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RESEARCH ARTICLE

The role of hyoid muscles in biotremor production in *Chamaeleo calyptratus*

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ABSTRACT

The production of biotremors has been described in veiled chameleons (*Chamaeleo calyptratus*), but the mechanism by which they are produced is unknown. We gathered muscle activation data via electromyography (EMG), with simultaneous recordings of biotremors using an accelerometer, to test for the role of hyoid muscles in biotremor production. We recorded a mean biotremor frequency of 150.87 Hz for females and 136.01 Hz for males. The durations of activity and the latencies to onset and offset for the M. sternohyoideus profundus (SP), M. sternohyoideus superficialis (SS), Mm. mandibulohyoideus (MH) and M. levator scapulae (LS) were all significantly correlated with biotremor durations and biotremor onset and offset, respectively. Linear mixed-effect regression model comparisons of biotremor duration indicated that models containing either the MH and/or the SP and LS account for the most variation in biotremor duration. Twitch times for the SP (100 ms) and the SS (132 ms) at field active body temperature, however, were individually too slow to produce the biotremors at the observed frequency without alteration after production by other anatomical structures. These results implicate the SP, SS, MH and LS in the production of biotremors, but the exact mechanism of production requires further study.

KEY WORDS: Biotremor, Chameleons, Communication, Vibration, Muscle physiology, Biotremology

INTRODUCTION

Biotremors are Rayleigh surface waves that occur at the boundary between two distinct media (e.g. air and solid), where particles are oscillated both perpendicular and parallel to the direction of the wave's propagation (Hill and Wessel, 2016). Biotremors are transmitted through solid substrates, such as soil or plant matter, more efficiently than through air because less attenuation occurs through denser media (Hill and Wessel, 2016). Vibrational signaling, through the production of biotremors, has been observed in numerous taxa throughout the animal kingdom (Senter, 2008; Hill and Wessel, 2016). Insects (Cokl and Virant-Doberlet, 2003; Cocroft and Rodriguez, 2005), arachnids (Barth, 2002), elephants (O'Connell-Rodwell et al., 2000, 2001; O'Connell-Rodwell, 2007) and some frogs (Lewis et al., 2001; Narins et al., 2018) utilize biotremors to communicate. There is also evidence that species of chameleons can produce biotremors in various contexts (Brygoo, 1971; Hillenius, 1986; Raxworthy, 1991; Tilbury, 1992; Barnett et al., 1999; Lutzmann, 2004); however, less is understood about the mechanism by which chameleons produce biotremors and the contexts in which they are utilized (Barnett et al., 1999). The aim of the present study was to identify the muscles that produce these biotremors.

According to Tornier (1905), and further supported by Huskey et al. (2020), chameleons lack the functional vocal cords that are thought to be needed to facilitate biotremor production. Barnett et al. (1999) recorded vibrations emanating from the throat region, and all authors observed a head click during biotremor production (personal observations), suggesting that muscles situated in the throat and neck are likely the source of the biotremors. Huskey et al. (2020) further observed that the M. sternohyoideus superficialis (SS) and M. sternohyoideus profundus (SP) are secured by connective tissue directly to the surface of the gular pouch, an expansive modification of the trachea that is present in at least 21 species of chameleons (Huskey et al., 2020). The Mm. mandibulohyoideus (MH) also contact the rostral surface of the inflated gular pouch. It was hypothesized by Huskey et al. (2020) that the gular pouch serves as an amplifier of the vibrations produced by the tongue and hyoid muscles.

As reviewed by Anderson and Higham (2014), the MH originate adjacent to the symphysis of the lower jaw and insert on the basihyoid and distal portions of the ceratohyal and ceratobranchial, depending on the division (represented in Fig. 1). The MH pull the hyobranchial apparatus anteriorly during tongue protrusion and protraction. The SS originates on the posteroventral surface of the xiphisternum and inserts on the ventral side of the basihyoid. It draws the basihyoid posteriorly during hyobranchial retraction. The SP originates on the midbody connective-tissue band anterior to the xiphisternum and inserts onto the ceratobranchial. The SP pulls the distal end of the ceratobranchial in a posteroventral direction during tongue protrusion. The M. levator scapulae (LS), thought to be responsible for the observed head click (personal observations), originates from the transverse processes of the first cervical vertebra. The LS insertion is marginal and dorsal to the acromial region. There is an additional LS origin from the basioccipital condyle of the skull, which is shared with the cervical axial muscles. The LS also allows scapular rotation in the parasagittal plane.

We hypothesized that the timing (duration and time of peak activity) of the SS, SP, MH and LS would directly correlate with the timing of biotremor production, whereas that of the Mm. triceps (TR; control) would not. We also hypothesized that the physiological characteristics of the SS, SP, MH and LS would allow the muscles to produce a biotremor of the observed frequencies. Muscles exhibiting such characteristics may be implicated in biotremor production. For a muscle exhibiting activation timing that correlates with biotremor production to produce a vibratory signal on its own, however, the muscle must cyclically activate and deactivate at a similar frequency to the vibration. In other words, the muscle's twitch time, the time required for the muscle to generate and dissipate force following a single stimulation, must equal the duration of a single vibratory cycle.

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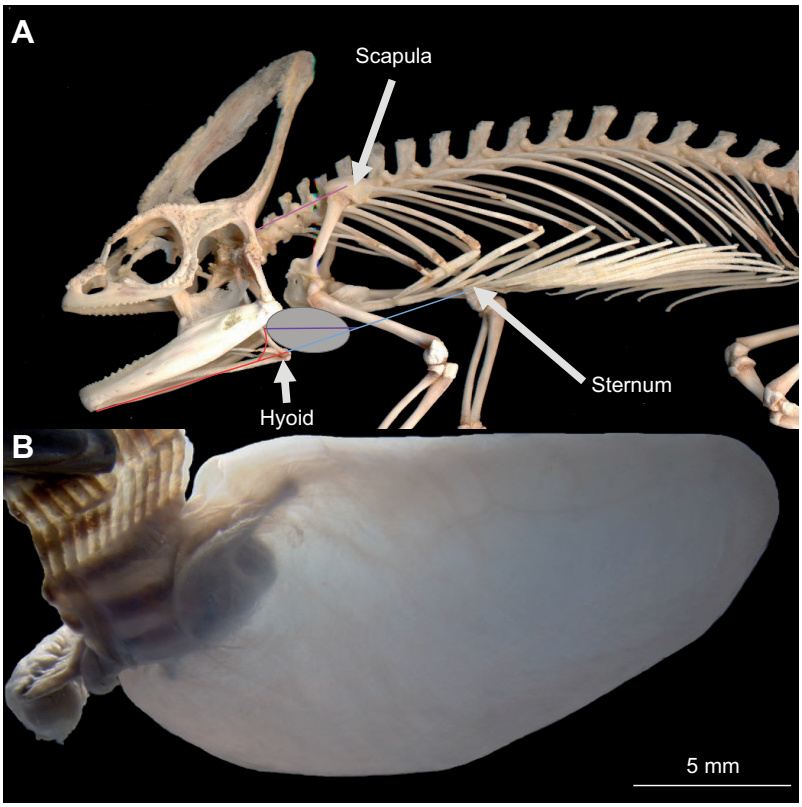

