

## RESEARCH ARTICLE

# Acoustic and postural displays in a miniature and transparent teleost fish, *Danionella dracula*

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## ABSTRACT

Acoustic behavior is widespread across vertebrates, including fishes. We report robust acoustic displays during aggressive interactions for a laboratory colony of *Danionella dracula*, a miniature and transparent species of teleost fish closely related to zebrafish (*Danio rerio*), which are hypothesized to be sonic based on the presence of a hypertrophied muscle associated with the male swim bladder. Males produce bursts of pulsatile sounds and a distinct postural display – extension of a hypertrophied lower jaw, a morphological trait not present in other *Danionella* species – during aggressive but not courtship interactions. Females show no evidence of sound production or jaw extension in such contexts. Novel pairs of size-matched or -mismatched males were combined in resident-intruder assays where sound production and jaw extension could be linked to individuals. In both dyad contexts, resident males produced significantly more sound pulses than intruders. During heightened sonic activity, the majority of the highest sound producers also showed increased jaw extension. Residents extended their jaw more than intruders in size-matched but not -mismatched contexts. Larger males in size-mismatched dyads produced more sounds and jaw extensions compared with their smaller counterparts, and sounds and jaw extensions increased with increasing absolute body size. These studies establish *D. dracula* as a sonic species that modulates putatively acoustic and postural displays during aggressive interactions based on residency and body size, providing a foundation for further investigating the role of multimodal displays in a new model clade for neurogenomic and neuroimaging studies of aggression, courtship and other social interactions.

**KEY WORDS:** Sound production, Aggression, Fish, Resident, Intruder

## INTRODUCTION

Fishes are known to use acoustic communication to mediate complex social interactions during courtship and aggression, with calls differing in one or more parameters ranging from amplitude, duration and fundamental frequency, to the time interval between repetitive components within a call and between calls (e.g. Amorim, 2006; Ladich and Myrberg, 2006; Myrberg and Lugli, 2006; Sebastianutto et al., 2008). From an ecological perspective, sound

production can influence aggressive competition for mates, nest sites, shelters and territories (Andersson, 1994; Amorim, 2015; Ladich and Myrberg, 2006), and can enable contests to be resolved through less costly measures before escalating to more intense stages of engagement involving direct contact and possible physical damage (e.g. Davies and Halliday, 1978; Clutton-Brock and Albon, 1979; Hsu et al., 2008; Oliveira et al., 2011; Green and Patek, 2018). It should be evolutionarily favorable for contest rivals to gather information on the possible costs and benefits of continued aggressive engagement (Parker, 1974). Behavioral tactics such as acoustic displays may communicate relevant information about a contestant's identity (e.g. age and sex), motivation to fight for a resource, which is often associated with higher signaling display rate (Burmeister et al., 2002; Arnott and Elwood, 2007; Triefenbach and Zakon, 2008), and physical attributes such as body size. All of these attributes can contribute prominently in determining the outcome of aggressive conflicts (e.g. Davies and Halliday, 1978; Clutton-Brock and Albon, 1979; Bradbury and Vehrencamp, 2011; Amorim, 2015; Conti et al., 2015; Billings, 2018).

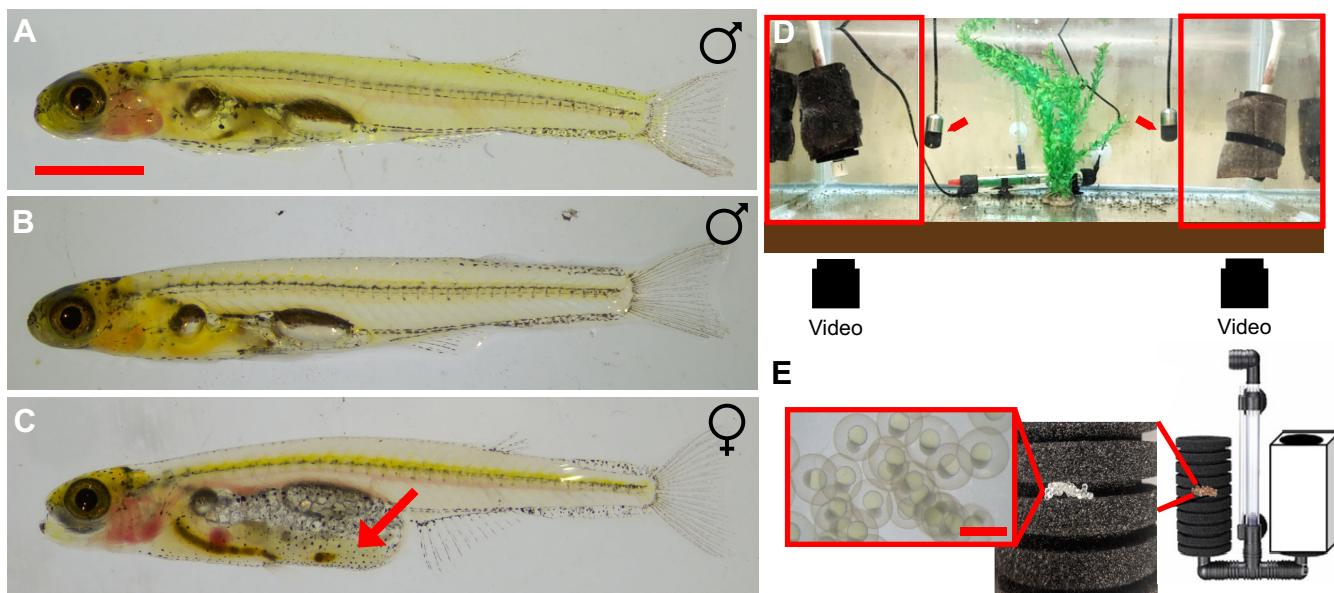
Having a robust description of context-specific displays in a species is essential for future investigations aimed to determine how and whether display features may be attended to across differences in social dominance, as well as sex, among the members of a social group (Amorim et al., 2019; McKibben and Bass, 1998, 2001; Abril-de-Abreu et al., 2015; Nunes et al., 2020). With this in mind, we provide a behavioral baseline for studies of acoustic behavior during social interactions for *Danionella*, a genus of miniature cypriniform teleost fishes (Roberts, 1986; Britz et al., 2021). More specifically, we report that *Danionella dracula* is sonic, and can be readily bred in laboratory settings, and studied for stereotyped patterns of sound production and postural displays during aggressive interactions. This species was first described in 2009, and inhabits small streams of the Kachin state in northern Myanmar (Britz et al., 2009, 2021). Nothing is known regarding the specifics of its behavior in the wild, largely because its natural habitat is currently relatively inaccessible as a result of political unrest (e.g. Goldman, 2021; UN Human Rights Council, 2018). Like other members of its genus, *D. dracula* exhibits developmental truncation, as they retain many larvae-like traits as adults (pedomorphosis) and only reach 12–18 mm in standard length (Britz, 2003, 2009; Britz et al., 2009, 2021; Britz and Conway, 2016; Conway et al., 2021; this report). Their especially small size combined with their transparency as both larvae and adults (e.g. Fig. 1A–C) offers multiple advantages for behavioral neuroscience, facilitating non-invasive, optical imaging of the brain using multiphoton microscopy (Schulze et al., 2018; Chow et al., 2020; Akbari et al., 2020; Akbari et al., 2021). *Danionella* is also closely related to zebrafish (*Danio rerio*), further making them especially amenable to genetic manipulation using the zebrafish molecular toolbox (Schulze et al., 2018). Despite possessing these attractive

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**Fig. 1. Male and female *Danionella dracula* and the controlled community tank.** (A) Adult male with green to yellow-green coloration (standard length, SL, 17.2 mm). Scale bar: 3 mm. (B) Adult male (SL 16.8 mm) and (C) female (SL, 17.1 mm; red arrow indicates ovary) without green coloration. (D) Tank setup (75×30×28 cm, ~64 l; details in Materials and Methods); red boxes indicate the video camera field of view. External video cameras are indicated below the tank. Red arrows indicate hydrophones. (E) Right: schematic diagram of the nest site used for egg collection. Insets: developing eggs (scale bar: 1 mm; left) and egg cluster in crevice of sponge filter nest site (right).

features for genetic and neural studies of social behavior, such behaviors have not yet been described in a comprehensive manner for any species of *Danionella*.

We describe sound production in *D. dracula* within the context of aggressive interactions because we found that males produce relatively simple, broadband sounds apparently solely during aggressive but not courtship encounters; females appear to be silent during all such interactions. Morphological studies predicted a sex difference in soniferous behavior for *Danionella* based on documentation of a putative sonic ‘drumming apparatus’ associated with the swim bladder in adult males, but not females (Britz and Conway, 2016). Sound production was reported earlier for male *Danionella cerebrum* (Schulze et al., 2018; as *Danionella translucida*, see Britz et al., 2021). In male *D. dracula*, a large muscle extends between the hypertrophied outer arm of the os suspensorum and the fifth rib, surrounding a fibrocartilage core attached to a connective tissue mass on the wall of the swim bladder (Britz and Conway, 2016). At this time, it is unclear how this structure may be producing sounds. Unlike other species within the genus so far described, *D. dracula* males also have a hypertrophied lower jaw and a series of odontoid processes that resemble fangs; females lack both of these features (Britz et al., 2009; Britz and Conway, 2016).

We report the establishment of a laboratory-based breeding colony of *D. dracula* to study courtship and aggressive behavior over the use of nest sites. Furthermore, we present the results of a resident–intruder assay to examine acoustic and postural displays during dyadic aggressive interactions in size-matched and size-mismatched male encounters. Previous research in aggression has utilized resident–intruder assays (see Koolhaas et al., 2013) and other dyadic designs to study how two individual males can compete over a resource and what display parameters correlate with differences in the ability of an animal to win a contest, i.e. an animal’s resource-holding potential (RHP; see Bradbury and Vehrencamp, 2011; Arnott and Elwood,

2009). Residency is a factor that can contribute to an animal’s overall motivation, or continued agonistic engagement and escalation over resources, and it can be influenced by internal physiology and perceived resource quality (Bradbury and Vehrencamp, 2011; Arnott and Elwood, 2007, 2008; Brown et al., 2007; Lindström, 1992). In our study of *D. dracula* social behavior, we investigated the effects of residency status and body size on dyadic interactions, two factors that contribute to fighting ability in other taxa (see Hack et al., 2010; Jennions and Backwell, 1996; Jackson and Cooper, 1991). Our original intent was to only investigate acoustic displays. However, we noticed early on that males often extended their lower jaw during sound production. This suggested that sonic activity may depend biomechanically on jaw extension, reminiscent of the involvement of jaw movement in clownfish (*Amphiprion clarkii*) sound production (Olivier et al., 2015). Thus, we focused our quantitative analyses on two prominent behavioral characters that we show distinguish *D. dracula* from others within the genus: temporal features of acoustic displays and extension of the hypertrophied lower jaw. We hypothesized that the amount or temporal pattern (e.g. inter-pulse interval) of sound production and jaw extension by an individual male would be influenced by residency and/or body size.

## MATERIALS AND METHODS

### Colony formation and breeding

To create a laboratory colony of breeding tanks, adult *Danionella dracula* Britz, Conway and Rüber 2009 were originally purchased from commercial dealers (The Wet Spot Tropical Fish, Portland, OR, USA; Invertebrates by Msjinkzd, York, PA, USA) and bred in environmental control rooms at Cornell University, Ithaca, NY, USA. Fish were kept at water temperatures of 23–25.5°C and on a 16 h:8 h light:dark cycle. We found that *D. dracula* breeds best with direct overhead lighting (e.g. Fig. S1). Fish were kept in rooms with artificial or mixed natural and artificial overhead lighting. There

were no obvious differences in social behavior, breeding or rates of development between the two lighting conditions. All animals were fed twice a day, with *Artemia nauplii* in the morning and ground fish flake (TetraMin Tropical Flake) in the evening. Plastic plants were added to each tank for fish to acclimate to the aquarium setting. We also found that *D. dracula* is a diurnal species, with more sonic activity during daylight hours, like their congener *D. cerebrum* (Schulze et al., 2018). All procedures used throughout our studies were approved by the Institutional Animal Care and Use Committee of Cornell University.

Fish were bred in 2.5–125 gallon (~9.5–473 l) aquaria that housed populations of varying density based on tank size, in ratios of 1:2 males:females, with at least three males per tank (Fig. 1D). Nest sites were made from double sponge water filters (XY-2822 Air Pump Double Sponge Water Filter, Xinyou) that contained nine 4 mm crevices for spawning (Fig. 1E). The sponges were placed at opposite ends of the tank (Fig. 1D) and covered with a BIO-CHEM ZORB filtration cartridge (API Fishcare CRYSTAL Filtration Cartridge), as the crevices must be enclosed for breeding (Fig. 1E). Eggs were collected daily by removing the nest, unrolling the filtration cartridge, and gently moving the clusters of eggs to acrylic cylinders (10 cm diameter) with a mesh bottom that rested within a 50 gallon (~189 l) aquarium (see eggs in Fig. 1E). Larval fish were fed AP100 Dry Larval Diet (Zeigler Bros, Inc.) twice a day for 10 days in these smaller cylinders before being moved to 5 gallon (~19 l) tanks, where they were fed adult diet and raised in the colony settings described. Larvae became adults in 3 months, visually determined by the presence of eggs in the abdomen of females and the hypertrophied jaw in males. All fish used for behavioral observations were 6–12 months in age.

