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Development of sound production in *Danionella cerebrum*

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Abstract

Acoustic signalling, integral to intraspecific communication and reproductive behaviour, undergoes notable changes during an animal's ontogenetic development. The onset and progression of this maturation in fish remains poorly understood. Here, we investigate the ontogeny of acoustic communication in the miniature teleost *Danionella cerebrum*, one of the smallest known vertebrates and an emerging model organism. Its adult males produce audible clicks that appear in sequences with a repetition rate of ~60 or ~120 Hz, caused by consecutive unilateral or alternating bilateral compressions of the swim bladder. To investigate the maturation of this ability, we performed long-term sound recordings and morphological studies of the sound production apparatus in *D. cerebrum* throughout its ontogenetic development. We found that fish start producing clicks during the second month of their lives and continually increase their abundance and structured repetition over the course of the following one to two months. The sound production machinery, including specialised bone and cartilage structures, start to form in males after approximately four weeks and prior to reaching sexual maturity. While clicks increase in amplitude as animals mature, click repetition rates of 60 and 120 Hz are stable throughout development. This suggests fully mature pattern generation in juvenile males, yet a continued development of the drumming apparatus capable of creating louder sounds.

Summary statement

Danionella cerebrum is one of the smallest vertebrates known to perform acoustic communication. This study describes how juvenile fish develop their sexually-dimorphic ability for sound production.

Introduction

Acoustic communication can be found throughout the animal kingdom in rich spectral and temporal diversity. While the mechanisms of sound production are manifold, from bone stridulation to larynx vibrations (Suthers et al., 2016), a common theme is that sounds are associated with reproductive and territorial behaviour.

In sound producing species with parental care, acoustic signals are typically produced already in early developmental stages, for instance as retrieval calls upon maternal separation, i.e. in bats (Bohn et al., 2007), squirrel monkeys (Symmes & Biben, 1985), rats (Brudzynski et al., 1999) and seals (Sauvé et al., 2015) or as food calls of nestlings (Briskie et al., 1999). Vocalizations change throughout the lifespan of an animal, especially around the time of sexual maturation. A well-known example is the change in pitch in human males during puberty. In many vocal learner species, individuals acquire a new vocal repertoire upon sexual maturation, as prominently studied in songbirds (Nottebohm, 1970). The trajectory of sound development of vocal learners can vary. For instance, in the greater sac-winged bat, pups already start vocal imitation of male territorial song (Knörnschild et al., 2010), while pups of neotropical singing mice are highly vocal right after birth, but stop vocalising before weaning and then produce their mature advertisement song *de novo* (Campbell et al., 2014).

In soniferous egg-laying species without parental care, acoustic communication is present in adults and tends to commence in juvenile stages. For instance, tadpoles do not emit sounds, but vocalisations are found in juvenile spadefoot toads (Ten Hagen et al., 2016). In several sound producing species of the Sciaenidae, the sound generating muscles only form in juvenile fish in parallel with gonad development (Hill et al., 1987). In contrast, it has been reported that coral reef grey snapper already produce nocturnal sounds as pre-settlement larvae with a body length of a mere centimetre (Staaterman et al., 2014). This suggests that within the teleostean fishes, there is variability when and how the sound production systems develop in an animal's lifetime. In addition, there is limited information to date linking the morphological ontogenesis of the sound producing organs to sound production and resulting sound characteristics across development.

Here, we investigate the interplay between morphological ontogenesis and the development of sound production in the transparent miniature teleost *Danionella cerebrum*. This fish has an adult body length of only ~12 mm and the smallest known vertebrate brain (Britz et al., 2021; Penalva et al., 2018; Schulze et al., 2018). Its small size, together with its life-long transparency has made *D. cerebrum* a useful model system for neuroscience, suitable for whole-brain optical measures of neuronal activity (Schulze et al., 2018; Kadobianskyi et al., 2019; Hoffmann et al., 2023; Lam, 2022; Zada et al., 2023; Lee & Briggman, 2023). Adult fish have a male-specific trait of acoustic signalling (Schulze et al., 2018; Cook et al., 2024; Vasconcelos et al., 2024). Sounds can be observed in social contexts, including during courtship and male aggression (Schulze et al., 2018; Vasconcelos et al., 2024). They are composed of discrete pulses with a broad frequency spectrum (Cook et al., 2024; Vasconcelos et al., 2024), hereafter referred to as clicks. These clicks are produced by the contraction of a drumming muscle, causing a piece of cartilage to snap against and compress the anterior swim bladder (Cook et al., 2024). Sequences of clicks appear at repetition rates of either ~60 or ~120 Hz, caused by unilateral or alternating bilateral muscle contractions (Cook et al., 2024). The highly specialised drumming apparatus is only present in males and absent in females (Britz et al., 2021). To date it is unknown when *D. cerebrum* develops its sexually dimorphic sound production apparatus and at which developmental stage acoustic signalling

begins. We addressed these questions through a combination of longitudinal sound recordings and morphological comparisons.

Methods

Animal husbandry

All animal experiments conformed to Berlin state, German federal and European Union animal welfare regulations. *D. cerebrum* were housed in water circulated Tecniplast aquaria with artificial fish water (Instant Ocean at a conductivity of 350 µS/cm, adjusted to a pH of 7.3 using $NaHCO_3$) at 27°C and a 14:10 hours light-dark cycle, with lights on from 8 AM until 10 PM preceded by a 30 min dimming period. For all behavioural experiments wild type (WT) fish were used. For morphological stainings a tyrosinase knock-out line (*tyr-/-*) was used to remove melanin pigmentation for increased transparency to see bone and cartilage structures.

Raising conditions

Fertilised eggs from communal breeding in the regular home tanks were collected and kept in 10 cm petri dishes containing embryo water (600 mg/L Instant Ocean, 150 mg/L $NaHCO_3$, 50 mg/L $CaCl_2$ $2H_2O$, 0.5 mg/L Methylene Blue) at a density of < 20 eggs per dish. At 5 days post fertilisation (dpf) larvae were transferred to 1 L plastic tanks (Tecniplast breeding tank) with A2000 water (2 g/L Instant Ocean - Aquarium system, 150 mg/L $NaHCO_3$, pH 7.4, conductivity 1.5 mS/cm) and L-type rotifers (Brachionus plicatilis, planktovie.biz). For regular facility rearing, larvae were transferred to the water circulation system at 10 dpf and fed twice daily with artemia, as well as dry food (Gemma micro 75). For rearing in experimental tanks, larvae were transferred at 3 weeks post fertilisation (wpf) and fed once daily with artemia. Water was either circulated by a pump or manually exchanged twice a week in the small tanks. All recording rooms were temperature controlled at 27°C.