Fig. 1. Muscle locations relative to the gular pouch in *Chamaeleo calyptratus* and a representative image of a gular pouch. (A) Representation of the M. levator scapulae (LS; pink), Mm. mandibulohyoideus (MH; red), M. sternohyoideus profundus (SP; purple), M. sternohyoideus superficialis (SS; blue) and gular pouch (gray) locations on an image of a skeletonized *C. calyptratus*. (B) Image (modified from Huskey et al., 2020) of an inflated gular pouch of a male *C. calyptratus*.

However, the muscle twitch time and sound production frequency do not need to have a 1:1 relationship in organisms in which a resonator or other anatomical structure is modulating the signal. For example, the melon of whales (Blomberg, 1976; Kloepper et al., 2011) and the swim bladder and swim bladder plate of some fish (Fine and Parmentier, 2015) modulate the signals produced by these animals.

To explore the hypothesis that the SS, SP, MH and LS produce the biotremors observed in the veiled chameleon, *Chamaeleo calyptratus*, we examined the timing of electrical activity and contractile physiology of muscles associated with the neck and hyoid during biotremor production. We employed electromyography (EMG), accelerometry and *in vitro* muscle contractile physiology experiments to: (1) establish whether these muscles were associated with biotremor production, (2) correlate the electrical activity of the muscles with the biotremor, (3) determine the order of muscle activity during biotremor production, (4) elucidate which muscles play an important role in the production of biotremors, and (5) test the contractile properties of the selected muscles (SP and SS) for the capacity necessary for biotremor production without the influence of other muscles or anatomical structures.

MATERIALS AND METHODS

Specimens

Veiled chameleons (*Chamaeleo calyptratus* Duméril and Bibron 1851) were used in this experiment as Barnett et al. (1999) demonstrated that contact-elicited biotremors are easily provoked from this species. We acquired six adult *C. calyptratus* specimens (three male and three female) from retail distributors and housed them individually in large, ventilated, glass terrariums with live plants. Ambient temperatures were maintained at 20–23°C with incandescent basking lamps and fluorescent ultraviolet (UV-B) light sources on a 12 h:12 h day:night cycle. Chameleons were fed a diet of gut-loaded crickets and watered three times per day using a misting system (MistKing, Carnation, WA, USA). All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of South Dakota (AUP 17-12) and Western Kentucky University (#15-07). The care and use of the experimental animals complied with all relevant local animal welfare laws, guidelines, and policies.

Electrode construction and surgery

Bipolar hook electrodes were constructed with formvar-insulated, nichrome wire (0.0020 in bare and 0.0026 in coated; A-M Systems, Carlsborg, WA, USA). Electrodes consisted of two 125 cm wire strands glued at their terminal ends with veterinary-grade cyanoacrylate. The wires were then threaded through a 27-gauge hypodermic needle. One millimeter of insulation was removed from the glued tips, and the wires bent away from each other in an arrowhead shape, according to Anderson and Deban (2012). The constructed electrodes were autoclaved prior to surgery.

Chameleons were anesthetized in an induction chamber with 2.5–5% isoflurane/oxygen mix with a flow rate of 1 l min^{-1}. The chameleon was then positioned with a nosecone receiving the same concentration of isoflurane throughout the surgical procedure. Chameleons were positioned on their left side on a stage under a dissecting microscope, and electrodes were implanted via a hypodermic needle into the muscle, either through a small incision in the skin (hyoid and shoulder muscles) or through the skin (ground and control electrodes). Electrodes were implanted into the SS, SP, MH, LS and TR (Fig. 2). An electrode was also inserted under the skin on the lateral surface of the body (Fig. 2) as a reference/ground baseline for measurement of electrical activity within the body. Surgical incisions were sutured closed, and veterinary-grade cyanoacrylate was applied to the implantation sites to help secure the electrodes in place.

The electrode wires were then stuck together using rubber cement, ~5 cm from their implantation site along the remaining length of the

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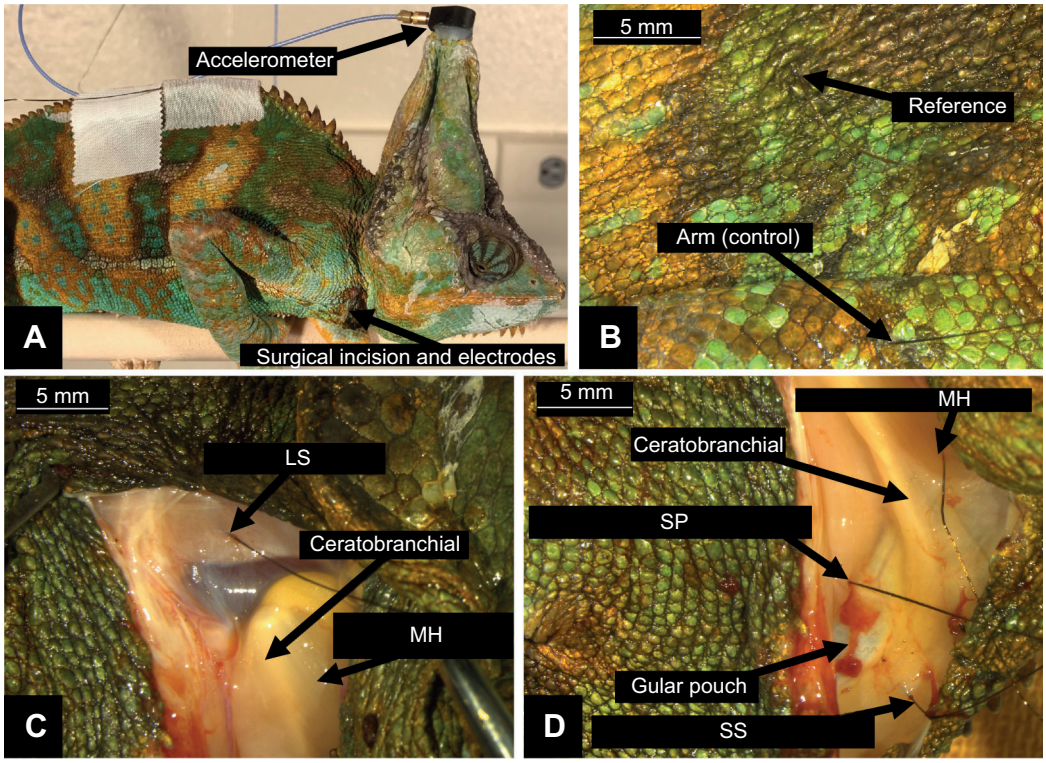

