

## RESEARCH ARTICLE

# *Gekko gecko* as a model organism for understanding aspects of laryngeal vocal evolution

Ruth Gutjahr<sup>1,\*‡</sup>, Loïc Kéver<sup>2,\*</sup>, Thorin Jonsson<sup>1</sup>, Daniela Talamantes Ontiveros<sup>2</sup>, Boris P. Chagnaud<sup>1,\*</sup> and Anthony Herrel<sup>2,3,4,5,\*</sup>

## ABSTRACT

The ability to communicate through vocalization plays a key role in the survival of animals across all vertebrate groups. Although avian reptiles have received much attention relating to their stunning sound repertoire, non-avian reptiles have been wrongfully assumed to have less elaborate vocalization types, and little is known about the biomechanics of sound production and their underlying neural pathways in this group. We investigated alarm calls of *Gekko gecko* using audio and cineradiographic recordings. Acoustic analysis revealed three distinct call types: a sinusoidal call type (type 1); a train-like call type, characterized by distinct pulse trains (type 3); and an intermediate type, which showed both sinusoidal and pulse train components (type 2). Kinematic analysis of cineradiographic recordings showed that laryngeal movements differ significantly between respiratory and vocal behavior. During respiration, animals repeatedly moved their jaws to partially open their mouths, which was accompanied by small glottal movements. During vocalization, the glottis was pulled back, contrasting with what has previously been reported. *In vitro* retrograde tracing of the nerve innervating the laryngeal constrictor and dilator muscles revealed round to fusiform motoneurons in the hindbrain–spinal cord transition ipsilateral to the labeled nerve. Taken together, our observations provide insight into the alarm calls generated by *G. gecko*, the biomechanics of this sound generation and the underlying organization of motoneurons involved in the generation of vocalizations. Our observations suggest that *G. gecko* may be an excellent non-avian reptile model organism for enhancing our understanding of the evolution of vertebrate vocalization.

**KEY WORDS:** Functional morphology, Sound production, Neurobiology, Neural pathways, Biomechanics

## INTRODUCTION

For vertebrates, the ability to communicate through vocalization is a key feature for survival for many species. The behavioral role of this communication channel has been investigated for every major vertebrate group (Amorim, 2006; Hollén and Radford, 2009; Ryan

and Rand, 2001; Slabbekoom and Smith, 2002). Signaling phenotypes are central to most animal life and variations in their features have promoted bursts of speciation (Laiolo, 2010; Strelman and Danley, 2003). Insights into the sound production apparatuses and the mechanisms by which the neuromuscular systems control sound production have been provided for diverse taxa including fish, amphibians, avian reptiles and mammals (Suthers et al., 2016). While detailed anatomical descriptions and a few observations of laryngeal movements and vocal cords during sound production (Paulsen, 1967; Russell and Bauer, 2021) are present, the biomechanics and the neural pathways controlling vocalizations in non-avian reptiles remain poorly studied. Although vocalizations with different levels of complexity have evolved repeatedly among non-avian reptiles (Jorgewich-Cohen et al., 2022), the imprint of acoustic communication on their evolutionary success and diversity has been generally underappreciated (Russell and Bauer, 2021). Compared with other vertebrate groups, the role, control and tuning of their communication, as well as the role acoustic communication has played in their diversity and evolutionary history, remain much less well known.

Most non-avian reptiles have long been considered to be silent or to possess a poor vocal repertoire (Russell and Bauer, 2021), which probably explains why they are the only large vertebrate group in which the central and peripheral mechanisms controlling the sound features remain largely obscure. This is regrettable because it is now undisputed that vocalizations with different levels of complexity have evolved repeatedly among non-avian reptiles (Russell and Bauer, 2021). An in-depth understanding of the mechanisms controlling their sound production system(s) would be helpful to understand basic mechanisms of complex vocalizations in general.

Studies conducted on the sound production apparatus of non-avian reptiles date back to the 19th century (von Humboldt, 1811; Moore et al., 1991; Russell and Bauer, 2021; Vergne et al., 2009), but have focused almost exclusively on the gross morphology of the laryngotracheal complex. Among reptiles, ‘true’ vocal cords are present only in some gekkotans, even though some other lizard species and turtles have ligament-like structures that could play a similar role (Moore et al., 1991; Russell and Bauer, 2021). Henle (1839) described an impressive diversity in laryngeal morphology across the Gekkota. However, only a single study has described the laryngeal movements during distress calls in a gecko (Paulsen, 1967), limiting our understanding of the relationship between the observed morphological diversity and the diversity in sound production.

To date, it is generally accepted that non-avian reptiles generate and modulate sounds at the level of the glottis, most likely by involving constrictor and dilator muscles acting on the cricoid and arytenoids (Colafrancesco and Gridi-Papp, 2016). This sound production apparatus is located at the cranial end of the trachea, in contrast to the syrinx of avian reptiles, which is positioned at its

<sup>1</sup>Department of Biology, University of Graz, 8010 Graz, Austria. <sup>2</sup>UMR 7179 C.N.R.S./M.N.H.N., Département Adaptations du Vivant, Bâtiment d’Anatomie Comparée, 55 rue Buffon, 75005 Paris, France. <sup>3</sup>Department of Biology, Evolutionary Morphology of Vertebrates, Ghent University, Ghent 9000, Belgium. <sup>4</sup>Department of Biology, University of Antwerp, Wilrijk 2610, Belgium. <sup>5</sup>Naturhistorisches Museum Bern, 3005 Bern, Switzerland. \*These authors contributed equally to this work

‡Author for correspondence (ruth.gutjahr@uni-graz.at)

© R.G., 0000-0003-0750-3092; L.K., 0000-0003-3672-5348; T.J., 0000-0002-5049-7612; B.P.C., 0000-0001-5939-8541; A.H., 0000-0003-0991-4434

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0>), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.

caudal (or posterior) extremity. This anatomical feature makes the sound production apparatus of non-avian reptiles much easier to access and may thus facilitate *in vivo* analyses. Despite the fact that most vocal reptiles generate relatively simple sounds that may not be species specific (e.g. hissing), other species are known to generate several call types with species-specific features (Rohtla et al., 2019; Yu et al., 2011). In the gecko *Chondrodactylus turneri* (Vedenin et al., 2008), for example, the features of the courtship call are more conserved than those of the distress call, suggesting that it contains species-specific information.

Here, we investigated sound production aspects in the tokay gecko (*Gekko gecko*). We chose this species as it is highly vocal and produces several easily elicited sound types (alarm calls; Fig. 1), even in captivity (Brumm and Zollinger, 2017; Paulsen, 1967; Tang et al., 2001; Yu et al., 2011). We investigated the acoustic properties of these readily inducible vocalizations in a laboratory context and explored the motion of the jaws and the position of the larynx during respiration and vocal behavior. We further studied the innervation of the laryngeal constrictor and dilator muscles by tracing the motor neurons using an *in vitro* preparation. Combined, our data highlight the suitability of these animals for in-depth neurophysiological

studies (Kennedy, 1975, 1981) and as a model for our understanding of vocal communication in non-avian reptiles.

