 s before attacking; Fig. 6D).

EOD modulations were temporally related across fish

Like the 'echo response' in other apteronotids (Hupé and Lewis, 2008; Zupanc et al., 2006), one fish was more likely to produce a chirp just after the chirp of the other fish (Fig. 7A). Compared with the null distribution, subordinate fish were more likely to chirp between 1.5 s before and 2.7 s after the dominant fish chirped, with a peak probability at 0.3 s after the dominant (Fig. 7A). Rises were also often concurrent. Subordinate fish were more likely to produce a rise within 0.7 s before or after a dominant fish's rise (Fig. 7C). These modulations often occurred in bouts lasting 1–2 min and were more common during periods of intense aggression. Subordinates also produced rises at a higher probability 1.4–0.2 s before a dominant attacked, peaking 0.9 s before being attacked (Fig. 7D). However, subordinate chirping was unrelated in time to dominant attacks (Fig. 7B). Subordinates attacked significantly less than dominants, and temporal relationships between subordinate attacks and dominant chirps were not significant.

Chirping during long-term overnight pairings

EOD modulation rate varied across the night and week

A negative binomial GLMM with chirp rate as the dependent variable was fitted using jaw length, EODf, hour of the night, week of recording, and the interaction between hour of the night and week of recording, with fish ID as a random effect. Hour of night, week of

Table 1. Paired differences in behavior and morphology between dominant and subordinate fish

	Dominant	Subordinate	P	Effect size
Chirps per minute	4.38±0.92	1.68±0.37	**<0.01**	0.521
Rises per minute	1.90±0.28	0.48±0.22	**<0.001**	0.620
Baseline EODf (Hz)	1084.34±6.37	1085.85±5.91	0.551	0.101
Attacks per minute	5.80±0.48	0.97±0.21	**<0.001**	0.872
Attack latency (s)	118.03±14.38	231.76±22.23	**<0.001**	0.721
Body length (cm)	20.83±0.25	20.10±0.37	0.342	0.172
Jaw length (cm)	2.38±0.11	1.92±0.10	**<0.01**	0.498

Dominant and subordinate values are means±s.e.m. P-values and effect sizes are from Wilcoxon tests with Benjamini–Hochberg correction (N=38 pairs for attacks min^{-1}, attack latency, body length, and jaw length; N=37 pairs for chirps min^{-1} and rises min^{-1}; N=36 pairs for baseline EODf). Bold indicates statistically significant pairwise differences.

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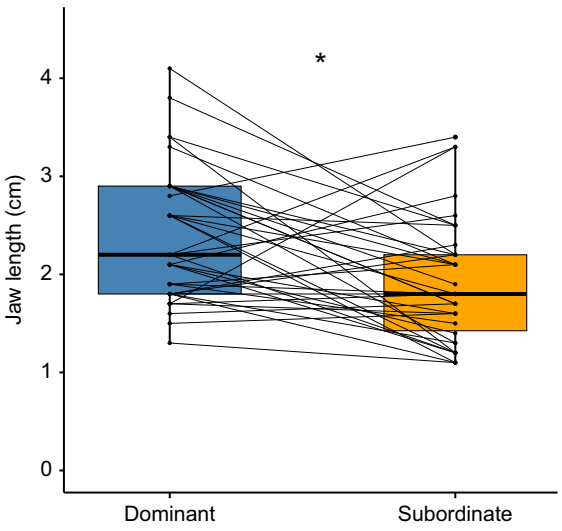

Fig. 4. Jaw length (median±IQR) of dominant and subordinate males. Lines connect males in each pairing (N=38 pairs). Dominant males had longer jaws than subordinate males in 26/38 trials (Wilcoxon: *P<0.01). Whiskers extend to the smallest and largest values, at a maximum of 1.5×IQR. Outlier represented as single point outside whiskers.

recording and their interaction were significant (all P<0.001), while jaw length (P=0.40) and EODf (P=0.91) were not. To simplify the number of contrasts, the temporal pattern of chirping across the night was analyzed by hour just on the first night of pairing, when chirp rate was highest. Chirp rate varied across hour (P<0.001), but was unrelated to jaw length (P=0.53) or EODf (P=0.35). Except for a few individuals, chirping was negligible while the lights were on. Chirp rate was highest in the hour after lights went off, decreased in the middle of the night, and increased moderately in the hour before lights-on (Fig. 8A). To examine how chirp rate changed across isolated and social housing weeks, variation in chirp rate across the night was accounted for by including hour in the random effect. Chirp rate varied across housing conditions (P<0.001), but was unrelated to jaw length (P=0.15) or EODf (P=0.42). Fish chirped more on the first night of social pairing and after 1 week paired compared with in isolation (Fig. 8A). Fish also chirped more on the first night of social pairing than 1 week later (Fig. 8A).