Repeated measure sound recordings

For the single recording of a large group (Fig. 1A) 100 larvae at 3 wpf were transferred to a standalone glass aquarium (30x60x30 cm) filled to a height of 10 cm with artificial fish water with a circulating pump. Three times per week 1 L of tank water was replaced with fresh artificial fish water. Water temperature was maintained at 27°C with three aquarium heater thermostats (Sera). Once a week in the morning between 9 and 11 AM a hydrophone (H3x, Aquarian Audio) was inserted into the tank and 1 hour sound recordings were acquired with a datalogger (Zoom field recorder - Fn8) at a sampling rate of 48 kHz, while the circulating pump was switched off.

For the multiple group recordings (Fig. 1B-J) 10 larvae at 3 wpf were kept inside 15x15x10 cm acrylic tanks filled to a height of 8 cm with artificial fish water (at 27°C). This led to a higher fish density than in the large aquarium or the fish facility, but it was a trade-off between recording quality and fish number. Because a higher fish density can lead to developmental delays, we measured body size in addition to biological age. To obtain length measurements without disturbing the recordings, we raised five additional groups of 10 fish in equivalent acrylic tanks, kept in the same recording room and treated under the same conditions apart from hydrophone recordings. Five of these sample fish were collected per week (one fish per tank), euthanised and photographed under a brightfield microscope (Olympus MVX-10 with a MV PLAPO 1X objective, connected to a FLIR usb colour camera). Standard length measurements were manually performed post hoc on the collected images, as measured from

snout to the base of the tail, excluding the tail fins. Due to the sampling the fish density in the extra tanks was decreasing per week, which could have led to inflated length measurements. We considered this factor to be negligible, in comparison with the length variability at each time point and because the values for weeks 11 to 14 are within the known range of adult male and female *D. cerebrum*.

To avoid disturbance of the fish prior to sound recordings, a hydrophone (AS-1, Aquarian Audio; linear frequency range from 1 Hz to 100 kHz) was placed inside each tank the evening prior to a 2 hour recording from 9 to 11 am the following morning, a time of the day when *D. cerebrum* have previously been shown to be sonically active (Schulze et al., 2018; Vasconcelos et al., 2024). Juvenile fish follow a similar circadian pattern of sound production during the light hours, with activity peaks during the first half of the day (Fig. S1). Recordings were repeated twice a week over the course of 11 weeks, to record ages 4 through 15 wpf. Sound recordings were obtained with a sampling rate of 51.2 kHz via a National Instruments data acquisition card (NI-9231) controlled through a custom written python script. Each AS-1 hydrophone was amplified with a PA-4 hydrophone preamplifier at an inverted wiring state (Aquarian Audio), powered by a 12V battery.

Close-range click recordings

Three age-matched males were placed in a 5x5x5 cm box made out of transparent acrylic and walls of blue cling film. This box was placed in a holder in the centre of a 27x27x30 cm glass aquarium filled to ca. 27 cm. Five AS-1 hydrophones were positioned around the four vertical walls and the bottom of the inner box, with a fixed distance of 3.5 cm to the centre of the box. Sound recordings were acquired for several hours at a sampling rate of 102.4 kHz using a National Instruments data acquisition card (NI-9250).

Clicks that were detected on at least four hydrophones at coinciding or adjacent samples were considered centre clicks, produced by fish at the centre of the box and hence an equal distance of ca. 3.5 cm. To control for the different amount of recorded centre clicks per group, 100 centre clicks were randomly selected and out of those the ten loudest were determined. For each of these ten clicks per age the sound trace from the channel with maximum amplitude was used for further analysis. We used the average of the ten clicks for statistical comparison of sound amplitude and peak-to-peak time across age, to avoid pseudo-replication and thus inflated p-values. Regardless of age, not all groups of fish produced sounds in this configuration. We only considered groups with at least 100 detected centre clicks for the analysis.

The fish used in this experiment were raised in the facility and had a faster growth rate than the repeated-measure experimental fish. Therefore, the colour-code for fish maturation (Fig. 2C-E, Fig. S2) is estimated based on swim bladder height as measured after the sound recordings and expressed as the mean between the three fish of each group. The matching between biological ages, SL and swim bladder height (Sb-height) were: 5 week old fish SL = 9.3 ± 0.4 mm, Sb-height = 0.68 ± 0.04 mm; 6 week old fish SL = 9.7 ± 0.6 mm, Sb-height = 0.75 ± 0.06 mm; 8 week old fish SL = 10.5 ± 0.2 mm, Sb-height = 0.85 ± 0.03 mm and adult (older than 3 months) SL = 10.5 ± 0.6 mm, Sb-height = 0.92 ± 0.07 mm. Because we did not assign clicks to individual fish, low within-group variation of Sb-height is required to meaningfully relate Sb-height to click parameters. We therefore excluded one group of juvenile fish with an unusually large variation in Sb-height.

Click detection

Clicks were detected with a custom-written python code. Waveform traces were bandpass-filtered between 3 and 20 kHz for repeated measure recordings and 1 and 20 kHz for the close-range click recordings. Peaks were detected using the waveform-envelope and a refractory period of 5 ms between peak times. Filtering parameters and detection thresholds were always kept constant for all recorded ages within one experiment. Bursts were defined as sequences of clicks appearing at less than 25 ms between two clicks.

Morphological measures

Tyr-/- specimens of different ages were euthanized in ice water and fixed overnight in 4% paraformaldehyde in 1x PBS at 4°C. After brief washing in 1x PBS, samples were stained for 24 hours with a double staining solution of 0.001% Alcian Blue (Roth) and 0.005% Alizarin Red S (Sigma-Aldrich) with 200 mM $MgCl_2$ x6 H_2O in 70% EtOH (Walker & Kimmel, 2007). After two brief washes in pure H_2O, samples were kept in 20% glycerol with 0.25% KOH for 2 hours and 50% glycerol with 0.25% KOH overnight. Thereafter, samples were kept in a commercial refractive index matching solution (RI=1.465, EasyIndex Life Canvas) for 2-3 days and imaged under a brightfield illuminated microscope (Olympus MVX-10). All steps after fixation were performed at room temperature. Standard length measurements were taken post-mortem prior to fixation to account for possible shrinkage due to sample dehydration.

Results

First clicks appear in juvenile *D. cerebrum*

To probe at what developmental stage *D. cerebrum* first emit sounds, we raised a group of 100 fish in a stand-alone community aquarium and performed underwater sound recordings once a week. The first time point at which click sounds were clearly distinguishable from background noise was 6 weeks post fertilisation (wpf). From 6 to 8 wpf we observed an increase in the abundance, as well as the amplitude of clicks (example sound traces shown in Fig. 1A).

