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

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Key Words:

Acoustic communication, aggression, teleost fish, residency, intruder

ABSTRACT

Individuals can reveal their relative competitive ability or mate quality through acoustic communication, varying signals in form and frequency to mediate adaptive interactions including competitive aggression. We report robust acoustic displays during aggressive interactions for a laboratory colony of *Danionella dracula*, a recently discovered miniature and transparent species of teleost fish closely related to zebrafish (*Danio rerio)*. Males produce bursts of pulsatile, click-like sounds and a distinct postural display, extension of a hypertrophied lower jaw, during resident-intruder dyad interactions. Females lack a hypertrophied lower jaw and show no evidence of sound production or jaw extension under such conditions. 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. Resident males produce significantly more sound pulses than intruders in both dyad contexts; larger males are consistently more sonic in size-mismatched pairs. For both conditions, males show a similar pattern of increased jaw extension that frequently coincided with acoustic displays during periods of heightened sonic activity. These studies firmly establish *D. dracula* as a sound-producing species that modulates both acoustic and postural displays during social interactions based on either residency or body size, thus providing a foundation for investigating the role of these displays in a new model clade for neurogenomic studies of aggression, courtship and other social interactions.

INTRODUCTION

Animals across taxa have distinct behavioral repertoires utilizing a variety of signaling channels and modalities to mediate complex social interactions (Bradbury & Vehrencamp 2011). Signal structure within a modality can further vary in a species depending on the social context where these signals are used. In addition, individuals within a social group may differ in signal parameters depending on the context (e.g., Bradbury & Vehrencamp 2011; McIver et al. 2014; Elias et al. 2003). Acoustic communication provides many such examples, such as advertisement and agonistic calls (e.g., Amorim et al. 2015; Amorim et al 2013; Pereira et al 2014). Across vertebrate taxa, acoustic signaling repertoires often differ between the sexes (Gerhardt and Huber, 2002; Bradbury & Vehrencamp 2011). These signals may further differ in parameters ranging from amplitude, duration and fundamental frequency to interval between repetitive components within a call and overall call rate (Davies and Halliday, 1978; Clutton-Brock and Albon, 1979; Sebastiannutto et al. 2008; Bradbury & Vehrencamp 2011; Odom et al., 2020). In many species of teleost fish, the focus of this report, males often exhibit more robust acoustic repertoires than females, although detailed investigations of female sound production are scarce (Amorim, 2006; McIver et al. 2014; Amorim et al., 2015; Ladich, 2015). This difference in behavior can reflect underlying dimorphisms, where males may possess hypertrophied muscles often associated with vibration of the swim bladder or stridulation of pectoral fin rays to drive sound production (Rice et al., in revision).

Like other vertebrate taxa, soniferous behavior in teleost fish can figure prominently into aggressive competition for mates, nest sites, shelters, and territories (Andersson, 1994; Amorim refs above). Acoustic signaling can enable these contests to be resolved through less costly measures before escalating to more intense stages of engagement involving direct contact and possible physical damage (Davies and Halliday, 1978; Clutton-Brock and Albon, 1979; Hsu et al., 2008; Oliveira et al. 2011; Green and Patek, 2018). These signals can communicate information about a contestant's physical attributes, such as body size which can figure prominently into determining the outcome of aggressive conflicts (Davies and Halliday, 1978; Clutton-Brock and Albon, 1979; Lee and Bass, 2006; Bradbury and Vehrencamp, 2011; Amorim et al., 2015; Conti et al., 2015; Billings, 2018). It is within these contexts that we describe the acoustic signaling behavior of *Danionella dracula*, a miniature species of cypriniform fish that we found produces relatively simple, broadband sounds apparently solely during aggressive

encounters. *Danionella dracula* was first described in 2009 and known only from a small stream in the Kachin state in northern Myanmar (Britz and Conway, 2016, Britz et al., 2009, Britz et al., 2021). Nothing is known regarding the specifics of its behavior in the wild, largely because its natural habitat is relatively inaccessible currently due to political unrest (e.g., see https://www.bbc.com/news/world-asia-55902070) . *Danionella dracula*, like other members of its genus, exhibits developmental truncation, leading to the retention of some larvae-like traits as miniature adults (progenesis), reaching only 12-17 mm in standard length (Britz and Conway refs above). So far, adult males in the genus, but not females, have a large muscle mass associated with the swim bladder and hypertrophied elements of the Weberian apparatus and fifth rib, which together form a sound-producing, "drumming" structure (Britz and Conway refs above, Judkewitz personal communication). *Danionella dracula* further possess a hypertrophied lower jaw and a series of odontoid processes which resemble teeth (Britz and Conway, 2016). Acoustic signaling and extension of the lower jaw are the two focal characters in the current investigation.

Our main intent here is to provide a behavioral baseline for a species that can be readily bred in captivity and studied for the effects of genetic manipulations on display behaviors during social interactions and the neuronal populations driving these behaviors. Given their close phylogenetic relationship to zebrafish (*Danio rerio*), *D. dracula* are amenable to genetic manipulation using the same molecular toolbox (see Schulze et al). Species within the genus *Danionella* further offer multiple advantages for behavioral neuroscience given their especially small size as adults (see above) and transparency through their entire lifespan (e.g., Fig. 1A-C), thus facilitating non-invasive, optical imaging of the brain using multiphoton microscopy (e.g., see Chow et al., 2020; Akbari et al. 2020,21; Schulze et al., 2018+erratum). Despite possessing these attractive features for neurogenomic studies of social behavior, these behaviors have not yet been described in a comprehensive manner for *D. dracula* or its sister species.

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 and present the results of a resident-intruder assay to examine acoustic and postural signaling during dyadic aggressive interactions in size-matched and size-mismatched male encounters. Our quantitative analyses focus on two display characters that distinguish this species from others within the genus: temporal features of acoustic signals and extension of a hypertrophied lower jaw. Previous research in aggression has

utilized behavioral tests like resident-intruder assays and other dyadic designs to study how two individual males can compete over a resource and what signaling parameters correlate with differences in the ability for an animal to win a contest, which is termed as the animal's resource-holding potential (Bradbury & Vehrencamp 2011). We hypothesized that in size-matched encounters, resident fish would exhibit increased sound production and jaw extension directed against the intruder fish, as well as possibly exhibit differences in the structure of their acoustic signals. However, in size-mismatched encounters, we hypothesized the larger fish regardless of residency would exhibit increased sound production and jaw extension against the smaller fish. For both sets of contests, we expected higher sound producing animals to exhibit similar acoustic signal structure differences.

MATERIALS AND METHODS

Colony formation and breeding

Adult *D. dracula* were originally purchased from commercial dealers (The Wet Spot Tropical Fish, Portland, OR; Invertebrates by Msjinkzd, York, PA) and bred in environmental control rooms at Cornell University, Ithaca, NY. Fish were kept at water temperatures of 23-25.5°C and on a 16:8 light: dark cycle. We found that *D. dracula* requires direct overhead lighting to breed. Thus, breeding tanks were maintained in rooms with natural overhead lighting via a skylight that was supplemented with artificial lighting during the short days of late fall and winter or in rooms that only had the artificial lighting year-long. There were no obvious differences between fish under the two housing conditions. All dyad contests were conducted in rooms supplemented with artificial lighting on a 16:8 light: dark cycle. All animals were fed twice a day, *Artemia nauplii* in the morning and ground fish flake (TetraMin Tropical Flake) in the evening. Plastic plants were added to the tank for fish to acclimate to the aquarium setting.

Fish were bred in 2.5-125 gal aquaria and were housed in populations of varying density based on tank size, in adult ratios of 1:2 males: females, with at least three males per tank (Fig. 1D). Nest sites were made from double sponge water filters (XINYOU XY-2822 Air Pump Double Sponge Water Filter, Amazon) that contained nine 4-mm crevices for spawning (Fig. 1E). The sponges were covered with a BIO-CHEM ZORB filtration cartridge (API Fishcare CRYSTAL Filtration Cartridge), as it is required for the crevices to be enclosed for breeding

(Fig. 1E). Breeding sponges were placed at opposite ends of the tank (Fig. 1D). 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, resting within a larger 50-gal aquarium (see eggs in Fig. 1E). Larval fish were fed AP100 Dry Larval Diet (Zeigler Bros, Inc.) twice a day. Larvae were kept in these smaller cylinders for 10 days before being moved to 5-gallon tanks where they are fed adult diet. Larvae become adults in 3 months, determined by the presence of eggs in females and the hypertrophied jaw in males.

Community tank observations

A 16.8-gal community tank with dimensions 75 x 30 x 28 cm was set up using the same parameters for colony system tanks to allow for behavioral observations in a reproductive context (1D). A heater in the tank kept the tank at 25°C. Males (3) and females (6) were placed into the tank 30 minutes after dye labeling. Each male had a muscle segment labeled in the tail with either 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). 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 Audio H1a) placed next to each nest site. Hydrophones were connected to 30-fps video cameras (Canon Vixia HFR500) using a mono to stereo adaptor to synchronize the audio collection with the video. *Danionella dracula* males are more sonic during daylight hours, like males of their sister species, *D. cerebrum* (Schulze 2018; initially designated *D. cerebrum* as *D. translucida*; see Britz et al., 2021). All observations in community tanks were carried out between 9:00 – 17:00 h.

Fish were allowed an acclimation period, which concluded after one week at the onset of courtship. Then, three independent observers used focal sampling and live observation (BORIS) 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 were observed, between 09:00 and 17:00 for each of seven days. Fish received the same diet regime as colony tanks, with the first feeding at 10:00-10:15 and the second at 17:00. 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

states and point events in the focal male. These point and state events are detailed in Table 1. These behavioral characterizations around the nest site were verified with the video camera and sound data collected.