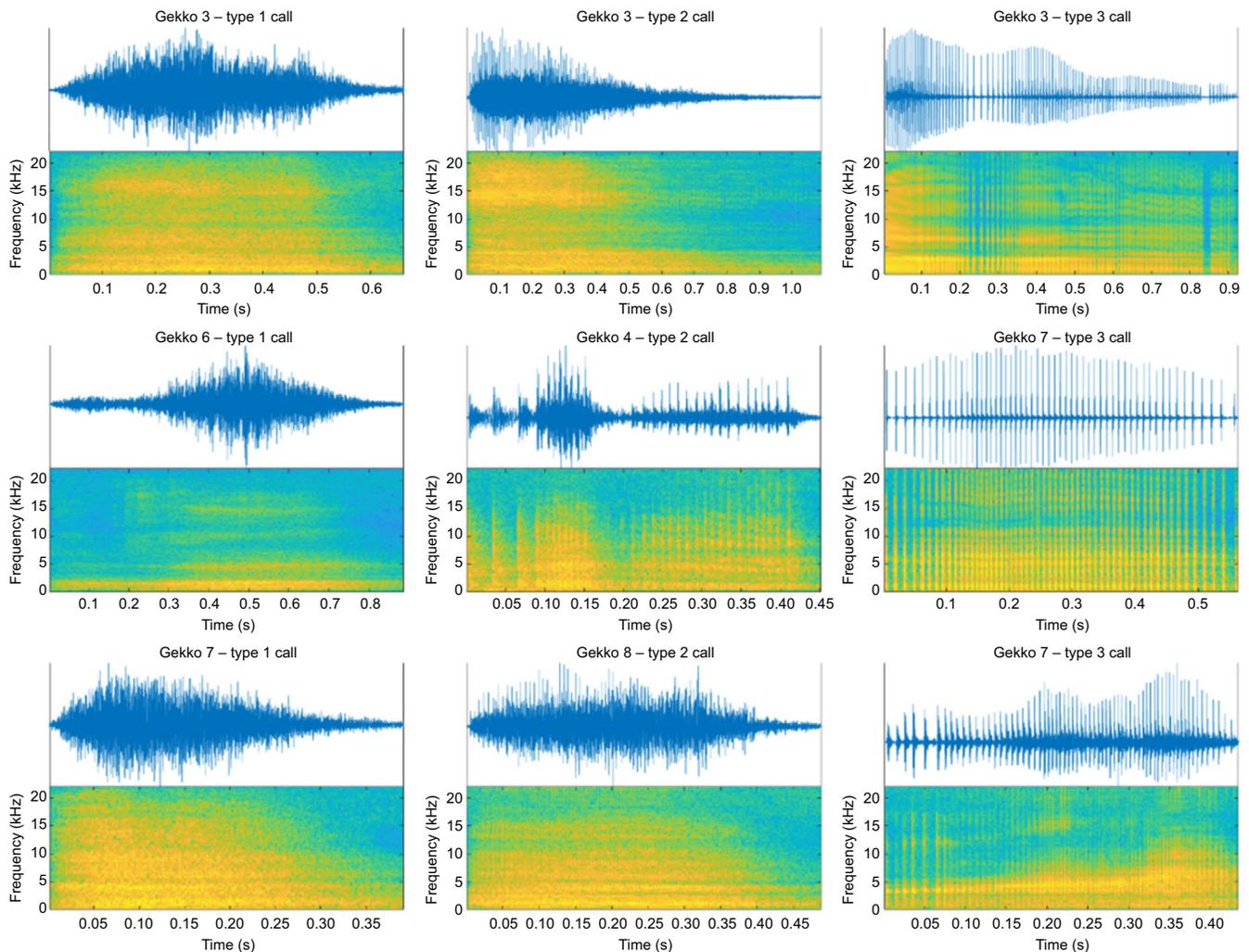
## MATERIALS AND METHODS

### Animal care and maintenance

Tokay geckos [*Gekko gecko* (Linnaeus 1758)] of both sexes (8 males and 2 females) were acquired from the commercial trade (Paris: La Ferme Tropicale, and Graz: Zoo Muser) and ranged in size from 119.0 to 148.2 mm (snout–vent length) and 28.30 to 50.07 g in body mass. Animals were held in terraria under a 12 h:12 h dark:light cycle in temperature-controlled rooms at 25°C. Animals were sprayed daily with tap water and fed with live grasshoppers and crickets. Observations in Paris were conducted in accordance with the ethics guidelines of the Comité Cuvier at the Muséum national d’Histoire naturelle under the animal protocol #68.

### Sound recordings

For acoustic analysis, six adult *G. gecko* individuals were used (for details on the exact sample sizes, see Table S1). Animals were hand-held during animal maintenance procedures, during which they readily elicited vocalizations. We focused on alarm calls as a class of



**Fig. 1.** Examples of the three visually identified types of alarm call vocalizations ( $n=156$ ) produced by individual *Gekko gecko* ( $N=6$ ). Left column: type 1 calls. Middle column: type 2 calls. Right column: type 3 calls. Upper part shows the amplitude waveform of the call, the lower part the matching spectrogram (see Materials and Methods for spectrogram details). Amplitudes are normalized for each call, and spectrogram colors indicate power at the corresponding frequency and moment in time (blue, low power; yellow, high power).

vocal signals as these were most easily elicited and could be analyzed both in terms of kinematics, as well as for sound signal properties. Sounds were recorded with a microphone (model 472049; MicW, Beijing, China) placed 25 cm in front of the respective animal. Microphone signals were digitized with an external sound card (Motu UltraLite mk3, Motu Inc., Cambridge, MA, USA) at 44.1 kHz sampling rate using Audacity (v. 3.2.4, audacityteam.org). A 100 Hz high-pass filter was applied to the recorded calls before sequences were saved on a PC as 16-bit wave files. Two recording sessions were performed at 25°C for each animal, resulting in 12 recorded call sequences ranging in duration from ~1 to 2 min.

### Acoustic analysis

Individual alarm call vocalizations were excised from the recorded call sequences, visually categorized into one of three call types (Fig. 1) and saved as separate wave files (using Audacity). This resulted in a total of 156 calls recorded from six animals ( $N=6$ ,  $n=156$ ; see Table S1 for details about the number of calls per type and individual animal). All subsequent acoustic analyses were carried out using custom written code in MATLAB (R2021b, The MathWorks Inc., Natick, MA, USA). To visualize the frequency composition of calls in time, spectrograms of selected recordings were calculated using MATLAB's *spectrogram* function with 4096 fast Fourier transform (FFT) points and a Hamming window of variable size (window size=total number of samples per signal/100; resulting in 100 time bins per signal with a frequency resolution of 10.8 Hz). To investigate the frequency composition of the gecko calls, spectral analysis was carried out on each individual vocalization by first applying a 250 Hz high-pass filter (6th order Butterworth) to the acoustic signals, followed by Welch's power spectral density (PSD) estimate with 4096 FFT points and a 256-point Hanning window. Resulting spectra for call types were averaged per individual, after which the mean over the individual averages was calculated to show the mean PSD of each call type. Averages over all calls produced by an individual were calculated as well.

### Principal components analysis of vocalizations

For each recorded vocalization [ $N=6$ ,  $n=147$ ; nine recordings were not included in the principal components analysis (PCA) because of their short duration], acoustic feature vectors in the form of 15 mel-frequency cepstral coefficients (MFCCs) and corresponding delta coefficients for 20 segments (window size=total number of samples per signal/20) were calculated using *melfcc* (see Clink and Klinck, 2021 and <http://www.ee.columbia.edu/~dpwe/resources/matlab/rastamat/> for a detailed description of the method). To cover the hearing range of *G. gecko*, the minimum and maximum band edges of the mel filter were set to 250 and 5000 Hz, respectively (Manley et al., 1999, 2014). The first MFCC (also denoted as 0th coefficient or MFCC 0) and its deltas were omitted from the analysis as these relate to the overall energy of the signals only (Clink and Klinck, 2021; Clink et al., 2019). Subsequently, PCA was performed on the resulting vocalization feature vectors to calculate the first three component coefficients and scores.

### High-speed cineradiography

Prior to videography, two adult males ( $N=2$ , for details on the sample sizes, see Table S2) were implanted with small metal markers to help quantify movements of the jaws and the larynx. Markers were placed subcutaneously at the anterior and posterior extremities of the upper and lower jaws using a hypodermic needle. One marker was placed next to the laryngeal cartilages, allowing the tracking of movements of the anterior-most part of the respiratory system. All markers

were inserted under light anesthesia achieved by intramuscular injection into the hind limbs of a mixture of ketamine (80 mg kg<sup>-1</sup>) and medetomidine (115 µg kg<sup>-1</sup>; see Barrillot et al., 2018; Chai et al., 2009). After implantation, anesthesia was reversed by an intramuscular injection of atipamezole (115 µg kg<sup>-1</sup>).

X-ray videos were recorded at 200 to 400 frames s<sup>-1</sup> at a resolution of 1280×800 pixels. Two Phantom Miro cameras (model R311, AMETEK, Berwin, PA, USA) mounted on the image intensifiers of a custom-designed biplanar X-ray system (RST Medical, <https://rstmedical.nl/>) were used to record a side and a top view of the geckos (see Movies 1 and 2). The cameras were mounted on Philips Imagica 38 cm imaging systems (Koninklijke Philips N.V., Amsterdam, Netherlands) mounted on Alp Lift L lift-trolley systems (Alp Lift B.V., Utrecht, Netherlands). X-rays were generated at 57 kV at 80 mA and 60 kV at 80 mA using Philips Super 50CP X-ray generators and Philips SRM 1550 ROT350 X-ray tubes also mounted on Alplift L lift-trolley systems. Sequences were recorded for 6 s simultaneously in lateral and dorso-ventral views. Geckos were positioned inside a transparent acrylic tube (diameter: 5 cm) and were incited to vocalize by approaching them with a piece of black foam. Recordings were performed in an environmentally controlled room set at 25°C. During the X-ray video recordings, sounds were acquired at a distance of 1.5 m with a laptop sound card and internal microphone using the Audacity software.