To examine sound production in more detail across age, we next raised six groups of ten fish in 15x15x10 cm^3 tanks, equipped with one hydrophone each, and recorded twice a week over the course of 11 weeks starting at 4 wpf. To accurately measure the growth rate without disturbing the sound recording tanks, we raised additional individuals under identical conditions and measured the standard length (SL, distance from snout to tail-base). Fish grew over the course of three months until they reached a SL of 11.9 ± 1 mm (Fig. 1B, N = 5 per age). These measurements were drawn from a random pool of a mixed sex fish population. Females are generally larger than males at 15 wpf (11.8 ± 0.7 and 10.8 ± 0.6 mm, respectively, two-sided t-test: $t = 5.3$, $p < 0.001$, N = 37 females, N = 23 males). Growth in body size was accompanied by gross morphological changes, including a change in the shape of the swim bladder complex (Fig. 1C).

Similar to the communal aquarium recording, the number of recorded clicks increased between 6 and 9 wpf (Fig. 1D), corresponding to 8.1 ± 1.3 mm SL at 6 weeks and 9.1 ± 0.8 mm SL at 9 weeks (Fig. 1B). The exact age when groups started producing clicks varied between 6.5 and 8 weeks in the different groups (see grey lines in Fig. 1D). The average age with more than 20 recorded clicks per group was 7 ± 0.5 weeks.

Bursts of clicks appear at stable repetition rates and become longer with age

Adult clicks appear with a repetition rate of either ~60 or ~120 Hz (Schulze et al., 2018; Vasconcelos et al., 2024), corresponding to repeated unilateral and alternating bilateral contractions of the drumming muscles (Cook et al., 2024). Both click repetition rates were present in juveniles from the earliest sound-producing age on (Fig. 1E) and the peak of the bimodal distribution did not change with age (Fig. 1F). Groups of repeated clicks, hereafter referred to as bursts, tend to occur with a click repetition rate of either 60 Hz or 120 Hz (example bursts shown in Fig. 1G). Similar to the total number of clicks (Fig. 1D), the number of bursts increased between 6 and 9 wpf (Fig. 1H). In total, bursts with a single click repetition rate (i.e. either 60 Hz or 120 Hz) make up approximately 80% of all detected bursts throughout all ages tested. At younger ages, *D. cerebrum* tend to produce more 60 Hz bursts, while at later stages bursts of either click repetition rate occur at a similar scale (Fig. 1I). Finally, we measured the length of each burst as the number of clicks in the sequence (Fig. 1J). The maximum number of clicks observed in a burst reached up to several hundred for 120 Hz bursts in older fish, while for 60 Hz bursts it did not surpass ten.

Clicks become louder with age while preserving the sound profile

In the communal aquarium recordings (Fig. 1A), as well as the group recordings (Fig. 2A), the amplitude of the clicks increased with age. Because clicks are generated by compressing the anterior swim bladder (Cook et al., 2024) and the swim bladder size increases with age (Fig. 2B), we next asked how click features change as a function of swim bladder size. Since in the previous sound recordings the distance of the sound source to the hydrophone was unknown, the sound attenuation and thus the actual sound volume could not be reliably established.

We therefore performed multi-hydrophone recordings of clicks at a fixed distance at four developmental timepoints (see Methods for details). These click recordings allowed us to compare the click profile, which was stereotyped within each developmental group (Fig. 2C). While the shape of the pulse was similar across stages, with its peaks aligned in time, click amplitude was positively correlated with swim bladder height (Fig. 2D; Pearson $r = 0.99$, $p = 0.013$, $N = 4$; one data point per group), and reached up to 140 SPL (dB re 1 µPa), matching previous reports of *D. cerebrum* sound pressure levels (Cook et al., 2024). In contrast, the peak-to-peak time of the main click pulse was not correlated with swim bladder height (Fig. 2E; Pearson $r = 0.20$, $p = 0.80$). Because clicks are produced by an ultrafast and discrete strike of the drumming apparatus (Cook et al., 2024), resulting sounds are broadband. When keeping the tank geometry, as well as the distance between the fish and hydrophone stable, clicks show a similar frequency power spectrum across development (Fig. S2). Taken together, this suggests that the sounds of individual clicks are stable, but get louder with age.

Drumming apparatus begins to form and matures in juvenile males

We next investigated the development of the *D. cerebrum* sound production apparatus via bone and cartilage staining. In adults the drumming apparatus and the skeletal structures associated with the anterior swim bladder are sexually dimorphic (Fig. 3 A,B). Adult males have a hypertrophied 5^{th} rib, a globular cartilage and two extra components of the os suspensorium (OS), a bone structure that supports the anterior swim bladder anteriorly and dorsally (Britz et al., 2021). The inner part of the male OS (iOS) has a posterior extension on the dorsal side that extends over the swim bladder. The male outer OS (oOS) extends further rostral and has an additional connection to the transverse process of the second vertebra. An additional feature of the sexual dimorphism is the position of the vent of the digestive tract,

which is shifted anteriorly to the pelvic fin in males (Britz et al., 2021). This rerouting of the vent is the last sexually dimorphic developmental change in juvenile males, occurring after all components of the drumming apparatus have developed (Fig. 3A,B, bottom). However, the connecting flanges between the outer and inner arms of the OS, as well as the connection between the oOS and the vertebral column continue to grow in adult males.

In 2–4-week-old juveniles, sexes cannot yet be distinguished morphologically. Undifferentiated juveniles resemble the adult female morphotype in their skeletal anatomy, with a thin 5th rib and only the lower extension of the iOS present (Fig. 3C, SL: 7 mm). At 7.5 mm the still undifferentiated juvenile develops the short, female version of the oOS (Fig. 3C, SL: 7.5 mm). The first male-specific component that emerges at a SL of 8.0 mm is a hook-like outgrowth of the oOS, which starts to form around the same time of the increase in hypertrophy of the 5th rib (Fig. 3C, SL: 8 mm). The next component is the posterior extension of the iOS over the swim bladder, which coincides with a faint first staining of the drumming cartilage (Fig. 3C, SL: 8.8 mm). The final step in the emergence of the male drumming apparatus is the connection of the oOS to the vertebral column (Fig. 3C, SL: 9.4 mm). The oOS continues to grow and becomes increasingly ossified during further maturation (Fig. 3A,B). In summary, the drumming apparatus components form in juvenile males at a SL between 8 and 10 mm. This is in line with the ontogeny of recorded clicks sounds in our behavioural experiments (Fig 1 and 2).

Discussion

D. cerebrum is a miniature fish with a male-specific trait of acoustic signalling. Here we showed that its males start producing sounds after 1 to 1.5 months of development, at a SL of ca. 9 mm. In line with this, we found that the sound-producing drumming apparatus develops in male specimens between 7 and 10 mm of SL. A full set of drumming components is present at approximately 9 mm SL, while the OS continues to ossify and the rerouting of the vent is yet to occur. We also found that click repetition rate, sound profile and broadband frequency spectra were consistent across age, while sound intensity increased with maturation.