Rises followed a similar pattern to chirping. In a negative binomial GLMM with number of rises as the response variable and fish ID as a random effect, hour of night (P<0.001) and week of recording (P<0.001) were significant, while their interaction (P=0.32), jaw length (P=0.31) and EODf (P=0.98) were not. On the first night paired, rise rate varied by hour (P<0.01), but was unrelated to jaw

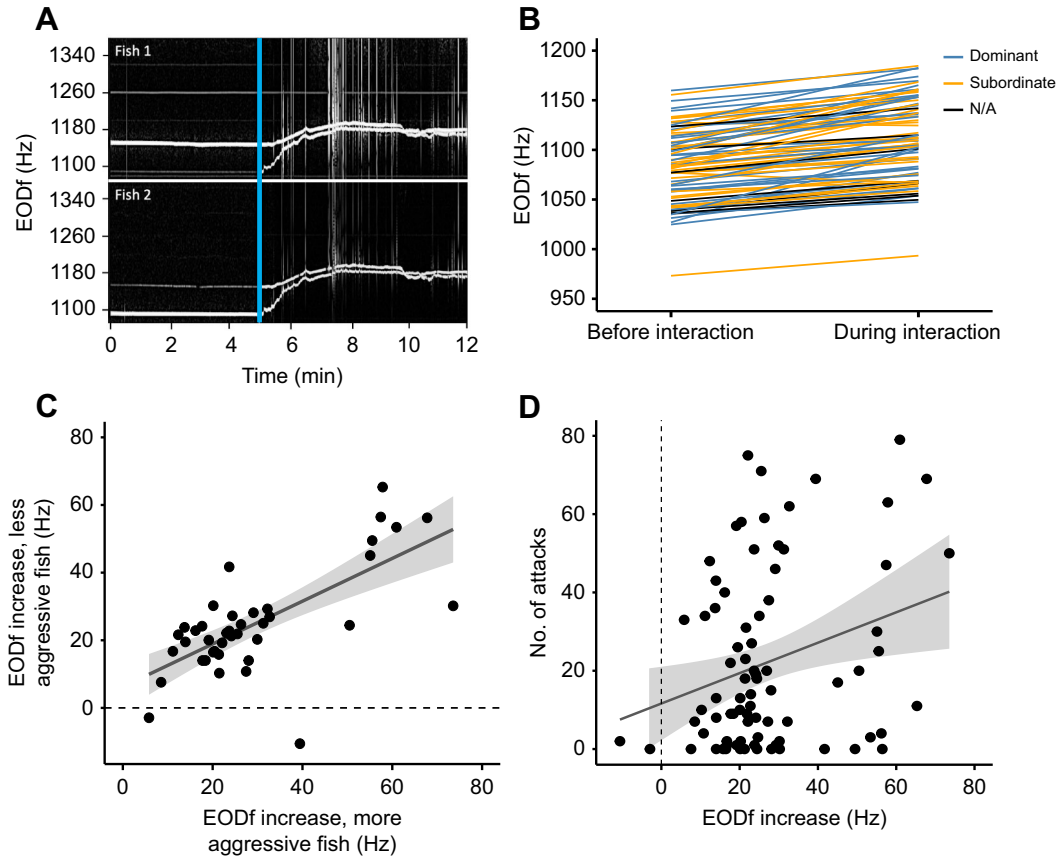

Fig. 5. EODf increases were correlated between fish and related to aggression. (A) EOD traces from two fish showing EODf increases during a dyadic trial. The first 5 min was a baseline recording. At 5 min (blue vertical line), the dividers were raised, and both fish increased their EODf. Top panel shows the higher EODf fish (Fish 1); lower panel shows the lower EODf fish (Fish 2). When dividers were removed, both channels detected EODs of both fish. The abrupt vertical lines on EOD traces are chirps. (B) After 1 min of social interaction, EODf increased in both dominants and subordinates (Wilcoxon: P<0.001, effect size=0.864). Each line represents one fish during one trial (N=80). N/A, fish in trials with no established hierarchy. (C) Magnitude of EODf increase was correlated between males in a dyad, with the fish that attacked more as the reference (N=40 trials) (LRM: β=0.766, R^2=0.471, P<0.001). (D) The number of times a fish attacked was slightly higher when their EODf increase was higher (LRM: β=0.388, R^2=0.066, P=0.012). Light gray shading indicates the 95% CI.

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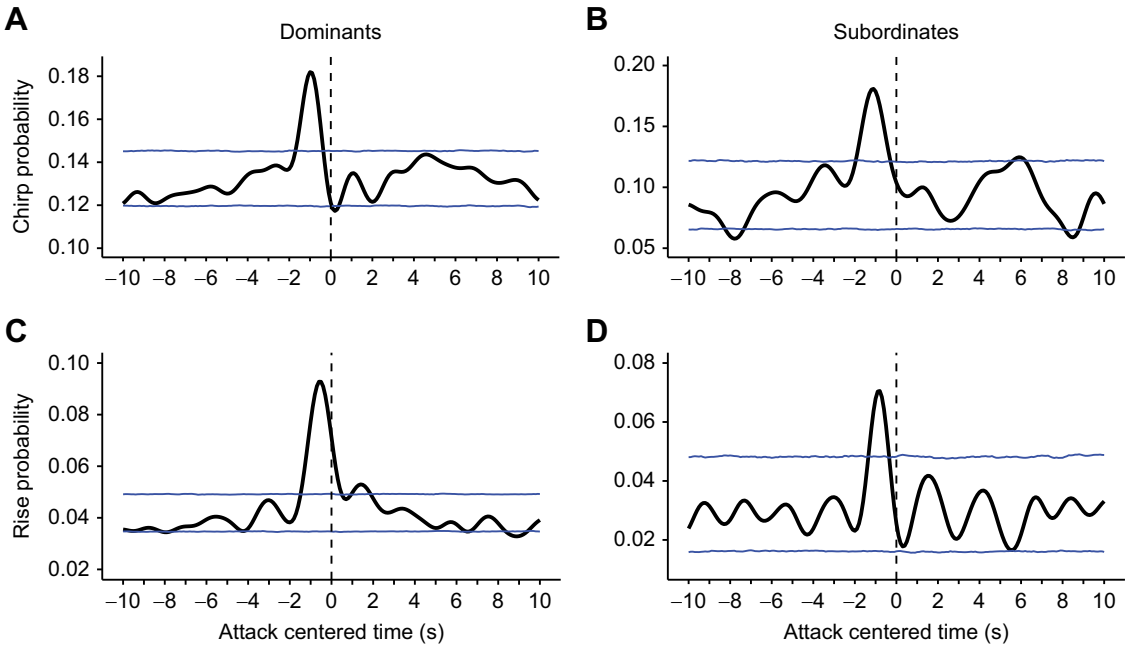