### X-ray video analysis

For each individual ( $N=2$ ; for details on the sample sizes, see Table S2), five vocalization and two breathing sequences were analyzed. In each 6-s sequence, multiple breathing or vocalization events were included. Both camera views were analyzed separately owing to a problem with camera synchronization during recording. Markers were tracked using the ProAnalyst software (Xcitex Inc., Woburn, MA, USA) and coordinates were exported to Microsoft Excel, smoothed using a low-pass Butterworth filter and scaled. Based on the marker coordinates, the distance between the two anterior jaw markers was calculated and is referred to hereafter as gape distance. We also calculated the distance between the laryngeal marker and the anterior marker on the lower jaw in dorsal view (i.e. a measure of antero-posterior displacement of the glottis relative to the lower jaw) and the distance between the posterior marker on the upper jaw and the laryngeal marker in lateral view (i.e. a measure of the dorso-ventral displacement of the glottis relative to the upper jaw). From the kinematic profiles we extracted the total movement of the glottis and the duration of the movement cycle for each vocalization and breathing event recorded ( $N=2$  animals, Gecko 1 lateral view:  $n_{\text{breathing}}=5$ ;  $n_{\text{vocalization}}=14$ ; Gecko 2 lateral view:  $n_{\text{breathing}}=6$ ;  $n_{\text{vocalization}}=6$ ; Gecko 1 dorsal view:  $n_{\text{breathing}}=8$ ;  $n_{\text{vocalization}}=8$ ; Gecko 2 dorsal view:  $n_{\text{breathing}}=5$ ;  $n_{\text{vocalization}}=17$ ). To test for differences in laryngeal movement between breathing and vocalization, we first log<sub>10</sub>-transformed the absolute displacements and then ran a general linear model testing for differences between the two behaviors. We then also ran a similar analysis on the log<sub>10</sub>-transformed duration of the movement cycle. Finally, we ran simple linear regressions between the jaw gape profile and the laryngeal movement profile to explore whether jaw and laryngeal movements were coordinated. From these regressions we extracted the  $R^2$  value as a measure of the strength of association between the jaw and laryngeal movement. All analyses were run in IBM SPSS statistics V.29.

### Neuronal tracing

We used 4 male animals ( $N=4$ ; for details on the sample size, see Table S2) (119.0–127.7 mm snout–vent length, 28.30–34.72 g) for

*in vitro* nerve backfills. For these tracings, animals were deeply anesthetized using ketamine (150 mg kg<sup>-1</sup>, Richter Pharma, Wels, Austria), perfused transcardially using a carbogenized, ice-cold snake Ringer's solution (Bothe et al., 2018) and were subsequently decapitated, leaving the target nerves intact. We removed excess tissue to gain access to the glossopharyngeal nerve, which served as the target nerve for our retrograde tracings. Ringer's solution was removed and the nerve was severed using microscissors. We used tracer-coated insect needles to place a small BDA-crystal (biotinylated dextran amine, 3 kDa; Invitrogen, Eugene, OR, USA) onto the proximal end of the severed nerve and incubated the preparation for 10 min. After this period, excess dye was rinsed off using fresh, ice-cold Ringer's solution. For the incubation period, the tissue was placed into a 1 l glass beaker filled with Ringer's solution, which was continuously carbogenized. The total tracer incubation time was 48–72 h at 4°C and Ringer's solution was exchanged every 12 h.

After incubation, the tissue was immersion-fixed in 4% paraformaldehyde (PFA) in 0.1 mol l<sup>-1</sup> phosphate buffer (PB) overnight and washed in 0.1 mol l<sup>-1</sup> PB before removing the brain from the skull. Brains were embedded in 2% agarose (ROTH, Karlsruhe, Germany) in 0.1 mol l<sup>-1</sup> PB and transversely sectioned at 100 µm on a vibratome (7000smz-2, Campden Instruments, Loughborough, UK). Sections were mounted onto chrome alum-gelatin coated slides and left to dry overnight. Next, the sections were washed in 0.1 mol l<sup>-1</sup> PB for 5 min, permeabilized with 0.5% Triton X-100 (TX-100) in 0.1 mol l<sup>-1</sup> PB for 10 min and developed with a streptavidin 488 solution (1:500 in 0.5% TX-100 in 0.1 mol l<sup>-1</sup> PB, Life Technologies, Carlsbad, CA, USA). The tissue was washed three times in 0.1 mol l<sup>-1</sup> PB and once in 0.025 mol l<sup>-1</sup> PB. Sections were mounted using VECTASHIELD Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

### Imaging and quantification

Slices were inspected and imaged on an epifluorescence microscope (Thorlabs Cerna Confocal 208 CM712, Newton, NJ, USA and ZEISS Axio Imager 2, ZEISS, Jena, Germany). For better visualization of the labeled neurons we adjusted brightness and contrast as necessary. Imaged slices were adjusted and analyzed using Fiji (v. 1.52, Schindelin et al., 2012). We determined minimal (min.) and maximal (max.) soma diameter and the surface area of the traced neurons. Analyses were performed using a custom script in python 3 (v. 8.3), building on the libraries 'pandas' (v. 2.0.1;

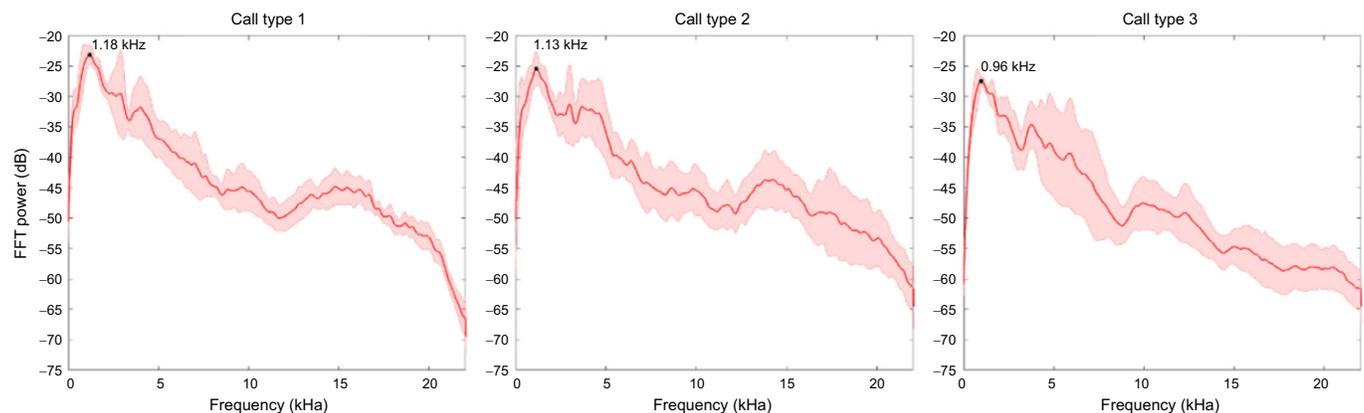
McKinney, 2010) and 'seaborn' (v. 0.11.2; Waskom, 2021). We calculated the minimum, maximum, mean and s.e.m. of the neuroanatomical measurements. Histograms of minimum and maximum soma diameter and surface area of the neurons were plotted, using the following bin sizes: minimum and maximum diameter=4 µm; surface area=100 µm<sup>2</sup>.