Changes in click characteristics across development

Acoustic allometry, a relationship between body size and sound features, has been described across species of primates and carnivore mammals (Bowling et al., 2017), as well as anurans (Gingras et al., 2013). While within-species allometry is more complex in vocal learning species and possibly species with the capability of volitional sound modulation (Garcia & Ravignani, 2020), it has been described in several fish species with differing sound production mechanisms. For instance, larger fish of the cichlid species *Metriaclima zebra* produce lower frequency and louder sounds (Bertucci, Attia, et al., 2012), a trend that was also found when comparing juvenile and adult fish (Bertucci, Scaion, et al., 2012). Similar results have been reported for Lusitanian toadfish *Halobatrachus didactylus* (Vasconcelos & Ladich, 2008), grey gurnard *Eutrigla gurnardus* (Amorim & Hawkins, 2005), mochokid catfish *Synodontis schoutedeni* (Lechner et al., 2010) and croaking gourami *Trichopsis vittata* (Wysocki & Ladich, 2001). A study performed on the weakfish *Cynoscion regalis* described a negative correlation between pulse duration and dominant frequency (Connaughton et al., 2000). By comparing individual sizes, the authors found that larger males produce louder sounds with a longer pulse duration and lower dominant frequency, without any clear change in pulse repetition rate. This is mostly in line with our results in *D. cerebrum*, where we found an increase in sound amplitude with size and age (Fig. 2A,D), yet no change in pulse repetition rate (Fig. 1F). Unlike

in weakfish and other species, we found no evidence of changes in pulse duration or frequency spectra with maturation (Fig. 2C,E). Most sonic fish with a swim bladder mechanism, including the weakfish, use direct muscle contractions to compress the swim bladder and produce sounds. In contrast, *D. cerebrum* employ an indirect sound production mechanism where a drumming cartilage snaps out and strikes the anterior swim bladder upon muscle contraction (Cook et al., 2024). Therefore, unlike in weakfish, the strength or size of the sonic muscle in *D. cerebrum* seems less likely to influence pulse duration and thus the frequency spectrum. Here we show a positive correlation between sound amplitude and swim bladder height (Fig. 2D), while the temporal click profile remained similar (Fig. 2C,E). The correlation between swim bladder height and click amplitude is unlikely to be a causal link, but rather a by-product of increased maturity of the structures of the drumming apparatus, such as the inner arm of the OS with its dorsal and ventral extension around the swim bladder and likely the mass of the sonic muscle. In line with this, a study comparing sonic muscle mass in the weakfish across spawning seasons showed a positive correlation with sound pressure levels (Connaughton et al., 1997).

Maturation of sound structure in *D. cerebrum*

Adult males produce clicks at repetition rates of ~60 and ~120 Hz by repeated unilateral or by alternating bilateral contractions, respectively (Cook et al., 2024). The presence of both repetition rates in juveniles suggests that the pattern generating neuronal circuitry that controls the alternating contractions is fully functional at the developmental onset of acoustic signalling. Similar results have been reported in weakfish, where pulse repetition rate did not differ with body size (Connaughton et al., 2000). Our dataset shows a trend for older fish to have proportionally more clicks at 120 Hz, as seen in the higher peaks of the histogram (Fig. 1E). This can be explained by the increasing length of 120 Hz bursts with age, of up to multiple hundred clicks. In contrast, 60 Hz bursts typically do not comprise more than 10 clicks (Fig. 1J). Furthermore, we show that throughout all ages tested, 80% of all bursts consist of only either 60 or 120 Hz click repetition rates. However, younger ages show a higher likelihood of 60 Hz bursts over 120 Hz (Fig. 1I). We note that the high variability in burst types and durations between groups could be a result of not having the same sex ratio in each tank, due to the repeated-measure design of the experiment. Given the positive relationship between maturation and sound amplitude, there is a possibility that younger fish produced sounds below the detection threshold of our setup. However, since our morphological analysis showed that the first cartilage staining appeared in fish with a SL of at least 8 mm (matching an age of 6-7 weeks in the repeated measure sound recordings), it is unlikely that males are able to produce sounds earlier than that. Indeed, in the repeated measure experiments first clicks appeared at 8.1 ± 1.3 mm SL (Fig. 1B and D). Since there is individual variability in growth rate, the larger males in the group recordings could have been the ones first producing sounds. In the close-range click recordings with fewer fish per group and only males, body size measurements align well with the morphological results; the youngest clicks we recorded were at 9.3 mm SL (Fig. 2C), a stage at which all components of the drumming apparatus have formed (Fig. 3C).

Development of sound-related anatomy

To date there is limited knowledge about the link between morphology of the sound producing machinery and resulting sound features across ontogenetic development. Several studies have measured sound production throughout development in fish (Vasconcelos & Ladich, 2008, Amorim & Hawkins, 2005, Lechner et al., 2010), yet without morphological measures

beyond body size. In contrast, few studies have described the morphological development of sound-related structures (Hill et al., 1987, Fine, 1989, Brantley et al., 1993), however, without measuring sound output. One exception is a study by Kéver and colleagues in *Ophidion rochei*. Unlike female *D. cerebrum*, females of *O. rochei* can produce sounds. The first juvenile sounds that were recorded in *O. rochei* resembled female sounds rather than adult male sounds (Kéver et al., 2012). Similar to *D. cerebrum*, the sound apparatus of *O. rochei* is sexually dimorphic and juvenile males initially resemble the female morphotype before differentiating new sound-producing structures during gonadal maturation (Kéver et al., 2012).

***Danionella* as a model for development of acoustic communication**

To date our understanding of the neuronal processes underlying the ontogenetic development of sound production and acoustic communication is limited due to methodological constraints of large-scale neuronal recordings across different developmental stages. *D. cerebrum,* with optical accessibility during its entire life, offers a unique opportunity as a vertebrate model to study the ontogeny of acoustic communication. An essential part of studying the development of acoustic communication is sound perception. Several studies have applied auditory brainstem recordings to probe hearing capabilities across age and related it back to the sound parameters individuals were able to produce (Lechner et al., 2010; Vasconcelos & Ladich, 2008; Wysocki & Ladich, 2001). The small, transparent *D. cerebrum* will enable whole-brain imaging of sound perception across development to establish not only when sounds are transduced to neural signals in the brainstem, but also when they start to trigger activity in brain regions that are associated with social behaviours across vertebrates, such as the preoptic area of the hypothalamus (Goodson, 2005). Notably, the Weberian apparatus shows an accelerated development in the *Danionella* species (Conway et al., 2021) and our morphological results also showed its fully ossified components, i.e. tripus and scaphium, prior to the development of any of the sexually dimorphic components of the sonic apparatus (Fig. 3C). This suggests that hearing develops prior to sound production in *D. cerebrum* and it will be an interesting future study to measure the perception of social sounds in non-sonic juveniles. Furthermore, an ancestrally shared development of the neuronal vocal-motor circuits between fish and vocal terrestrial mammals has been proposed (Bass et al., 2008). *D. cerebrum* may therefore serve as a suitable model to study both the development of sound production circuitry, as well as auditory perception of acoustic social signals.