Fig. 6. EOD modulations preceded attacks in both dominants and subordinates. Probability that fish produced chirps or rises within 10 s before or after they attacked at time 0 [*N*=41 trials, 1612 dominant attacks (A,C), 239 subordinate attacks (B,D)]. Both dominants (A,C) and subordinates (B,D) were more likely than expected to produce chirps (A,B) or rises (C,D) just prior to attacking. Blue lines indicate the 95% CI of the null distribution.

length (P=0.10) or EODf (P=0.13). Rise rate was significantly higher in the hour before lights on only when compared with the hour before lights off (Fig. 8B). When hour was included in the random effect, rise rate changed across social housing conditions (P<0.001), but was unrelated to jaw length (P=0.35) or EODf (P=0.95). Rise rate was lower in isolated fish than on the first or seventh night of social pairing (Fig. 8B). Rise rate was also higher on the first night socially paired than after 1 week paired (Fig. 8B).

Chirp rate in the hour after lights-off on the first night (when chirp rate was highest) was unrelated to the difference in jaw length between fish (LRM: β=–81.59, R^2=0.057, P=0.14), but was higher when the two fish had closer EODfs (Fig. 8C). The change in chirp rate in the first hour after lights-off following 1 week of social pairing was also unrelated to the difference in jaw length (LRM: β=109.69, R^2=0.072, P=0.11). Very few rises were produced during overnight recordings, and we were unable to assess relationships between rise rate and jaw length or EODf.

Chirps were clustered across fish

As in live dyads, chirping was temporally related in an echo response in overnight recordings. Compared with the null distribution, the shorter-jawed fish was more likely to chirp between 7.4 s before and 7.8 s after the longer-jawed fish (Fig. 8D). The peak of the distribution was +0.3 s, indicating there may have been a slightly higher probability that the shorter jawed fish chirped after the longer jawed fish (Fig. 8D). Fish did not produce enough rises to analyze the temporal relationship between them in this context.

Communication signals differed between artificial and live stimuli

Fish chirped more during their first live dyadic interactions than in response to artificial playback (Fig. 9A). Chirp rates elicited by artificial playbacks were unrelated to chirp rates in live interactions (LRM: β=–0.03, R^2=0.004, P=0.76). We also compared the JAR-like EODf increases produced by fish in response to artificial playback with those produced during live interactions. EODf increases were larger in live interactions than in response to playback (Fig. 9B). EODf increases in response to artificial playback were not correlated with EODf increases during live interactions (LRM: β=0.27, R^2=0.04, P=0.63).

DISCUSSION

Morphological variation across males is often related to variation in androgen levels, agonistic behavior and communication signals (Brantley and Bass, 1994; Emlen, 1997; Hoskin et al., 2009; McGlothlin et al., 2008). In *C. samueli*, relationships between jaw length, aggression and electrocommunication signals were context-dependent. EODf correlated with jaw length when fish were brought in from the wild, but not after weeks of captivity. Longer-jawed males attacked more in dyadic interactions, suggesting jaw length is related to social dominance. Chirps and rises functioned as aggressive signals in both dominants and subordinates. Jaw length, however, was unrelated to chirp rate in response to artificial playbacks or when fish were separated by a mesh barrier during overnight interactions. Males also chirped more in live interactions than in response to playbacks. Androgens, however, were not correlated with morphology, chirping, EODf or dominance.

EODf, but not chirping, is related to male morphology

Some apteronotid species produce distinct chirp types (Turner et al., 2007). Two chirp types in *A. leptorhynchus* (Bastian et al., 2001; Hagedorn and Heiligenberg, 1985; Zupanc and Maler, 1993) are differentially produced in response to artificial playbacks of male versus female EODfs and have been proposed to serve different functions: big chirps for courtship and small chirps for agonistic encounters (Bastian et al., 2001; Hagedorn and Heiligenberg, 1985). During live interactions, however, subordinate *A. leptorhynchus* males sometimes produce big chirps, and small chirps are also produced during courtship, suggesting less clear-cut functions