Table 1. Community Tank Behavioral Ethogram for *D. dracula*

Point Event	**Definition**
Lunge at male	Focal male orients head towards and swims rapidly towards male. Trajectory directed at target male , but does not pursue as target swims away.
Lunge at female	Focal male orients head towards and swims rapidly towards female. Trajectory directed at female, but does not pursue as female swims away.
Courtship	Focal male swims beneath female and vibrates its head and fins back and forth underneath egg vent
Chase Female	Focal male swims in the direction towards female, while female swims in direction away from male. Trajectory may change depending on movement of female.
Lead Female	Focal male swims in front of female; female swims in direction towards male.
Enter nest	Focal male swims into crevice of nest head-first.
State Event	**Definition**
Nest Circling Left Nest	Focal male fish swims around left nest site within 2-3 body lengths
Nest Circling Right Nest	Focal male fish swims around right nest site within 2-3 body lengths
Male Interaction	2 male fish associate closely with each other, performing aggressive behaviors including lunge, sound production, and/or jaw extension
Alone	Focal male fish swimming outside nest zone alone
Shoaling	Focal male fish swimming in coordination with at least one other fish within 2-3 body lengths of other fish for 3-5 seconds, mirroring movements of other fish

162

163 **Resident-intruder assays**

164 Males were removed from group colony tanks 22-24 h before the resident-intruder assay. The
165 resident male was housed in the experimental rectangular tank, which was 14 cm x 5 cm x 5 cm
166 (Fig. 2A). The intruder male was housed separately in an 8 cm x 8 cm x 8 cm tank. Both tanks
167 contained the same volume of water, 315 cm^3, with a similar depth of 4.5-5 cm. Taking
168 advantage of male color variation (Fig. 1A-B), residents and intruders were selected to be
169 different colors in size-matched assays, to readily distinguish them in the videos. One fish was
170 greener in coloration than the other, which was more pale yellow; half of the size-matched
171 residents were pale yellow, and half were green. There was no apparent effect of color on
172 outcome of contests. Fish were fed their community tank diet in the evening and morning before
173 testing. Both tanks were aerated with an air stone, and water was novel system water that had not
174 housed adult fish previously. Three of the four tank walls were black and opaque, allowing for
175 better contrast for later fish identification. The experimental tank used to run resident-intruder
176 trials alone contained a hydrophone. The size of the hydrophone was chosen to best resemble a
177 *D. dracula* nest site; this type of hydrophone has been observed to elicit crevice-seeking
178 behaviors in males (R. Tatarsky, unpublished observations). Each resident-intruder trial lasted 2
179 h, beginning at 11:30 (Fig. 2B). The intruder male was added to the experimental tank with a
180 small net to begin the resident-intruder assay (Fig. 2C). Sound production and video were
181 recorded through the hydrophone attached to a 30-fps video camera (Canon Vixia HFR500).
182 There were 10 size-matched dyads and 10 size-mismatched dyads. Two of the size-matched
183 dyads were removed from analysis due to fish swimming continuously against the sides of the
184 miniature tank, as this appeared as if the fish did not acclimate to the experimental chamber. Two
185 of the size-mismatched dyads were removed from analysis, one due to fish swimming
186 continuously against the sides of the miniature tank, and the other because both fish were
187 intensely engaged in an escalated aggressive interaction where it was not possible to ascertain
188 identity of the sound producer, similar to extended aggressive interactions in community tanks.
189 In size-matched assays, the relative size difference between males ranged from 0.2 to 0.5 mm
190 (standard length). In size-mismatched assays, males differed by 1.6 to 4.4 mm. Five single-male
191 control trials were conducted following the same procedure, but only one male was in the

experimental chamber and an intruder male was not added to the chamber at the start of the 2-h trial. Single male controls did not produce sounds and solely swam around the tank.

Five additional control dyads were conducted using male-female pairs. 5 males and 5 females were combined in single pairs in the experimental tank following the same procedure, varying in size differences between males and females. 2 of the residents were male and 3 were female. There was no evidence in these recordings that females made any sounds similar to males, consistent with the reported sexual dimorphism in the putative sonic "drumming" muscle (Britz and Conway, 2016). A hydrophone placed in a 20-gal tank of 10 female fish for 16 hours also did not provide any evidence of sound production by females.

Audio recording sampling and analysis

After the intruder male was added to the experimental tank for resident-intruder trials using a small net, the fish were given 5 min to expectedly acclimate to the experimental scenario. The hydrophone recording for each dyad was for the entire 2 h long trial. Due to the large number of sounds observed in the 2 h period, the oscillogram from each hydrophone recording after the initial acclimation period was split into six 5 min long time-periods separated by 15 min intervals (Fig. 2D).

Sound characteristics definition and measurement criteria

Sound characteristics in Table 1 were measured using Raven Pro 1.6 (K. Lisa Young Center for Conservation Bioacoustics, Cornell Laboratory of Ornithology). Recordings typically displayed a high signal to noise ratio (e.g., Fig. 2D). Pulse duration was determined by first measuring the maximum amplitude of an individual sound pulse's waveform. This value was then divided 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. Pulse peak frequency was measured for each pulse after selecting the pulse using the pulse duration criteria above, using Raven's Peak Frequency measurement. This measurement in Raven is calculated from the spectrogram of the sound and is the frequency at which the maximum/peak power occurs within the selection. On the recording, we observed individual pulses occurring very close to each other in time, forming apparent clusters composed of multiple pulses. We measured the duration of time between neighboring individual pulses, which we call the inter-pulse interval

223 (IPI). We measured the duration of time between all individual pulses and pooled all the male's
224 inter-pulse interval (IPI) data together and found a distinct peak value of 34 ms. We used this
225 value to set boundaries for individual burst types, where pulses that composed a burst had to
226 possess an IPI within two standard deviations of the peak, be less than 70 ms apart. This criterion
227 allowed us to identify burst types ranging up to 7 pulses in length. All IPIs greater than 70ms
228 were defined as inter-burst intervals (IBI), or the distance between bursts composed of individual
229 pulses. We did not measure sound amplitude given our inability to measure sounds from
230 individual sound producing fish that were always moving in community tanks and during dyadic
231 interactions in small volumes, at most 7 cm away from the hydrophone.
232
233 Table 2. Sound Characteristics and Definitions in *D. dracula*

Sound Characteristic	Definition
Pulse Duration	Total time of one individual sound pulse.
Peak Pulse Frequency	Frequency at which the maximum peak power occurs within one individual sound pulse.
Burst	A train of individual pulses (1-7 observed), where pulses are separated by ≤ 70 ms.
Inter-Pulse Interval	Time between individual pulses in a single burst of pulses, where pulses are separated by ≤ 70 ms.
Inter-Burst Interval	Time between individual bursts, where interval between two successive pulses is ≥ 70 ms.

234
235 **Analysis of a Male's Sound Production at Tank Reflection**

For determining near-cooccurence of sound production and lunging behavior, a 4 minute segment of a 23 min recording of a *D. dracula* male continuously lunging at its reflection and producing simultaneous multi-pulse bursts and jaw extensions was analyzed for lunging behaviors and sound production (Supplementary Video 1). To examine the timing of sound production and lunging, the video was coded in BORIS for lunging behavior by the focal fish towards its reflection by an observer at 0.3x the normal speed without sound. After the observer completed the lunging behavior analysis, the observer went back to the time points marked for lunging behavior and recorded yes or no if sound production nearly occurred with the lunging behavior observed, determining a percentage of how many lunges were concurrent with sound production.

We also wanted to determine what percentage of sound production was near-cooccurent with lunging behaviors. An observer coded in Raven every sound pulse in the same 4-minute time period analyzed for lunging behaviors in BORIS, and then the start value for a burst of sound production was compared to the start value of a lunge. To determine how close a burst and lunge could be associated to be determined as near-cooccurent, we calculated the distance in seconds between the start times of subsequent lunges and saved these into a new dataset. From the dataset, the minimum distance between individual lunges was determined as 0.2s, and the cutoff for near-cooccurence was determined as the minimum distance + 1 standard deviation (0.424s), or 0.624s. These operations were performed using the Python coding language and the package numpy (Python 3.8; numpy 1.18.5).

Video analysis of sound production and jaw extension behavior

For the two different contexts of size-matched and size-mismatched assays, an observer blind to resident-intruder status verified instances of male sound production by watching the synchronized video at 0.3X the normal speed. Sound bursts were attributed to an individual male based on associated lunging movement, which was noted but not quantified. This assumption was based on our analysis of the 23 min recording of the *D. dracula* male continuously lunging at its reflection.

Observers distinguished fish based on coloration: green or pale yellow (see above), as well as other identifying features such as body girth. Hinged extension of the lower jaw can also occur during sound production. Coding of jaw extension was done by a third observer in BORIS

who watched the video at 0.3X 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. We did not collect the end point of jaw extension as the start point was readily visualized, but finer resolution video would be needed for quantifying the duration, and hence end point, of jaw extension.

Statistical analyses

Statistical analyses were performed in R. To first test the hypothesis that multipulse burst usage may vary between residents or contexts, we reduced each animals multipulse pulse bursts (2 – 7 pulses/burst) into a single metric. As such, we performed a principal component analysis (PCA), using the *psych* package and *principal* function. In these models, we loaded the number of the different burst types for each male, and generated PCs separately for size matched and mismatched contexts. Higher PC scores represented greater multipulse burst usage. To next investigate whether PC1 (multipulse burst usage) varied between residents and intruders across each contexts we used two linear mixed model (LMM) using the *lme4* package with pair identity as a random effect. In size mismatched contests, we also used a LMM to investigate whether mutlipulse burst usage differed between large and small individuals size. Finally, we subsetted animals into high, medium, and low sound producers. Using this categorical variable we tested whether overall call production (e.g., high, medium, low sound producers) was related to multipulse burst usage (PC1).