## RESULTS

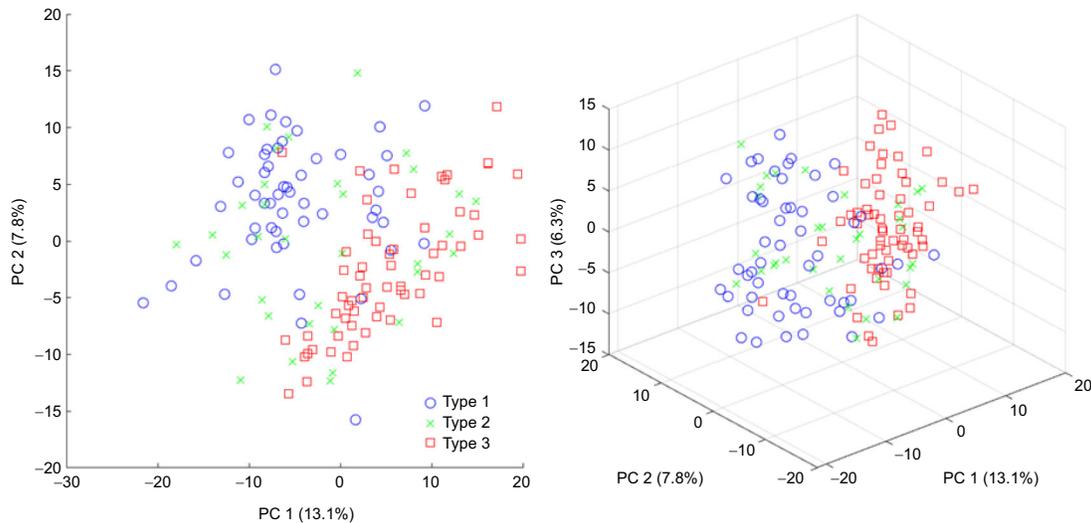
### Sound quantification

A total of 156 vocalizations were obtained from six individuals. Geckos did not emit their characteristic 'to-keh' advertisement call under hand-held conditions. An initial visual analysis of the waveforms of the recorded call sequences suggested that the geckos produced three different types of vocalizations: a sine-like call (type 1 call; Fig. 1 left column), a train of irregularly spaced individual pulses (type 3 call; Fig. 1 right column), and a mixture of both, where pulse trains are visible within sinusoidal events (type 2 call; Fig. 1 middle column). Of the 156 calls ( $N=6$ ), 54 vocalizations were of type 1, 34 of type 2, and 68 of type 3 (see Table S1 for a distribution of call types by individual). Sounds were characterized by a relatively broad frequency range (~85% of all power between 500 Hz and 5 kHz), with a mean peak frequency around 1 kHz for all call types and were similar in frequency content across call types and individuals (Fig. 2; Fig. S1).

To test whether the visually identified vocalization types do indeed represent distinct types of alarm calls, a principal component analysis of the signals' MFCCs was carried out. Cepstral coefficients are a form of acoustic feature vectors and can be extracted from the signal as shape descriptors of the spectral envelope (Clink and Klinck, 2021). Here, the non-linear mel scale was chosen for frequency mapping instead of linearly spaced frequency bands because most terrestrial vertebrates do not perceive sound frequency in a linear manner (Deecke and Janik, 2006; Manley et al., 1999). For each vocalization ( $n=147$ ,  $N=6$ ; nine recordings were excluded due to short duration), 15 MFCCs and delta coefficients were calculated and PCA was performed on those feature vectors. The first three component scores for all vocalizations and their corresponding call types were then plotted in 2D and 3D to investigate whether the visually determined call types correspond to observable clusters in the principal component space (Fig. 3; Fig. S2). Two separate clusters delineating type 1 and type 3 calls were seen with type 2 calls broadly overlapping call types 1 and 3. Thus, we concluded that type 1 and type 3 calls are indeed different call types, and that type 2 calls represent an



**Fig. 2.** Mean frequency spectra of the three *G. gecko* alarm call types. Solid line shows the average ( $N=6$  animals,  $n=156$  calls), shaded areas show  $\pm 1$  s.d. Black dot marks the frequency with the greatest amplitude. FFT, fast Fourier transform.



**Fig. 3. Results of principal components analysis (PCA) of mel-frequency cepstral coefficients (MFCCs) of alarm calls ( $n=147$ ) produced by *G. gecko* individuals ( $N=6$ ).** Left: 2D plot of the first two principal components (PCs), explaining 20.9% of the total variance. Blue circles, green crosses and red squares represent the call types (type 1, 2 and 3, respectively) assigned to the vocalizations after visual inspection of the wave forms. Right: 3D plot of the first three principal components of the same data, explaining 27.2% of the total variance.

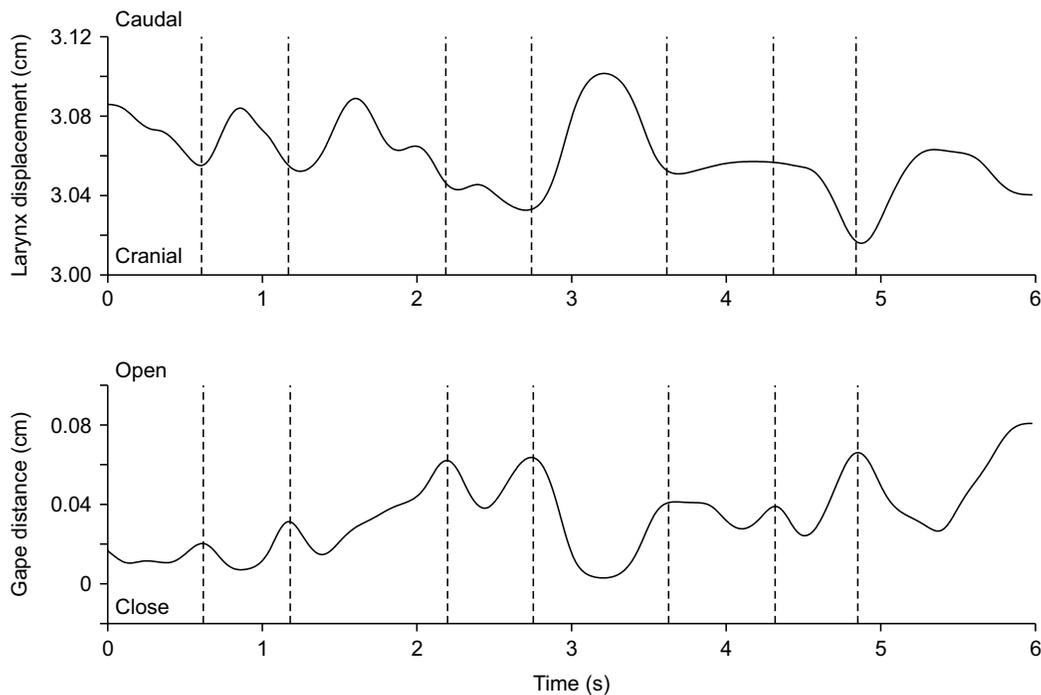
intermediate call type sharing properties of both type 1 and type 3 vocalizations.

### Kinematic analysis

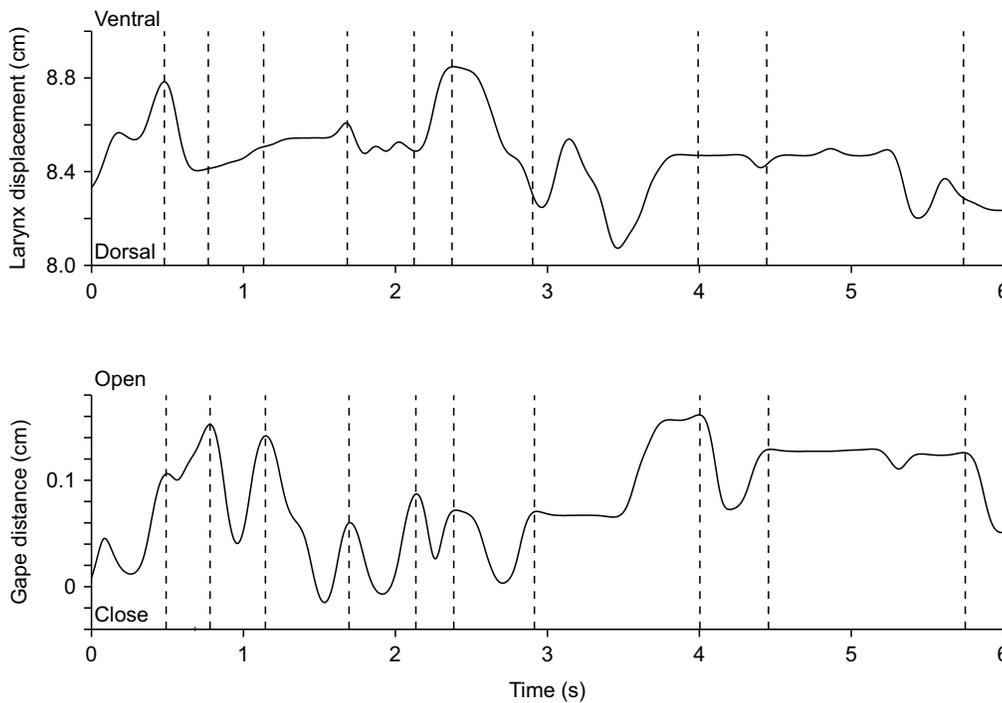
#### Breathing

During breathing, the mouth opens slightly in a repeated pattern roughly associated with laryngeal movements (Figs 4 and 5). More specifically, as the mouth opens slightly, the larynx is pulled rostrad, closer to the tip of the lower jaw. However, most of the movements occur in the vertical plane, with the larynx being

pulled ventrally relative to the largely stationary lower and upper jaws (Movie 1 and see Fig. S3 for schematic positioning of the markers). Displacements of the jaws are very small ( $<1$  mm; Figs 4 and 5). Although displacements of the larynx in the horizontal plane are also very small (typically less than 1 mm, mean =  $0.06 \pm 0.03$  cm), displacements in the vertical plane during breathing are larger and can be up to 3 mm (mean =  $0.26 \pm 0.14$  cm,  $N=2$  animals, Gecko 1 lateral view:  $n_{\text{breathing}}=5$ ; Gecko 2 lateral view:  $n_{\text{breathing}}=6$ ; Gecko 1 dorsal view:  $n_{\text{breathing}}=8$ ; Gecko 2 dorsal view:  $n_{\text{breathing}}=5$ ).