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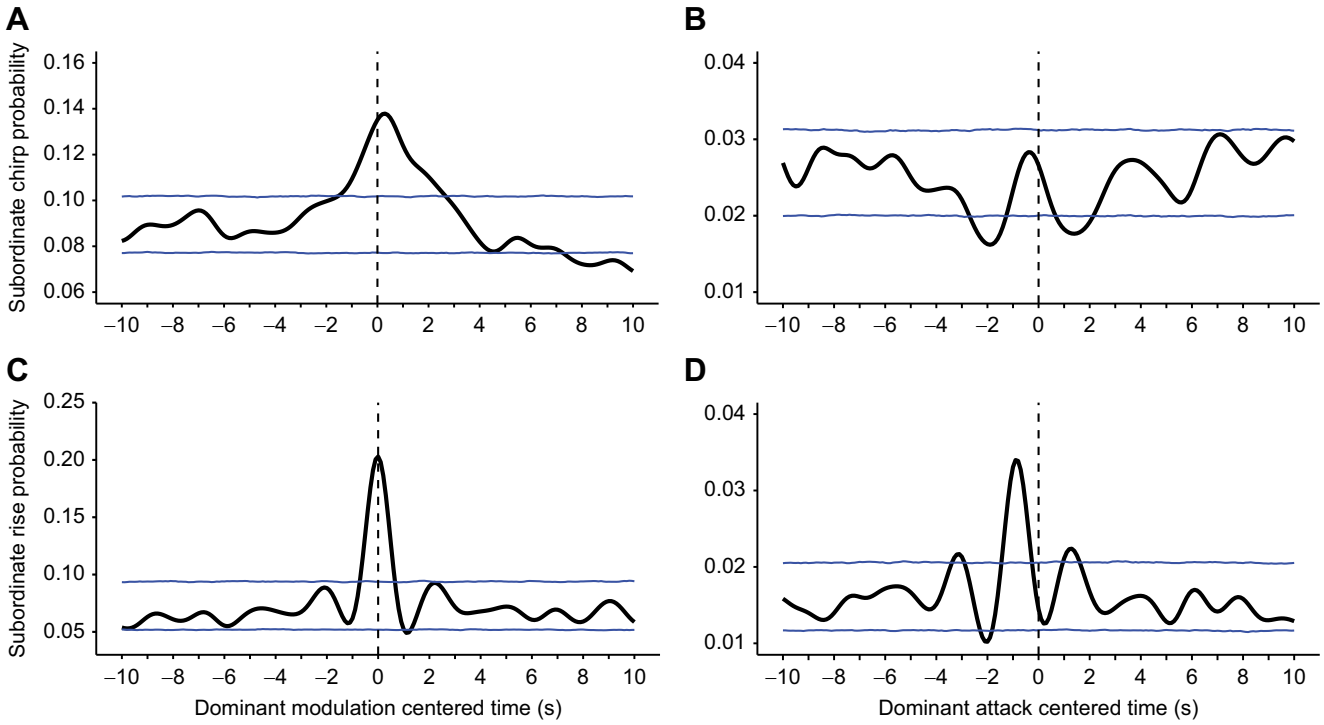

Fig. 7. EOD modulations were clustered in time across dominants and subordinates. Probability that subordinate fish produced chirps or rises within 10 s before or after dominant fish produced an EOD modulation (A,C) or attacked (B,D) at time 0 [*N*=41 trials; 1183 dominant chirps (A); 1612 dominant attacks (B,D); 329 dominant rises (C)]. (A) Subordinates were more likely to chirp around the time that dominants chirped. (B) Subordinate chirps were not related in time to dominant attacks. (C) The dominant and subordinate fish produced rises in sync with each other. (D) Subordinates were more likely to produce a rise just before a dominant attacked. Blue lines indicate the 95% CI of the null distribution.

for these signals (Cuddy et al., 2012; Henninger et al., 2018). In response to artificial EOD playbacks, *C. samueli* produced chirps with more than 480 Hz FM that interrupted the EOD and chirps with less FM that did not. Chirps that interrupt the EOD and those that do not might be functionally distinct signals like chirp types in *A. leptorhynchus*, or they could represent ends of a continuum of variation in FM and amplitude modulation. The five fish that produced the large chirps had intermediate jaw lengths, so the large chirps are not differentially produced by long-jawed fish. Distinguishing whether these putative chirp types have distinct functions requires examining contexts in which they occur and their effects on receivers. We were unable to discriminate between these putative chirp types in the live, dyadic and long-term recordings and thus could not determine how they were used during social encounters. Even if the large and small chirps are not discrete types, continuous variation in chirp parameters might still convey information. Graded variation in signals can encode motivation, urgency or escalation (Mager et al., 2012; Manser, 2001; Patricelli et al., 2004; Reichert and Gerhardt, 2013).

Chirp FM, duration and rate in response to playbacks did not vary with jaw length. Thus, chirp structure does not contain information about the signaler's jaw morphology. Relationships between communication signals and variation in male morphology have been studied in other knifefishes. In *S. nattereri*, males with teeth have higher EODfs and produce chirps with greater FM than toothless males (Cox-Fernandes et al., 2010; Ho et al., 2013). In *P. hasemani*, jaw length varies substantially across males, but EODf and chirping did not differ between long- versus short-jawed males (Petzold and Smith, 2016). In *C. samueli*, EODf varied with male morphology as in *S. nattereri*. However, unlike *S. nattereri*, but like *P. hasemani*, chirping did not vary with morphology. Thus, associations between electrocommunication signals and male morphology vary across species.

Relationships between androgens, jaw length and electrocommunication

Although longer-jawed males initially had higher EODf than shorter-jawed males, this correlation disappeared after fish were in captivity. A possible explanation for this is that relationships between EODf and jaw length might be maintained through social interactions. EODf, dominance, aggression and body size are sometimes correlated in other apteronotid species, and social interactions often influence EODf (Dunlap, 2002; Fugère et al., 2011; Hagedorn and Zelick, 1989; Oestreich and Zakon, 2005; Smith, 2013). The relationships between EODf, morphology and social rank could thus be an emergent property of long-term social interactions. Because the fish in this study were housed individually before experiments, the lack of social interaction might have disrupted relationships between dominance-related morphological traits (i.e. jaw length) and EODf.