#### Controlled community tank observations

A 16.8 gallon (~64 l) controlled community tank with dimensions 75×30×28 cm was set up using the same parameters for colony system breeding tanks to allow for behavioral observations in a reproductive context (Fig. 1D). Fish were kept at 25°C and on a 16 h:8 h light:dark cycle in rooms with artificial overhead lighting. Males (3) and females (6) were placed into the tank 30 min after dye labeling. Each male had a muscle segment labeled in the tail with red, green or black dye to allow three independent observers to determine identity while watching the tank and in video recordings (Tissue Marking Dye Kit, MDT100, Sigma-Aldrich). We did not observe noticeable changes in behavior from injecting muscle dye. Females were also labeled using the same method; thus, all fish in the tank went through the same injection process. Sounds were recorded with a hydrophone (Aquarian Hydrophones, H1a; frequency response range: ±4 dB, 20Hz to 4 kHz) placed next to each nest site and connected to a 30 frames s<sup>-1</sup> video camera (Canon Vixia HF R500; audio sampling frequency: 48 kHz) using a mono to stereo adaptor to synchronize the audio collection with the video. Videos were recorded as MP4s, using ACC codec. The sound recording from the video was exported as MP3s for analysis. To determine any effects of compression on our measurements, we recorded sound production in a resident–intruder assay containing both an Aquarian Hydrophone and a calibrated hydrophone (8013, Brüel & Kjaer) connected to a conditioning amplifier (2635, Brüel & Kjaer) captured on a digital recorder (LS-12, Olympus) which did not use lossy compression (WAV files, audio sampling frequency: 44.1 kHz). We found in comparing compressed with uncompressed sounds from the different recording devices that pulses had the same pulse duration values and comparable spectral properties.

**Table 1. Community tank behavioral events for *D. dracula***

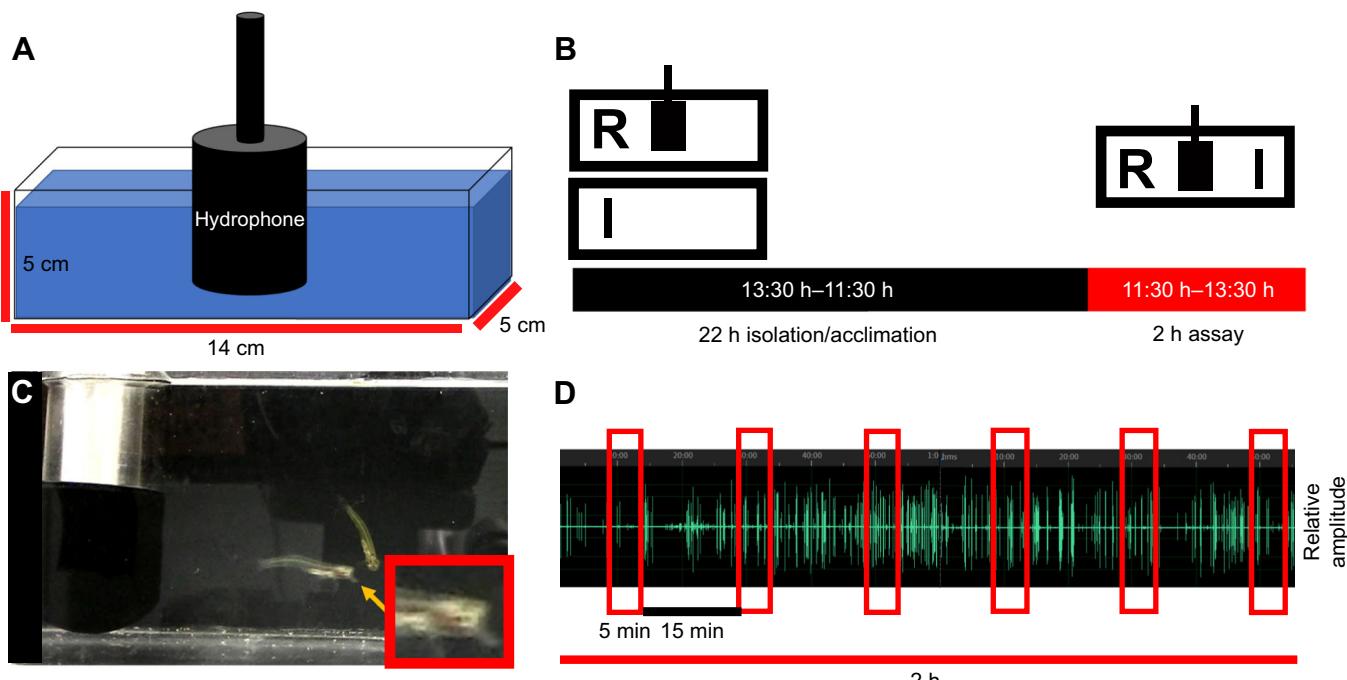
| Event         | Definition  |
|---------------|---|
| Lunge at male | Focal male orients head towards and swims rapidly towards target male. Trajectory directed at target male but does not pursue as target swims away. |
| Courtship     | Focal male swims beneath female and vibrates its head and fins back and forth underneath the egg vent.  |
| Enter nest    | Focal male swims into crevice of nest head-first.   |

Fish were allowed an acclimation period, which concluded after 1 week at the onset of courtship. Then, three independent observers used the software BORIS (Friard and Gamba, 2016) to conduct focal sampling (behaviors in Table 1) and live observation, alongside collecting video and acoustic data centered on the two nest sites, to observe each of the three labeled males. Behavioral observations were based on three randomly selected, 15 min periods, made up of three 5 min periods where each of the different focal males was observed, between 09:00 h and 17:00 h for each of 7 days. Fish received the same diet regime as colony tanks, with the first feeding at 10:00–10:15 h and the second at 17:00 h. Live observations were synchronized to the video with a red LED pressed at the start of the observation period by the observer. Observers sat 46 cm in front of the tank and used keystrokes to signify behavioral events of interest in the focal male (Table 1). These characterizations were verified with the video camera and sound data collected.

#### Resident–intruder assays

All dyad contests were conducted in rooms maintained on a 16 h:8 h light:dark cycle using artificial lighting. Males were removed from colony tanks 22 h before the resident–intruder assay. All resident–intruder pairs were age matched in both size-matched and -mismatched contexts. The resident male was housed in the experimental rectangular tank, which was 14 cm×5 cm×5 cm (Fig. 2A). The intruder male was housed separately in an 8 cm×8 cm×8 cm tank. The two tanks contained the same volume of water, 315 cm<sup>3</sup>, with a similar depth of 4.5–5 cm. A 6500K white LED lamp (Full Spectrum SkyLED Plus Aquarium Light, NICREW) on the same 16 h:8 h light:dark cycle as the room was placed 38 cm above the tanks. For size-matched dyads, males were selected to be as close as possible in size (standard length, SL, 13.19–17.94 mm), with the size difference between males in any dyad being less than 0.5 mm (measured with calipers). Taking advantage of adult male color variation (Fig. 1A–C), residents and intruders were selected to be different colors in size-matched assays to make them readily obvious in the videos, as they could not be readily distinguished based on size. One male was greener in coloration than the other, which was more pale yellow; half of the size-matched residents were pale yellow, and half were green (Fig. 1A,B). Fish coloration did not have an apparent effect on total sound production in size-matched contests. However, color is among the many possible variables that could be controlled and/or manipulated in future studies, especially with a large sample size.

Resident–intruder assays were also performed with size-mismatched dyads. For these dyads, males were selected to investigate the effects of large differences in size on aggressive interactions in dyads, with the size difference between males in any dyad being more than 1.5 mm (SL larger fish 14.64–17.81 mm; SL smaller fish 12.08–13.86 mm). The size difference between males in the dyads ranged from 1.6 to 4.4 mm. Residency and relative size status (larger or smaller compared with their counterpart) were counterbalanced across dyads; half of the residents were larger and



**Fig. 2.** Dyad testing tank for male *D. dracula*. (A) Schematic diagram of the experimental tank (14 cm×5 cm×5 cm), showing the hydrophone. (B) Dyad interaction timeline. R, resident; I, intruder. Black line represents 22 h isolation/acclimation period (13:30 h–11:30 h) before the 2 h assay (11:30 h–13:30 h), which is indicated in red. (C) Two males in the testing tank, one with its jaw extended (orange arrow) as it lunges at the other. Inset: closeup of the anterior body region and jaw. (D) Behavior sampling regime. Red boxes on the oscillogram (sounds in green) indicate six 5 min time periods sampled at 15 min intervals (black line) over the 2 h assay.

half were smaller. In these contests, males were easily distinguished from each other in the videos based on size, given one fish was distinctly larger than the other in each of the assays.

Before testing, fish were fed their colony tank diet in the evening and morning. Both tanks were aerated with an air stone, and water was novel system water that had not housed adult fish previously. Three of the four tank walls were black and opaque, allowing for better contrast for later fish identification. The experimental tank used to run resident–intruder trials alone contained a hydrophone (Aquarian Audio H1a) (Fig. 2A). The size of the hydrophone was chosen to best resemble a *D. dracula* nest site; this type of hydrophone has been observed to elicit crevice-seeking behaviors in males. We used a mono to stereo adaptor to synchronize the audio collection with the video through attaching this hydrophone to a 30 frames s<sup>-1</sup> video camera in front of the experimental tank (Canon Vixia HFR500). Following a 22 h acclimation period lasting from 13:30 h on day 1 until 11:30 h on day 2, each resident–intruder trial lasted for 2 h, beginning at 11:30 h (Fig. 2B). We decided on a 2 h assay time rather than one that was more brief, in part, to possibly observe multiple extended aggressive interactions and to examine any variability over time of individual animal use of behavioral tactics, which could allow for observations of possible stages of aggressive escalation in this species (Green and Patek, 2018). Our assays were also 2 h long to allow for acclimation after fish addition and tank disruption. The intruder male was transferred to the experimental tank with a small net to begin the resident–intruder assay (Fig. 2B,C). Two of the 10 size-matched dyads were removed from analysis as the fish did not acclimate, swimming continuously against the sides of the miniature tank. Two of the 10 size-mismatched dyads with smaller residents were also removed from analysis. In one dyad, both fish were swimming continuously against the sides of the miniature tank. In the second dyad, both fish

were intensely engaged in an escalated aggressive interaction, making it impossible to ascertain the identity of the sound producer (similar to extended aggressive interactions in the controlled community tank). Therefore, 8 dyads were analyzed for each context, size matched and mismatched. Five of the residents in size-mismatched assays were larger fish and three were smaller fish.

Five single-male control trials were conducted following the same procedure, but only one male was placed in the experimental chamber and an intruder male was not added to the chamber at the start of the 2 h trial. Five additional control dyads were conducted using male–female pairs. Five males and five females (SL males 14.06–17.08 mm; SL females 13.73–17.12 mm) were combined in single pairs in the experimental tank following the same procedure; male–female pairs varied in size by 0.3–3 mm. Two of the residents were male and three were female.

#### Stress considerations

We recognize that stress might have been a factor contributing to our results, which show that residents and larger males exhibit higher levels of sound production and jaw extension. The principal concern here would be that we did not do a net control in which the resident fish in all assays was also netted just before introducing the intruder into the test arena. While some studies include a net control by physically handling the resident when an intruder is introduced into a test arena (Desjardins et al., 2005), others do not physically net the resident (Alward et al., 2021). Our goal was to minimize any disruption of the resident's sense of 'ownership' of the test arena (Koolhaas et al., 2013; Alward et al., 2021, 2019). We observed that following the addition of the intruder male to the assay tank, the resident and intruder often both froze, which we define as when a fish stood immobile near the bottom of aquaria or under the hydrophone for at least 2 s. We started our sound analysis as

described below after a 5 min period following addition of the intruder fish, after most fish acclimated to the start of the assay. Fish were not lunged at by the other fish while they were frozen, even when a fish was frozen beyond the acclimation period noted here. Thus, sound production associated with lunging, as well as any jaw extension behaviors, did not occur when the other male was frozen. After freezing, fish returned to conventional swimming behavior in all of the assays that we analyzed. Using *Q*–*Q* plots and Shapiro–Wilk tests, we found that freezing time was not normally distributed in the two contexts. In the size-matched context, this was largely due to many time periods having little to no freezing behavior (3 residents and 3 intruders did not freeze after tank addition). We thus ran a zero-inflated Poisson mixed model with pair identity as a random effect to examine whether time of freezing after intruder addition differed between residents or intruders in the size-matched context. However, the size-mismatched context was not zero-inflated (only 1 small intruder did not show freezing). Given that the size-mismatched data were not normally distributed but not zero-inflated, we used  $\log(1+x)$  transformation to normalize the data. Shapiro–Wilk tests confirmed that transformation resulted in normally distributed data. As such, we then ran a linear mixed model (LMM) with pair identity as a random effect using the log-transformed data to examine whether time of freezing after intruder addition differed between residents or intruders in the size-mismatched context. In size-matched and size-mismatched contexts, there was no effect of residency on freezing time, suggesting that intruders did not freeze significantly more than residents ( $W=0.0004$ ,  $P=0.9834$ ;  $F_{1,14}=1.4695$ ,  $P=0.2455$ , respectively). Residents and intruders had very similar mean freezing times in the size-matched context (resident  $2.9\pm1.3$  min, intruder  $3.1\pm1.2$  min; all data are means $\pm$ s.e.m., unless otherwise specifically noted), and intruders had higher mean freezing times in the size-mismatched context compared with residents (intruder mean  $4.9\pm1.6$  min, resident mean  $2.4\pm0.7$  min). This suggests that netting and introduction of a new fish to the assay tank could be a stressful event for both resident and intruder fish.