We next wanted investigated whether agonistic behaviors varied between residents and intruders across each behavioral context. To do this, we ran a repeated measures LMM with pair identity as a random effect to examine agonstic behaviors differed between residents or intruders across each context, and whether this usage 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). As in the previous models, we also investigated overall behavior usage varied between large and small fish in size mismatched contests.

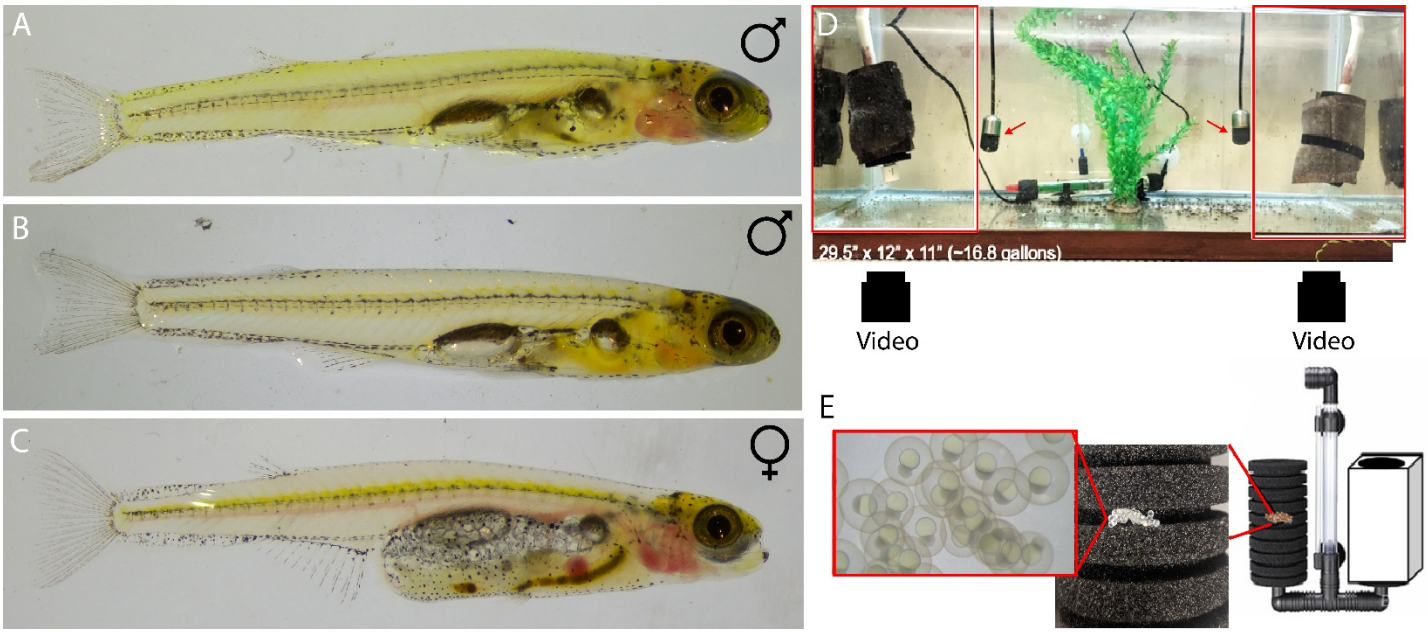


Fig. 1: Male and female *Danionella dracula* and community tank. A. Male fish with green coloration, especially on dorsum; SL = 17.2 mm B. Male fish without green coloration, SL = 16.4 mm. C. Female fish, SL = 16.7 mm. D. Community tank setup described in Methods. Red boxes at either end of tank indicate video camera field of view. External video cameras indicated by black boxes below tank and red arrows indicate hydrophone placement. E. Eggs in nest site. Zoom-in insets from left to right: developing *D. dracula* eggs; cluster of eggs in crevice of sponge filter nest site, schematic of nest site we use for egg collection.

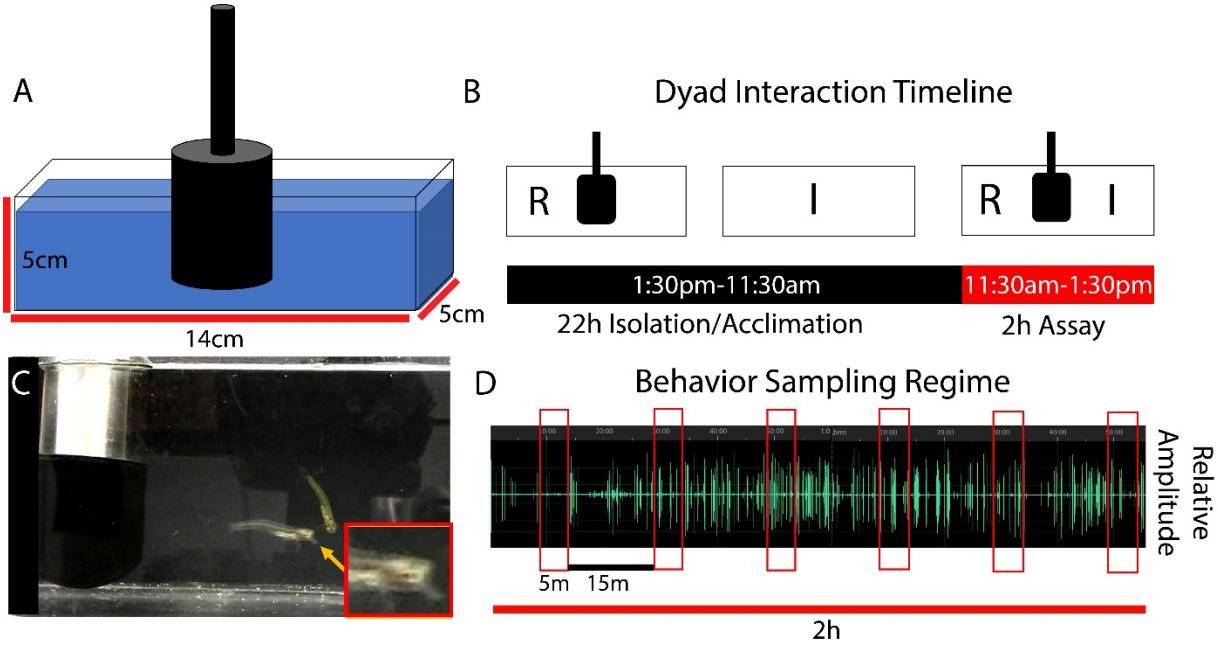


Fig. 2: Dyad testing tank for male *Danionella dracula*. A. Experimental tank schematic. Dimensions:14 cm x 5cm x 5cm. B. Dyad interaction timeline. R stands for Resident and I for Intruder male. The black line indicates the 22 h Isolation/Acclimation period (1:30pm-11:30am) that the fish undergo before the 2 h assay (11:30am-1:30pm), which is indicated by a red line. C. Two males in miniature tank, one has its jaw extended as it lunges at the other. Extended jaw is indicated with an orange arrow and insert zooms in on anterior region of body and jaw. D. Behavior sampling regime. Red boxes indicate on oscillogram 5 min sampled time periods over 2 h assay. Sounds on oscillogram in green. Black line indicate 15 min intervals between sampled time periods.

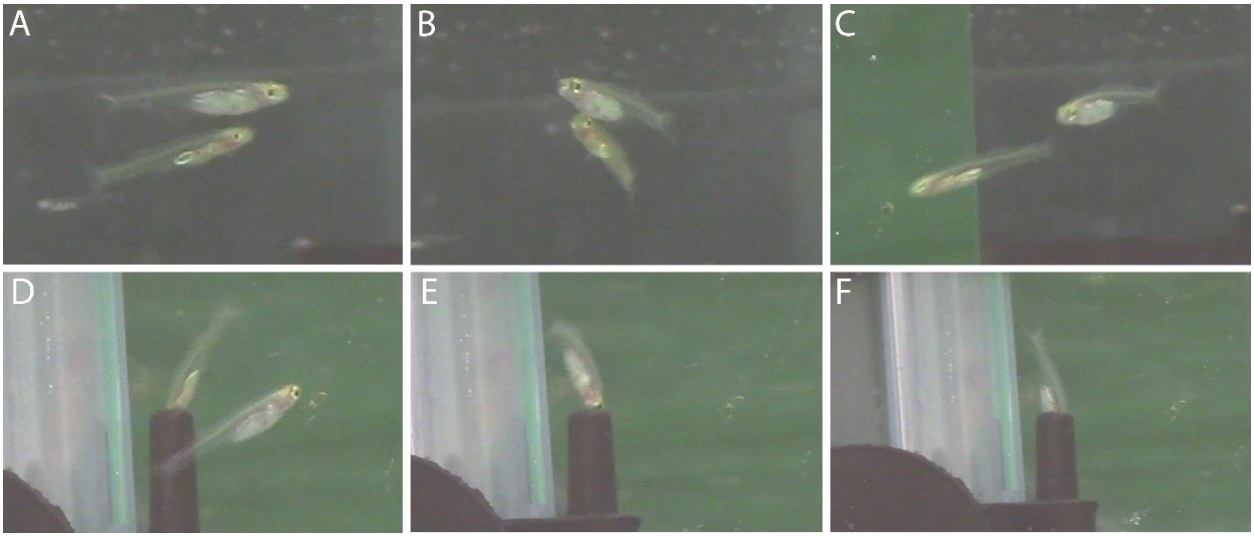


Fig. 3: Video frames of *Danionella dracula* courtship behavior. A. Male swimming below female B. Male vibrating his body back and forth beneath female egg vent. C. Male swimming back to nest entry crevice. Female swims behind male to same nest entry crevice. D. M male swimming headfirst into nest entry crevice E. Female orienting head towards nest entry crevice. F. Female swimming headfirst into nest entry crevice.