Fig. 2. Implantation of electrodes and placement of the accelerometer in experimental *C. calyptratus*. (A) The incision and electrode implantation site, and the location of the accelerometer during trials. (B) The location of the reference and control electrodes. (C) The internal location of the electrode inserted into the M. levator scapulae (LS), and its proximity to the ceratobranchial and Mm. mandibulohyoideus (MH). (D) The electrodes inserted into the MH, M. sternohyoideus profundus (SP) and M. sternohyoideus superficialis (SS), and their location relative to the gular pouch and ceratobranchial.

nichrome wire. As the individuals recovered from anesthesia, 1 mm of insulation was removed from the opposite end of the electrodes and soldered to a plug (Anderson and Deban, 2012). This plug was attached to a differential amplifier (3500; A-M Systems) amplifying the signal 1000 times and filtered to remove 60 Hz line noise. The conditioned signals were sampled at 4 kHz with a PowerLab 16/35 analog-to-digital converter (ADInstruments, Dunedin, New Zealand) to record EMG data in LabChart (V8.1.6; ADInstruments).

Trials

Chameleons were placed on a 12.7 mm-diameter wooden dowel after surgical recovery was complete, and an accelerometer (352A24; PCB Piezotronics, Depew, NY, USA) was attached to their casque with beeswax (Fig. 2) and connected to the same data acquisition system to which the EMG electrodes were attached for synchronization. Biotremors were elicited by gently and briefly poking the elbow with a pointed probe as a form of tactile stimulation. The contralateral forelimb that was not implanted with the control electrode was poked to avoid any accidental stimulation of the implanted electrode in the ipsilateral TR. Over each 43 s recording period, chameleons would be gently poked every few seconds, depending on the activity level of the individual, in order to elicit biotremor responses. These recording processes were then repeated multiple times per individual to a maximum of 59 biotremor recordings or until the chameleon would no longer remain perched on the dowel. Chameleons were then euthanized after trials were completed to surgically verify the position of electrode implantation, as some electrodes were displaced during or between trials.

Statistical methods

The accelerometer and EMG data were analyzed for correlation, latency to onset and offset (the time between muscle activation or cessation and biotremor production and termination), and effects of individual muscles or interactions between muscles on the duration of the biotremor. The duration of the biotremor and muscle electrical activity were measured in seconds. The start of the biotremor and electrical activity were determined to be when the accelerometer and EMG recordings were 25% greater than the previous 0.25 s of baseline activity. The biotremor and muscle activity were considered finished when the recordings returned to the baseline of the 0.25 s prior to the onset of accelerometer and EMG activity. The time of peak activity (s) was also recorded and was provided by LabChart.

A fast Fourier transformation (FFT) in LabChart was utilized to examine the fundamental frequencies of a subset of 75 biotremor recordings. We digitized biotremor signals via LabChart at 44.1 kHz and exported them as .wav files. These files were imported into Raven Pro (V1.5.23; Cornell Lab of Ornithology, Ithaca, NY, USA), and representative biotremors were visualized and analyzed via oscillograms, spectrograms and power spectra. Temporal features were visualized from oscillograms, and frequency parameters were obtained by power spectra (3 dB filter bandwidth, 5.62 Hz; FFT size, 256 points; time overlap, 50%; Hanning window).

The correlation analyses were performed in R (http://www.R-project.org/) using linear regressions. The duration (s) and time of peak activity (s) of the biotremors and muscles were regressed to determine which muscles were most tightly correlated with biotremor production. The latencies to onset and offset of the muscles were calculated using the mean time of activation and cessation in relation to the biotremors. For latency to onset, negative numbers indicate activity before the biotremor activation, and, conversely, for latency to offset, negative numbers indicate activity after biotremor cessation.

Mixed-effect linear modeling was performed using the lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), and car

Journal of Experimental Biology (2020) 223, jeb227603. doi:10.1242/jeb.227603

(Fox and Weisberg, 2011) packages in R (http://www.R-project.org/). Within these mixed-effect linear models, biotremor duration (s) was regressed against the duration of muscular activity (s) of the LS, SS, SP and MH. The LS, SS, SP and MH durations were included as fixed-effect parameters in the models. Individual (IND) was included as a random-effect parameter. The models used for analyses were the full model and Models I–XIV (see Table 5). All models consisted of a combination of the LS, MH, SP, SS and IND as model parameters. Akaike's information criterion (AIC; Sakamoto et al., 1986), corrected AIC (AICc; https://cran.r-project.org/web/packages/AICcmodavg/index.html) and Bayesian information criterion (BIC; Sakamoto et al., 1986) yielded the same results for model selection. Therefore, we utilized only BIC for our analyses and further description. The full model was used as the baseline to understand how removing parameters from the model affected its fit. Parameters were then removed from the full model in a stepwise manner to determine which muscles or combination of muscles most influenced the variation in biotremor duration. An ANOVA was then performed on the two-, three- and four-parameter models with the lowest BIC values and the three models with the lowest BIC values overall. An alpha value of 0.05 was used for all statistical analyses.

Muscle physiology

We performed *in vitro* muscle contractile experiments on four male *C. calyptratus* on a single hyoid muscle. A leg muscle from each individual was used to compare the twitch times of a muscle associated with biotremor production with the twitch times of a locomotor muscle. For these experiments, we used the SP (two individuals), the SS (two individuals) and the M. iliotibialis (all four individuals). Immediately prior to *in vitro* muscle experiments, *C. calyptratus* were euthanized by isoflurane overdose followed by decapitation and double pithing. For each muscle, its origin was secured to the rigid base of an experimental chamber and its insertion was attached to a servomotor (305C dual mode; Aurora Scientific, Aurora, ON, Canada) above via a single silver chain (Anderson and Roberts, 2020).