**Fig. 4. Laryngeal and lower jaw displacement profiles illustrating the coordination between jaw and anteroposterior larynx movements during breathing.** The top graph illustrates the horizontal laryngeal displacement relative to the anterior lower jaw marker, and the bottom graph illustrates the gape profile (jaw opening distance) in this exemplary sequence of one animal. Dashed lines indicate moments of maximal mouth opening, which are often associated with maximal protraction of the larynx (lowest distance between the anterior jaw marker and the larynx).



**Fig. 5. Laryngeal and lower jaw displacement profiles illustrating the coordination between jaw and dorsoventral larynx movements during breathing.** The top graph illustrates the dorsoventral larynx displacement relative to the posterior upper jaw marker, and the bottom graph illustrates the gape profile (jaw opening distance) in this exemplary sequence of one animal. Dashed lines indicate moments of maximal jaw opening. No clear synchronization between the dorso-ventral movements of the larynx and jaw opening are apparent.

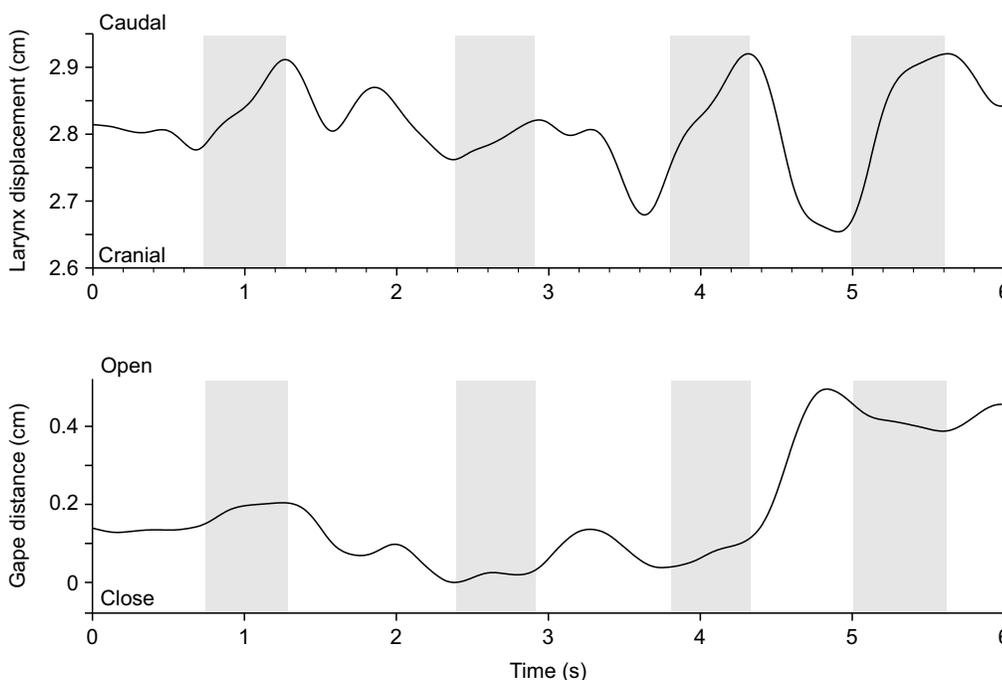
### Vocalization

During vocalization, the movement of the larynx relative to the jaw is the reverse of that seen in breathing, with the larynx being pulled caudad and the distance between the tip of the lower jaw and the larynx increasing (Figs 6 and 7). Moreover, movements occur both in the horizontal and the vertical planes, different from breathing movements (Movie 2). These movements are less regular and the posteroventral displacement of the larynx is generally associated with a slight increase in gape. However, in some instances gape increases before sound production and decreases during sound production, as illustrated in the last cycle in Fig. 6. Laryngeal

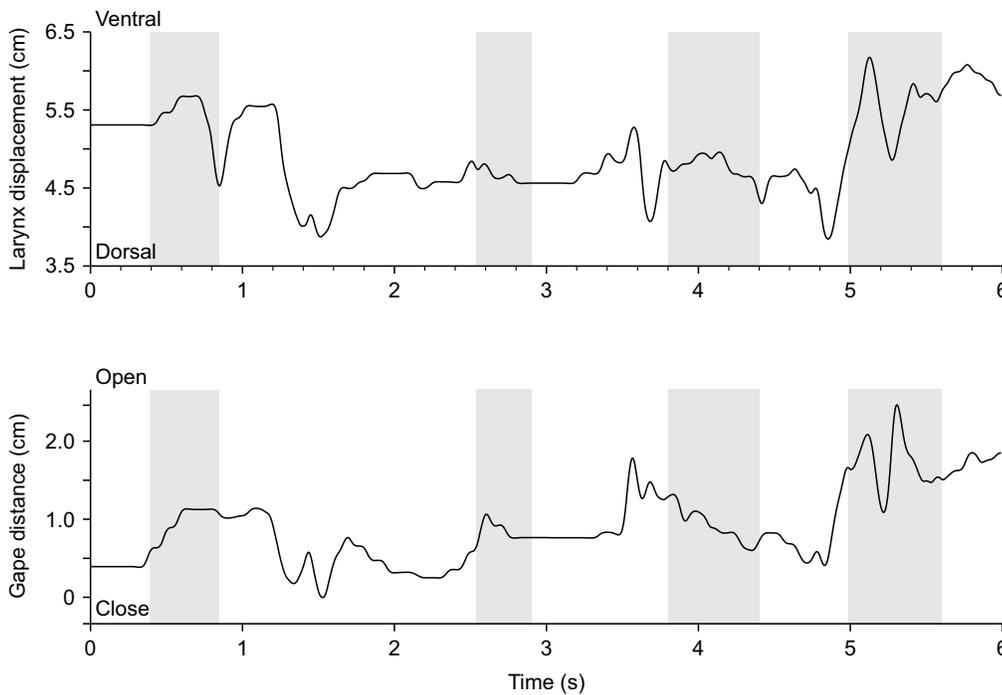
movements are larger during vocalization and may encompass up to 5 mm in the horizontal plane (mean=0.54±0.40 cm) and 8 mm in the vertical plane (mean=0.80±0.71 cm). Jaw movements are also more pronounced and can be larger than 5 mm ( $N=2$  animals, Gecko 1 lateral view:  $n_{\text{vocalization}}=14$ ; Gecko 2 lateral view:  $n_{\text{vocalization}}=6$ ; Gecko 1 dorsal view:  $n_{\text{vocalization}}=8$ ; Gecko 2 dorsal view:  $n_{\text{vocalization}}=17$ ).

### Statistical analysis

The univariate analysis of variance testing for differences in laryngeal movements between breathing and vocalization was



**Fig. 6. Displacement profiles of the larynx and lower jaws during vocalization.** Grey areas denote times at which sound is produced by the gecko in this exemplary sequence of one animal. During vocalization, the larynx moves posteriorly with the posterior-most position being achieved towards the end of the vocalization. No clear coordination between vocalization and jaw opening is apparent, however.