RESULTS

Courtship and spawning in colony and community tanks

Danionella dracula readily breed and produce courtship and aggressive behaviors in laboratory colony and community tank settings. Although we focus on male aggressive behaviors, we first

provide a description of courtship and spawning for both current and future broader contexts. Fig. 3A-F are single frames from a video record of courtship and spawning in the presence of a black vertical tube that was originally part of an underwater filtration system in a colony tank. After entering a nest site, males swim from the nest to court females who are often schooling around the tank (Supplementary Video 2). A male will approach a female and swim beneath her body, directly hovering beneath her egg vent (Fig. 3A, B). The male rapidly moves his pectoral fins and vibrates his body and head back and forth beneath the female. No sound production has been recorded during this behavior in our colony and community tank settings. Males swim back to the nest site that they were observed to swim closely around during the day, and the female swims behind the male to the same nest site (Fig. 3C). Males beat their tails back and forth rapidly as they enter nest crevices (Supplementary Video 3). The male enters the spawning crevice head-first (Fig. 3D), followed by the female (Fig. 3E, F). Spawning cannot be directly observed as fish require the spawning crevices to be enclosed, but it is inferred to occur in the crevices due to the presence of eggs in the crevices of the artificial sponge nest. Clutches of 12-24 eggs are often found in nests (see Fig. 1E) following these male-female interactions. Furthermore, during daily nest checks, we often find a male and/or a female on top of a cluster of eggs within the sponge grooves. After spawning occurs, the female leaves the nest. The male then emerges from the nest and swims closely around the nest, which we refer to as nest circling, courting additional females throughout the day. Multiple females appear to be courted over the course of the day, as multiple clutches are found per nest daily and eggs can be found at any time of the light cycle.

Male aggressive displays in colony and community tanks

In colony tanks, we often observed solo males swimming throughout the day around artificial nest sites that are dark-colored objects containing crevices for spawning (see Fig. 1D, E). By observing dye-marked males in a controlled community tank over a two week-long period, we found that the same male swims 2-3 body lengths around the same nest site for 1-8 days, suggesting nest fidelity. These males also lunge at other males who swim into the perimeter surrounding the nest site, often producing a burst of stereotyped sound pulses at the same time as the lunge (Fig. 4A, B; Supplementary Videos 4, 5). These observations combined with a recording of a male lunging continuously against its own reflection (Fig. 4C) led us to conclude

that sound production is primarily coupled to lunges at intruder fish or the visual presence of another male in the contexts critically assessed here (Fig. 4A, B). In response to directed agonistic signals, rival males either flee or respond with sound production (Fig. 4B-D). Male interactions can escalate and include mutual sound production and lunging, extension of the lower jaw, and vertical lateral displays until one male flees (Fig. 4C-F; Supplementary Videos 5,6). Extended aggressive interactions have been observed to span up to 30 mins before one of the males flees. Males will swim around the entire community tank after leaving the vicinity of the nest.

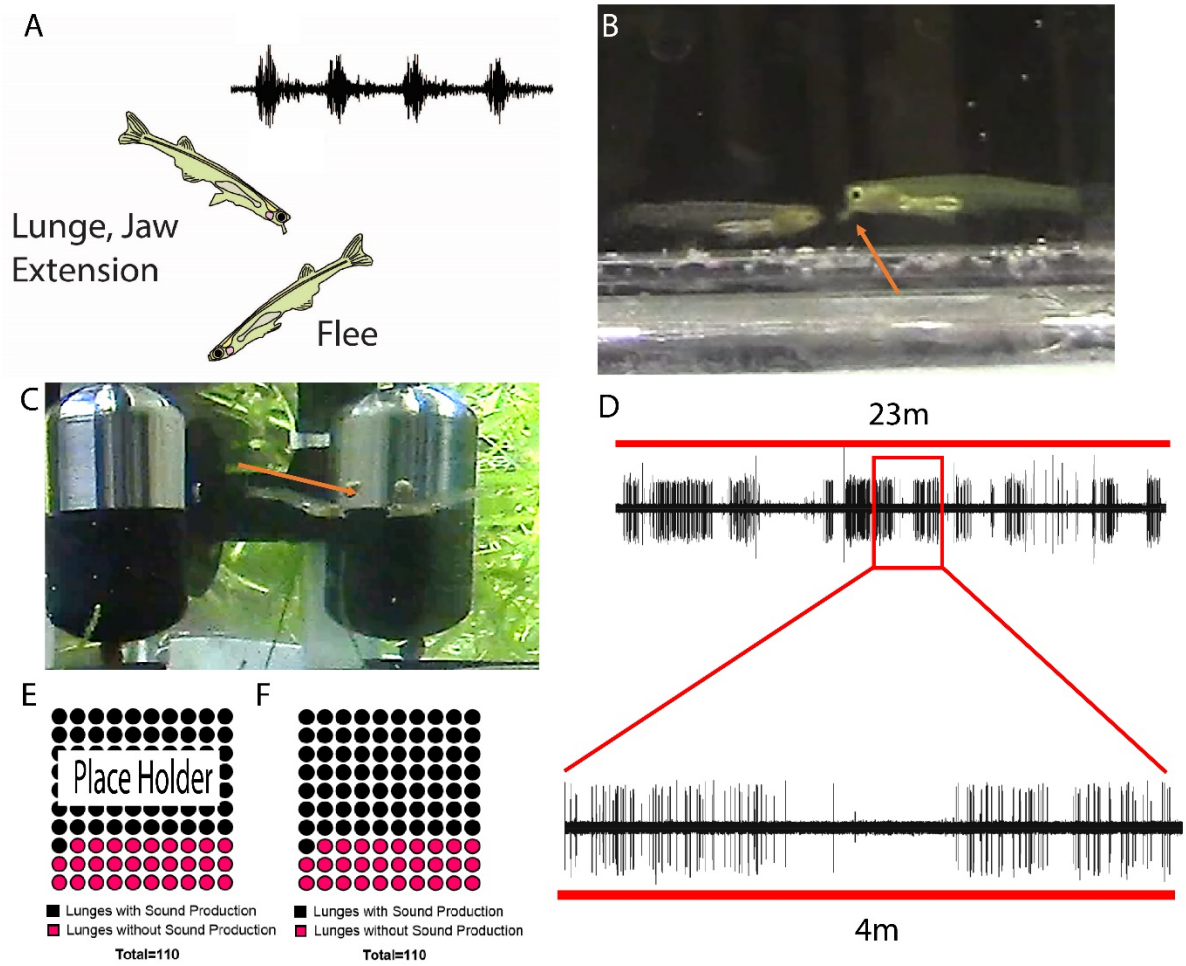


Fig. 4: Schematic and video frames of *Danionella dracula* male-male aggressive interaction. A. Schematic of male lunging at other male who flees. The resident or intruder male can be the one that lunges and produces sound pulses. The lunge can be associated with sound production as well as extension of the lower jaw. The male lunged at can flee from the other male after the

lunge. B. Frame from resident-intruder assay of male lunging at other male and extending its lower jaw, indicated by orange arrow. C. Frame from male lunging at its own tank reflection. Direction of lunge towards tank glass indicated by orange arrow extending above entire dorsum of lunging male. D. Oscillogram from 23 min recording of male lunging at its reflection in tank wall.

Sound production by dyadic males

To characterize male sound production during aggressive interactions in *D. dracula*, we simplified the community tank setting and created a dyadic resident-intruder assay to generate aggressive interactions between males (Fig. 2). The assay was in a miniature tank that constrained two fish within the field of view of the camera and allowed enough resolution to determine the identity of individual sound producing males in the dyad. Dyad interactions are primarily one-sided, where one fish emerges as the more apparent aggressor towards the other. It is the pulsatile sounds of the more aggressive male that are always concurrent with a lunge and sometimes with jaw extension that are both directed at another fish (Fig. 5A,B).

Individual sound pulses have a mean pulse peak frequency of 1988.6±14.0Hz in size-matched and 1961.6±11.5 Hz in size-mismatched contexts (see Fig. 5A, B - example spectrogram in 5A). Mean pulse duration is 2.0 ± 0.02 ms in all sound producing size-matched males and 2.6±0.03 ms in all sound producing size-mismatched males (Fig. 5C, D).

Individual males vary widely in the total number of sound pulses directed towards another fish, with some time periods for individual males having more sound production than others over the course of the 2 h dyad trial. To investigate the hypothesis that sonic characteristics might vary depending on the amount of total sound production, we first placed males into one of three groups based on the quartiles of the dataset. In size-matched assays, 5 out of 16 males were in the upper 75% of the observed male total sound production and designated high sound producing males in the size-matched assays, with high sound producers ranging up to 318 total sound pulses in the 30 mins sampled over the 2 h trial for each dyad (Fig. 6A, C, E). In size-mismatched assays, 5 out of 16 males were in the upper 75% of the observed total sound production and designated high sound producing males, with high sound producers ranging up to 428 total sound pulses (Fig. 6B, D, E). Four out of 16 males in the size-matched assays and 4 out of 16 males in the size-mismatched assays were designated medium sound producers as their sound production values were around the median of the dataset (Fig. 6E). Seven out of 16 males in size-matched assays and 7 out of 16 males in size-mismatched assays were in the lower 25%

of the observed male total sound production, and these fish were designated low sound producers (Fig. 6E).