For all experiments, reptilian Ringer's solution (Marsh, 1988) saturated with 100% oxygen was recirculated from a container suspended in a temperature-controlled water bath. For three individuals, temperatures were set to maintain the muscle chamber at 31.7°C, the average selected body temperature for *Chamaeleo calyptratus* across two studies (30.4°C and 32.9°C; Zari, 1993; Andrews, 2008). Data from the fourth individual was collected at 25°C owing to a setup error. Muscles were allowed to equilibrate to the experimental temperature and setup for 30 min prior to beginning the *in vitro* muscle contractile experiments.

Muscles were then supramaximally stimulated (0.2 ms square wave pulses) using platinum electrode bars (805A; Aurora Scientific) attached to a stimulator (Grass S48; Grass Medical Instruments, Quincy, MA, USA) and amplifier (Crown DC-300A II; Crown International, Elkhart, ID, USA). Muscle force and length were sampled from the servomotor at 10 kHz (NI USB-6361; National Instruments, Austin, TX, USA; Igor Pro 7, Wavemetrics, Lake Oswego, OR, USA).

A length–tension curve for twitch contractions was constructed for each muscle. To do so, muscle length was initially set to a short length and twitch contractions were performed at incrementally increasing lengths. Passive force was measured as the tension recorded prior to the onset of muscle stimulation, and maximal force was measured as the maximum tension following stimulation. Active force at each muscle length was calculated as the difference between passive and maximal force within a contraction. The length corresponding to maximal active force produced was defined as the optimal length for twitch contractions ($L_{0,\ Twitch}$). Twitch kinetics were measured from two twitch stimulations ~350 ms apart with the muscle held at $L_{0,\ Twitch}$. Twitch times were measured as the duration from the onset of each stimulus to the time of 50% relaxation of each twitch. The shortest duration for each muscle was then used as the twitch time for that muscle in each individual.

RESULTS

Correlation of muscular electrical activity and biotremor activity

A total of 186 biotremors with corresponding EMG data were recorded from the six individuals (see Table 1 for individual breakdowns). The number of EMG recordings, and thus the degrees of freedom (d.f.), differed among muscles owing to the removal of electrodes by some individuals. Prior to the completion of individual trials, the EMG electrodes implanted into the SS became dislodged in two individuals, while the electrodes implanted into the SP became dislodged in three individuals. All other electrode implantations (n=25) remained intact throughout the duration of data collection. A mean biotremor peak frequency of 150.87 Hz was produced by the *C. calyptratus* females, and the males generated a mean of 136.01 Hz (Table 1). The signals were tonal with a sigmoidal waveform, with most of the energy at the fundamental frequency (ranging from ~120 Hz to 160 Hz; Table 1; Fig. 3). First and second harmonics were also evident, with frequency peaks of consecutively declining amplitudes (Fig. 3). We found significant correlations between the EMG and biotremor durations for the SP (r^2=0.9644; P≤0.001; s.e.=0259; Fig. 4A,B; Table 2), SS (r^2=0.8245; P≤0.001; s.e.=0.0545; Fig. 4A,B; Table 2), MH (r^2=0.6785; P≤0.001; s.e.=0.0583; Fig. 4A,B; Table 2) and LS (r^2=0.5109; P≤0.001; s.e.=0.0889; Fig. 4A,B; Table 2). The TR was not correlated to biotremor duration (r^2=0.0022; P=0.24; s.e.=0.0088; Fig. 4A; Table 2). Owing to the lack of correlation, the subsequent data from the TR were omitted. The times of peak activity for all muscles (excluding the TR) were strongly correlated with the times of peak biotremor activity (Table 3; Fig. 4C).

Mechanistic description

For latency to onset, negative numbers indicate activity before biotremor activation, and, conversely, for latency to offset, negative numbers indicate activity after biotremor cessation. The mean latency to onset and offset were calculated as follows. The SS onset was at 0.016 s, and the offset was at 0.075 s. The SP onset occurred at 0.014 s and offset was at −0.137 s. The MH onset was at −0.040 s, and its offset was at −0.011 s. LS onset occurred at −0.196 s and offset at −0.045 s. The TR onset was at 0.021 s, and its offset was at −0.682 s. The TR was removed from all further

Table 1. Experimental data on biotremors in *Chamaeleo calyptratus*

		Frequency (Hz)			
Individual	*N*	Mean±s.e.m.	Range	Minimum	Maximum
Female 1	30	149.05±1.59	35.95	133.06	169.01
Female 2	38	158.44±13.63	474.91	80.65	555.56
Female 3	5	145.12±3.10	14.93	140.00	154.93
Female mean	3	150.87±3.95	13.32	145.12	158.44
Male 1	33	146.87±1.66	36.53	128.42	164.95
Male 2	59	122.69±3.41	188.24	97.47	285.71
Male 3	21	138.48±5.20	59.86	112.36	172.22
Male mean	3	136.01±7.09	24.18	122.69	146.87

Data on the biotremors produced by all individuals and the mean biotremor peak frequencies of males and females.