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Competing interests

Authors declare that they have no competing interests.

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Author contributions

Conceptualization: AG, BJ

Methodology: AG, LD, MK, JM

Investigation: AG, LD, MK, JM

Visualisation: AG

Funding acquisition: BJ, AG

Supervision: BJ

Writing – original draft: AG, BJ

Writing – review & editing: AG, LD, MK, JM, BJ

Data availability

Click data and code used to generate the figures are available at https://github.com/danionella/groneberg_et_al_2024. Underlying raw audio data and click detection code are available from the authors upon request.

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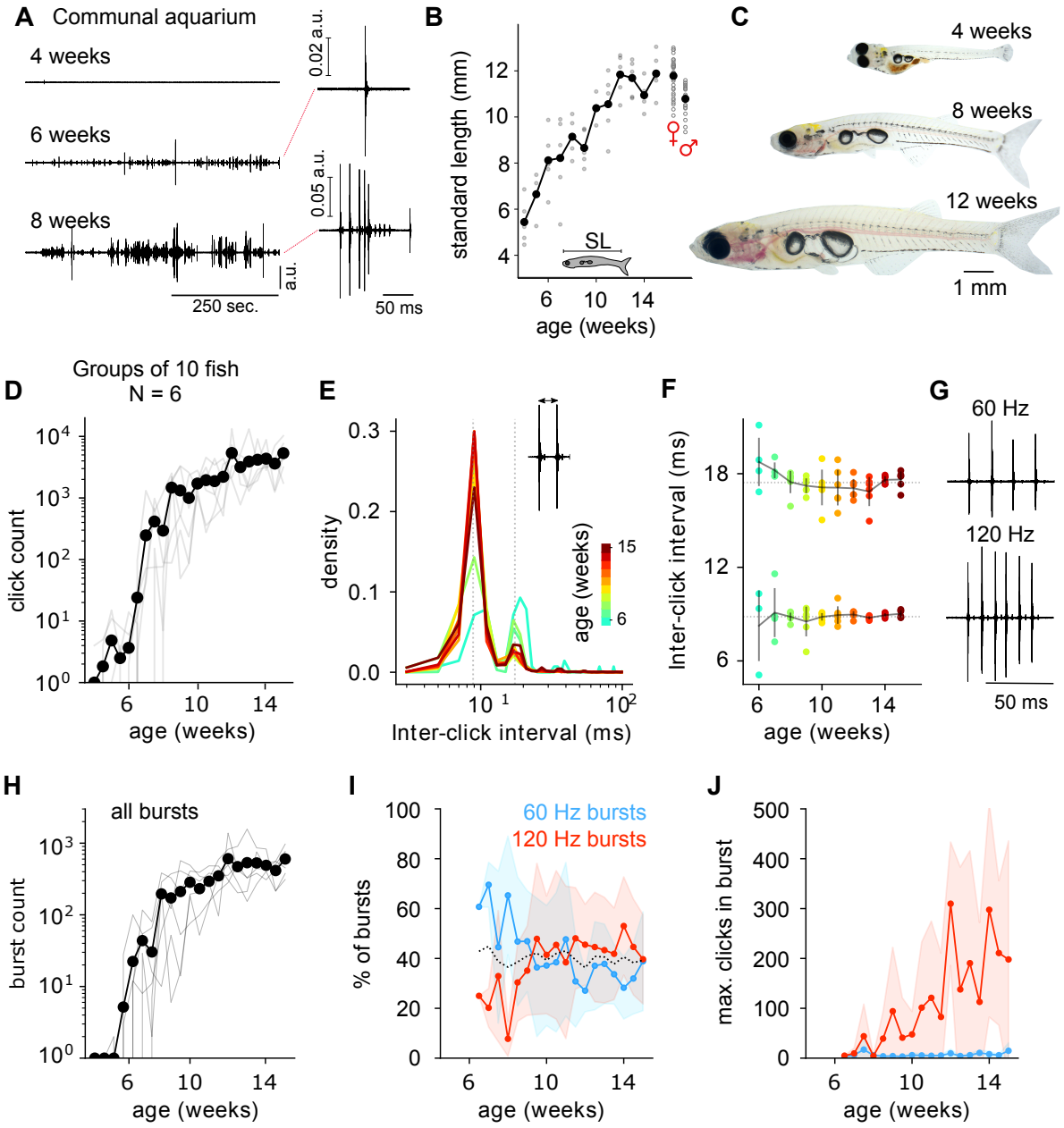

Figure 1: Emergence of click sounds in juvenile *D. cerebrum*. **A**) Example sound traces from hydrophone recordings across different stages of development in a communal aquarium containing 100 fish. Scale bars of time and sound amplitude in arbitrary units (a.u.) refer to all three traces on the left and were adjusted as stated for the zoom-in on the right. **B**) Standard length measures over the course of three months, as five samples per age (shown as grey dots) and mean between samples (connected black dots). At 15 weeks-post fertilisation females are larger than males (two-sided t-test: t = 5.3, p < 0.001, N = 37 females, N = 23 males). **C**) Example images of larval, juvenile and male adult according to stated age and scale bar. The sex of the larval and juvenile fish could not be determined without bone staining. **D-J** Six groups of 10 fish were raised in individual tanks and recorded twice a week with a hydrophone. **D**) Number of clicks detected per recording time point, shown as connected lines for each recording group (grey lines) and mean across groups (black dots and line). **E**) Inter-click interval refers to the time between two adjacent clicks (schematic in inset). Density distribution of inter-click intervals colour-coded for ages 6 to 15 weeks. **F**) The peaks of the bimodal distribution in E are shown for each recorded age. Circles show each recording group, colour-coded by age as in E, and black lines and error indicate mean ± SD values across recording groups per age. Dashed, horizontal lines indicate means across age and groups (8.8 ± 0.8 and 17.4 ± 1.0 ms). **G**) Example sequences of clicks with inter-click intervals of 60 and 120 Hz. **H**) Number of bursts detected per recording time point. Bursts are defined as groups of consecutive clicks with a max. interval of 25 ms. Mean across recording groups and individual recording groups are shown as black dots and grey lines, respectively. **I**) The percentage of 60 and 120 Hz bursts of all detected bursts per recording point, shown as blue and red mean values with shaded SD, respectively. In total these single frequency burst types make up approximately 80% of all bursts across age (dashed black line). **J**) The maximum number of clicks detected in a single burst shown across age for 60 and 120 Hz bursts as blue and red mean values with shaded SD.