#### Audio recordings: limitations and analysis

We first note that we used small tanks for both video and sound recordings because they proved best for visualizing miniature, transparent and fast-moving fish. This allowed us to assign sonic behavior to individual males by resolving associated lunging behavior from the video made in front of the tank, as well as to visualize details of behavioral tactics such as jaw extension. We recognize, however, that using small tanks offers distinct limitations for assessing the physical attributes of sound (Parvulescu, 1964; Akamatsu et al., 2002; Rogers et al., 2016). For one, it is impossible to have aquarium walls anechoic across all frequencies, which will affect sound reverberation and resonance (Parvulescu, 1964; Rogers et al., 2016). Reverberation affects measurements of individual sound pulse duration, and resonance distorts dominant frequency, sound-pressure level and power spectrum (Akamatsu et al., 2002). Given these limitations, we focus our comparisons between individual males primarily on total output of sound production and temporal patterning of the sounds, as we could reliably resolve individual pulses with a high signal to noise ratio using this assay; individual differences would be best assessed using recording conditions optimized for duration and spectral content (Akamatsu et al., 2002).

Hydrophone recordings of resident–intruder trials lasted for the full 2 h. Because of the large number of sounds observed in the 2 h period, the oscillogram from each hydrophone recording after the

initial 5 min acclimation period described above was split into six 5 min long time periods separated by 15 min intervals (Fig. 2D). Sound characteristics were measured using Raven Pro 1.6 using a Hann window with 50% overlap and DFT 512 samples (<https://ravensoundssoftware.com/>). Recordings typically displayed a high signal to noise ratio (e.g. Fig. 2D).

To approximate pulse duration, we first measured a sound pulse's maximum peak amplitude from baseline (0 in Raven) for the highest peak, which is always at the beginning of the waveform. The amplitude values are in 'kilounits' in Raven and are the actual sample values in the sound signal, which are proportional to the sound pressure at the hydrophone when the sound was recorded (see Charif et al., 2010). We do not use this maximum peak amplitude measurement to comment on individual variation in pulse duration or amplitude (see below) or to compare it with that of other species given its relative nature and limitations of sound recordings in small tanks (see below), although the pulse duration values are very similar to those measured for *D. cerebrum* (Schulze et al., 2018) despite different recording conditions and hydrophone (see Discussion). We divided the maximum amplitude value by 4 to get a quarter amplitude value, and the pulse duration was determined as the duration of the pulse where waveform peaks were all greater than the quarter amplitude value. We also measured pulse peak frequency for each pulse after selecting the pulse and using Raven's Peak Frequency measurement. This measurement in Raven is calculated from the spectrogram of the sound (spectrogram parameters: DFT size: 512 samples; grid spacing: 93.8 Hz) and is the frequency at which the maximum/peak power occurs within the selection.

Individual pulses occurred close to each other in time, forming apparent clusters that we refer to as bursts. To define a burst, we measured the duration of time between all neighboring individual pulses, the inter-pulse interval (IPI), and pooled all the IPI data together. We used the mode value of 34 ms (Fig. S2) to set boundaries for individual pulses per burst, where pulses that composed a 'burst' had to possess an IPI within 2 s.d. of the mode, or be less than 70 ms apart. This criterion allowed us to identify bursts ranging up to 6 pulses in length. All IPIs greater than 70 ms were defined as inter-burst intervals (IBI), i.e. the duration of time between bursts composed of multiple pulses.

To characterize the amplitude range of sound pulses, we recorded sound production using a calibrated hydrophone (8013, Brüel & Kjaer) connected to a conditioning amplifier (2635, Brüel & Kjaer) captured on a digital recorder, which did not use lossy compression (LS-12, Olympus; Wav files, audio sampling frequency: 44.1 kHz). We first recorded sounds in a large colony tank ( $122\times46\times74$  cm,  $\sim100$  gallons,  $\sim379$  l) with 75 fish and 4 nest sites (one in each corner of the tank). The calibrated hydrophone was suspended 15 cm beneath the water surface and equidistant (15.24 cm) between the two nest sites on the left side of the tank. The hydrophone was 23 cm displaced from the tank walls. The maximum distance of an individual fish from the hydrophone was approximately 150 cm, although sounds were most likely captured from fish interacting close to the nests and hydrophone. We analyzed sounds from 2 h of audio recorded at 10:00–12:00 h. We next recorded sounds in the resident–intruder assay in an acoustic isolation chamber (Industrial Acoustics). In the dyad assay tank, the Brüel & Kjaer hydrophone was suspended in one corner of the tank, 1 cm displaced from the walls and water surface. Therefore, the maximum distance an individual fish could be relative to the hydrophone was estimated as the space diagonal of the tank,  $\sim14$  cm. Two size-matched males were introduced into the tank as previously

described and 1 h of audio was captured. Sound pulses were isolated and analyzed in Raven Pro 1.6 using a custom-written script. Each pulse was measured separately, using Raven's Peak Amplitude measurement, which finds the waveform's greatest absolute and instantaneous (i.e. non-time-weighted) change from baseline.

### Video analysis of sound production and jaw extension behavior

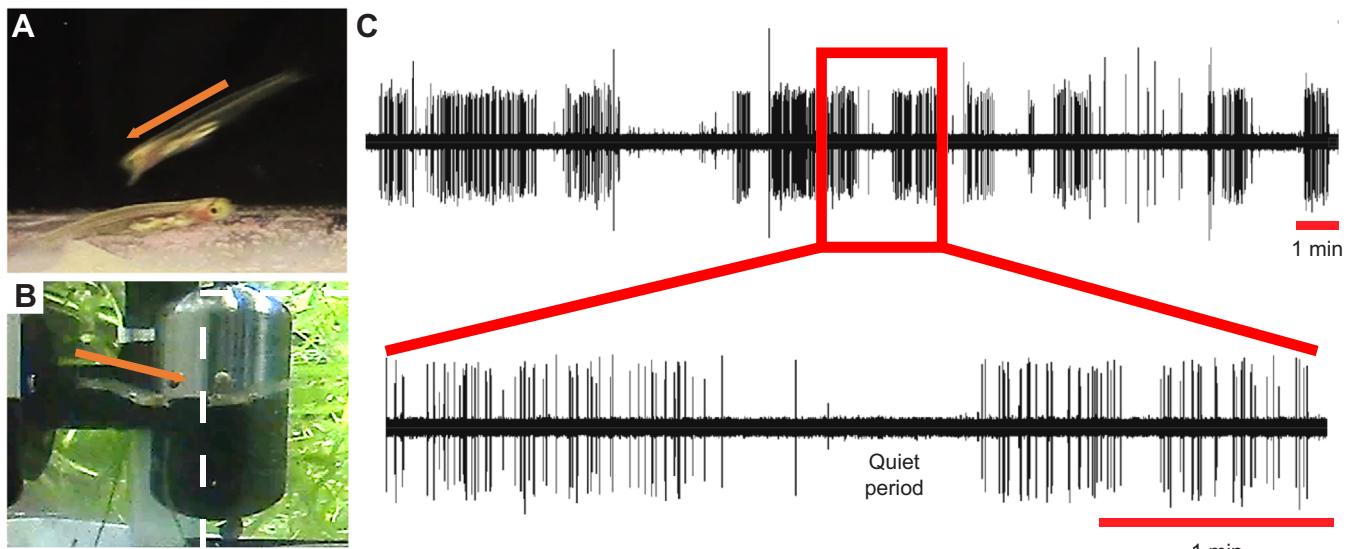
For the resident–intruder assays (Fig. 3A), an observer blind to resident–intruder status verified instances of male sound production by watching the synchronized video at  $0.3\times$  the normal speed. Sound bursts were attributed to an individual male based on associated lunging movement. This association between lunging and sound production was established based on our analysis of a 4 min portion of a 23 min recording of a *D. dracula* male continuously lunging at its reflection in the wall of the tank (Fig. 3B, see oscillogram in Fig. 3C; Movie 1). For this recording, an observer first coded in BORIS all lunges directed at the reflection, without sound. The time point of an instance of lunging was determined as the first frame where the male fish oriented its head towards and swam rapidly towards its reflection. Burst and pulse start times from the same portion of the recording were measured using Raven Pro 1.6, so the time between a lunge and a burst could be examined for temporal proximity, as is described in Results (see Fig. S3A).

Observers distinguished fish in the size-matched assays based on coloration: green or pale yellow (see above), as well as other identifying features such as body girth. In size-mismatched assays, one fish was distinctly larger than the other and the two fish could be readily identified based on relative size. Coding of jaw extension was done by a third observer in BORIS who watched the video at  $0.3\times$  the normal speed without sound. The time point of an instance of jaw extension was determined as the first frame where the lower jaw was first extended from the head.

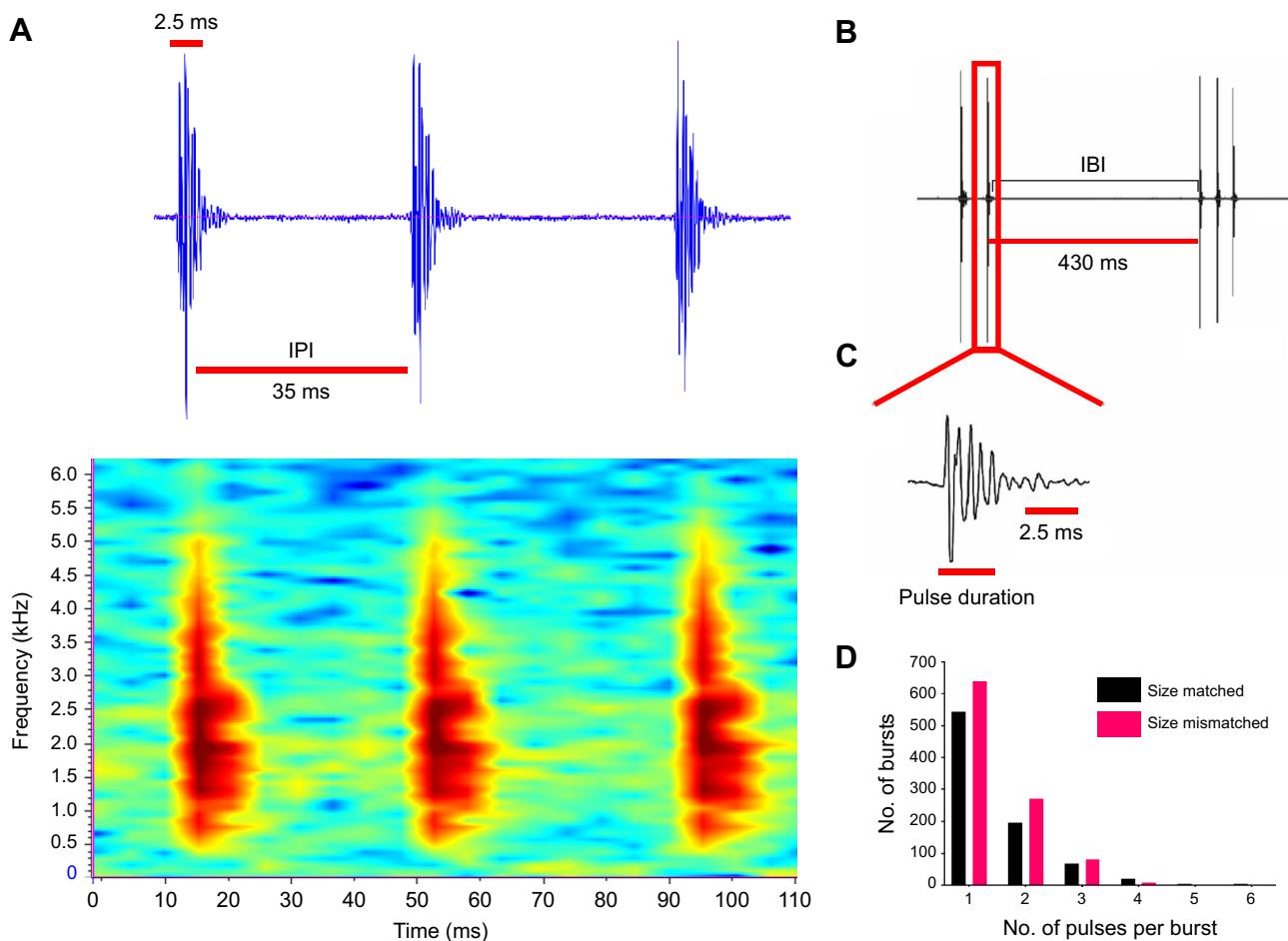
### Statistical analyses

Statistical analyses were performed in R version 4.1.2 (<http://www.R-project.org/>). To determine whether each variable conformed to a normal distribution, we used *Q–Q* plots and Shapiro–Wilk tests. These analyses showed that all count data (e.g. sound production and jaw extensions) were not normally distributed. Count data were either summed across all sampled time periods for each male or separated by individual time periods 1–6 for analyses described below investigating changes in behavior over the length of the behavioral trial. In analyses where count data were separated into time periods (described below), the lack of normality was largely due to many time periods having little to no scored behavior. To account for this, we used zero-inflated Poisson mixed models (ZIPMM) in those analyses. When count data were summed, the data were no longer zero inflated and thus ZIP models do not apply; therefore, we used LMMs with pair identity as a random effect using  $\log(1+x)$  transformed data in analyses, as these transformations yielded more normally distributed data according to Shapiro–Wilk tests (described in detail below for relevant analyses). Our sample sizes were selected to be similar to those in other studies that used resident–intruder assays to study male aggression in fish (Alward et al., 2021; Desjardins et al., 2005; Oliveira et al., 2011).

We investigated whether agonistic behaviors varied between residents and intruders across each behavioral context. To do this, we ran ZIPMM using the *glmmTMB* package (Brooks et al., 2017) with pair identity as a random effect to examine whether agonistic behaviors differed between residents or intruders in both contexts, and whether the production of acoustic displays and jaw extensions may change across the length of the behavioral trial (e.g. time periods 1–6 of behavioral sampling; see above for more details about sampling). In the case of significant main effects or interactions, we followed these up with *post hoc* tests using the *lsmeans* package (Lenth, 2016). We corrected for multiple comparisons using the Benjamini–Hochberg method.