Androgen levels were not correlated with jaw length. This parallels findings in *P. hasemani*, in which long-jawed males did not have greater androgen levels than short-jawed males (Petzold and Smith, 2016), but contrasts with findings in *S. nattereri*, in which males with toothed jaws had higher androgen levels than toothless males (Cox-Fernandes et al., 2010). The lack of a correlation between androgens and jaw length in the present study might reflect either that jaw length is not regulated by androgens in *C. samueli* or that jaw length might be influenced by androgens during jaw development, but not after jaws have grown.

Other than a weak correlation between testosterone and chirping in dyadic trials, androgen levels were unrelated to EODf or to chirping in any of the experimental paradigms. In species with sex differences

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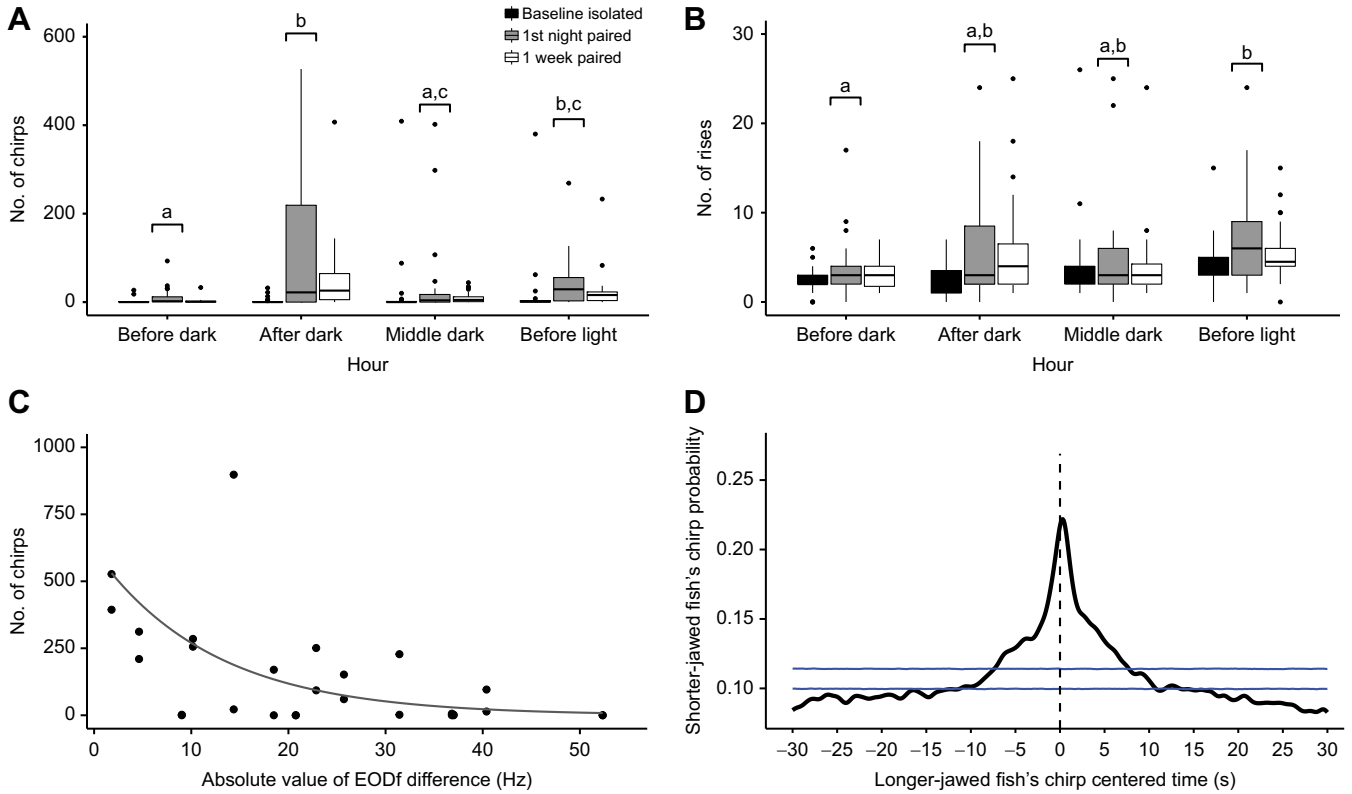

Fig. 8. EOD modulation rate varied across overnight recordings. Number of chirps and rises while isolated, first night socially paired and after 1 week paired, counted in 1 h segments throughout the night (N=28 fish). (A) On first night paired, chirp rate varied by hour (P<0.001). Significant differences across hours on first night paired are denoted by letters. Fish first-night paired (P<0.001) and 1-week paired (P<0.001) chirped more than isolated fish. Chirp rate was higher on first night paired than 1-week paired (P<0.01). Boxplots indicate median and IQR. Whiskers extend to the smallest and largest values, at a maximum of 1.5×IQR. Single points outside whiskers are outliers. Six outliers outside y-axis range not shown. (B) On first night paired, rise rate varied by hour (P<0.01). First-night paired (P<0.001) and 1-week paired (P=0.04) fish produced more rises than isolated fish. Rise rate was higher on first night paired than 1-week paired (P=0.04). Four outliers outside y-axis range not shown. (C) After dark on first night paired, fish that were closer in EODf chirped more (negative-binomial regression: P<0.01). (D) Probability that the shorter-jawed fish chirped within 30 s of when the longer-jawed fish chirped at time 0 (N=4328 longer-jawed fish chirps). Shorter-jawed fish were more likely chirp around the time the longer-jawed fish chirped. Blue lines indicate the 95% CI of the null distribution.