**Fig. 7. Displacement profiles of the larynx and the jaws during vocalization.** Grey areas denote times at which sound is produced by the gecko in this exemplary sequence of one animal. During vocalization, no clear directionality of larynx movement can be observed and movements often include both ventral and dorsal displacements of the larynx. Similarly, no clear association between jaw opening and vocalization is observed.

significant (antero-posterior displacement:  $F_{1,29}=5.33$ ;  $P<0.001$ ; dorso-ventral displacement:  $F_{1,36}=6.34$ ;  $P<0.001$ ) with geckos producing greater movements of the larynx during vocalization (antero-posterior:  $0.54\pm 0.40$  cm; dorso-ventral:  $0.80\pm 0.71$  cm) compared with breathing (antero-posterior:  $0.06\pm 0.03$  cm; dorso-ventral:  $0.26\pm 0.14$  cm). The cycle duration of the larynx movement was also significantly different between vocalization and breathing (antero-posterior displacement:  $F_{1,29}=7.63$ ;  $P=0.01$ ; dorso-ventral displacement:  $F_{1,36}=8.55$ ;  $P=0.006$ ), with cycles being shorter during vocalization (antero-posterior:  $0.89\pm 0.31$  s; dorso-ventral:  $0.70\pm 0.36$  s) compared with breathing ( $1.28\pm 0.38$  s; dorso-ventral:  $0.93\pm 0.18$  s). On average, the  $R^2$  value indicating the strength of the association between jaw and laryngeal movements during breathing was greater than during vocalization for the dorsal view sequences (breathing:  $0.30\pm 0.23$ ; vocalization:  $0.14\pm 0.19$ ) but not the lateral view sequences, where the pattern was opposite (breathing:  $0.26\pm 0.40$ ; vocalization:  $0.50\pm 0.32$ ). However, the statistical analysis suggests that the two types of behaviors were not significantly different in their coordination (dorsal view:  $F_{1,11}=1.80$ ;  $P=0.21$ ; lateral view:  $F_{1,11}=1.40$ ;  $P=0.26$ ). For statistical analyses of breathing and vocalizations, we could only record data from 2 animals ( $N=2$  animals, Gecko 1 lateral view:  $n_{\text{breathing}}=5$ ;  $n_{\text{vocalization}}=14$ ; Gecko 2 lateral view:  $n_{\text{breathing}}=6$ ;  $n_{\text{vocalization}}=6$ ; Gecko 1 dorsal view:  $n_{\text{breathing}}=8$ ;  $n_{\text{vocalization}}=8$ ; Gecko 2 dorsal view:  $n_{\text{breathing}}=5$ ;  $n_{\text{vocalization}}=17$ ).

### Anatomy of laryngeal motoneurons

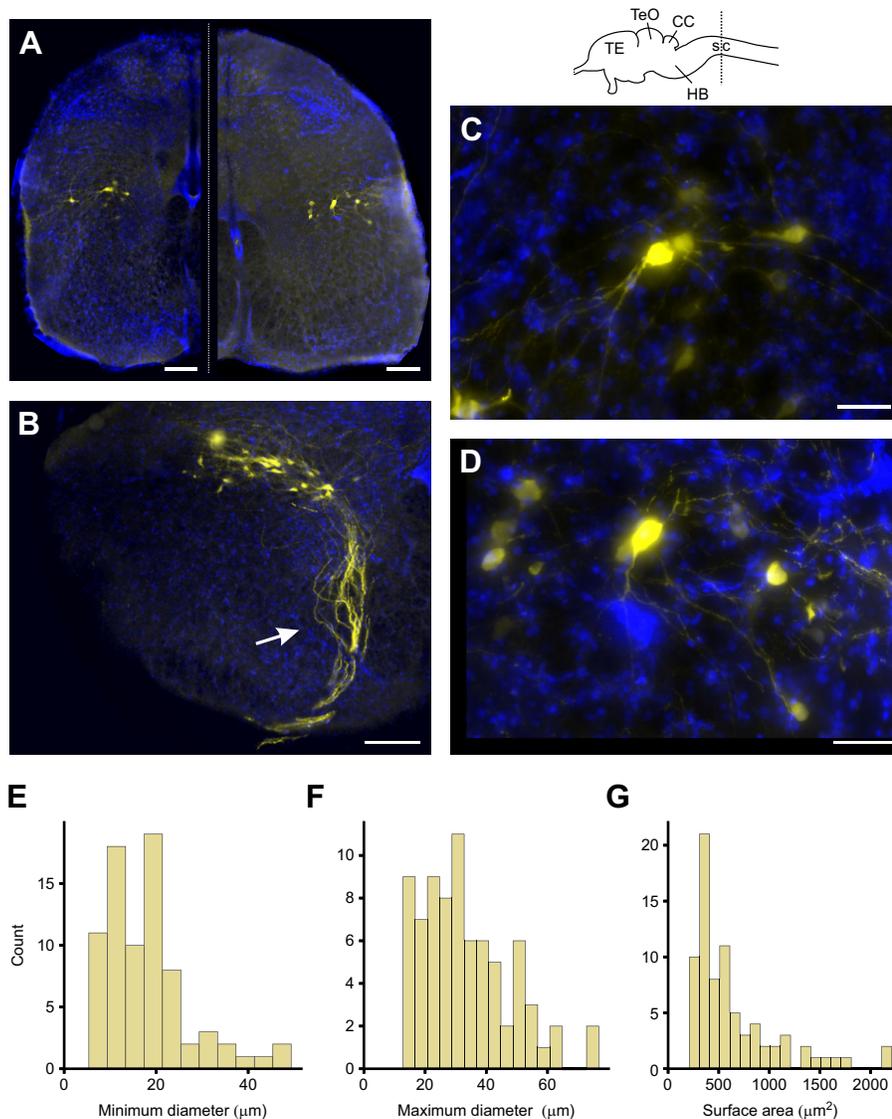
After retrograde tracing of the glossopharyngeal nerve, we found labeled motoneurons exclusively located ipsilaterally within the hindbrain–spinal cord transition. Neurons were located at the level of the central canal or slightly more dorsally along the dorsoventral axis, in a central position along the dorso-ventral axis (Fig. 8A). Axons could be followed from the nucleus ventrally (Fig. 8B, arrow), where they exited the central nervous system as part of the glossopharyngeal nerve (N.IX). The shape of the individual neurons ( $N=4$  animals,  $n=73$  neurons) within the nucleus ranged from round

to oval and fusiform (Fig. 8B–D), with a mean minimum soma diameter of  $18.1\ \mu\text{m}$  (s.e.m.:  $1.1\ \mu\text{m}$ ; range= $5.4\text{--}48.8\ \mu\text{m}$ ), mean maximum soma diameter of  $33.2\ \mu\text{m}$  (s.e.m.:  $1.6\ \mu\text{m}$ ; range= $12.8\text{--}74.1\ \mu\text{m}$ ) and a mean surface area of  $646.2\ \mu\text{m}^2$  (s.e.m.:  $50.2\ \mu\text{m}^2$ ; range= $209.5\text{--}2202.2\ \mu\text{m}^2$ ). The histograms depicting the minimum soma diameter, maximum soma diameter and surface area show a left-skewed distribution (Fig. 8E–G).

## DISCUSSION

### Kinematics of movement during breathing and sound production

To our knowledge, the only prior study of the movements of the gecko larynx and glottis during sound production is that of Paulsen (1967), in which direct observations of handheld tokay geckos were employed. Paulsen stated that during the production of sounds, the larynx is pulled anteriorly and rotated to a more upright position, remaining in this orientation for several seconds before being retracted and returning to its resting position. Using an *in vitro* preparation, in which air was driven through the trachea, Paulsen then filmed the movements of the vocal cords at  $8000\ \text{frames s}^{-1}$  and showed that these move at a frequency between 300 and 400 Hz. Our results obtained from voluntarily emitted alarm calls were different: although we were unable to directly observe the movement of the glottis, the marker positioned next to the larynx allowed us to infer its movements. First, our results showed that movements of the larynx during sound production are significantly different from movements observed during breathing. Whereas during breathing, movements are mostly in the vertical plane (dorso-ventral movements), during vocalization, the glottis moved to a more posterior and ventral position, rather than to a more anterior and dorsal position, as suggested by Paulsen (1967). Moreover, movements of the larynx during sound production are faster when compared with those when breathing and are of 600 and 700 ms in cycle duration, considerably shorter than what was observed by Paulsen (1967). The combination of cineradiography and sound recordings allowed us to determine the onset of sound production and link it to the movements of the larynx,

**Fig. 8. Anatomy of laryngeal motoneurons.**

Top: sketch of the *G. gecko* brain. (A) Left: overview image of motoneurons within a transverse spinal cord section; right: overview of another section with backfilled motoneurons. (B) Laryngeal motoneurons with axons (arrow) protruding towards the ventral horn of the spinal cord. (C, D) Magnified image of motoneurons depicted in A (C: left side; D: right side). (E) Histogram of minimum soma diameter, bin size=5 μm. (F) Histogram of maximum soma diameter, bin size=5 μm. (G) Histogram of surface area, bin size=100 μm<sup>2</sup>. For histograms in E–G: *N*=4 animals/tracings, *n*=73 neurons. For fluorescent images in A–D: blue, DAPI; yellow, neurobiotin+streptavidin 488. Scale bars: (A, B) 200 μm; (C, D) 50 μm. TE, telencephalon; TeO, optic tectum; CC, cerebellum; HB, hindbrain; sc, spinal cord.

an advantage over the visual observations conducted by Paulsen (1967). Interestingly, movements of the larynx were, on average, not coordinated with jaw movements, even though in some cases strong correlations were observed. This suggests that tokay geckos rely strongly on movements of the larynx, which may be either coupled to or decoupled from movements of the jaws. These complex coordination patterns may be what allows them to produce different types of alarm calls as described here, but this remains to be investigated further.