**Fig. 3. Video frames and oscillogram of *D. dracula* male–male aggressive interaction.** (A) Frame from a resident–intruder assay of a male lunging at another male and extending its lower jaw in the direction indicated by the orange arrow. Both resident and intruder males lunge and produce sound pulses. (B) Frame from a male lunging at its own tank reflection, with the direction of the lunge towards the tank glass indicated by the orange arrow extending above the entire dorsum of the lunging male. The white dashed line indicates the reflective surface of the tank wall. Note jaw opening and extension. (C) Oscillogram from a 23 min recording of the male in B lunging at its reflection in tank wall on two time scales. The lower trace is a 4 min section of the recording to show individual sound pulses. 'Quiet period' indicates when the focal fish was not oriented towards its reflection and was swimming around the nest.



**Fig. 4. Multi-pulse sound bursts of male *D. dracula*.** (A) Top: oscillogram of three-pulse burst made by a lunging male. The red line above the first pulse indicates pulse duration, ~2.5 ms. Pulses were separated by an inter-pulse interval (IPI) of ~35 ms (bottom red line). Bottom: corresponding spectrogram. Deep red indicates peak power frequency (1968.750 Hz). (B) Two-pulse (left) and three-pulse (right) bursts separated by an inter-burst interval (IBI) of 430 ms. (C) One pulse from B on an expanded time scale with a duration of ~2.5 ms. (D) Bar graph showing the number of pulses per burst of multi-pulse bursts from size-matched (black,  $n=16$ ) and size-mismatched (pink,  $n=12$ ) dyads.

We also investigated whether overall behavior usage varied based on size in the different contexts, examining the effects of absolute size (SL, mm) in both contexts and size difference (mm) in size-mismatched interactions. Count data (e.g. sound production and jaw extensions) were not normally distributed. Shapiro–Wilk tests confirmed that  $\log(1+x)$  transformation made data significantly more normally distributed for all datasets except size-mismatched jaw extension counts. While the Shapiro–Wilk test confirmed that this transformation resulted in more normally distributed data, it still suggested that the distribution was not entirely normal ( $P=0.04$ ). However, we chose to prioritize consistency in our statistical approach and thus used LMM with pair identity as a random effect for all data.  $R^2$  values for LMM regressions were calculated using the *rsq* package (<https://CRAN.R-project.org/package=rsq>). Size difference (SD) for each male was calculated as:  $SD = (\text{male's SL} - \text{its counterpart's SL})$ .

We investigated whether the number of sound bursts produced was significantly correlated with the number of jaw extensions exhibited, in both size-matched and -mismatched contexts. We used the number of sound bursts produced, rather than the number of individual pulses, as a variable in this analysis to account for the difference in sampling rate of sound production and jaw extension data. Count data (number of sound bursts and jaw extensions) were

not normally distributed. Shapiro–Wilk tests confirmed that  $\log(1+x)$  transformation made data significantly more normally distributed for all datasets except size-mismatched jaw extension counts. While the Shapiro–Wilk test confirmed that this transformation resulted in more normally distributed data, it still suggested that the distribution was not entirely normal ( $P=0.04$ ). We again chose to prioritize consistency in our statistical approach and thus used LMM regressions with transformed sound burst and jaw extension data as variables, using pair identity as a random effect.  $R^2$  values for LMM regressions were calculated using the *rsq* package (<https://CRAN.R-project.org/package=rsq>).

To test the hypothesis that multi-pulse burst usage may vary between residents or intruders in the different contexts, we reduced each animal's multi-pulse bursts (2–6 pulses per burst) into a single metric. As such, we performed a principal component analysis (PCA), using the *psych* package (<https://CRAN.R-project.org/package=psych>) and *principal* function. In these models, we entered the number of bursts for each category of burst length (1–6 pulses) for each male into R, and generated PCs separately for size-matched and -mismatched contexts. Higher PC scores represented greater usage of multi-pulse bursts. To next investigate whether PC1 (multi-pulse burst usage) varied between residents and intruders in the size-matched and -mismatched

contexts, we used a repeated measures LMM using the *lme4* package (Bates et al., 2015), with pair identity as a random effect for each context. We also ran a multiple linear regression model for each context, using absolute size and total sound production as continuous variables for size-matched contexts, and using absolute size, size difference and total sound production as continuous variables for size-mismatched contexts, to test the effect of these variables on multi-pulse burst usage (PC1).

For two different sound characteristics – IPI and IBI – the data were not normally distributed but not zero inflated. Simple transformations did not make the data more normal. We ran Poisson mixed models with pair identity as a random effect for IPI and IBI to examine whether these characteristics differed between males in the two different contexts, size matched and size mismatched.

## RESULTS

As stated in the Introduction, our main goal here was to provide a behavioral baseline for studies of acoustic behavior during social interactions in *D. dracula*. We focused on males, as we found that they are the sonic sex in this species and apparently only make sounds during aggressive encounters. Quantitative assessments of acoustic displays during resident–intruder male interactions are presented, including a description of the spectral and temporal properties of sound pulses. We also assessed a prominent postural display, extension of the hypertrophied lower jaw, which is an additional display behavior that we found occurs along with sound production during aggressive interactions. No sound production was recorded in direct association with 43 instances of courtship or presumed spawning by three focal males towards females, which occurred inside a nest, in colony and controlled community tanks (Fig. S1, Movie 2). All behavioral observations for colony, controlled community and dyad tanks were carried out between 09:00 h and 17:00 h.

### Male aggressive displays

In colony tanks, we often observed males swimming throughout the day around artificial nest sites, which were dark-colored objects containing crevices for spawning (see Fig. 1D,E). By observing dye-marked males in a controlled community tank setting over a 2 week period, we often recorded the same male swimming within 2–3 body lengths around the same nest site for 1–8 days, suggesting nest fidelity. These males also lunged at other males which swam within 2–3 body lengths around the nest site, often producing a burst of stereotyped sound pulses close to the same time as the lunge (Fig. 3A).

We concluded that sound production is primarily coupled to either lunges at intruder fish or the visual presence of another male in the contexts critically assessed in our study. We reached this conclusion after combining these colony tank observations with another colony tank video recording of a male lunging continuously against its own reflection at the same time as apparent sound production (Fig. 3B; see Fig. 3C for oscillogram from recording; see Movie 1). For a representative 4 min portion of this recording where the male lunged continuously at his reflection, we found that sounds were produced when the male was within one body length of its reflection in the tank wall, with its head and body oriented and moving towards its reflection in the tank wall (Movie 1). By contrast, there was essentially no evidence of sound production in a portion of the recording when the male was swimming around the nest and not lunging at its reflection (labeled ‘quiet period’ in Fig. 3C); the two pulses one can see during the quiet period are

timed with the fish lunging at another fish (Fig. 3C). We pooled together all the times between lunges and sounds and found a mean value of  $572 \pm 27$  ms; 88.4% of sound bursts and lunges ( $n=107/121$ ) were less than 600 ms apart, closely temporally related (Fig. S3A).

In colony tanks, we also observed extended aggressive interactions, with individual contests lasting up to 30 min before one male fled, although males could re-engage in these aggressive interactions multiple times over the course of the day. These extended aggressive interactions escalated from sound production coordinated with lunging behavior, to stages that included mutual lunging and increased sound production and lower jaw extensions until one male fled. Males also performed these displays at the same time as swimming vertically in parallel (sparring) in the water column. It was not possible to readily assign sound production to either male during such interactions.

### Sound production during resident–intruder assay

To characterize sound production during aggressive interactions, we examined dyads in a resident–intruder context (Fig. 2). Single male controls did not produce sounds and swam around the experimental chamber. There was also no evidence in male–female dyads that females made any sounds similar to males, consistent with absence of the putative sonic ‘drumming’ muscle in females (Britz and Conway, 2016). A hydrophone placed in a 20 gallon ( $\sim 76$  l) tank of 10 female fish for 10 h also did not provide any evidence of sound production by females.

Of the total sounds made during resident–intruder assays, 94.7% (2705/2858) were associated with one male’s distinct lunge at the other fish (see example in Movie 3) and 5.3% were not readily associated with either male. The latter were not included in any statistical comparisons as sound pulses could not be attributed an identity because the two fish were oriented towards each other and lunging at the same time as sound production (3.0%, 87/2858), swimming next to each other closely with their heads oriented towards each other but with no distinct lunges during sound production (1.4%, 41/2858), or obscured by either the hydrophone or being next to each other in a tank corner (0.9%, 25/2858).

Individual males produced trains of sound pulses composed of repetitive bursts of up to 6 pulses per burst (Fig. 4). In the dyad assay tank, individual sound pulses had a mean peak frequency of  $1988.6 \pm 14.0$  and  $1961.6 \pm 11.5$  Hz in size-matched and -mismatched contexts, respectively (see example spectrogram in Fig. 4A). Mean pulse duration was  $2.0 \pm 0.02$  and  $2.6 \pm 0.03$  ms for size-matched and -mismatched males, respectively (Fig. 4C). We did not do statistical comparisons between these two groups of males for peak frequency and pulse duration because of limitations measuring these values in small tanks (see ‘Audio recordings: limitations and analysis’ in Materials and Methods). The mean IPI was  $42.9 \pm 0.6$  and  $39.5 \pm 0.5$  ms in size-matched and -mismatched contexts, respectively (see two- and three-pulse bursts in Fig. 4B). Males in the two different contexts did not differ significantly in IPI ( $z=-1.29$ ,  $P=0.196$ ). Variation in IPI was not explained by absolute body size, as we did not see any correlation between IPI and standard length (mm) or size difference (mm) between competitors in a dyad (Fig. S4). In both dyad contexts, males primarily used single pulses (size matched, 65.3% of all bursts; size mismatched, 64.1%) and two-pulse bursts (size matched, 23.4%; size mismatched, 27.1%) that overlapped with lunging at other males (Fig. 4D). Three and higher pulse bursts occurred more rarely, with three-pulse bursts making up 8.1% and 7.9% for size-matched and mismatched male dyads, respectively (Fig. 4D). Four pulse and higher bursts made up only 3.2% and 0.9% for size-matched and

-mismatched males, respectively (Fig. 4D). Individual fish produced multiple lengths of bursts containing differing numbers of pulses throughout the interaction (e.g. Fig. 4B). Mean IBI was  $12.6 \pm 1.6$  and  $10.5 \pm 0.7$  s for size-matched and -mismatched contests, respectively. Males in the two different contexts did not differ significantly in IBI ( $z = -0.572$ ,  $P = 0.567$ ).

We did not measure sound amplitude given our inability to measure sounds from individuals that were always moving around one hydrophone during dyadic interactions in small volumes. In a separate set of tests designed explicitly to characterize the amplitude range of *D. dracula* sound pulses, we recorded 365 sound pulses in a 100 gallon ( $\sim 379$  l) colony tank that had an amplitude range of 141.5–153.09 dB re. 1  $\mu$ Pa. When recording males in the miniature resident–intruder assay tank, we found an amplitude range of 119.09–153.46 dB re. 1  $\mu$ Pa from 451 pulses. The background noise (root mean square, RMS) in the colony tank was  $137.81 \pm 0.46$  dB (mean  $\pm$  s.d.) re. 1  $\mu$ Pa, mostly composed of low frequency noise [peak  $\sim 20$  Hz, all power below  $\sim 200$  Hz; when filtered, background is  $113.1 \pm 0.57$  dB (mean  $\pm$  s.d.) re. 1  $\mu$ Pa]. In the assay tank, the background noise (RMS) was  $105.43 \pm 1.22$  dB (mean  $\pm$  s.d.) re. 1  $\mu$ Pa.

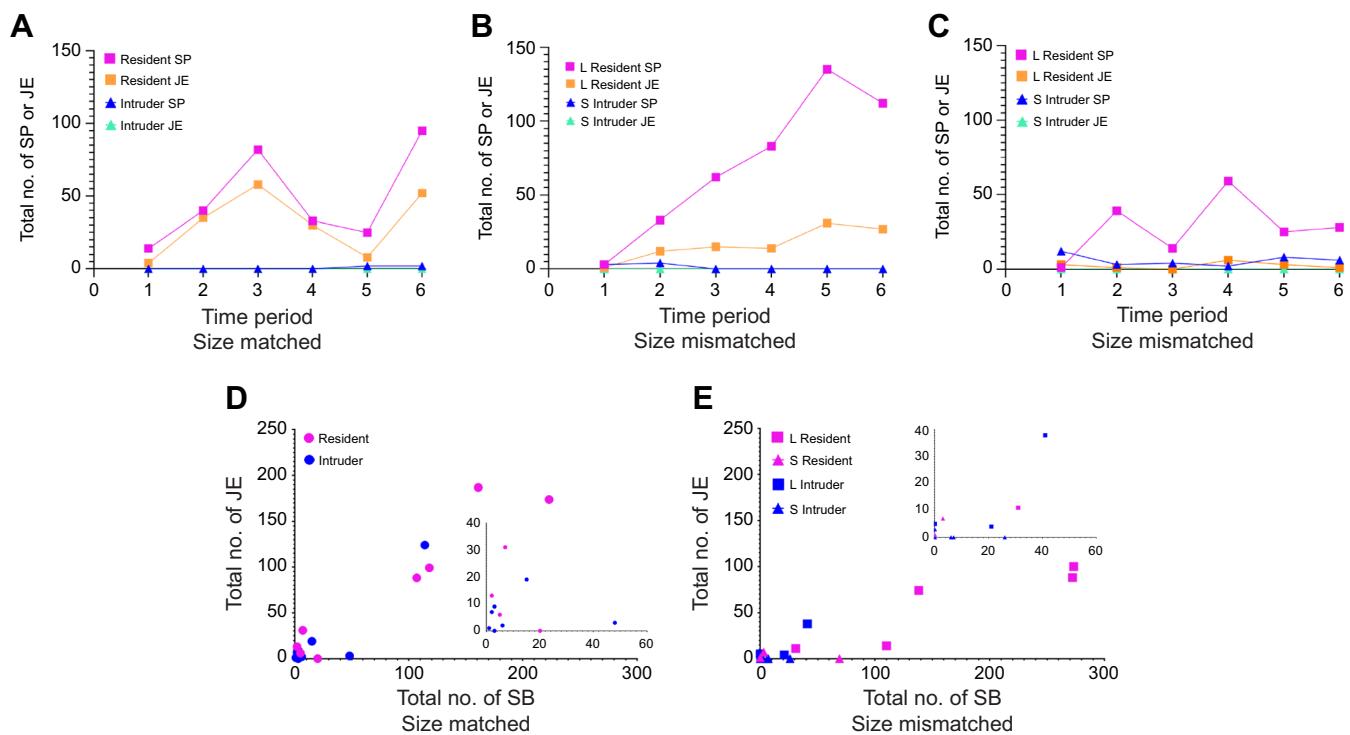
Regarding total acoustic display rate, males varied widely in the total number of sound pulses directed towards another male over the six time periods measured (see Fig. 2D), with some time periods having more sound production than others over the course of the 2 h dyad trial (see Fig. 5A–C; Fig. S5A–D). In size-matched assays,

males that produced sounds above the median of observed male total sound production were categorized as high sound-producing males, and those exhibiting below the median were categorized as low sound-producing males (Fig. S5A–E). High sound producers ranged up to 318 total sound pulses in the 30 min sampled over the 2 h dyad trial (Fig. S5E). In size-mismatched assays, we used the same criteria as with size-matched males and found that high sound producers ranged up to 428 total sound pulses (Fig. S5E).