in EODf and/or chirping, these signals are androgen-sensitive and often correlated with 11-KT (reviewed in Smith, 2013). This is the first study of EODf and chirping in *C. samueli*. Because we examined only one female, we do not know whether these signals differ between sexes. The paucity of correlations between androgens and EODf or chirping might suggest that these signals are sexually monomorphic and/or androgen-insensitive in *C. samueli*. In apteronotid species with sexually monomorphic EODf and/or chirp rate, these signals are insensitive to and/or uncorrelated with androgens (Dunlap et al., 1998; Ho et al., 2013, 2010; Petzold and Smith, 2016). Alternatively, because many blood samples were taken a month or more after behavioral trials, androgen levels might have changed during this time and obscured relationships between chirping and hormone levels. Additionally, most males were not in reproductive condition. Only two males had GSIs or 11-KT levels indicative of breeding condition. Androgen levels might be correlated with EODf and/or chirping only in breeding males, in which case we would not have observed such a correlation in the non-reproductive males in this study. Additional studies in both sexes and in breeding *C. samueli* would be needed to test these hypotheses.

Jaw length is related to dominance, but indirectly related to behavior

Weapons or ornaments that communicate fighting ability can serve as status badges (Tibbetts and Dale, 2004). However, weapon or ornament size does not always accurately predict fighting outcomes (Graham et al., 2020). In *C. samueli*, jaw length was associated with, but did not always predict, contest outcome. Males with longer jaws more often dominated shorter-jawed males, but shorter-jawed males did sometimes win contests. Subordinates avoid fights with dominants to prevent injury, whereas closely matched rivals are often more aggressive (Barki et al., 1997; Jennions and Backwell, 1996; Tibbetts and Lindsay, 2008). In *C. samueli*, however, the difference in jaw length between opponents did not correlate with attack rate. This may be partly explained by substantial variation in aggression across dominants, whereas subordinates were less aggressive, regardless of who they were paired with. Thus, although jaw length varies with status, the difference in jaw length between two competitors does not necessarily predict how aggressive they will be. Indeed, the competitive interactions during dyadic trials of *C. samueli* were qualitatively less overtly aggressive than those of some other apteronotids (Serrano-Fernández, 2003; Triefenbach and Zakon, 2008). This may be consistent with the fact that long jaws in *C. samueli* function as an ornament in ritualized agonism rather than as a weapon as they do in some other apteronotids (Evans et al., 2019; Keeffe et al., 2019).

The difference in jaw length was also not associated with differences in chirp or rise rate. Dominant fish did, however, produce more chirps and rises. As with attacks, the lack of a direct relationship between the difference in jaw length and difference in

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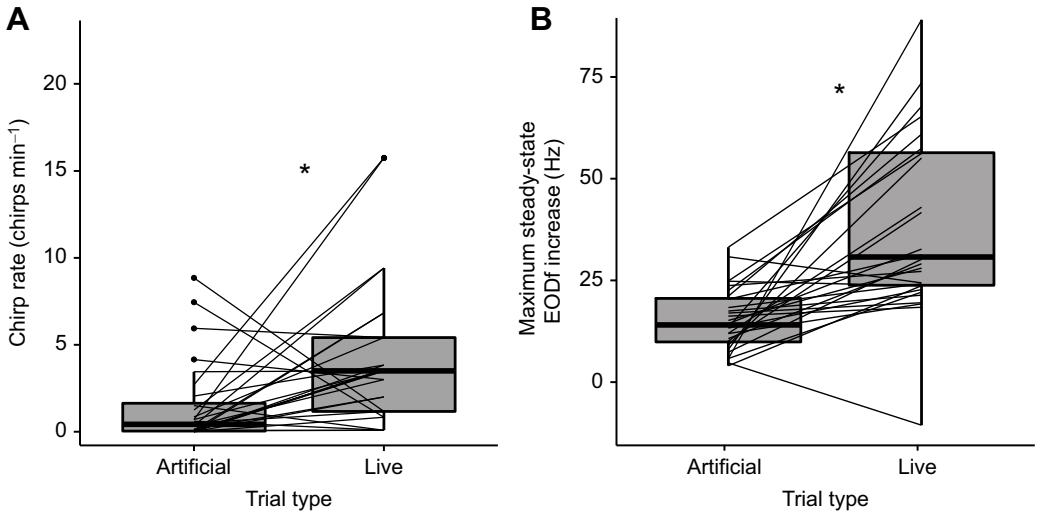

Fig. 9. Chirp rate and steady-state EODf increases differed across trial types. (A) Points connected by a line indicate chirp rates of the same fish (*N*=27) in response to artificial playback versus during live dyadic interactions. Chirp rate was higher during live interactions (Wilcoxon: **P*=0.03, effect size=0.47). Boxplots show median±IQR. Whiskers extend to the smallest and largest values, at a maximum of 1.5×IQR. Single points outside whiskers are outliers. (B) Points connected by a line indicate the steady-state EODf increase of the same fish (*N*=26) in response to artificial playback versus during live dyadic interactions. Steady-state EODf increases were larger during live interactions (Wilcoxon: **P*<0.001, effect size=0.41).

chirps or rises may be a consequence of individual variation. Importantly, jaw length is a static signal, whereas behavior is dynamic. Although jaw morphology is fixed and context-independent, individual pairing, internal state, experience or reactivity could influence communication as social context changes (Butler et al., 2018; Dingemanse and Réale, 2005). Therefore, these results indicate jaw length alone is not related directly to aggression or chirp and rise production, but is instead related indirectly, as an emergent property of status.