Males produce burst trains composed of up to 6 pulses per burst (see Table 2 for definition), with mean IPI of 42.9±0.6 ms in size-matched contexts and 39.5±0.5 ms in size-mismatched contexts (e.g., see 2- and 3-pulse burst in Fig. 5B). In both contexts, males primarily use single (65.3% of all bursts size-matched; 64.1% size-mismatched) and 2-pulse bursts (23.4%, size-matched; 27.1%, size-mismatched) that are concurrent with lunging at other males (Fig. 5F). Three and higher pulse bursts occur more rarely, with 3-pulse bursts making up 8.1% for size-matched males and 7.9% for size-mismatched males (Fig. 5F). Four pulse and higher burst types make up only 3.2% for size-matched males and 0.9% for size-mismatched males (Fig. 5F). Individual fish can produce multiple types of bursts containing differing numbers of pulses, with mean inter-burst intervals of 12.6±1.6 s in size-matched contests and 10.5±0.7 s in size-mismatched contests.

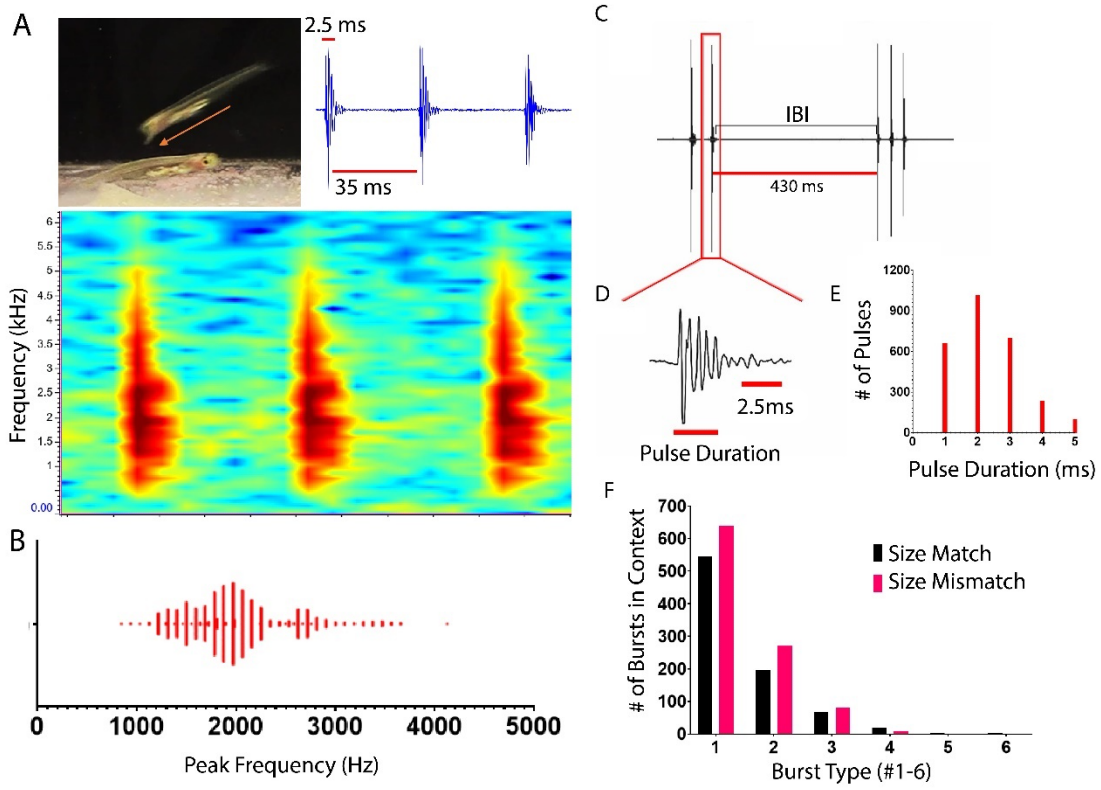
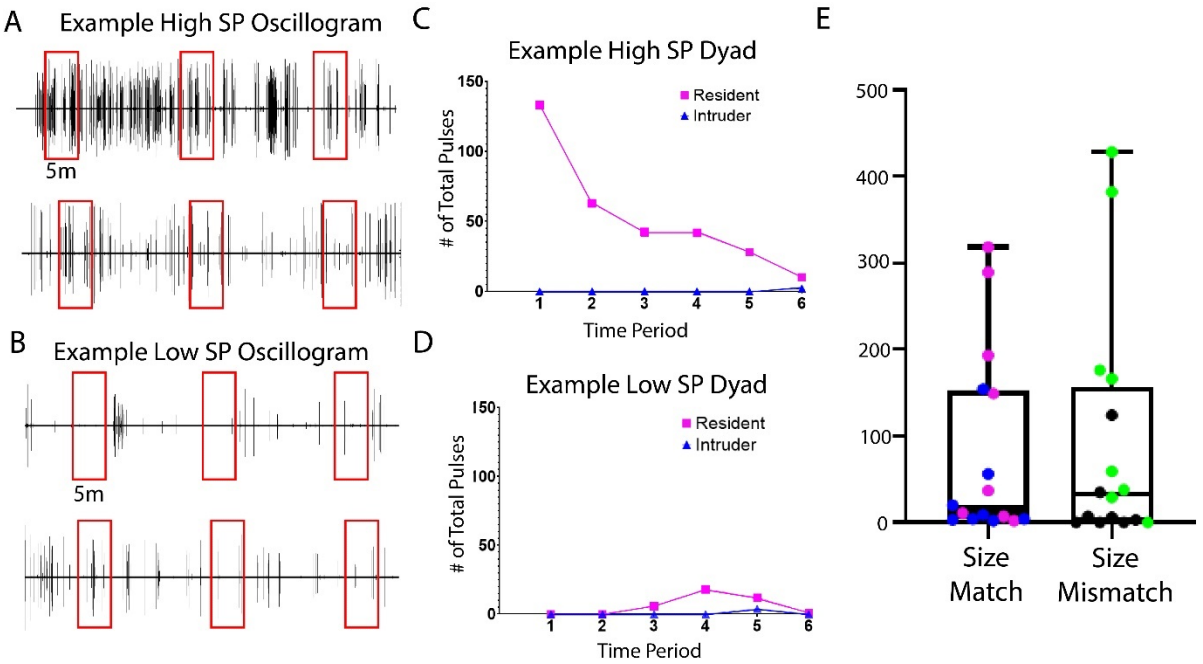


Fig. 5: Multi-pulse sound bursts of male *Danionella dracula*. A. Left: Male lunges at other male in a size-matched dyad during 3-pulse burst. Lunging male also has its jaw extended in this instance. Orange arrow indicates direction of lunge toward target male. Right: Oscillogram of 3-pulse burst made by lunging male to the left. Red lines indicate that pulses were 2.5 ms in this burst and were separated by around 35 ms. Below: spectrogram of 3-pulse burst. Dark red

426 indicates peak power frequency, which in this burst was 1968.750 Hz. B. Example 2- and 3-
427 pulse bursts separated by inter-burst interval (IBI) of 430 ms. C. Zoom-in indicates one of the
428 single pulses that composes the example 2-pulse burst in C. This pulse was 2.5 ms in duration
429 (see criteria in Materials and Methods).
430



431

432 Fig. 6: Total sound production by *Danionella dracula* males in dyadic contests. A. Boxplots of
433 total individual sound production in size-matched dyads (left boxplot; magenta = resident fish,
434 black = intruder fish) and size-mismatched dyads (right boxplot; green = larger fish, black =
435 smaller fish). Black horizontal line within box indicates median. B. Example high dyad sound
436 production from size-matched dyad, showing the total number of pulses for individual fish over
437 the 6 5-minute sampled time periods (spaced 15 minutes apart). Magenta squares indicate the
438 resident fish's sound production values and black triangles indicate the intruder fish's sound
439 production, which is at or near zero during all time periods. C. Example high sound production
440 oscillogram; rows 1 and 2 are from one continuous 2h record. D. Example low dyad sound
441 production from size-matched dyad, showing the total number of pulses for individual fish over
442 the 6 sampled time periods. Magenta squares indicate the resident fish's sound production
443 values, which peaks at 20 sound pulses, and black triangles indicates the intruder fish's sound
444 production, which in this case is also at or near zero during all time periods. E. Example low
445 sound production oscillogram; rows 1 and 2 are from one continuous 2h record.
446

447 **Principal components analysis of sound bursts**

448 To test the hypothesis that there are differences in multi-pulse burst usage among the three
449 groups of sound producers, we ran a principal components analysis where the number of
450 different burst types per male was loaded as different variables (number of 2 - 7 pulse bursts for
451 each male in size-matched contests and number of 2 - 5 pulse bursts for each male in size-

mismatched contests as fish in that context did not make 6 and 7 pulse bursts in the sampled time periods). One principal component (PC1) explained 73% of the variation of male usage of multi-pulse bursts in size-matched males and 70% of the variation in size-mismatched males (Fig. 7A-C). We compared the different groups of sound producers in each context: high (upper 75%), medium (median), and low (lower 25%). In both contexts, high sound producers generate more multi-pulse bursts than medium (p=0.002163; size-matched; p=0.003596; size-mismatched) and low (p=0.000342; size-matched; p=0.0003194; size-mismatched) sound producers (Fig. 7D, E). Medium sound producers in both contexts also produced more multi-pulse bursts than low sound producers (p=0.001036; size-matched; p=0.003316; size-mismatched) (Fig. 7D, E).