Journal of Experimental Biology (2020) 223, jeb227603. doi:10.1242/jeb.227603

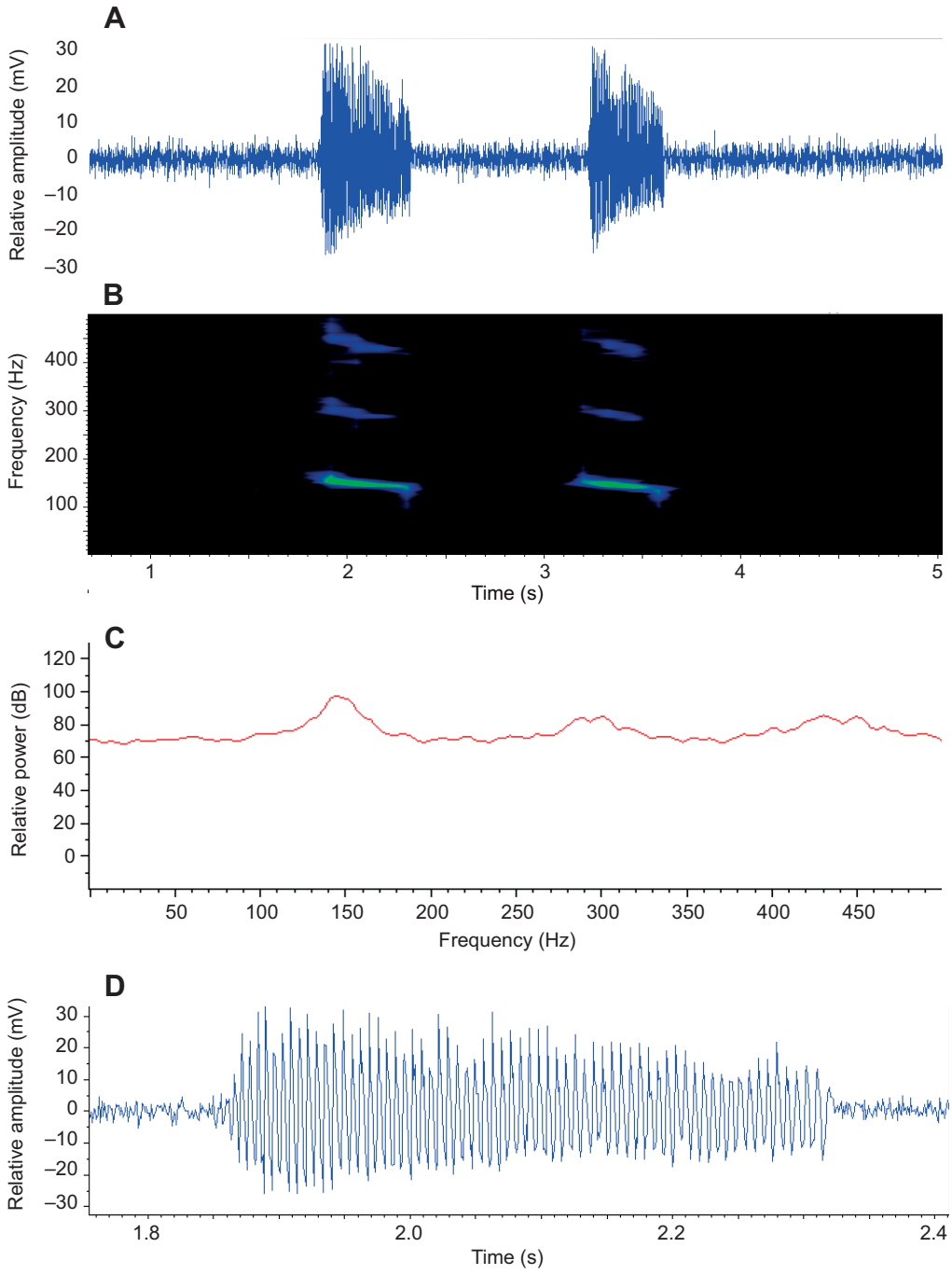

Fig. 3. Bioacoustics analysis of two representative biotremors in *C. calyptratus*. (A–C) Oscillogram (A), spectrogram (B) and power spectrum (C) of two vibratory signals of one of the experimental *C. calyptratus*. (D) Oscillogram of the first signal, enlarged to show the waveform of the vibration.

latency analyses because it was only activated when the chameleons moved their legs during a trial and was not correlated with the biotremors.

The calculated latency to onset and offset depicts the mechanistic interactions of the muscles before, during and after the biotremor (Table 4). During all biotremors, the LS and MH were activated just prior to the biotremor onset, and the SP and SS were activated immediately after the biotremor onset. The SS then ceased activity just prior to vibration cessation, and the SP, MH and LS ceased activity immediately after the vibration ended. There was no variation in the order of these events during the 186 recorded trials.

Linear mixed-effect regression model comparisons (Table 5) for biotremor duration indicate that Model IX (MH, LS and IND) best explains the observed variation in biotremor duration (Table 5). When compared within groups composed of models with the same number of parameters, Model III (SP, MH, LS and IND) is the best four-parameter model, Model IX (MH, LS and IND) is the best three-parameter model and Model XII (MH and IND) is the best two-parameter model. ANOVA of Model III and Model IX reveals that the SP explains the most variation in the duration of the biotremor (P<0.001). ANOVA of Model VI and Model XIII shows that the MH and LS significantly contribute to the variation in biotremor (P<0.001).