### Neural networks controlling vocal behavior in non-avian reptiles

The neural control of sound production in non-avian reptiles is virtually unknown. A pair of exploratory studies conducted on *G. gecko* support the assumption of a generally conserved vocal pathway organization (Kennedy, 1975, 1981). In those studies, Kennedy managed to label vocal-associated motoneurons and was able to elicit vocalization by electrical stimulation of the midbrain, reportedly the periaqueductal gray. His tracings revealed motoneurons localized exclusively in the ipsilateral nucleus ambiguus in the caudal part of the medulla and the rostral part of the spinal cord (Kennedy, 1981). Laryngeal motoneurons showed a

medio-lateral orientation but were not densely packed within the nucleus. These findings are in accord with our tracing results (Fig. 8). Although Kennedy's (1981) study showed neurons to be round or fusiform in shape and to exhibit maximal soma diameters in the range of 12–15 (round) to 20–30 μm (fusiform), motoneurons in our neural backfills (while revealing similar shapes and dendritic extends) span a broader size range of 12.8–74.1 μm.

Owing to their conserved location at the caudal hindbrain–spinal cord boundary across vertebrates, vocal motoneurons were first regarded as having originated from a single developmentally or evolutionarily conserved progenitor pool (Bass and Baker, 2008; Bass et al., 2008). This classical view was recently challenged by data showing that the highly conserved *Phox2b* gene (presumed to be expressed in all vertebrate hindbrain branchial motoneurons) is present in the vocal motoneurons of frogs, mice and humans, but not in fish or avian reptiles (Albersheim-Carter et al., 2016). Generally, vocal motoneuron axons exit the brain via the twelfth cranial nerve in songbirds and the occipital nerve (potential homolog of the N.XII) in fish, and via the ninth and tenth cranial nerves in frogs and mammals (Barkan and Zornik, 2020). Although evolutionary considerations previously suggested convergent evolution (Barkan and Zornik, 2020) of vocal traits, recent evidence suggested that a

common ancestor in choanate vertebrates was already vocal (Barkan and Zornik, 2020; Jorgewich-Cohen et al., 2022). Because of their rich vocal behavior, gekkotans, turtles and crocodylians offer a unique opportunity for improving our understanding of vocal pathway(s) and the control and modulation of sound features.

Our *in vitro* tracings further revealed that the gecko hindbrain remains stable in Ringer's solution for multiple days at appropriate temperature. Although we have not yet recorded from neurons in such preparations, these first results indicate that *in vitro* electrophysiological and anatomical studies, similar to the ones performed on diverse motor patterns, including locomotor activity, respiration and vocal behavior (Kelley et al., 2017; Rhodes et al., 2007; Zornik and Kelley, 2008), are likely to be successful in *G. gecko* as well. If an *in vitro* whole brain preparation of *G. gecko* would allow for the production of fictive calls (i.e. action potential volleys with features specific to the *G. gecko* sounds in the nerves associated with the control of sound production), then such a preparation would greatly facilitate studying vocal networks irrespective of respiratory activation, as already shown for the amphibian *Xenopus laevis*. The ability of *G. gecko* to produce different sound types in different contexts might thus allow for the study of the neural substrates of context-dependent sound production on easily identifiable fictive motor patterns (i.e. nerve activity).

As non-avian reptiles have their sound production organ (i.e. the larynx) located at the cranial end of the trachea (in contrast to the syrinx of avian reptiles), this facilitates the study of its biomechanics and allows for more direct comparisons with mammalian sound production. Consequently, *G. gecko* could serve as a reference taxon for comparative studies of sound production as the reptile counterpart of the midshipman fish among teleosts (Bass and Ramage-Healey, 2008; Chagnaud et al., 2021), the African clawed frog among amphibians, and the zebra finch among birds (Brenowitz et al., 1997; Düring et al., 2013; Elemans, 2014).

#### Acknowledgements

We acknowledge the help of Birgit Rönfeld in histological processing and Renaud Boistel for lending audio equipment.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

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

#### Funding

This work was co-funded by grants by the OeAD-GmbH (FR10/2022) and the Partenariat Hubert Curien' Amadeus. T.J. received funding from the Austrian Science Fund (FWF) (P 35792-B). The authors acknowledge the financial support by the University of Graz. Open Access funding provided by University of Graz. Deposited in PMC for immediate release.

#### Data availability

Raw data obtained during sound recordings, cineradiography and nerve tracings, as well as code used during the analysis, are available on the Zenodo open repository: <https://doi.org/10.5281/zenodo.12188321>.

#### ECR Spotlight

This article has an associated ECR Spotlight interview with Ruth Gutjahr.

#### References

Albersheim-Carter, J., Blubaum, A., Ballagh, I. H., Missaghi, K., Siuda, E. R., McMurray, G., Bass, A. H., Dubuc, R., Kelley, D. B., Schmidt, M. F. et al. (2016).