#### Jaw extension during resident–intruder assay

As noted in the Introduction, jaw extension often appeared to overlap with sound production when we first observed *D. dracula* males in colony tanks, suggesting that sonic activity may depend on jaw extension. Thus, we also assessed the total number of jaw extensions in the dyads. Jaw extension often (Fig. 5A,B), but not always (Fig. 5C), increased during the same 5 min time period samples of high sound production in both contexts. Using LMM regressions, we found that the number of sound bursts was significantly correlated with the number of jaw extensions in both size-matched and -mismatched contexts ( $t_{14}=4.090$ ,  $R^2=0.544$ ,  $P=0.0011$ ;  $t_{14}=3.047$ ,  $R^2=0.39871$ ,  $P=0.0087$ , respectively) (Fig. 5D,E).

When directly examining the timing of the two behaviors to each other, we observed many instances where jaw extension appeared immediately before or after instances of sound production (Fig. S3D) as well as many instances of sound production without



**Fig. 5. Temporal relationship between total number of male sound pulses, sound bursts and jaw extensions for *D. dracula* males.** (A–C) Total number of sound pulses (SP) and jaw extensions (JE) for individual fish over the six sampled 5 min time periods from one size-matched and two size-mismatched dyads. (A) High SP and JE from a size-matched dyad. Pink squares and blue triangles indicate resident and intruder SP, respectively; orange squares and turquoise triangles indicate resident and intruder JE, respectively. The intruder did not extend its jaw during time periods 1–6. (B) High total number of SP and JE from a size-mismatched dyad. Pink squares and blue triangles indicate larger (L) resident and smaller (S) intruder SP, respectively; orange squares and turquoise triangles indicate larger resident and smaller intruder JE, respectively. The smaller intruder did not extend its jaw during time periods 1–6. (C) High total number of SP, but low JE from a size-mismatched dyad. Color code same as in B. The smaller intruder did not extend its jaw during time periods 1–6. (D) Scatterplot of total number of sound bursts (SB) by JE in size-matched contests ( $n=16$ ). Pink and blue circles indicate resident and intruder, respectively. Inset is a close-up of the origin. (E) Scatterplot of total number of SB by JE in size-mismatched contests ( $n=16$ ). Pink and blue squares indicate larger resident and intruder, respectively; pink and blue triangles indicate smaller resident and intruder, respectively. Inset is a close-up of the origin.

the presence of jaw extension, and vice versa. In aggregate, the evidence suggested that sound production and jaw extension were not obligatorily linked. Nonetheless, we wanted to more rigorously examine the temporal relationship between sound production and the start of jaw extension. We did this, in part, to provide baseline data for future studies assessing the impact of genetic manipulations on this temporal relationship, as well as for comparative studies of multimodal displays between the different species of *Danionella*. To maximize the sample size, these values were measured in males that exhibited both high sound production and jaw extension in size-matched ( $n=5$ ) and -mismatched ( $n=3$ ) contexts. For each context, we pooled together all times less than 2 s (considering events more than 2 s apart to be independent) between sonic bursts and jaw extensions and found that mean time intervals between the two actions were  $492\pm20$  and  $590\pm33$  ms in the size-matched and -mismatched contexts, respectively (Fig. S3B,C). We found that the temporal separation between sounds and jaw extensions directed by the high sound-producing and jaw extending male toward the other male could occur over a wide range, although a large number occurred within less than 100 ms in both dyad contexts and within as little as 2.5 and 1.3 ms of each other in size-matched and size-mismatched dyads, respectively (Fig. S3B,C). These results provided convincing quantitative evidence that sound production and jaw extension were independent events, although temporal gaps between the two actions could be very brief.

### Effects of residency status and size on display rate

We next investigated the effects of residency status on sound production and jaw extension by examining the effect of being the resident or intruder in both dyad contexts. Residents produced significantly more sound pulses than intruders in dyads that were size matched (resident mean/time period= $21.0\pm4.5$  pulses, intruder mean/time period= $5.1\pm1.4$  pulses, means $\pm$ s.e.m.;  $W=39.71$ ,  $P<0.001$ ) (Fig. 6A) and size mismatched (resident mean/time period= $27.4\pm5.3$  pulses; intruder mean/time period= $3.0\pm0.8$  pulses;  $W=218.38$ ,  $P<0.001$ ) (Fig. 6B). We also found a significant effect of time period on the total amount of sound production in size-matched and -mismatched contexts ( $W=114.37$  and  $42.54$ , respectively;  $P<0.001$  for both), indicating that there were time periods (1–6) during the 2 h trial in which fish increased sound production (Fig. 6C,D). The interaction between residency and time period was also significant in size-matched and -mismatched contexts ( $W=32.39$  and  $101.73$ , respectively;  $P<0.001$  for both). *Post hoc* tests showed that sound production differed significantly between residents and intruders in both contexts for all time periods (Fig. 6C,D). Residents produced more sounds than intruders in time periods 1–6 in size-matched dyads, with the largest difference occurring in time period 3 (Fig. 6C). In size-mismatched contexts, intruders produced more sounds than residents in time period 1, but residents then produced many more sounds than intruders in subsequent time periods (Fig. 6D).

Resident fish also performed more JE than intruders in dyads that were size matched (resident mean/time period= $12.6\pm2.6$  jaw extensions; intruder mean/time period= $3.6\pm1.0$  jaw extensions;  $W=9.72$ ,  $P=0.0018$ ), but not in ones that were size-mismatched (resident mean/time period= $6.1\pm1.4$  jaw extensions; intruder mean/time period= $1.1\pm0.4$  jaw extensions;  $W=0.75$ ,  $P=0.3864684$ ) (Fig. 6E,F). There was also a significant effect of time period on the total number of jaw extensions in size-matched and -mismatched contexts ( $W=76.63$  and  $21.32$ , respectively;  $P<0.001$  for both) indicating there were time periods (1–6) during the 2 h trial in which fish used an increased number of jaw extensions (Fig. 6G,H). The

interaction between residency and time period was also significant in both size-matched and -mismatched contexts ( $W=22.96$  and  $29.43$ , respectively;  $P<0.001$  for both). *Post hoc* tests showed that residents in size-matched contexts produced more jaw extensions compared with intruders for multiple time periods: time period 1 and then later time periods 3 and 4, with time period 3 again having the largest difference between residents and intruders (Fig. 6G). Intruders in size-mismatched contexts produced more jaw extensions compared with residents in time period 1 (Fig. 6H).

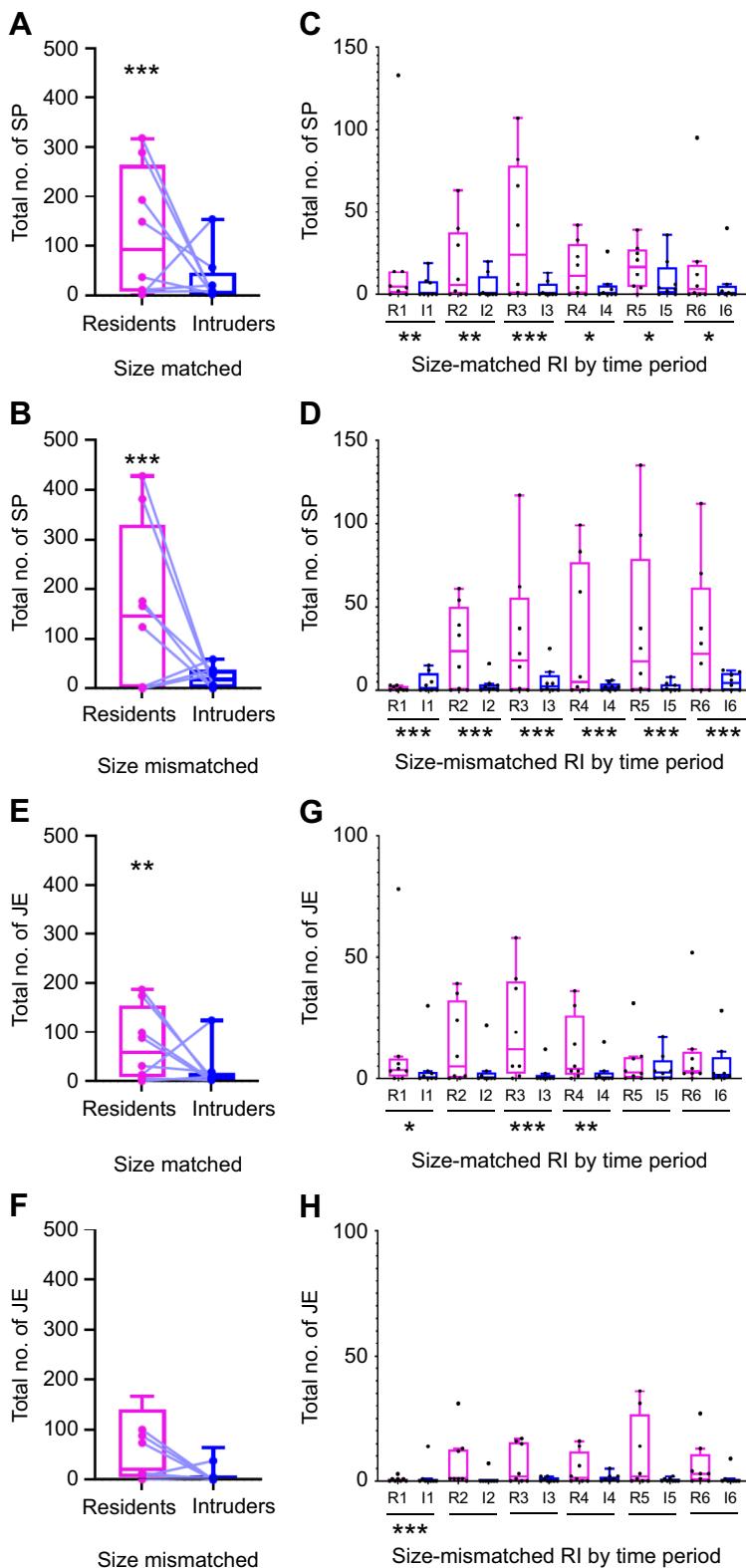
Given these effects with residency, we also wanted to investigate the effects of size on sound production and jaw extension in each context, irrespective of fish being size matched or mismatched. We examined the effects of absolute size (SL) on either the total number of sounds produced or the total number of jaw extensions in both contexts, as well as any effects of size difference in size-mismatched contexts using LMM. There was a significant effect of absolute size in size-mismatched but not-matched contexts ( $t_{14}=2.900$ ,  $R^2=0.375$ ,  $P=0.0116$ ;  $t_{14}=0.796$ ,  $R^2=0.04$ ,  $P=0.439$ , respectively), where the number of sound pulses produced increased with increasing absolute body size. Regarding jaw extension, there was no effect of absolute size on total jaw extensions in the size-matched context ( $t_{14}=1.496$ ,  $R^2=0.138$ ,  $P=0.157$ ), but there was an effect in the size-mismatched context ( $t_{14}=5.300$ ,  $R^2=0.667$ ,  $P<0.001$ ), where the number of jaw extensions increased with increasing absolute body size. In addition, there was an effect of size difference in size-mismatched pairs, where males that were larger than their counterpart produced significantly more sounds ( $t_{14}=2.844$ ,  $R^2=0.366$ ,  $P=0.013$ ) and extended their jaw more often ( $t_{14}=5.258$ ,  $R^2=0.664$ ,  $P<0.001$ ).

### Effects of residency status and size on multi-pulse burst use

Recognizing that other teleosts make multi-pulse bursts comparable to those observed here in a variety of contexts, including aggression (e.g. Myrberg, 1972; Crawford et al., 1986; McIver et al., 2014; Tricas and Boyle, 2015), we tested the hypothesis that there were residency- and/or size-dependent differences in multi-pulse burst usage. We used a PCA where the number of different lengths of bursts per male was entered as different variables (2–6 and 2–5 pulse bursts for each male in size-matched contests and size-mismatched contests, respectively). A single principal component (PC1) explained 77% of the variation of male usage of multi-pulse bursts in size-matched males and 65% of the variation in size-mismatched males (Table S1; Fig. 7). In both models, higher PC1 scores represent animals that produced a greater number of multi-pulse bursts rather than only producing single pulses, often with these animals also using different lengths of bursts (2–6 pulses).