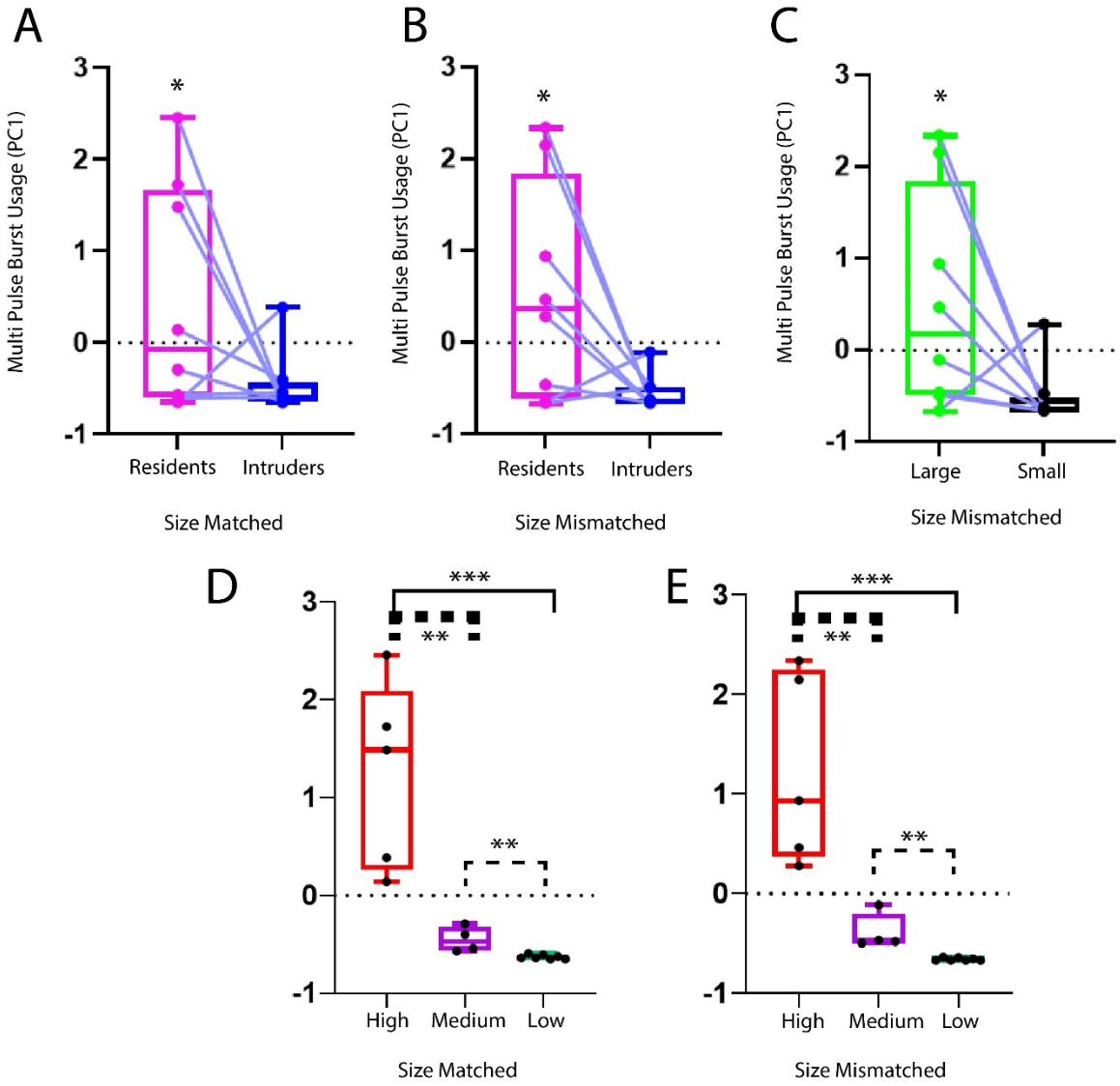


Fig. 7: Multi-pulse burst use for *Danionella dracula* males in dyadic contests**.** A. Pie chart detailing percentages of multi-pulse bursts used by all males in size-matched contests. Legend signifies type of multi-pulse burst, from single to 7-pulse burst. B. Pie chart detailing percentages of multi-pulse bursts used by all males in size-mismatched contests. Legend signifies type of multi-pulse burst, from single to 5-pulse burst. C. Boxplots of PC1 describing individual male multi-pulse burst usage in size matched contests. Residents are in magenta, left and intruders in black, right. Individuals in the same dyad are connected with light blue lines. D. Boxplots of PC1 describing individual male multi-pulse burst usage in size mismatched contests. Residents are in magenta, left and intruders in black, right. Individuals in the same dyad are connected with light blue lines. E. Boxplots of PC1 describing individual male multi-pulse burst usage in size mismatched contests. Larger fish are in green, left, and smaller fish in black, right. Individuals in the same dyad are connected with light blue lines. F. Boxplots of PC1 describing individual male multi-pulse burst usage in size matched contests. High sound producing fish are in red-orange, leftmost; medium sound producing fish are in purple, middle; and low sound producing fish are in sea green; rightmost. Black undashed bracket indicates comparison between high and low sound producing fish; Black small, dashed bracket indicates comparison between high and medium sound producing fish; and black large, dashed bracket indicates comparison between medium and low sound producing fish. Dashed axis indicates 0 axis. Line on boxplots indicates median. G. Boxplots of PC1 describing individual male multi-pulse burst usage in size mismatched contests. High sound producing fish are in red-orange, leftmost; medium sound producing fish are in purple, middle; and low sound producing fish are in sea green; rightmost. Black undashed bracket indicates comparison between high and low sound producing fish; Black small, dashed bracket indicates comparison between high and medium sound producing fish; and black large, dashed bracket indicates comparison between medium and low sound producing fish. Dashed axis indicates 0 axis. Line on boxplots indicates median. C-G: ** indicates significance as p compared to 0.01; *** indicates significance as p compared to 0.001.

Jaw extension

Beyond sound production, we also observed that *Danionella dracula* males extending their lower jaw during aggressive interactions, often in the context of lunging and sound production. During our initial observations of colony tanks, jaw extension always appeared to be nearly coincident with sound production. This led us to test the hypothesis that sound production is dependent on jaw extension. Males who produce more sounds in size-matched and size-mismatched contexts exhibit more jaw extension behaviors during the assay, where all high sound producers in size-matched contexts also exhibit high jaw extension and 4 of the 6 high sound producers in size-mismatched contexts exhibit high jaw extension (Fig. 8A, B). Jaw extension is often increased as well during the same 5 min time period samples of high sound production for all size-matched males and the same 4 of 6 size-mismatched males (e.g., Fig. 8C, D).

By comparing video time points for sound production and jaw extension, we found that jaw extension can happen just before or after instances of high sound production. To more rigorously examine if sound production and jaw extension are near-concurrent events in high sound producing males in both size-matched and mismatched contexts, we pooled time intervals separating sound bursts and jaw extension that were less than 5 s apart for high sound producing males that also exhibited high jaw extension (Fig. 8E). We compared bursts to jaw extension instead of individual pulses to account for the difference in sampling frequency for the different behaviors. The time intervals of jaw extension occurring right before or after sound production have median values ranging from 140.8 to 393.5 milliseconds in high sound producing fish. Together, the results indicate that sound production and jaw extension are not obligatorily linked, but often closely coordinated in time of occurence.