Our findings suggest that jaw length and chirping may function as assessment cues during non-breeding aggressive interactions. Jaw length and chirping might also function in other social contexts. We were able to investigate relationships between jaw length and chirping only during agonistic encounters between males, most of whom were in low-to-marginal reproductive condition. During the breeding season, longer jaws and/or chirping could also be attractive to females during courtship. We could not assess the role of chirping during breeding because little is known about reproduction in *C. samueli*, and we could only obtain one female. Future studies with breeding males and females are needed to assess reproductive functions of ornaments and signals in this species.

EOD modulations during agonistic interactions

Chirps are classically associated with aggression, but may signal submission (Cuddy et al., 2012; Henninger et al., 2018; Hupé and Lewis, 2008; Zupanc et al., 2006). The function of EOD modulations varies across species and contexts. In non-apteronotid electric fish, chirps signal submission in the territorial species *Gymnotus omarorum*, but not in the gregarious species *Brachyhypopomus gauderio* (Batista et al., 2012; Zubizarreta et al., 2012). In live dyads in *C. samueli*, males that attacked more also chirped more. Moreover, fish were most likely to chirp ∼1 s before they attacked, regardless of status. These findings suggest that chirps function as aggressive signals in both dominants and subordinates. Chirping was temporally clustered, and fish performed an echo response, where one fish chirped in response to chirps of the other. Subordinate chirps followed dominant chirps with a preferred latency of 0.3 s, suggesting that subordinates might be slightly more likely to echo dominants than vice versa. Echo responses have been observed in both agonistic and courtship interactions in other apteronotids (Henninger et al., 2018; Hupé and Lewis, 2008; Zupanc et al., 2006). The echo response here likely serves a communicative function during aggression and may help reinforce status during contests.

In *A. leptorhynchus* and *A. albifrons*, rises are more often produced by subordinate fish (Raab et al., 2021; Serrano-Fernández, 2003). In *C. samueli*, dominants produced more rises than subordinates. As with chirps, fish that attacked more produced more rises. *Compsaraia samueli* males also produced rises just before they attacked. Both fish also produced rises concurrently in several trials during periods of intense aggression. These results suggest that rises, like chirps, are associated with aggression. Subordinate fish were also more likely to produce a rise just before the dominant attacked it. One possible explanation is that this pattern is simply a consequence of concurrent rises during periods of intense aggression. When a dominant fish produces a rise before it attacks, the subordinate may also produce a rise to signal an intended, but unsuccessful, attack. Another alternative is that subordinate rises signal submission in anticipation of an attack. However, rises produced by dominants are associated with attacks, so a more likely interpretation is that rises are provocative signals that indicate a lack of submission. Consistent with these findings, a recent study in *A. leptorhynchus* found that rises provoke dominant attacks (Raab et al., 2021). However, unlike in *A. leptoryhnchus*, dominant *C. samueli* produced significantly more rises than subordinates, suggesting rises would not be successful in reducing relative dominance. Differences in experimental setup might also account for differences between our findings and those of Raab et al. (2021). That study used a slightly larger tank with more shelters and a much longer assessment period of 6 h. It is possible that subordinate *C. samueli* would have produced more rises during very long dyadic interactions. However, the substantial difference between the studies in the relative rise rates of dominants versus subordinates more strongly suggests that *A. leptorhynchus* and *C. samueli* differ inherently in how rises are used. Dominants produced four times as many rises as subordinates in the 7-min trials

in this study, whereas in the first 20 min of the interactions between *A. leptorhynchus*, losing fish emitted five times as many rises as winners (Raab et al., 2021). Together, these results suggest that the same signals might have been co-opted for different functions in different species.

Males also produced a JAR-like signal by increasing their EODf ∼10–80 Hz at the beginning of a trial. Traditionally, the JAR mitigates negative effects of jamming when two EODs of similar frequencies interfere (Metzner, 1993). However, JAR-like signals may also have social functions (Kramer, 1987). Some species produce an atypical JAR or an anti-jamming response to an EODf difference outside the range typical of jamming (Ho et al., 2010; Petzold et al., 2018). Although a JAR-like signal was observed in both the chirp chamber and dyads in *C. samueli*, the magnitude of EODf increase was higher during live interactions. This JAR-like signal could function to maintain dominance hierarchies or to coordinate aggression (Kramer, 1987). However, in *C. samueli*, neither EODf nor JAR-like increases differed between subordinates and dominants. The magnitude of increase was similar between competing males and correlated weakly with aggression. Interacting fish may maintain consistent EODf differences, and JAR-like signal magnitude might function in assessment during aggression.