We first investigated how multi-pulse burst usage (PC1) varied between residents and intruders across each behavioral context. In size-matched assays, the values for residents and intruders approached but did not reach significance ( $F_{1,14}=4.09$ ,  $P=0.06$ ; Fig. 7A), whereas residents and intruders significantly differed in size-mismatched contexts ( $F_{1,14}=6.50$ ,  $P=0.02$ ; Fig. 7B).

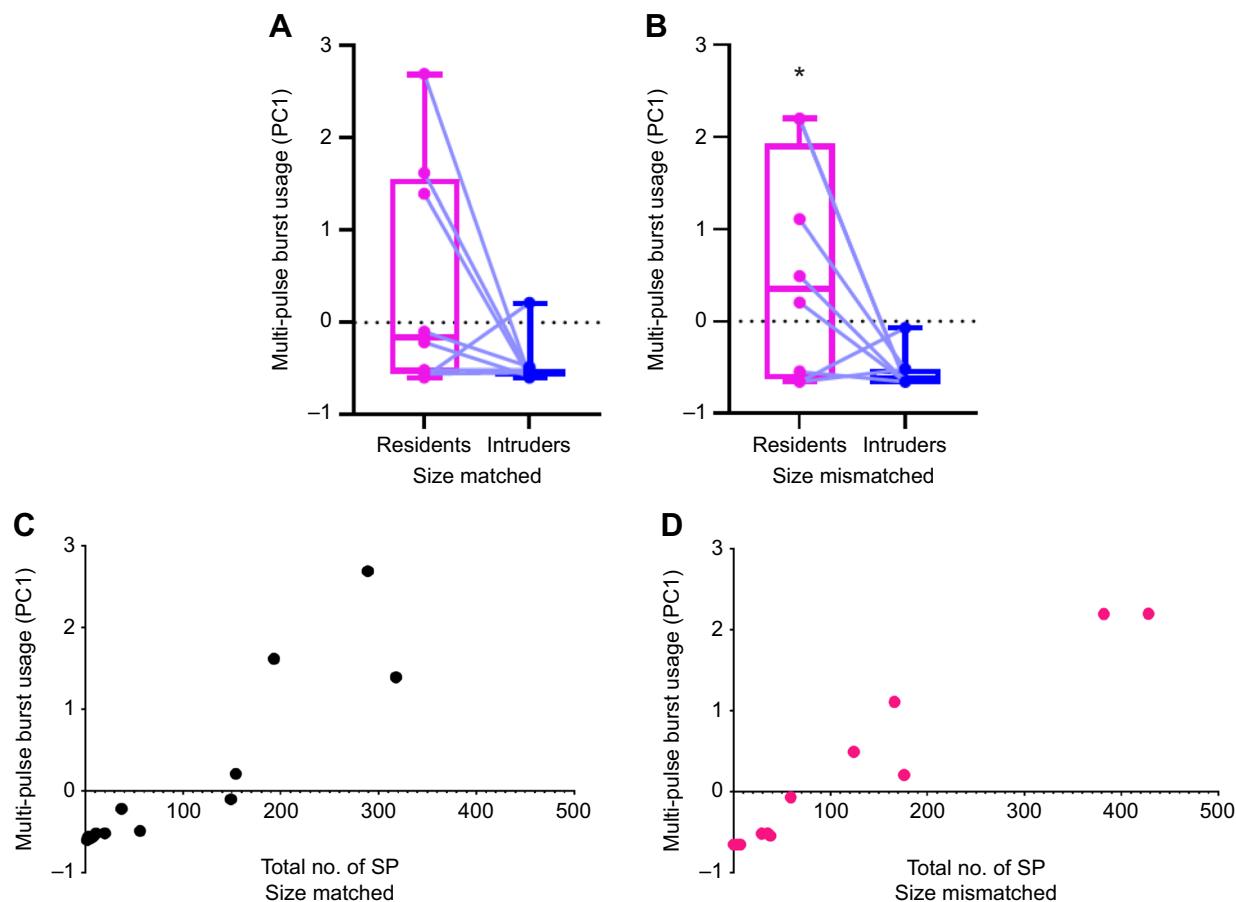
We next assessed the effects of absolute size (SL) on multi-pulse burst usage in both contexts, as well as the effects of size difference in size-mismatched contexts. We also wanted to test the hypothesis that when males exhibit an increased acoustic display rate (total number of pulses), they may group pulses into multi-pulse bursts more often than only using single pulses. We ran a multiple linear regression model for each context, using absolute size and total sound production as continuous variables for size-matched contexts, and using absolute size, size difference and total sound production as continuous variables for size-mismatched contexts, to



**Fig. 6. Effects of residency and time period on total number of sound pulses and jaw extensions for *D. dracula* male dyads.** Boxplots (median, upper and lower quartiles and 1.5× interquartile range) comparing the total number of SP (A–D) and JE (E–H) in size-matched and size-mismatched contexts ( $n=16$  males for each context, 6 time periods for each male). In A, B, E and F, individual residents are in pink and intruders are in blue. Individuals in the same dyad are connected by light purple lines. \* $P<0.05$ , \*\* $P<0.01$  and \*\*\* $P<0.001$ . (A,B) Boxplots of total number of SP for resident (left, pink) and intruder (right, blue) in size-matched (A) and size-mismatched (B) contests. (C,D) Boxplots of total number of SP for resident (R; left, pink) and intruder (I; right, blue) in time periods 1–6 in size-matched (C) and size-mismatched (D) contests. (E,F) Boxplots of resident (left, pink) and intruder (right, blue) total number of JE in size-matched (E) and size-mismatched (F) contests. (G,H) Boxplots of total number of JE for resident (R; left, pink) and intruder (I; right, blue) in time periods 1–6 in size-matched (G) and size-mismatched (H) contests. Black dots in C, D, G and H represent individual residents and intruders.

examine the effect of these variables on multi-pulse burst usage. Total sound production significantly predicted multi-pulse burst usage in both contexts (size-matched:  $t_{13}=10.409$ ,  $R^2=0.8939$ ,  $P<0.001$ ; size-mismatched:  $t_{12}=13.776$ ,  $R^2=0.9559$ ,  $P<0.001$ ; Fig. 7C,D). Absolute size also predicted multi-pulse burst usage in the size-matched context, as multi-pulse burst usage increased

with increasing absolute body size ( $t_{13}=-2.648$ ,  $P=0.0201$ ). In the size-mismatched context, absolute size and size difference did not predict multi-pulse burst usage (absolute size:  $t_{12}=-0.725$ ,  $P=0.483$ ; size difference:  $t_{12}=0.630$ ,  $P=0.541$ ). Therefore, the more sounds a fish produced during the course of the interaction, the greater the propensity to use multi-pulse bursts versus single pulses.



**Fig. 7. Multi-pulse burst usage of *D. dracula* males in dyadic contests.** (A) Boxplots of principal component 1 (PC1) describing individual multi-pulse burst usage in size-matched contests ( $n=16$  males) (see Table S1). (B) Boxplots of PC1 describing individual multi-pulse burst usage in size-mismatched contests ( $n=16$  males). In A and B, individual residents are in pink (left) and intruders are in blue (right). Individuals in the same dyad are connected by light purple lines.  $*P<0.05$ . (C) Scatterplot of total SP number by PC1 describing individual multi-pulse burst usage in size-matched contests. Black circles indicate individual fish. (D) Scatterplot of total SP number by PC1 describing individual multi-pulse burst usage in size-mismatched contests. Pink circles indicate individual fish.

## DISCUSSION

We provide the most comprehensive description to date of social behavior for any species of *Danionella*, a genus of miniature vertebrates. This includes a resident–intruder assay for size-matched and size-mismatched dyads of adult males that shows the effects of residency and body size on the performance of two prominent display behaviors, sound production and jaw extension. More broadly, the results show that both types of display can serve as robust behavioral benchmarks for comparative studies among species of *Danionella*, identifying neural, genomic and developmental mechanisms of social behavior evolution within a new model clade (cf. Jourjine and Hoekstra, 2021).

### Sound-producing displays

We report sound production by male *D. dracula* during aggressive but not courtship interactions; females are apparently not sonic. Individual sound pulses were broadband, short in duration and possessed a peak frequency close to 2 kHz in all social contexts observed here. The frequency spectrum of these pulses (Fig. 4A) is comparable to that of other ostariophysans (e.g. Ladich, 2000; Amorim, 2006) and well within the range of hearing reported for closely related genera that, like *Danionella*, have a Weberian apparatus for enhanced sound detection (Fay, 1988; Ladich, 2000; see also Braun and Grande, 2008; Johnston and Johnson, 2000; Stout, 1963; 1975; Popper and Sisneros, 2022). *Sundadanio*,

another genus of miniature cyprinids closely related to *Danionella*, exhibits similar sexual dimorphism of the Weberian apparatus, fifth rib and pectoral girdle (Conway and Britz, 2007; Conway et al., 2011; Britz and Conway, 2016), and while sound production is not described in detail, males are reported to repeatedly produce croaking sounds when removed from the water in a net and by aquarist observations (Kottelat and Witte, 1999; Conway and Britz, 2007). Males of the cyprinid genus *Barboides*, a group of miniature fishes from Africa and unrelated to *Danionella* and *Sundadanio*, also exhibit a hypertrophied muscle associated with the fifth rib that has been identified as a putative drumming muscle (Conway et al., 2017). For some teleost species, dominant frequency correlates with body size as well as with winning in dyadic contests (see Conti et al., 2015, for overview). It will be essential to describe the exact mechanism of sound production (e.g. Rice et al., 2022) and auditory sensitivity (e.g. Braun and Grande, 2008) to determine how *Danionella* and other miniature cyprinids such as *Sundadanio* and *Barboides* produce and perceive these higher frequency sounds used in aggressive contexts.

For both size-matched and size-mismatched dyads, males predominantly made single pulse bursts, alongside the generation of repetitive multi-pulse bursts ranging in length from two to six pulses. The single pulses produced by male *D. dracula* are reminiscent of damselfish and mormyrid ‘pops’ (Myrberg, 1972; Crawford et al., 1986) and single pulse sounds of butterfly fishes

(Tricas and Boyle, 2015). Many fish species produce multi-pulse sounds, commonly referred to as ‘grunts’, during reproductive and aggressive interactions (Lobel et al., 2010, 2021; Amorim and Almada, 2005; Ladich and Myrberg, 2006; Myrberg and Lugli, 2006; McIver et al., 2014). Several damselfish species, including *Stegastes partitus*, make multi-pulse ‘chirps’ during reproductive interactions (Myrberg et al., 1978). Future studies of *Danionella* building on the foundation set out here should examine these acoustic characteristics in the context of nest holding in controlled community tanks to determine whether nest-holding males differ in these characteristics compared with ‘loser’ males, similar to other species.

*Danionella* contains five species for comparative studies of both sound production and hearing (Britz et al., 2021). So far, sound production has only been reported in one other species, *D. cerebrum* (Schulze et al., 2018) (*D. priapus* are also sonic; R.L.T. and A.H.B., unpublished observations). Male *D. dracula* and *D. cerebrum* both make very short broadband, sharp onset pulses around 2 ms in duration. They also exhibit variation in how frequently they sequence bursts during male–male interactions. The sound pulses of both species can be grouped into bursts separated by a characteristic IPI, close to 35 ms in *D. dracula* and either 8 or 17 ms in *D. cerebrum* (Schulze et al., 2018). Beyond the difference in IPI duration, there is a large difference between the two species in the duration of individual bursts and burst trains. *Danionella dracula* primarily make single pulse sounds versus multi-pulse bursts typically of 2–3 and rarely 4–6 pulses, at least under the conditions tested here. By contrast, *D. cerebrum* have multi-pulse bursts lasting close to 1 s and repetitive bursts lasting of the order of minutes (Schulze et al., 2018). As pointed out recently by Britz et al. (2021), there are dramatic differences in the skeletal anatomy of the putative sound-producing mechanism between *D. dracula* and *D. cerebrum* (especially the os suspensorium) and this may explain the differences in the sounds produced by the two species. For both species, it would be of interest to compare behaviors that might be coordinated with sound production. The temporal relationship between sound production and lunging is not described for *D. cerebrum* (Schulze et al., 2018) as documented here for *D. dracula*. In addition, *D. cerebrum* (together with *D. mirifica*, *D. priapus* and *D. translucida*) lacks the hypertrophied jaw and thus the dramatic jaw extension of *D. dracula*.

### Jaw extension

Beyond acoustic behavior, animals use other sensory modalities to communicate in conflict scenarios, including visual mechanisms such as weapons and display postures (see Kodric-Brown et al., 2006; Lappin et al., 2006). As we have demonstrated, *D. dracula* males often extend their hypertrophied lower jaw during aggressive interactions. This morphological and behavioral characteristic appears to be unique to this species within the genus. Future studies should test whether jaw extension behavior functions as a visual agonistic signal, possibly allowing males to signal contest escalation and assessment information to their competitors.