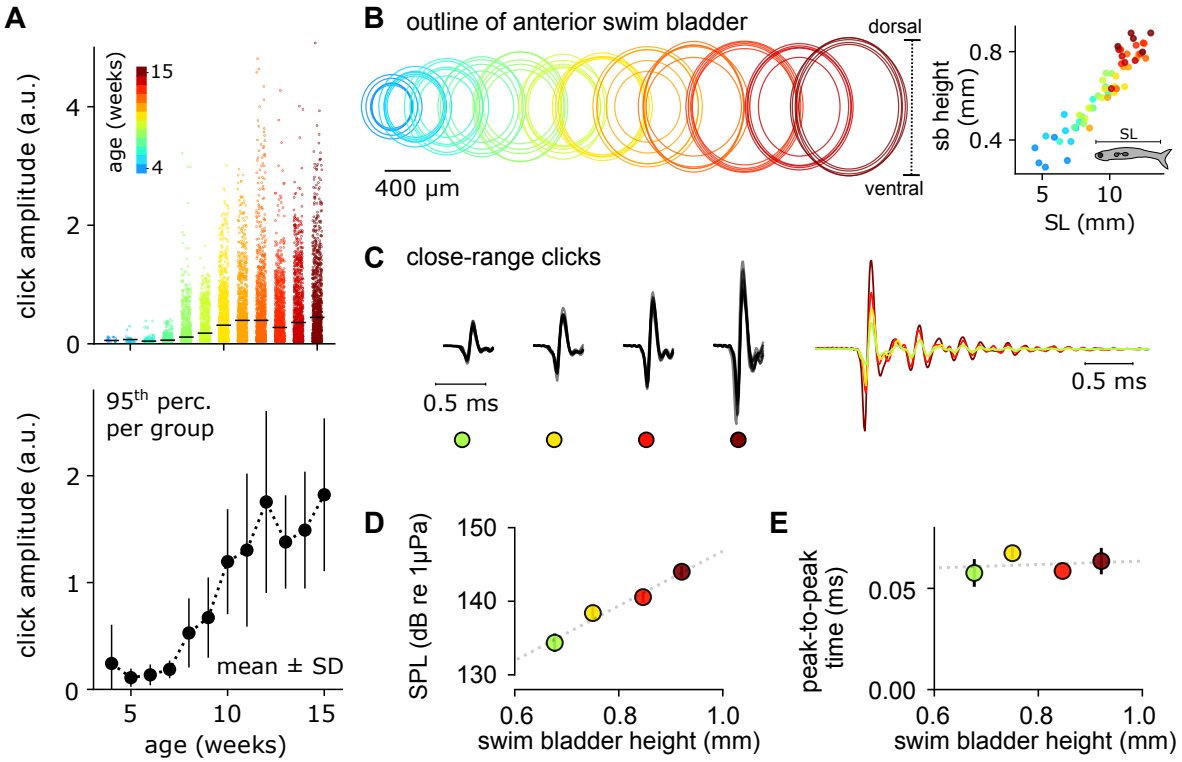

Figure 2: ***D. cerebrum*** **sound features across age. A**) Click amplitude in arbitrary units (a. u.) across age, measured in six groups of 10 fish as a repeated measure. Top: 100 randomly selected clicks from all recording groups, colour-coded by age, with median values as black lines. Bottom: the loudest 95th percentile of all recorded clicks per group is shown as the mean and standard deviation between the six recording groups. **B**) Outline of the anterior swim bladder according to the shown scale, colour-coded for age as in A. Inset on the right shows the standard length against swim bladder (sb) height, measured as the dorsal to ventral diameter, for each measure per age. Note that this data was collected from fish that were raised in parallel with the sound-recorded groups under the same conditions and sampled at a weekly rate as single measures, not repeated measures. **C-E** Groups of three males at different developmental stages we recorded in a small enclosure surrounded by five hydrophones at a distance of 3.5 cm. Because growth rates depend on the raising conditions, the relative age is colour-coded according to the measured sb-height **C**) Sound amplitude profiles of the ten loudest clicks recorded per developmental time point. Profiles of a single click per developmental stage are overlaid in colour on the right. **D,E** Maximum click amplitude converted to sound pressure level (**D**) and peak to peak time of the main sound pulse (**E**) is plotted against the swim bladder height of the recorded males. Shown are the mean values ± standard deviation of the ten clicks shown in C. Dashed lines show the slope of a Pearson correlation. Mean values for each recorded group were used for the correlation to avoid pseudoreplication. Pearson r = 0.99, p = 0.01 for SPL and r = 0.20, p = 0.80 for peak-to-peak times.