Chirping during long-term social housing

Electric fish are active and modulate EOD signals more at night (Henninger et al., 2020; Silva et al., 2007; Zupanc et al., 2001). Consistent with this, in long-term overnight recordings, *C. samueli* chirped less when isolated or when lights were on. As expected, chirp rate was highest in the hour after lights-out on the first night of pairing. During this hour, chirp rate was also higher in fish paired with a partner close in EODf. This effect is common in chirp chambers, where fish chirp more to playbacks close in frequency (Kolodziejski et al., 2007). As in dyadic interactions, chirping in overnight pairings was clustered in time across fish (Hupé and Lewis, 2008; Zupanc et al., 2006). Like the echo responses in dyads, shorter-jawed fish were slightly more likely to chirp after than before the longer-jawed fish. Chirp rate was lower after 1 week of pairing, probably reflecting social habituation, as reported in other apteronotids (Dunlap et al., 2011, 2002). Chirping might play a stronger role in establishing hierarchies than in maintaining them. Fish, however, were only able to communicate across a divider during long-term housing. Neither chirp rate nor difference in chirp rate were related to jaw length or the difference in jaw length between fish. In *A. leptorhynchus*, chirp rate is higher in free-swimming interactions than when fish are separated by a barrier (Dunlap and Larkins-Ford, 2003). It is possible that status cannot be resolved with electrocommunication signals alone, explaining why certain relationships emerged during dyadic, short-term physical interactions but not during longer interactions when separated by a mesh barrier.

Differences in signals produced in response to playback versus live interactions

Understanding trade-offs between studying signals elicited by artificial playbacks versus those produced in naturalistic contexts is critical for interpreting signal structure and function (D'eath, 1998; Hauber et al., 2015; Lahti, 2015; Morrell et al., 2008). Chirp chamber experiments provide a few advantages over live interactions. First, the stimulus can be presented with a constant, repeatable amplitude, frequency and geometry. In live interactions, chirp rate is influenced by EOD amplitude, which varies with distance, direction and orientation between interacting fish (Dunlap et al., 1998; Engler and Zupanc, 2001). Chirp chamber experiments thus facilitate standardized chirp rate comparisons across fish. Chirp structure can also be measured more precisely in chirp chambers, because the geometry of the fish and stimulus relative to recording electrodes remains constant and playback contamination can be minimized (Kolodziejski et al., 2007). For example, in chirp chamber recordings, we were able to distinguish two putative types of chirps in *C. samueli*, whereas these putative chirp types could not be distinguished reliably in live recordings.

Using an artificial stimulus also has disadvantages, especially because chirping is context-specific (Dunlap, 2002; Dunlap et al., 2002; Hupé and Lewis, 2008). EOD modulations in *C. samueli* differed between artificial settings and during naturalistic encounters. For example, larger JAR-like responses were observed in live interactions than in response to artificial playback. Moreover, chirp rate in the chirp chamber did not correlate to chirp rate during live interactions. In *A. leptorhynchus*, chirp rate is also higher when presented with a live conspecific versus an artificial playback (Dunlap and Larkins-Ford, 2003). Field studies have similarly highlighted behaviorally relevant stimulus frequencies and amplitudes not observed in laboratory settings (Henninger et al., 2018). Thus, understanding signal function requires examining signal structure and usage across experimental contexts.

Aggression persists with low levels of circulating androgens in *C. samueli*

Sustained aggression despite low levels of testosterone and 11-KT suggests that non-breeding aggression in *C. samueli* might not be regulated by circulating androgens. Several other species, such as Siberian hamsters (*Phodopus sungorus*), song sparrows (*Melospiza melodia*) and the banded knifefish (*Gymnotus omarorum*), also display high levels of non-breeding aggression. Species that remain aggressive during gonadal regression typically lack relationships between circulating androgens and aggression (Munley et al., 2018; Quintana et al., 2016; Silva et al., 2020). Several non-mutually exclusive mechanisms regulate non-breeding aggression. In Siberian hamsters, brain regions that mediate aggression become more sensitive to low hormone levels (Rendon et al., 2017). In song sparrows and banded knifefish, non-breeding aggression is facilitated by local steroid synthesis in the brain (Soma et al., 2000a,b; Zubizarreta et al., 2020). Neuromodulators such as arginine vasotocin and serotonin also affect aggression in *G. omarorum* (Silva et al., 2013). The robust non-breeding aggression in *C. samueli* might provide an additional model for studying mechanisms that link signaling and non-reproductive aggression.

Conclusion

Our findings on non-breeding agonistic interactions in male *C. samueli* suggest that male morphological variation may be used as an assessment tool that influences contest outcomes, but may also function independently of hormones and agonistic communication signals. Instead, individual variation in signal use during contests may be influenced by interactions of status and the social and temporal contexts in which non-breeding agonism occurs.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.K.F., M.R.P., G.T.S.; Methodology: M.K.F., M.R.P., G.T.S.; Software: M.K.F., G.T.S.; Validation: M.K.F., M.R.P.; Formal analysis: M.K.F., M.R.P.; Investigation: M.K.F., M.R.P.; Resources: G.T.S.; Data curation: M.K.F., M.R.P.; Writing - original draft: M.K.F., M.R.P.; Writing - review & editing: M.K.F., M.R.P., G.T.S.; Visualization: M.K.F., M.R.P.; Supervision: G.T.S.; Project administration: G.T.S.; Funding acquisition: G.T.S.

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Data availability
Raw data on fish morphology, hormones and communication signals and on counts and timing of chirps, rises and attacks in live interactions are available on figshare (https://doi.org/10.6084/m9.figshare.19365023).

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