Despite our pilot observations that jaw extension was always linked to sound production and hence the latter might depend on the former, we found them to independent events. The varied temporal separation between the two types of display, especially during periods of low sound production, as well as the independent occurrence of each during male aggressive encounters, indicate these two actions are separate displays. However, the overlap of the two behaviors during periods of heightened activity provides the opportunity to study a possible multimodal signaling repertoire (e.g. Elias et al., 2003; Amorim et al., 2019) that could maximize

robustness of the overall multichannel signal (Ay et al., 2007). Males in other sonic species of fish have multimodal acoustic signaling repertoires that generate sounds by more than one mechanism (e.g. Rice et al., 2022; Kéver et al., 2021). Sonic signals might also be combined with specific features of other sensory modalities, such as color and ornamentation as well as stereotyped visual dancing/movement displays (Hebets and Uetz, 2000; Elias et al., 2003; Soma and Garamszegi, 2015). Jaw extension by *D. dracula* males could serve as a modifier or amplifier of sound production as well, not contributing its own information but instead augmenting possible acoustic signaling (Bradbury and Vehrenamp, 2011; Gualla et al., 2008; Lappin et al., 2006). For a more rigorous investigation of possible temporal coupling/coordination of acoustic and postural displays in *D. dracula* and hence any possibility of multimodal signaling, the concurrency of sound production and jaw extension should be re-visited with higher resolution video than used here to examine the timing more precisely between these displays, as done for other sonic species (e.g. Bostwick and Prum, 2003; Fusani et al., 2007).

### Individual assessment

Resources such as shelters and territories are continually defended by an owner to gain fitness advantages (Conti et al., 2015; Arnott and Elwood, 2007). *Danionella dracula* males swim closely around nest sites that contain spawning crevices. Future studies would benefit from examining whether and how males might utilize acoustic communication and other signaling modalities (e.g. vision) to structure possible dominance hierarchies and determine nest ownership in a community (Conti et al., 2015; Chase et al., 2002; Amorim and Almada, 2005; Arnott and Elwood, 2009; Barata et al., 2007; Myrberg and Riggio, 1985). While the acoustic parameters we describe here can be correlated with features of agonistic displays, it is essential to first study what components, if any, of this information are perceived by members of a contest and any observers before characterizing a behavior as a signal or a cue, or to determine what features may or may not mediate mutual or self-assessment (Bradbury and Vehrenamp, 2011; Arnott and Elwood, 2009). It would be essential to determine how nest-holding males might differ from other males in complex acoustic parameters such as multi-pulse burst usage, as well as factors that contribute to fighting potential examined here such as residency and body size. How does ownership of a nest site in a community setting affect fighting strategy and escalation, as could be indicated by display rate and sonic characteristics in *D. dracula*? And does the largest male in the community hold this nest site? Body size is often predictive of winning an escalated fight in most animal species, especially those with high variation in body size (Andersson, 1994). A display posture can advantageously reveal body size, and large animals can produce or bear large weapons (Kodric-Brown et al., 2006). We saw a size effect in *D. dracula* where the largest males (in terms of absolute size) produced the most sounds and jaw extensions in the size-mismatched dyad context, and the larger male relative to its counterpart most often (7/8 dyads) produced more sounds and extended its jaw more often in size-mismatched dyads. We also found a size effect where larger males in the size-matched context (in terms of absolute size) produced significantly more multi-pulse bursts. In addition, we found a distinct resident effect in size-matched dyads, implying a potential strategy where residents are predicted to escalate aggression compared with intruders (Maynard Smith, 1979). Given the effects of residency and size we observed in the different contexts, there may be a possible interaction between residency and size asymmetry in *D. dracula* fighting strategies.

(Hack et al., 2010; Hofmann and Schildberger, 2001; Jennions and Backwell, 1996; Jackson and Cooper, 1991). Closer examination of the correlation between these variables and changes in behavioral sequencing, across a wide range of size differences in *D. dracula* males, would allow researchers to investigate whether this type of information is mutually or self-assessed (Parker, 1974; Arnott and Elwood, 2009; Bradbury and Vehrencamp, 2011).

### Concluding comments

We demonstrate that *D. dracula* is a sound-producing species and uncover initial residency and body size effects on sound production as well as a postural display (jaw extension) during aggressive interactions. Future physiological investigations of auditory sensitivity (see Bass and McKibben, 2003), as well as whole-brain neuroimaging of *Danionella* (Schulze et al., 2018; Chow et al., 2020), can determine the components of natural acoustic and visual/postural displays that could be attended to by both males and females. The results are also a basis for subsequent behavioral studies determining how and whether males of different species of *Danionella* in general may assess each other's fighting ability via multiple sensory modalities, such as audition and vision for observing sonic and postural (jaw extension) behaviors, respectively. The temporal relationship of sound production to other displays such as jaw extension could also allow researchers to determine how such actions may augment information conveyed through sound production, perhaps acting as a tactical threat or amplifier (see Bradbury and Vehrencamp, 2011). Last, our experiments provide a foundation on which to test established social behavioral models of conflict resolution such as fighting strategy models (see Bradbury and Vehrencamp, 2011), which altogether would make a comprehensive framework on which to study the neural and genetic drivers of social behaviors in the multiple species of *Danionella*.

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### Competing interests

The authors declare no competing or financial interests.

### Author contributions

Conceptualization: R.L.T., H.K.R., A.H.B.; Methodology: R.L.T., S.C.C., J.T.P., E.R.S., K.W.C., H.K.R., A.H.B.; Validation: R.L.T.; Formal analysis: R.L.T., E.R.S., H.K.R.; Investigation: R.L.T., Z.G., S.C.C., H.K., W.F., J.T.P.; Resources: A.H.B.; Data curation: R.L.T.; Writing - original draft: R.L.T., A.H.B.; Writing - review & editing: R.L.T., Z.G., S.C.C., J.T.P., E.R.S., K.W.C., H.K.R., A.H.B.; Visualization: R.L.T.; Supervision: A.H.B.; Project administration: A.H.B.; Funding acquisition: A.H.B.

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### Data availability

Data are available from figshare: <https://doi.org/10.6084/m9.figshare.20340876.v1>

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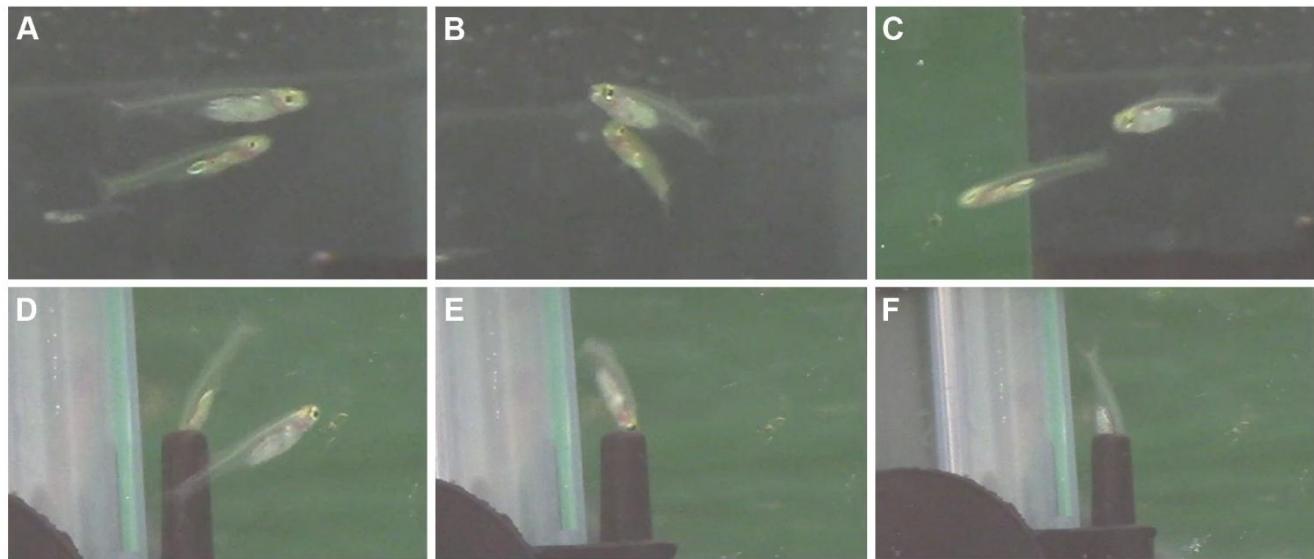
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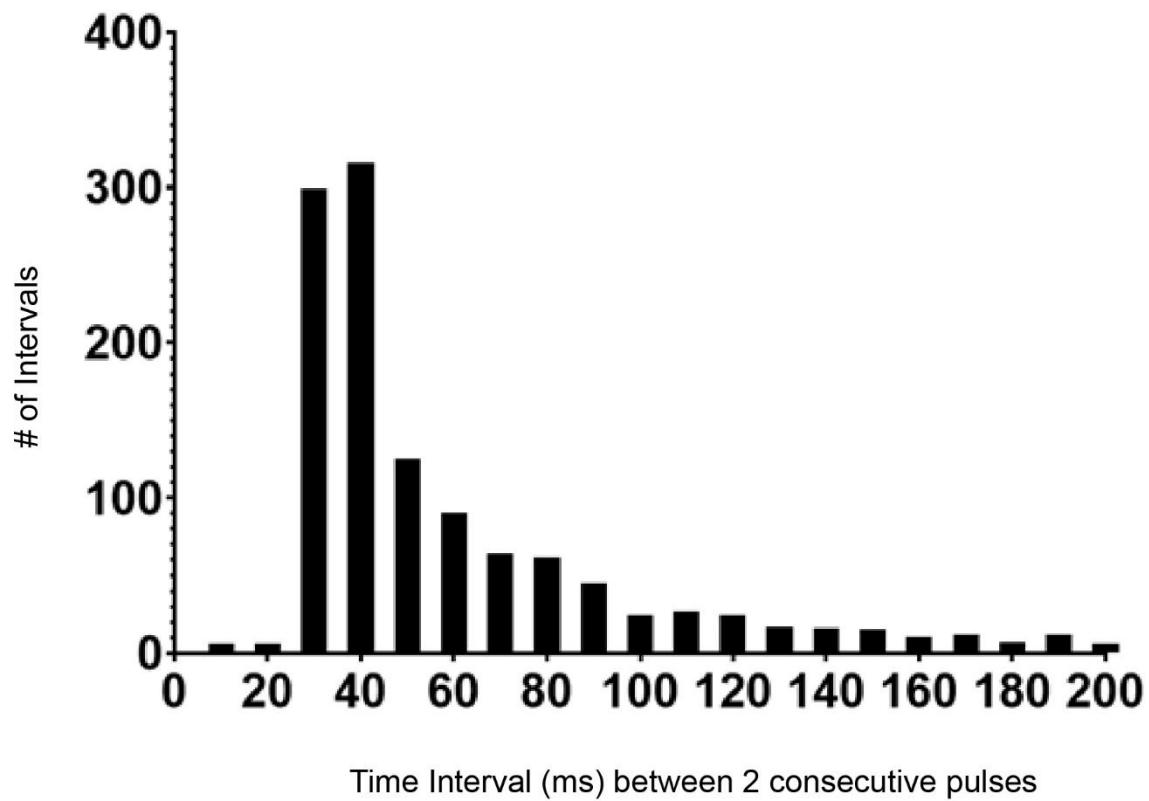
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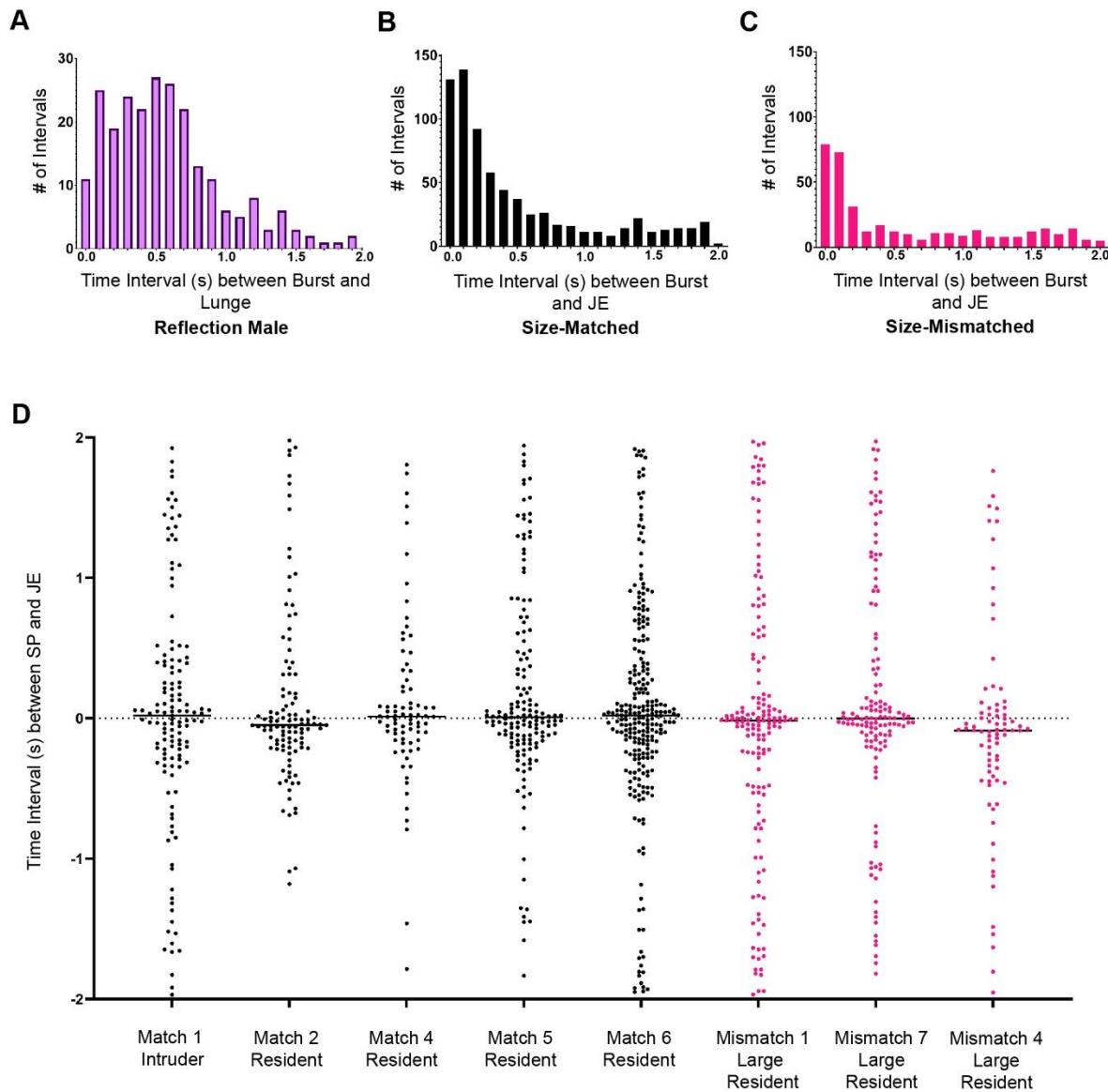
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**Fig. S1 (also see Movie 2). Video frames of *Danionella dracula* courtship behavior in the presence of a black vertical tube that was originally part of an underwater filtration system in a colony tank.** (A) Males typically swim from nest sites to court females who were often schooling around the tank. Male swims below female. (B) Male rapidly moves his pectoral fins and vibrates his body back and forth beneath female egg vent. (C) Female follows male back to nest entry crevice (part of the underwater filter) that he swam closely around during the day. (D) Male enters headfirst into spawning crevice. (E) Female orients head towards spawning crevice. (F) Female swims headfirst into spawning crevice. After spawning, the female left the nest and the male emerged from the nest, swam closely around the nest, and then courted additional females throughout the day. Spawning cannot be directly observed, but is inferred to take place in enclosed crevices for two reasons. First, clutches of 12-24 eggs are found in the grooves of the sponge nests (see Fig. 1E) at any time of the light cycle following spawning interactions. Second, a male and/or a female are often found on top of an egg cluster within the grooves during daily nest checks.