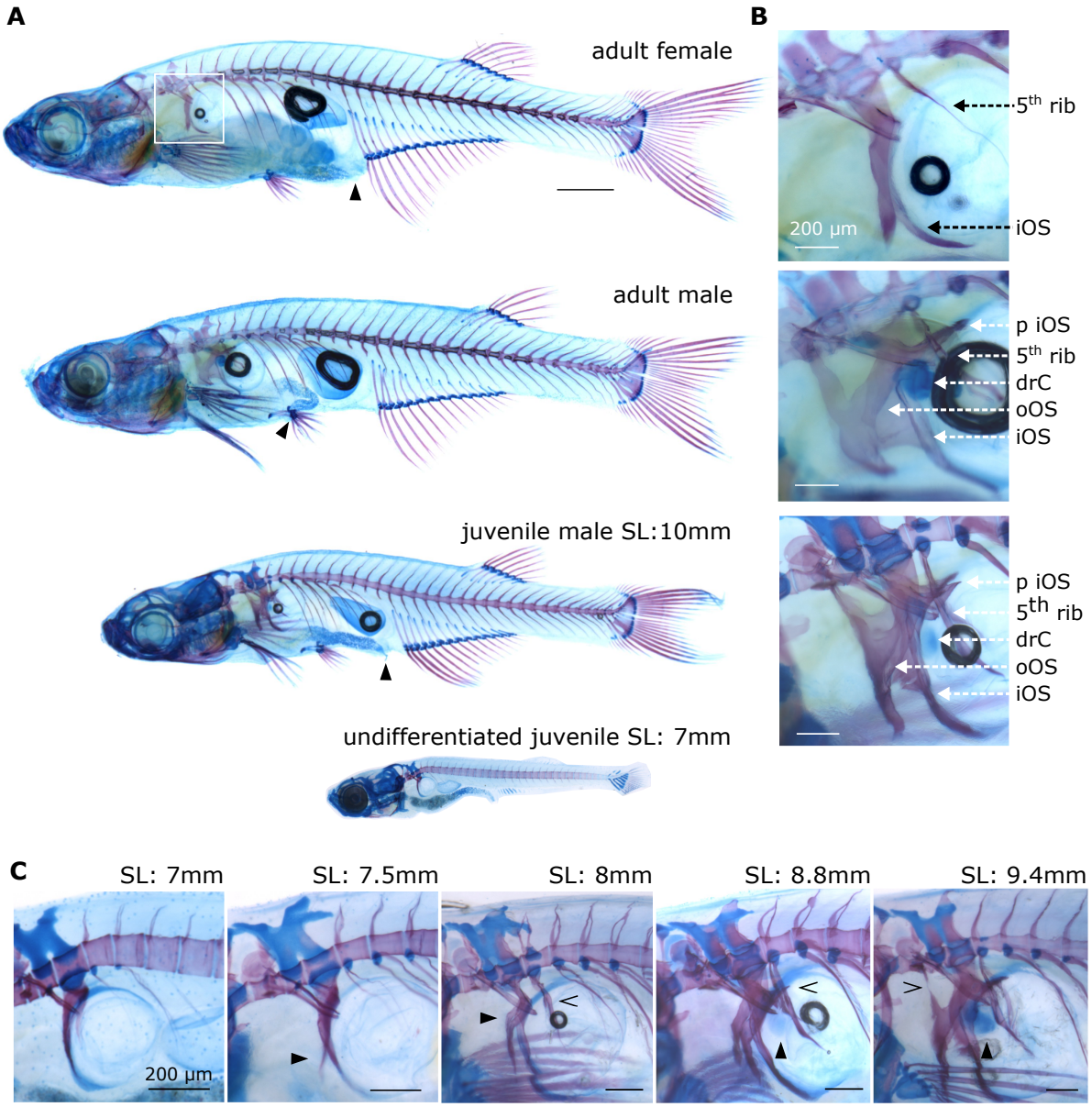

Figure 3: Development of the sound production apparatus in *D. cerebrum* males. Bone and cartilage staining via Alizarin Red S and Alcian Blue, respectively, in unpigmented fish (tyrosinase knock-out). **A**) Exemplary adult female, adult male, juvenile male and an undifferentiated juvenile fish. Black arrowheads point to the anal exit of the gastro-internal tract, which is rerouted in adult males, but not in juvenile males and females. Scale bar 1 mm. **B**) Zoom-in on the anterior swim bladder region in the samples shown in A (white box on female shows zoom-in region). **C**) Zoom-in on the anterior swim bladder region in juveniles at different developmental stages. Standard length (SL) as stated in the figure. Below a SL of 8 mm, the sex could not be determined. SL 8 mm and above are clearly identified male samples. Black arrowheads point towards: the developing oOS in SL 7.5 mm and 8 mm and the developing drumming cartilage in SL 8.8 mm and. 9.4 mm. Open arrowheads point towards the 5th rib in SL 8 mm and 8.8 mm; and to the connection of the oOS to the vertebral column in SL 9.4 mm. Abbreviations: inner arm of the os suspensorium (iOS), posterior extension of the iOS (p iOS), outer arm of the os suspensorium (oOS), drumming cartilage (drC). The black circles seen in some of the images are the remainder of the partially deflated swim bladder. SL was measured post-mortem, prior to fixation, to avoid distorted measures due to shrinkage.

Supplementary Information

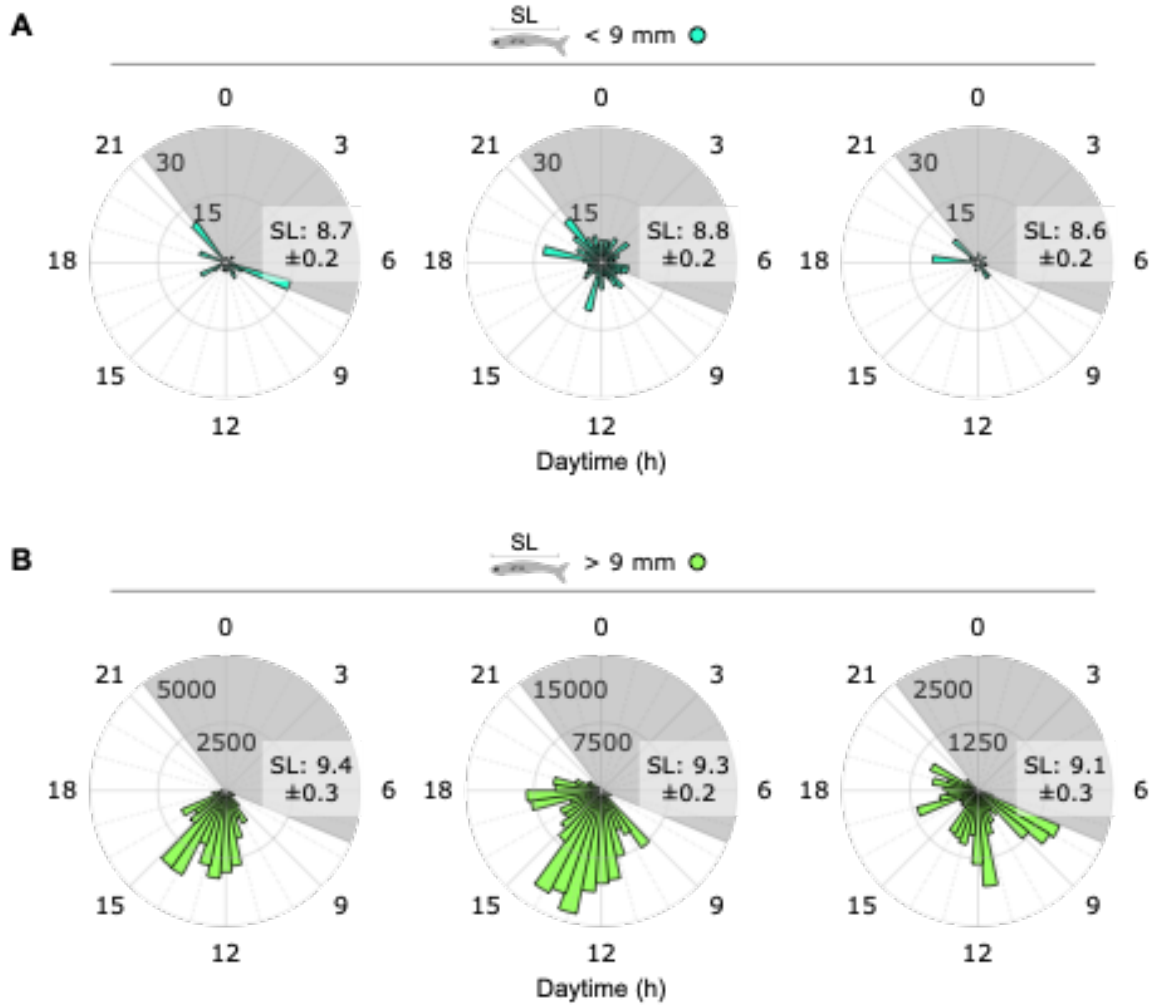

Figure S1: Circadian rhythm of sound production in juvenile *D. cerebrum*.