Journal of Experimental Biology (2020) 223, jeb227603. doi:10.1242/jeb.227603

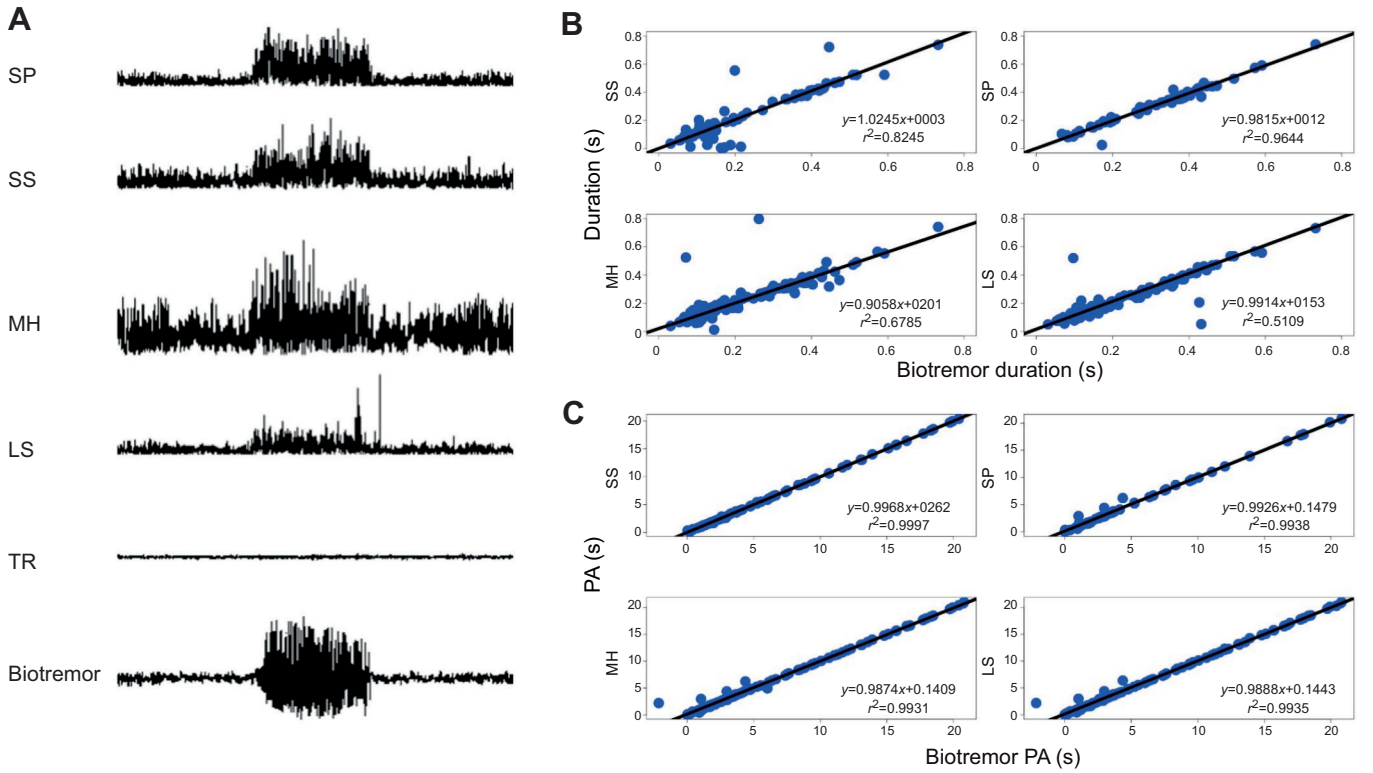

Fig. 4. Visualization of the timing and relationship of the biotremor and muscular activity in *C. calyptratus*. (A) Simultaneous rectified electromyographic recordings of the M. sternohyoideus profundus (SP), M. sternohyoideus superficialis (SS), Mm. mandibulohyoideus (MH), M. levator scapulae (LS) and Mm. triceps (TR), and accelerometer recording of the vibrations recorded during biotremor production in *C. calyptratus*. (B,C) Linear regressions of the duration (B) and time of peak activity (PA) (C) of SS, SP, MH and LS electrical activity, and the duration (B) and time of PA (C) of the biotremor. The blue circles indicate the duration of a single recorded biotremor and the corresponding duration of the muscular electrical activity. The black line is the line of best fit.

The twitch times recorded from the SP were 100 ms at average selected body temperature and 198 ms at 25°C, and were between 132–143 ms for the SS at average selected body temperature. Twitch times for the M. iliotibialis were between 67 ms and 69 ms at average selected body temperature and 104 ms at 25°C.

DISCUSSION

Chameleon researchers have observed numerous species of chameleons producing biotremors (e.g. Raxworthy, 1991; Barnett et al., 1999; Lutzmann, 2004). Our results confirm that *C. calyptratus* is also a species capable of producing biotremors as observed by Barnett et al. (1999). These results also support the hypothesis that the SS, SP, MH and LS play a role in the production of biotremors by *C. calyptratus* (Huskey et al., 2020).

Linear regression results confirm that there is a correlation between the duration and time of peak activity of the SS, SP, MH and LS and the biotremor (Fig. 3). The latency calculations further corroborate the muscle activity–biotremor correlation and illustrate the relationship over time (Table 4). The latency calculations allow for a more granular exploration of the muscle activity during the biotremor than the linear regressions. The MH activity is, on average, more closely associated with the biotremor duration (Table 4), despite the significant positive relationship with biotremor and SS and SP durations in the linear regressions (Table 2). The SP is also closely associated with the biotremor (Table 4), although there is a closer positive relationship between the SS and the biotremor (Table 3). Based on these results, it is clear that there is a significant contribution of the MH to the production of the biotremor, with contributions from both the SP and SS. The results of our latency calculations are further confirmed by the linear mixed-effects model comparisons. The optimal four (Model III; BIC=−205.92), three (Model IX; BIC=−245.31) and two (Model XIII; BIC=−236.33)-parameter

Table 2. Statistics from linear regression of biotremor and muscular activity duration in *C. calyptratus*

Muscle	r^2	s.e.	t	F	d.f.	P
SS	0.8245	0.0545	18.80	353.4	74	<0.001
SP	0.9644	0.0259	37.89	1436	52	<0.001
MH	0.6785	0.0583	15.54	241.6	113	<0.001
LS	0.5109	0.0889	11.15	124.3	117	<0.001
TR	0.0020	0.0088	1.19	1.4	186	0.24

Statistics for the linear regression of the duration of the biotremor and duration of the electrical activity of the M. sternohyoideus superficialis (SS), M. sternohyoideus profundus (SP), Mm. mandibulohyoideus (MH), M. levator scapulae (LS) and Mm. triceps (TR).

Table 3. Statistics from linear regression of the timing of biotremor and muscular peak activity in *C. calyptratus*

Muscle	r^2	s.e.	t	F	d.f.	P
SS	0.9997	0.0019	538.44	289,900	74	<0.001
SP	0.9938	0.0107	92.40	8,537	52	<0.001
MH	0.9931	0.0077	128.30	16,460	113	<0.001
LS	0.9935	0.0074	134.09	17,980	117	<0.001

Statistics for the linear regression of the time of peak activity of the biotremor and time of peak electrical activity of the M. sternohyoideus superficialis (SS), M. sternohyoideus profundus (SP), Mm. mandibulohyoideus (MH) and M. levator scapulae (LS).

Journal of Experimental Biology (2020) 223, jeb227603. doi:10.1242/jeb.227603

Table 4. Average timing of muscular activity during biotremor production in *C. calyptratus*

	−0.19 s	−0.04 s	0.00 s	0.01 s	0.02 s	0.05 s	0.08 s	0.09 s	0.13 s	0.22 s
SS	–	–	–	–	X	X	–	–	–	–
SP	–	–	–	X	X	X	X	X	X	–
MH	–	X	X	X	X	X	X	–	–	–
LS	X	X	X	X	X	X	X	X	–	–
BA	–	–	X	X	X	X	X	–	–	–
PBA	–	–	–	–	–	X	–	–	–	–

The time of M. sternohyoideus superficialis (SS), M. sternohyoideus profundus (SP), Mm. mandibulohyoideus (MH) and M. levator scapulae (LS) activation and cessation in relation to the biotremor activation (BA), peak biotremor activity (PBA) and biotremor cessation. Active muscles, PBA and BA are all indicated by 'X'; no activity is indicated by '–'. Time 0.00 s is the start of the biotremor.

models contain the MH, and, of the top three models overall, one contains the MH and SP (Model VI). Despite strong evidence from the linear regressions, latency calculations and linear model comparisons, the muscle physiology experiments determined that the SP and SS are physiologically incapable of producing biotremors on their own. Although these muscles are unable to produce the biotremors at the observed frequencies on their own, it is possible that anatomical structures (e.g. the gular pouch) can filter or amplify the vibrations produced by these muscles.