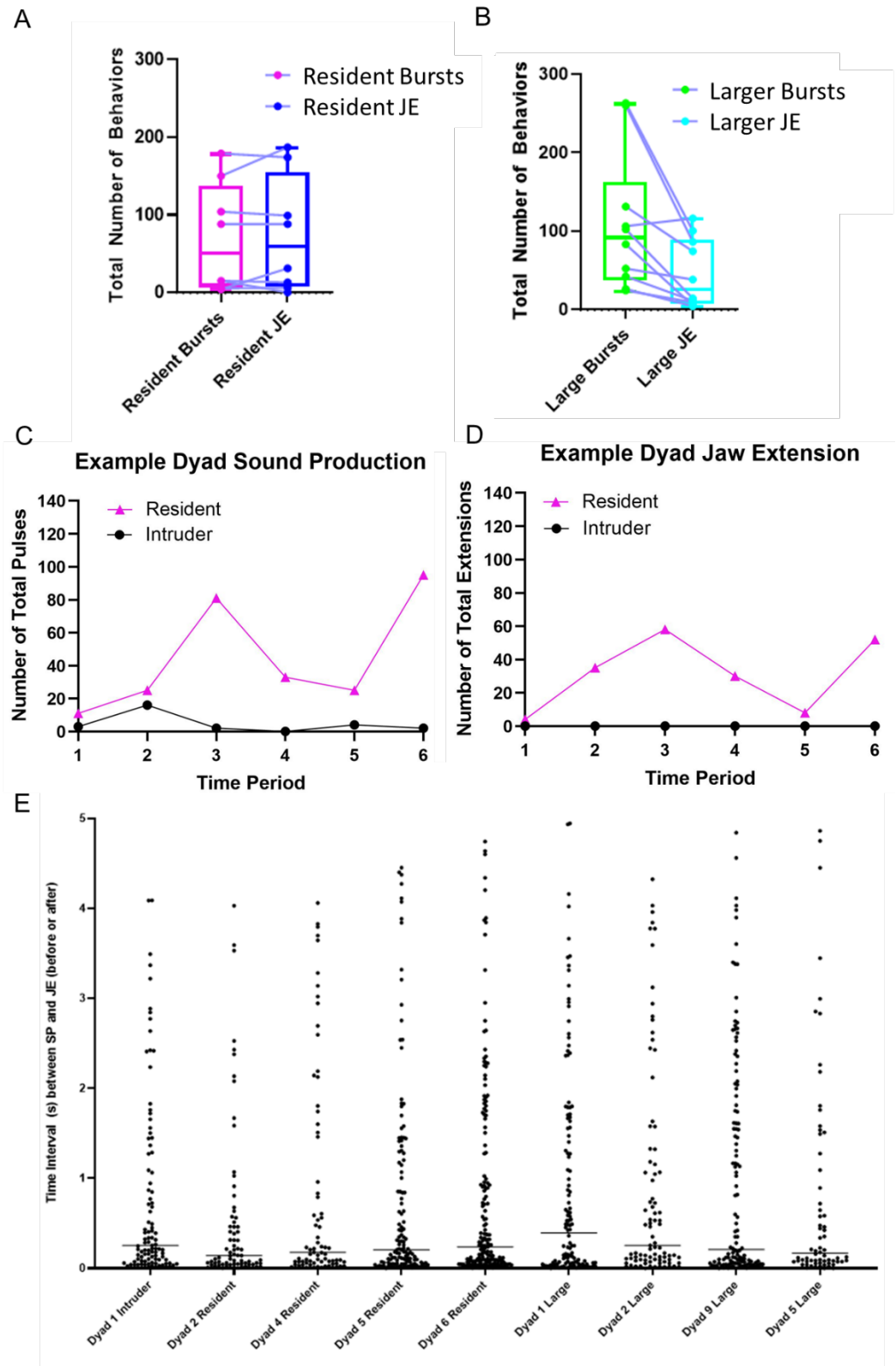


Fig. 8: Temporal relationship between sound production and jaw extension for *Danionella dracula* males. A. Boxplots of resident fish sound production (SP) and jaw extension (JE)

behaviors in size-matched contests; total number of sound production in bursts (pink) on left and total number of jaw extensions (blue) on right for individual resident males. Sound production and jaw extension totals for the same individual are connected with light blue lines. B. Boxplots of large fish sound production and jaw extension behaviors in size-mismatched contests; total number of sound production in bursts (green) on left and total number of jaw extensions (cerulean) on right for individual larger males. Sound production and jaw extension totals for the same individual are connected with light bluelines. C. Example high dyad sound production from size-matched dyad, showing the total number of pulses for individual fish over the 6 sampled time periods. Magenta triangles indicate the resident fish's sound production values and black circles indicates the intruder fish's sound production. D. Example dyad jaw extension from same size-matched dyad as C, showing the total number of jaw extensions for individual fish over the 6 sampled time periods. Magenta triangles indicate the resident fish's jaw extension values and black circles indicate the intruder fish's jaw extension. E. Pooled time intervals (black circles) separating sound production and jaw extension that were less than 5 seconds apart in high sound producing males in size-matched contests (upper 75% of sound producing males), labeled as residents or intruders, and high jaw extending males in size-mismatched contexts (upper 75% of sound producing males that also were high jaw extenders), labeled as large fish. Median indicated by black line.

Effects of Fish Identity on Variation in Behavior

We next investigated the effects of fish identity on sound production and jaw extension, as well as sonic characteristics, by examining the effect of being the resident or intruder in both assay contexts, and the larger or smaller fish in size-mismatched assays. We ran repeated measures linear mixed models for each behavior, with pair identity as a random effect. Residents produced significantly more sound pulses than intruders in size-matched ($p=0.00145$) and size-mismatched contexts ($p=0.002705$) (Fig. 9A, B). Larger fish, irrespective of their resident or intruder status, produced significantly more pulses than smaller fish ($p=1.321e-08$) in size-mismatched contexts (Fig. 9C). In size-matched contexts, the resident fish also extended its jaw more frequently than intruders ($p=0.001601$) (Fig. 9D). This was not the case in size-mismatched contexts ($p=0.1182$) (Fig. 9E), but larger fish did significantly extend their jaws more frequently than smaller fish ($p=0.000969$) (Fig. 9F). There was no significant effect of time period on the amount of sound production ($p=0.7163$), indicating there was not one specific time period (1-6) during the 2 h trial that fish across the dyads had increased sound production or jaw extension.

In examining specific sonic characteristics, there is a trend where residents produce more multi-pulse bursts than intruders in both contexts, but it is not significant ($p=0.07298$; size-matched; $p=0.09965$; size-mismatched) (Fig. 7A, B). In size mismatched contests, larger fish produced significantly more multi-pulse bursts than smaller fish ($p=0.007457$) (Fig. 7C).

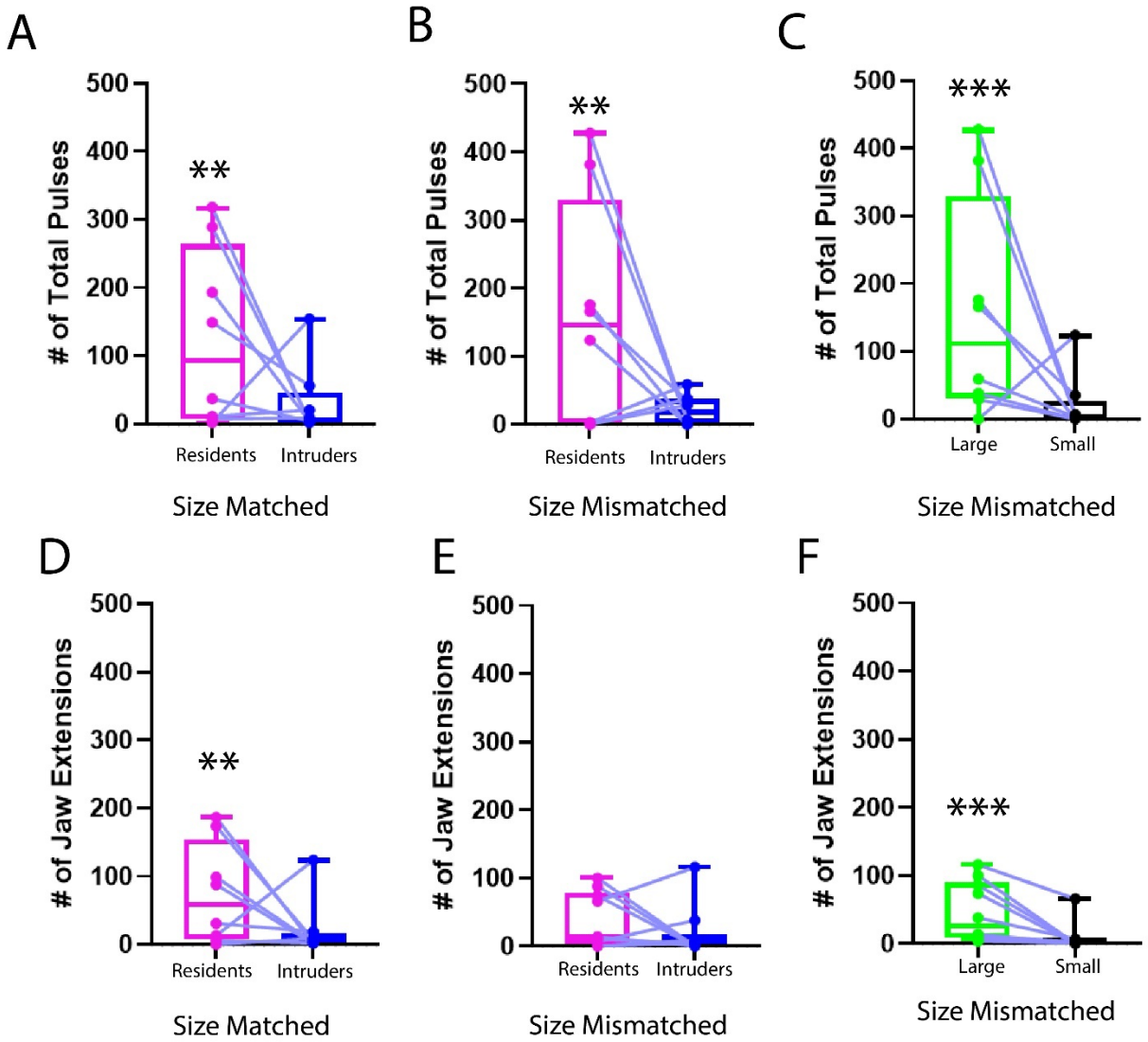


Fig. 9: Fish identify and sound production for *Danionella dracula* males in dyadic contests. Boxplots comparing total number of sound production (SP) in pulses (A-C) and jaw extension (JE) (D-F) in size matched and size mismatched contexts. Individuals in the same dyad are connected between the two boxplots compared with a light blue line. ** indicates significance as p compared to 0.01; *** indicates significance as p compared to 0.001. A. Boxplots of resident (left, magenta) fish sound production and intruder sound production (right, black) in size-matched contests. B. Boxplots of resident (left, magenta) fish sound production and intruder sound production (right, black) in size-mismatched contests. C. Boxplots of larger (left, green) fish sound production and smaller fish sound production (right, black) in size-mismatched contests. D. Boxplots of resident (left, magenta) fish jaw extension and intruder jaw extension (right, black) in size-matched contests. B. Boxplots of resident (left, magenta) fish jaw extension and intruder jaw extension (right, black) in size-mismatched contests. C. Boxplots of larger (left, green) fish jaw extension and smaller fish jaw extension (right, black) in size-mismatched contests.