Groups of three juvenile male were recorded in 15x15x10 cm tanks (equivalent to the repeated measure sound recordings, see Methods for details). All fish came from the same raising tank in the fish facility and were pre-selected by their sex and separated by body size and drumming apparatus maturity at 5 weeks post fertilisation. Click data of two 24h recordings per group of three were pooled and are displayed in a polar histogram with daytime indicated along the polar angle. The shaded grey area signals the light-off period (from 9:30 PM to 7:30 AM). Standard length (SL) measurements are indicated in each subplot as mean and SD for each group of three fish. **A)** Juvenile groups of less than 9 mm SL displayed fewer than 15 clicks per 30 min time bin. We consider this low of a click count to be indistinguishable from noise. No clear circadian pattern can be seen. All of these fish had a thin 5th rib and lacked a distinct cartilage structure; we therefore consider this developmental stage comparable with the staining shown in Fig. 3C for SL 8.8 mm. **B)** Juvenile groups with an SL of more than 9 mm showed more than 1000 clicks per time bin. These clicks were distributed throughout the daylight hours with peaks during the morning, midday or the early afternoon. Such distribution is in line with previous reports of the circadian rhythm of sonic activity in adult *D. cerebrum* (Schulze et al., 2018; Vasconcelos et al., 2024).

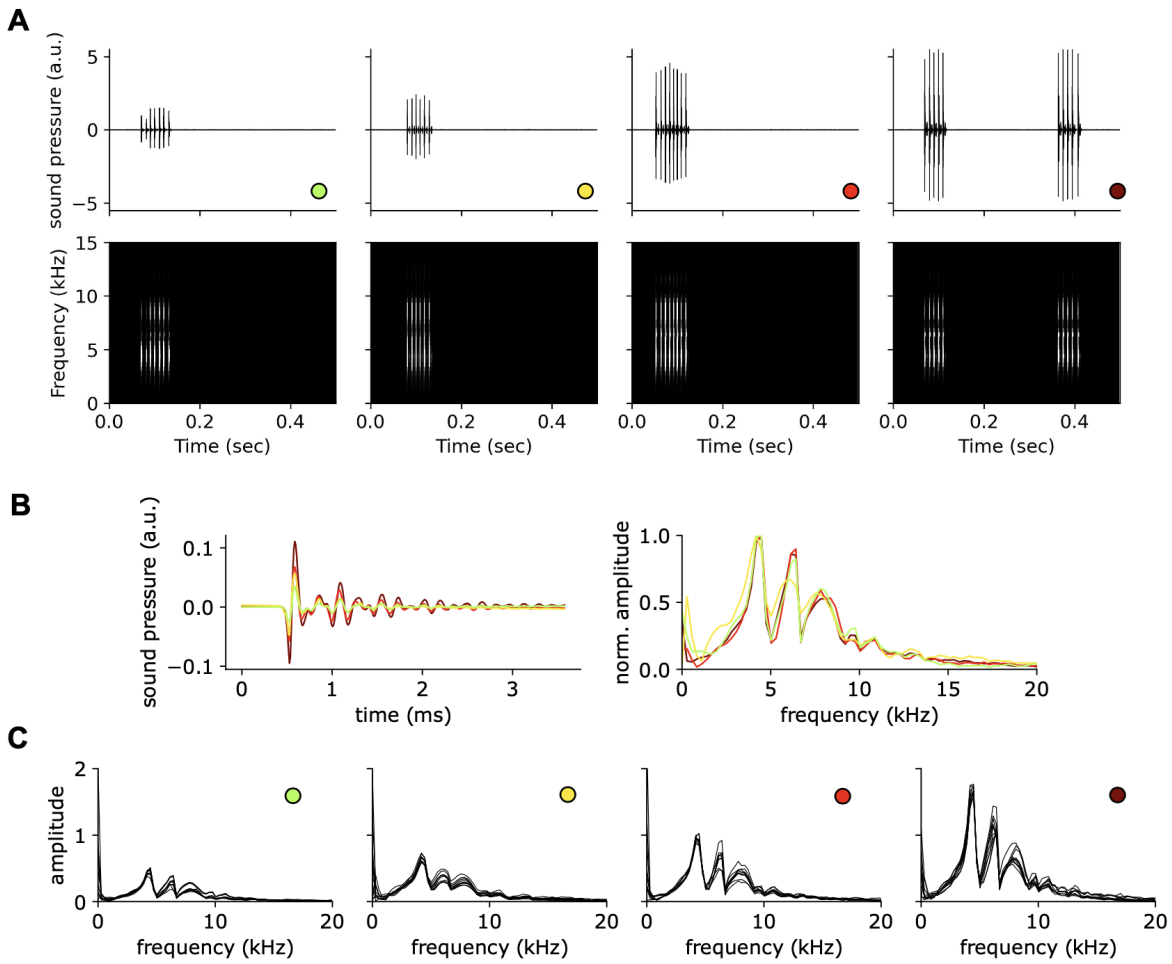

Figure S2: Frequency spectrum of *D. cerebrum* clicks across age.

Groups of three males were recorded in a multi-hydrophone design, which allowed for distance-controlled recordings (see Methods section 'Close-range click recordings' for details). **A)** Example sound traces (top) with the corresponding spectrogram (bottom) for bursts of clicks performed by groups of fish of increasing age from left to right. Developmental stage is indicated by colour, matching the previous data shown in Fig. 2, according to the height of the anterior swim bladder. The lookup table of each spectrogram is normalised within each age group. **B)** Example trace of the loudest detected click per age group and its corresponding normalised amplitude spectrum of the real fast Fourier transform (FFT). **C)** Spectrum of the ten loudest clicks overlaid for each age group (a.u., shared among subpanels). Note that the amplitude spectrum of clicks is broadband. All age groups were recorded in the same tank condition and can therefore be qualitatively compared. To avoid false conclusions that may depend on the tank geometry and spectral filtering caused by wall reflections (cavity resonances), we refrain from quantifying any measures related to specific peaks within the broadband frequency distribution. The overall characteristic of broadband clicks is in line with previous reports of adult *D. cerebrum* (Cook et al., 2024; Schulze et al., 2018; Vasconcelos et al., 2024).