- Testing the evolutionary conservation of vocal motoneurons in vertebrates. *Respir. Physiol. Neurobiol.* **224**, 2-10. doi:10.1016/j.resp.2015.06.010
- Amorim, M. C. P. (2006). Diversity of sound production in fish. In *Communication in Fishes* (ed. F. Ladich, S. P. Collin, P. Mollin and B. Kapoor), pp. 71-104. Enfield, NH: Science Publishers.
- Barkan, C. L. and Zornik, E. (2020). Inspiring song: the role of respiratory circuitry in the evolution of vertebrate vocal behavior. *Dev. Neurobiol.* **80**, 31-41. doi:10.1002/dneu.22752
- Barrillot, B., Roux, J., Arthaud, S., Averty, L., Clair, A., Herrel, A. and Libourel, P.-A. (2018). Intramuscular administration of ketamine-medetomidine assures stable anaesthesia needed for long-term surgery in the Argentine tegu *Salvator merianae*. *J. Zoo Wildl. Med.* **49**, 291-296. doi:10.1638/2017-0089.1
- Bass, A. H. and Baker, R. (2008). Phenotypic specification of hindbrain rhombomeres and the origins of rhythmic circuits in vertebrates. *Brain Behav. Evol.* **50**, 3-16. doi:10.1159/000113351
- Bass, A. H. and Ramage-Healey, L. (2008). Central pattern generators for social vocalization: androgen-dependent neurophysiological mechanisms. *Horm. Behav.* **53**, 659-672. doi:10.1016/j.yhbeh.2007.12.010
- Bass, A. H., Gilland, E. H. and Baker, R. (2008). Evolutionary origins for social vocalization in a vertebrate hindbrain-spinal compartment. *Science (1979)* **321**, 417-421.
- Bothe, M. S., Luksch, H., Straka, H. and Kohl, T. (2018). Synaptic convergence of afferent inputs in primary infrared-sensitive nucleus (LTTD) neurons of rattlesnakes (Crotalinae) as the origin for sensory contrast enhancement. *J. Exp. Biol.* **221**, jeb185611. doi:10.1242/jeb.185611
- Brenowitz, E. A., Margoliash, D. and Nordeen, K. W. (1997). An introduction to birdsong and the avian song system. *J. Neurobiol.* **33**, 495-500. doi:10.1002/(SICI)1097-4695(19971105)33:5<495::AID-NEU1>3.0.CO;2-#
- Brumm, H. and Zollinger, S. A. (2017). Vocal plasticity in a reptile. *Proc. R. Soc. B* **284**, 20170451. doi:10.1098/rspb.2017.0451
- Chagnaud, B. P., Perelmuter, J. T., Forlano, P. M. and Bass, A. H. (2021). Gap junction-mediated glycinergic inhibition ensures precise temporal patterning in vocal behavior. *eLife* **10**, e59390. doi:10.7554/eLife.59390
- Chai, N., Lecu, A., Petit, T., Sillero, L. N. C., Alves, C. and Wedlarski, R. (2009). Dexmedetomidine: premiers essais avec la kétamine. *Point vétérinaire* **40**, 25-30.
- Clink, D. J. and Klinck, H. (2021). Unsupervised acoustic classification of individual gibbon females and the implications for passive acoustic monitoring. *Methods Ecol. Evol.* **12**, 328-341. doi:10.1111/2041-210X.13520
- Clink, D. J., Crofoot, M. C. and Marshall, A. J. (2019). Application of a semi-automated vocal fingerprinting approach to monitor Bornean gibbon females in an experimentally fragmented landscape in Sabah, Malaysia. *Bioacoustics* **28**, 193-209. doi:10.1080/09524622.2018.1426042
- Colafrancesco, K. C. and Gridi-Papp, M. (2016). Vocal sound production and acoustic communication in amphibians and reptiles. In *Vertebrate Sound Production and Acoustic Communication* (ed. R. A. Suthers, W. T. Fitch, R. R. Fay and A. N. Popper), pp. 51-82. Cham: Springer International Publishing.
- Deecke, V. B. and Janik, V. M. (2006). Automated categorization of bioacoustic signals: avoiding perceptual pitfalls. *J. Acoust. Soc. Am.* **119**, 645-653. doi:10.1121/1.2139067
- Düring, D. N., Ziegler, A., Thompson, C. K., Ziegler, A., Faber, C., Müller, J., Scharff, C. and Elemans, C. P. H. (2013). The songbird syrinx morphome: a three-dimensional, high-resolution, interactive morphological map of the zebra finch vocal organ. *BMC Biol.* **11**, 1. doi:10.1186/1741-7007-11-1
- Elemans, C. P. H. (2014). The singer and the song: the neuromechanics of avian sound production. *Curr. Opin. Neurobiol.* **28**, 172-178. doi:10.1016/j.conb.2014.07.022
- Henle, J. (1839). *Vergleichend-anatomische Beschreibung des Kehlkopfs: mit Berücksichtigung des Kehlkopfs der Reptilien*. Leipzig: Leopold Voss.
- Hollén, L. I. and Radford, A. N. (2009). The development of alarm call behaviour in mammals and birds. *Anim. Behav.* **78**, 791-800. doi:10.1016/j.anbehav.2009.07.021
- Jorgewich-Cohen, G., Townsend, S. W., Padovese, L. R., Klein, N., Prashag, P., Ferrara, C. R., Ettmar, S., Menezes, S., Varani, A. P., Serano, J. et al. (2022). Common evolutionary origin of acoustic communication in choanate vertebrates. *Nat. Commun.* **13**, 6089. doi:10.1038/s41467-022-33741-8
- Kelley, D. B., Elliott, T. M., Evans, B. J., Hall, I. C., Leininger, E. C., Rhodes, H. J., Yamaguchi, A. and Zornik, E. (2017). Probing forebrain to hindbrain circuit functions in *Xenopus*. *Genesis* **55**, e22999. doi:10.1002/dvg.22999
- Kennedy, M. C. (1975). Vocalization elicited in a lizard by electrical stimulation of the midbrain. *Brain Res.* **91**, 321-325. doi:10.1016/0006-8993(75)90556-9
- Kennedy, M. C. (1981). Motoneurons that control vocalization in a reptile: an HRP histochemical study. *Brain Res.* **218**, 337-341. doi:10.1016/0006-8993(81)91311-1
- Laiolo, P. (2010). The emerging significance of bioacoustics in animal species conservation. *Biol. Conserv.* **143**, 1635-1645. doi:10.1016/j.biocon.2010.03.025
- Manley, G. A., Köppl, C. and Sneary, M. (1999). Reversed tonotopic map of the basilar papilla in *Gekko gecko*. *Hear. Res.* **131**, 107-116. doi:10.1016/S0378-5955(99)00021-0
- Manley, G. A., Köppl, C. and Siemknecht, U. J. (2014). The remarkable ears of geckos and pygopods. In *Insights from Comparative Hearing Research* (ed. C.

- Köppl, G. A. Manley, A. N. Popper and R. R. Fay), pp. 111-131. New York, NY: Springer New York.
- McKinney, W.** (2010). Data structures for statistical computing in Python. In Proceedings of the 9th Python in Science Conference (ed. van der Walt and Millman Jarrod), pp. 50-61.
- Moore, B. A., Russell, A. P. and Bauer, A. M.** (1991). Structure of the larynx of the tokay gecko (*Gekko gecko*), with particular reference to the vocal cords and glottal lips. *J. Morphol.* **210**, 227-238. doi:10.1002/jmor.1052100303
- Paulsen, K.** (1967). *Prinzip der Stimmbildung in der Wirbeltierreihe und beim Menschen*. Frankfurt am Main: Akademische Verlagsgesellschaft.
- Rhodes, H. J., Yu, H. J. and Yamaguchi, A.** (2007). *Xenopus* Vocalizations are controlled by a sexually differentiated hindbrain central pattern generator. *J. Neurosci.* **27**, 1485-1497. doi:10.1523/JNEUROSCI.4720-06.2007
- Rohtla, E. A., Jr, Russell, A. P. and Bauer, A. M.** (2019). Sounding off: relationships between call properties, body size, phylogeny, and laryngotracheal form of geckos. *Herpetologica* **75**, 175-197. doi:10.1655/D-19-00021
- Russell, A. P. and Bauer, A. M.** (2021). Vocalization by extant nonavian reptiles: a synthetic overview of phonation and the vocal apparatus. *Anat. Rec.* **304**, 1478-1528. doi:10.1002/ar.24553
- Ryan, M. J. and Rand, A. S.** (2001). Feature weighting in signal recognition and discrimination by túngara frogs. In *Anuran Communication* (ed. M. J. Ryan), pp. 86-101. Washington, DC: Smithsonian Institution Press.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B. et al.** (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* **9**, 676-682. doi:10.1038/nmeth.2019
- Slabbekoorn, H. and Smith, T. B.** (2002). Bird song, ecology and speciation. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **357**, 493-503. doi:10.1098/rstb.2001.1056
- Streelman, J. T. and Danley, P. D.** (2003). The stages of vertebrate evolutionary radiation. *Trends Ecol. Evol.* **18**, 126-131. doi:10.1016/S0169-5347(02)00036-8
- Suthers, R. A., Fitch, W. T., Fay, R. R. and Popper, A. N.** (2016). *Vertebrate Sound Production and Acoustic Communication*. Springer.
- Tang, Y.-Z., Zhuang, L.-Z. and Wang, Z.-W.** (2001). Advertisement calls and their relation to reproductive cycles in *Gekko gecko* (Reptilia, Lacertilia). *Copeia* **2001**, 248-253. doi:10.1643/0045-8511(2001)001[0248:ACATRT]2.0.CO;2
- Vedenin, A. A., Vedenina, V. Y. and Okshtein, I. L.** (2008). Vocal communication in thick-fingered gecko *Pachydactylus turneri*. In *The Problems of Herpetology. Proceedings of the 3rd Meeting of the Nikolsky Herpetological Society* (ed. N. B. Ananjeva, I. G. Danilov, E. A. Dunayev, V. G. Ishchenko, S. N. L. G. A. Lada, V. F. Orlova, E. M. Smirina, B. S. Tuniyev and R. G. Khalikov), pp. 89-94. Alexander Mikhailovich Nikolsky Herpetological Society of the Russian Academy of Sciences and Zoological Institute of Russian Academy of Sciences. ISBN 978-5-98092-021-0.
- Vergne, A. L., Pritz, M. B. and Mathevon, N.** (2009). Acoustic communication in crocodylians: from behaviour to brain. *Biol. Rev.* **84**, 391-411. doi:10.1111/j.1469-185X.2009.00079.x
- von Humboldt, A.** (1811). *Mémoire sur l'os hyoïde et le larynx des oiseaux, des singes et du crocodile*. Paris: F. Schoell.
- Waskom, M.** (2021). Seaborn: statistical data visualization. *J. Open Source Softw.* **6**, 3021. doi:10.21105/joss.03021
- Yu, X., Peng, Y., Aowphol, A., Ding, L., Brauth, S. E. and Tang, Y.-Z.** (2011). Geographic variation in the advertisement calls of *Gekko gecko* in relation to variations in morphological features: implications for regional population differentiation. *Ethol. Ecol. Evol.* **23**, 211-228. doi:10.1080/03949370.2011.566581
- Zornik, E. and Kelley, D. B.** (2008). Regulation of respiratory and vocal motor pools in the isolated brain of *Xenopus laevis*. *J. Neurosci.* **28**, 612-621. doi:10.1523/JNEUROSCI.4754-07.2008