For a muscle to produce a biotremor at a frequency of 150 Hz on its own, a single cycle of activation and deactivation must occur in 6.67 ms (1 s/150=0.006666 s). This means that a muscle predicted to produce a biotremor on its own must have a twitch time of 6.7–7.7 ms to generate vibrations between 130 Hz and 150 Hz, which we observed in *C. calyptratus*. The twitch times of the SP (100 ms at average selected body temperature) and SS (130 ms at average selected body temperature) are not individually fast enough to generate vibrations at the recorded frequencies. It is possible that these twitch times do not have a 1:1 relationship because other anatomical features are filtering the biotremor fundamental frequencies (Blomberg, 1976; Kloepper et al., 2011; Fine and Parmentier, 2015). This might occur as the biotremor is passing through other tissues. It is also possible that the inflated gular pouch of *C. calyptratus* acts as a resonator (Huskey et al., 2020), which could increase the dominant frequency of the call. The size-related shift in frequency, which is also dependent upon the sex, suggests that the resonance of the gular pouch is involved. For example, Female 2 has a mass of 147.5 g and a mean frequency of 158.44 Hz, while Male 2 has a mass of 256 g and a mean frequency of 122.69 Hz. If the frequencies were consistent across sizes, that would suggest a muscle-related phenomenon (Skoglund, 1961; Waybright et al., 1990; Fine et al., 2001). Because the frequencies are size and sex dependent, this suggests that the gular pouch is possibly amplifying the vibrations produced by the MH and SP, much like the swim bladder of some fish amplifies the sonic muscles' vibrations (Weston, 1967; Fine et al., 1990; Fine and Parmentier, 2015).

Table 5. Comparisons of linear mixed-effect regression models explaining biotremor duration in *C. calyptratus*

Model	Parameters	d.f.	BIC
Full	SS+SP+MH+LS+IND	7	−107.67
I	SS+MH+LS+IND	6	−194.14
II	SS+SP+LS+IND	6	−106.98
III	SP+MH+LS+IND	6	−205.92
IV	SS+SP+MH+IND	6	−129.03
V	SP+SS+IND	5	−127.19
VI	SP+MH+IND	5	−227.40
VII	SP+LS+IND	5	−214.47
VIII	MH+SS+IND	5	−213.04
IX	MH+LS+IND	5	−245.31
X	SS+LS+IND	5	−179.35
XI	SP+IND	4	−194.71
XII	SS+IND	4	−194.71
XIII	MH+IND	4	−236.33
XIV	LS+IND	4	−204.08

The M. sternohyoideus superficialis (SS), M. sternohyoideus profundus (SP), Mm. mandibulohyoideus (MH) and M. levator scapulae (LS) are the fixed-effect model parameters. Individual (IND) is included as a random-effect parameter. BIC, Bayesian information criterion.

Taken together, these data suggest that the SS, SP, MH and LS play a role in the production of biotremors in *C. calyptratus*, but the specific source of the biotremor cannot yet be confirmed as modification of the signal by other structures appears likely. However, given our results, a plausible mechanism by which the biotremors are produced can be suggested. In particular, we suggest that the LS is active to stabilize the head, while the MH and SS contract simultaneously to hold the hyoid in place. The MH, SP or both rapidly contract to produce a vibration. This vibration is then amplified by the gular pouch and/or modified by some other tissues.

Although our results cannot confirm the aforementioned mechanism at this time, we can deduce robust hypotheses about how these muscles may interact to generate the biotremor. For example, it is unlikely that the LS is the source of the biotremor because it is not tightly correlated with the onset and offset or time of peak activity of the biotremor. However, the LS might play a supporting role by stabilizing the head of the chameleon during biotremor production. The SS and MH may act antagonistically during the production of the biotremor as they are both attached to the hyoid and their lines of action are in opposite directions (Fig. 1). This would hold the hyoid in place, allowing either the MH, SP or both muscles to produce the biotremor. This would not be the first example of muscles playing a supporting role while another muscle, or suite of muscles, produces a sound or vibrations. In some species of Ophidiiformes, a combination of dorsal and ventral muscles is necessary to produce the observed sounds (Parmentier and Fine, 2016). Prolonged contraction of the dorsal muscles puts tension on the swim bladder, while the ventral muscles rapidly contract and relax to produce multiple pulses of sound. In fact, the vibrations produced by the muscles last longer than the short muscle contractions (Parmentier and Fine, 2016).

The SS, SP, MH, M. accelerator linguae and M. hyoglossus are in direct contact with, or in close proximity to, the inflated gular pouch and lie in the throat region of chameleons (Huskey et al., 2020). It is also possible that the combined action of these muscles produces the biotremor at the observed frequencies. This would be similar to the calls produced by sea robins, where their two intrinsic muscles contract alternatively to double the fundamental frequency of their calls (Connaughton, 2004).

To definitively say that a specific muscle is producing biotremors, all other hyoid and throat muscles must be ruled out. For example, the MH has emerged as a leading candidate muscle, given its importance in our latency calculations and model comparisons. Further

Journal of Experimental Biology (2020) 223, jeb227603. doi:10.1242/jeb.227603

physiological examination is necessary to determine whether this muscle is capable of producing the biotremor on its own. It would also be advantageous to determine whether the M. accelerator linguae and M. hyoglossus are also possible generators of the biotremors. The M. accelerator linguae surrounds the entoglossal process and is responsible for producing the energy underlying the ballistic action of the tongue, and the M. hyoglossus retracts the tongue after prey capture (Anderson and Higham, 2014). The M. omohyoideus is also a candidate muscle because of its location in relation to other muscles we have identified and its close proximity to the source of the biotremor. The M. omohyoideus originates on the anterior, ventral scapula, attaches to the basihyoid (Anderson and Higham, 2014), continues to dorsally wrap around the SS and SP, and ventrally curves under the M. episternocleidomastoideus toward the scapula (Anderson and Higham, 2014). If it is not directly involved in biotremor production, it could also be another stabilizing muscle in this mechanism as it is known to pull the basihyoid upward (Anderson and Higham, 2014). It has also been suggested that the intercostal muscles may be involved in biotremor production (Raxworthy, 1991); however, observations that the biotremor is localized in the throat area, near the gular pouch, would preclude intercostal muscles from consideration.