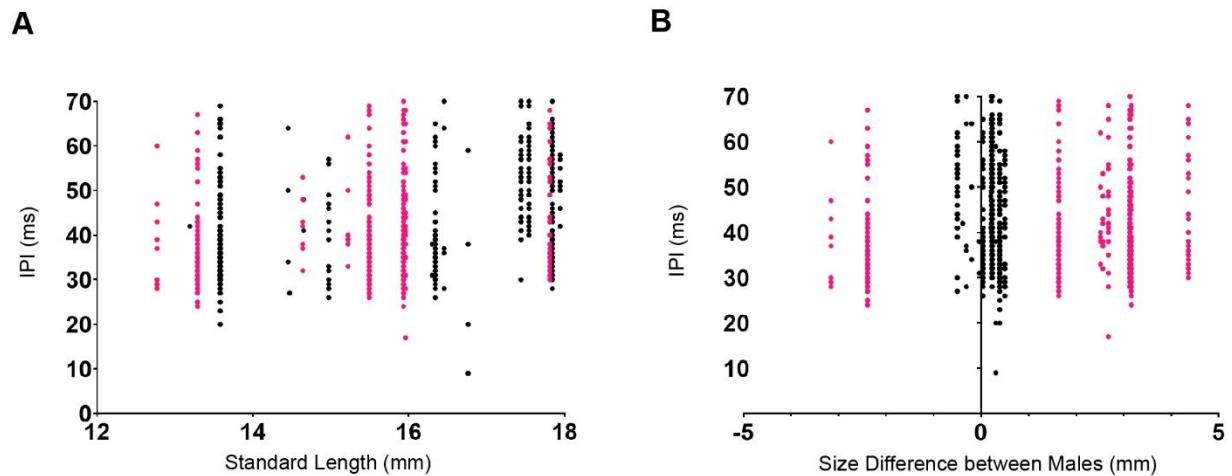


**Fig. S2.** Frequency distribution of intervals (ms) measured between two consecutive individual sound pulses (includes all males in both size-matched ( $n=16$ ) and size-mismatched ( $n=12$ ) dyads). The mode and peak are 34 ms.

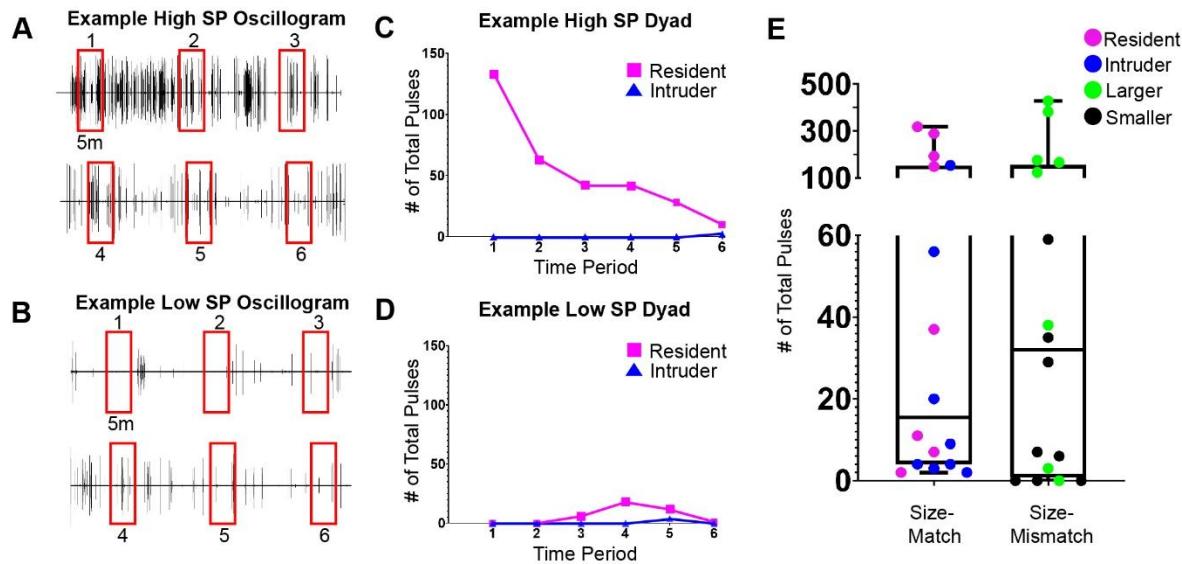


**Fig. S3. Frequency and scatterplot distributions of intervals measured between two consecutive individual behavioral events.** A. Frequency distribution of intervals (s) measured between a burst of sound and a lunge. B, C. Frequency distribution of intervals (s) measured between a burst of sound and an extension of the lower jaw (JE) in high sound producing and high jaw extending males in size-matched (B, n=5) and size-mismatched (C, n=3) dyads. D.

Scatterplots of intervals (s) measured between a burst of sound and an extension of the lower jaw (JE) in high sound producing and high jaw extending males in size-matched and size-mismatched dyads. Negative values indicate intervals between a burst of sound and a jaw extension that happened before the sound burst. Positive values indicate intervals between a burst of sound and a jaw extension that happened after the sound burst.



**Fig. S4. Scatterplots of individual male standard length (A) or size difference between competitors in a dyad (B) by inter-pulse interval (IPI).** Black circles indicate measurements from individual males in size-matched contests ( $n=16$ ) and salmon pink circles from individual males ( $n=12$ ) in size-mismatched contests ( $n=422$  and  $456$  pulses in size-matched and size-mismatched dyads, respectively, for both A and B). A. Individual male standard length (mm) plotted by IPI (ms). B. Size difference (mm) between two males in dyad plotted by IPI.

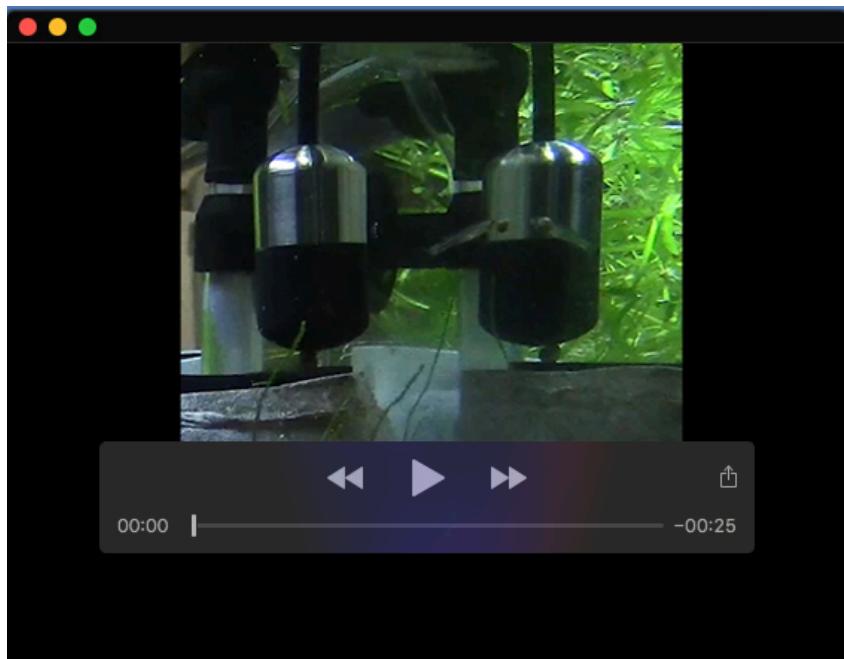


**Fig. S5. Total sound production (SP, pulse number) by *Danionella dracula* males in dyadic contests.** (A) Representative oscillogram of high SP; rows 1 and 2 from one continuous 2 h record. Red boxes indicate the six 5min sampled time periods (1-6) spaced 15 min apart. (B) Representative oscillogram of low SP; rows 1 and 2 from one continuous 2h record. Red boxes indicate the six sampled time periods. (C) Example high SP from size-matched dyad in 'A', showing total pulse number for individual fish over sampled time periods. Magenta squares indicate resident fish's SP values and blue triangles indicate intruder fish's SP values, which are at or near zero during all time periods (0 pulses in periods 1-5; 2 in period 6). (D) Example low SP from size-matched dyad in 'B', displayed as in 'C'. Magenta squares indicate resident fish's SP values, which peaks at 20 sound pulses in period 4, and blue triangles indicate intruder fish's SP, which is also at or near zero during all time periods (0 in periods 1-4, 6; 4 in period 5). (E) Boxplots of individual SP values in size-matched (n=16; left boxplot; magenta = resident fish, blue = intruder fish) and size-mismatched (n=16; right boxplot; green = larger fish, black = smaller fish). Break in y axis from 60-100 total pulses for best visualization of all SP values. Black horizontal line within box indicates median used to designate individuals as high or low sound producers

**Table S1.** Principal component analysis of multi-pulse burst in size-matched and size-mismatched dyadic contests: Loading Coefficients.

|                           | Size-matched | Size-mismatched |
|---------------------------|--------------|-----------------|
| Type of multi-pulse burst | PC1 (77%)    | PC1 (65%)       |
| Two pulse                 | 0.96         | 0.97            |
| Three pulse               | 0.99         | 0.92            |
| Four pulse                | 0.92         | 0.68            |
| Five pulse                | 0.86         | 0.59            |
| Six pulse                 | 0.59         | ---- *          |

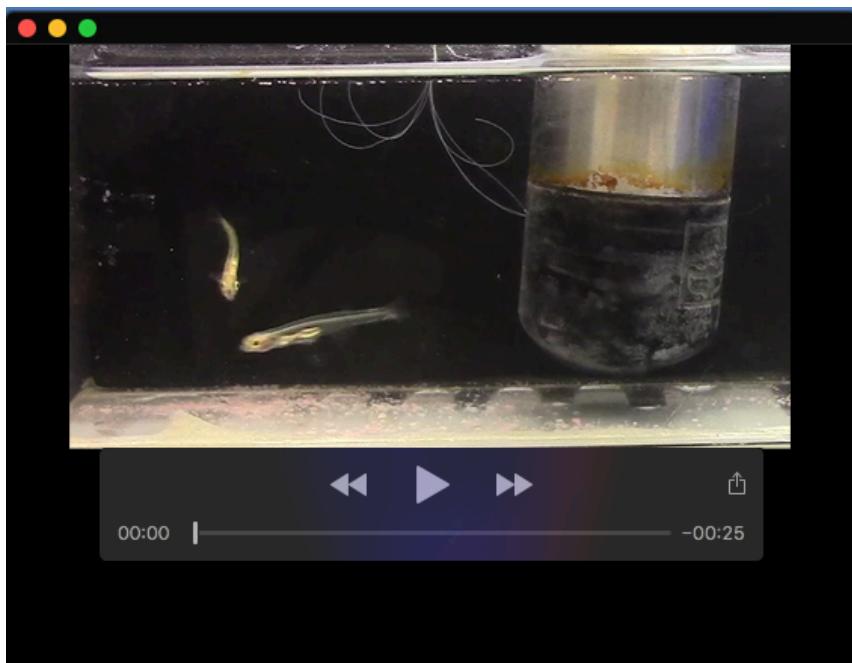
\*Six pulse bursts were never observed in size-mismatched contests



**Movie 1.** A male fish lunges at its own reflection in the tank wall, producing sound pulses and extending the lower jaw.



**Movie 2.** Male swims below female and vibrates his body and head back and forth beneath the female's egg vent. Male then swims back to nest entry crevice and female swims behind male to same nest entry crevice. Movie zooms in to show male as he swims headfirst into nest entry crevice and female orients head towards crevice before swimming headfirst into crevice.



**Movie 3.** In a dyadic interaction, a male orients head towards other male and lunges at the other male, producing a series of sound pulses and extending his lower jaw. The other male swims away from the lunging male.