DISCUSSION

We provide the most comprehensive descrption to date of social behavior for any species within the *Danionella* genus. Sound production and jaw extension of individual adult male *D. dracula* quantitatively characterized by developing a simplified resident-intruder assay for testing size-matched and size-mismatched male dyads. The results support the hypothesis that resident fish in size-matched and larger fish in size-mismatched assays, irrespective of resident or intruder status, exhibit increased total sound production directed towards the second fish in the dyad. During periods of high sound production, males in both size-matched and mismatched dyads also extend their hypertrophied lower jaw. Both actions can serve as behavioral benchmarks for comparative studies of *Danionella* species as a new model clade (see Jourjine and Hoekstra, 2021) for identifying neurogenomic mechanisms of social behavior.

Reproduction and courtship

We interpret the vibrating behavior and swimming back to the nest as related to courtship given the spawning activity inside the dark crevice of the tube that ensues these interactions (see Fig. 3). *Danionella dracula* may differ in their courtship and spawning behaviors from *D. cerebrum* (formerly designated *D. translucida*), based on the description available (Schulze 2018). Female *D. cerebrum* enter the nest tubes first, where upon males follow and spawning occurs (Schulze 2018). In *D. dracula*, the male is the first to venture into the spawning crevices, followed by the female. In addition, *D. cerebrum* nest tubes are transparent and allow light in, whereas *D. dracula* requires nest coverings to completely darken spawning crevices (R. Tatarsky, unpublished observations). There is also no description of nest site circling in *D. cerebrum,* whereas *D. dracula* resident males swim in close proximity to nest sites from where they court females. These possible differences in courtship, spawning, and nest localization could indicate differences in the natural habitats of the two species, as *D. cerebrum* is described in southern Myanmar and *D. dracula* in the north, which may possess different breeding mediums and facilitate different courtship and resource holding strategies.

Acoustic signal characteristics

We report that *D. dracula* males produce sounds during agonistic, but not courtship, interactions. Females were not found to be sonic in either context, consistent with their apparent lack of a sound producing apparatus (Britz and Conway, 2016). Males in the two aggressive contexts tested, size-matched and size-mismatched, have similar characteristics as their individual sound pulses were broadband, short in duration and possessed a peak frequency close to 2 kHz. The frequency spectrum is well within the range of hearing reported for other otophysans (Fay, 1988; Ladich, 2000). Individual males vary in the total number of sound pulses produced during male-male interactions and in their usage of multi-pulse bursts in both size-matched and size-mismatched contexts. For some teleost species, sound characteristics such as dominant frequency and amplitude correlate with body size as well as with winning dyadic contests (e.g., croaking gourami, *Trichopsis vittata*; Ladich, 1992; also see Conti et al., 2015 for overview).

For both size-matched and size-mismatched dyads, *D. dracula* males predominantly produced single pulse bursts, with multi-pulse burst trains appearing to be generated during periods of escalated sound production (and lunging). Perhaps bursts of three or more pulses signal aggressive escalation compared to single and two pulse bursts, which were more frequently used by all primary aggressor males. *Danionella dracula*'s single pulses are reminiscent of the agonistic "pops" of the bicolor damselfish, *Eupomacentrus partitus* (Myrberg, 1972). Many fish species produce multi-pulse, grunt-like sounds during reproductive and aggressive interactions (Lobel et al., 2010, Amorim 2005; Ladich & Myrberg, 2005; Myrberg & Lugli, 2005; McIver et al., 2014; Lobel et al., 2021; several damselfish species, including *E. partitus*, make multi-pulse chirps during reproductive interactions; Myrberg et al., 1978). Future studies of *Danionella* building on the foundation set out here should look at these acoustic characteristics in the context of nest-holding and determine if nest-holding males differ in these characteristics compared to loser males, similar to other species.

The *Danionella* genus contains multiple species for comparative studies of both sound production and hearing (Britz et al., 2021). Male *D. dracula* and males of its sister species *D. cerebrum* (Schulze 2018; initially designated *D. translucida*; see Britz et al., 2021) 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 sounds of both species can be grouped into bursts separated by a characteristic IPI, 38ms in *D. dracula* and 8 and 17 ms in *D. cerebrum* (Schulze 2018). Beyond the difference in IPI duration, there is a large difference

between the species when looking at the duration of individual bursts and burst trains between the species, as *D. dracula* appears to have individual bursts of only up to 7 pulses in length, whereas *D. cerebrum* are capable of producing single bursts lasting close to 1 sec and burst trains on the order of minutes (Schulze 2018). We also observe *D. dracula* primarily using single pulse signals compared to multi-pulse bursts, with burst types of 4-7 pulses also considerably rare. In the two species, it would be of interest to compare behaviors that might be concurrent with sound production. It is not described if *D. cerebrum* sound production is also coordinated tightly with lunging behaviors (Schulze 2018) as documented here for *D. dracula*, and *D. cerebrum* lacks the hypertrophied jaw and thus the dramatic jaw extension of *D. dracula*.

Jaw extension

Beyond acoustic signaling, animals use other sensory modalities to communicate in conflict scenarios, including visual ornaments in males and chemical signals (see Bradbury & Vehrencamp, 2011). As we show, *D. dracula* males often extend their hypertrophied lower jaw during aggressive interactions. This morphological character appears to be unique to this species within the genus *Danionella*. Jaw extension occurs in community tank settings when two males escalate their aggressive interaction and in periods of high sound production in dyadic assays. Future studies should test if jaw extension behavior functions as a visual agonistic signal, possibly allowing males to signal contest escalation and assessment information to their competitors. Jaw extension could also act to enhance characteristics of the acoustic signal, akin to the influence of vocal tract and mouth skeleton on sound attributes in songbirds (Podos et al., 2004).

During our initial observations of *D. dracula* in large colony tanks, we thought that jaw extension was always linked to sound production. However, their apparent temporal separation during periods of low and high sound production, as well as the independent occurrence of each during aggressive encounters between two males, indicate these two actions are very separate behavioral displays. The close temporal coupling of sound production and jaw extension during periods of heightened activity, provides the opportunity to study a possible multimodal signaling repertoire (Nihat et al 2007, Amorim et al 2019). Multimodal signals are composed of signals from different transmission channels and/or modalities, that when correlated are theorized to maximize robustness of the overall multichannel signal (Elias et al 2003). Males in other species

660 have evolved multimodal signaling repertoires that combine diverse features across sensory
661 modalities, such as combining stereotyped visual displays or visual signals such as color and
662 pattern changes, with acoustic signals generated by one or more mechanisms (Rice et al., in
663 press). For the investigation of possible coupling of postural and acoustic displays in *D. dracula*
664 and putative multimodal signaling, the exact concurrency of sound production and jaw extension
665 would be best re-visited with high resolution and high-speed video, to more precisely examine
666 the timing of jaw extension and retraction with sound production as done for other species (e.g.,
667 Fusani et al 2007, Bostwick et al 2003).
668
669 **Fish Identity**
670 Resources such as shelters and territories are continually defended by an owner to
671 continue to hold fitness advantages (Conti, Arnott et al 2007). *Danionella dracula* males swim
672 closely around nest sites that contain spawning crevices. Future studies would benefit from
673 examining how males utilize acoustic communication and other signaling types to structure
674 dominance hierarchies and determine nest ownership in a community (Conti, 2015; Chase et al
675 2002, Amorim et al 2005, Arnott et al 2009, Barata et al 2007, Myrberg et al 1985). It would be
676 essential to determine how nest-holding males differ from other males in acoustic parameters
677 like inter-pulse interval, but also in body size. Do the largest males in the tank hold the nest
678 sites? Body size is often a determinant of winning an escalated fight in most animal species,
679 especially those with high variation in body size (Andersson, 1994). A display posture can
680 advantageously reveal body size, and larger animals can produce or bear relatively larger
681 ornaments (Bro-Jorgenson, 2007). Other species can assess differences in body size (see
682 Introduction), and we see a size effect in *D. dracula* where the larger male regardless of
683 residency is the primary aggressor in size-mismatched dyads. In size-matched dyads, we found a
684 resident effect where the slightly larger fish did not necessarily become the primary aggressor
685 however, indicating possible trade-offs between residency and total body size (Jennions et al
686 1996, Jackson et al 1991).
687
688 **Concluding Comments**
689 We demonstrate that *D. dracula* is a sonic species and characterize the sounds produced by
690 individual males in dyadic assays for size-matched and size-mismatched contexts, uncovering

initial residency and body size effects on aggressive behaviors in this species. The different acoustic parameters described here serve as a foundation and could facilitate future behavioral playback studies, physiological investigations of auditory sensitivity, and neuroimaging of regions of the adult auditory system due to whole-brain neuroimaging access in these animals (Chow et. al., 2020; Akbari et al., in review), to determine what components of the acoustic signal are attended to by males as well as females. Beyond the males directly involved in an aggressive interaction, this dyadic assay should be built upon to include the potential for eavesdropping by females and other males to determine the interactions between acoustic signaling in *D. dracula* and fitness advantages, like the ability to defend a nest. Male *D. dracula*, like other fish species, could learn about each other's fighting experiences by observing male aggressive interactions in multiple modalities, perceiving sound production and jaw extension behaviors of individual males to facilitate assessment. The coupling of acoustic signals with a postural display, jaw extension, could provide additional information regarding male quality during aggressive encounters and other social interactions yet to be studied in comparable detail. The experiments reported here also provide a basis on which to test established social behavioral models (see Bradbury &Vehrencamp, 2011), which altogether would make a comprehensive framework on which to study the neural and genetic drivers of social behaviors in *Danionella* species in general.

ACKNOWEDGEMENTS

We sincercely thank Kevin Conway for his early and very generous guidance in setting up a breeding tank. We also thank Melissa Hoffman for animal care and Margaret Marchaterre for logistical support. Research support from NSF IOS-1457108 and IOS-1656664, and Cornell University (AHB).

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