Based on the ability of *C. calyptratus* to produce biotremors and the behavioral observations of Barnett et al. (1999), we also hypothesize that these biotremors are used by chameleons to communicate. Observations of live *C. calyptratus*, *Chamaeleo dilepis* and *Chamaeleo gracilis* interactions, both intraspecifically and interspecifically, revealed that these gular pouch-possessing species generate biotremors in numerous contexts (Barnett et al., 1999; Laslie, 2018; Kappel, 2020). We hypothesize that these vibrations are produced by tongue and hyoid muscles and then amplified by an inflated gular pouch (Huskey et al., 2020), and that the legs act as a conduit through which the vibration can travel to the branches of vegetation or earth for communication. The ballistic tongue of chameleons, with its associated muscles for projection and retraction, has been greatly studied (summarized in Higham and Anderson, 2014). Although these muscles evolved to optimize tongue projection for feeding, this does not preclude their being co-opted for communicative vibration production. In fact, it is probably the rule, not the exception, that sound- and vibration-producing muscles and other structures are exaptations (i.e. they originally evolved to serve non-communicative functions; Gould and Vrba, 1982). For example, in fishes, existing anatomical structures that were likely used in non-voluntary sound production before being selected for optimization for communication include the swim bladder, teeth, pharyngeal jaws, stretched ligaments and pectoral structures (Parmentier et al., 2017).

Some chameleon species do not possess gular pouches (Klaver, 1973, 1977, 1979, 1981; Klaver and Böhme, 1986; Huskey et al., 2020). Because the biotremors themselves are likely generated by muscles, the absence of a gular pouch would not preclude the generation of biotremors in these taxa. However, owing to the lack of amplification by the gular pouch, the biotremors might not be transmitted significant distances. The production of a biotremor without amplification by a gular pouch may be an anti-predator adaptation (Necas and Schmidt, 2004). For example, a vibration may startle a predator enough to drop the chameleon and has even been hypothesized to shake off ants in ground-dwelling chameleons (Necas and Schmidt, 2004). The evolution of a specialized biotremor-producing mechanism in all species, and a sophisticated gular pouch in multiple genera of chameleons, could have enhanced their anti-predation and/or communication capabilities.

Air-filled cavities, like the chameleon gular pouch, are associated with acoustic communication across all vertebrate taxa [mammals, e.g. siamang (*Symphalangus syndactylus*; Hill and Booth, 1957); birds, e.g. sage grouse (Dantzker, 2015); amphibians, e.g. túngara frog (Ryan, 1985); fish, e.g. red drum (*Sciaenops oscellatus*; Holt, 2002); reptiles, e.g. alligator (*Alligator mississippiensis*; Riede et al., 2015)]. The general term for such cavities is vocal sacs, which can be extensions of the esophagus, trachea or buccal cavity (Dantzker and Bradbury, 2006).

Although most birds utilize open-beaked vocalization, species with inflatable vocal sacs exhibit closed-beak vocalizations, and include species such as the greater prairie chicken (*Tympanuchus cupido*; Schwartz, 1945), the greater sage-grouse (*Centrocercus urophasianus*; Dantzker et al., 1999; Danztker, 2015), American bittern (*Botaurus lentiginosus*; Chapin, 1922), bustards (*Otididae*; Collar, 1996; Lichtenberg and Hallager, 2007), kakapo (*Strigops habroptilus*; Cockrem, 2002), ring dove (*Streptopelia risoria*; Riede et al., 2004), ostriches (*Struthio* sp.; Riede et al., 2016), rheas (*Rhea* sp.; Folch, 1992) and cassowaries (*Casuarius* sp.; Mack and Jones, 2003). Birds that have a vocal sac emanating from the trachea, similar to the gular pouch of chameleons, include the emu (*Dromaius novaehollandiae*; Murie, 1867) and the ruddy duck (*Oxyura jamaicensis*; Wetmore, 1918; McLelland, 1989). One common theme exhibited by closed-beak, vocal-sac bird species is their comparatively large size and the associated lower-frequency vocalizations (Riede et al., 2016). Similarly, the veiled chameleon examined in the current study is a comparatively large chameleon, which also produces low-frequency vibrations (Barnett et al., 1999). Another common theme that has not previously been recognized is that most of these species of birds are primarily ground dwelling and, often, the vocalizations are done by males in courtship displays that are performed on the ground, often in leks. All previous bioacoustics research on these courtship vocalizations have recorded the signals in terms of sound pressure, but because low-frequency Rayleigh waves travel with greater velocity propagation and decay more slowly than those at higher frequencies (Foti et al., 2018), it may be that these low-frequency bird vocalizations, sometimes referred to as ‘booms’, produce biotremors that are transmitted down the legs of these birds to the substrate in a similar manner to what we are proposing occurs in chameleons with gular pouches. This would produce a multimodal courtship display that would incorporate vision, hearing and touch. An alternative hypothesis is that these ground-dwelling species produce low-frequency sounds in order to minimize signal attenuation and degradation caused by ground foliage (Kirschel et al., 2009).

Recent ancestral state reconstruction revealed that closed-mouth vocalizations evolved independently 16 times within Aves, within mostly large-bodied taxa, and likely was also exhibited in their ancestors, the non-avian dinosaurs (Riede et al., 2016). Because birds are phylogenetically reptiles, it is not surprising to see closed-mouth vocalizations evolving independently multiple times in reptiles as it has in birds (i.e. in chameleons as well as in crocodilians). As communication between reptiles is often at close range, it is likely that researchers will find more examples of closed-mouth vocalizations when vibratory signals are examined in addition to the standard sound signals that are normally recorded during bioacoustic studies.

Conclusion

The present study provides the first experimental insight into a biotremor-producing mechanism in a reptilian species. The evidence produced here, in conjunction with the absence of vocal cords (Huskey et al., 2020) and external ears (Wever, 1968, 1969a,b; Anderson and

Higham, 2014), and the theoretical ability to detect biotremors (Hartline, 1971; Barnett et al., 1999) supports that biotremors may be utilized by *C. calyptratus* for communication (Barnett et al., 1999). However, courtship, territoriality and anti-predator trials (Barnett et al., 1999), accompanied by EMG, accelerometry and soft tissue imaging (fluoromicrometry or ultrasound), are necessary to explicitly demonstrate how the hyobranchial muscles and gular pouch may generate these biotremors in a communicative context. Further studies of other chameleon species will also reveal whether this ability is ubiquitous among all chameleons, merely a behavior exhibited by a few species or a novel adaptation of *C. calyptratus*.

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Author contributions
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