

# Active control of ultrasonic hearing in frogs

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Vertebrates can modulate the sound levels entering their inner ears in the face of intense external sound or during their own vocalizations. Middle ear muscle contractions restrain the motion of the middle ear ossicles, attenuating the transmission of low-frequency sound and thereby protecting the hair cells in the inner ear. Here we show that the Chinese concave-eared torrent frog, *Odorrana tormota*, can tune its ears dynamically by closing its normally open Eustachian tubes. Contrary to the belief that the middle ear in frogs permanently communicates with the mouth, *O. tormota* can close this connection by contraction of the submaxillary and petrohyoid muscles, drastically reducing the air volume behind the eardrums. Mathematical modeling and laser Doppler vibrometry revealed that the reduction of this air volume increases the middle ear impedance, resulting in an up to 20 dB gain in eardrum vibration at high frequencies (10–32 kHz) and 26 dB attenuation at low frequencies (3–10 kHz). Eustachian tube closure was observed in the field during calling and swallowing. Besides a potential role in protecting the inner ear from intense low-frequency sound and high buccal air pressure during calling, this previously unrecognized vertebrate mechanism may unmask the high-frequency calls of this species from the low-frequency stream noise which dominates the environment. This mechanism also protects the thin tympanic membranes from injury during swallowing of live arthropod prey.

audition | Eustachian tube | middle ear | tuning | eardrum

All major tetrapod groups possess middle ear muscles that can potentially modify the auditory input before it reaches the inner ear (1–3). In the stapedius reflex, the middle ear muscles contract in response to intense external sound (4) or in anticipation of a vocalization (5). Such contractions restrain the movement of the middle ear ossicles (6) and attenuate the sound transmitted to the inner ear, mostly at low frequencies (7), with no or slight amplification at high frequencies (8).

We found that the Chinese concave-eared torrent frog, *Odorrana tormota*, Ranidae (previously *Amolops tormotus*; refs. 9 and 10), can modify the auditory sensitivity of its middle ear by using a previously unrecognized mechanism. Unlike mammals, the mouth in frogs was believed to be permanently connected to the middle ear cavities through widely open Eustachian tubes (ETs; refs. 11–15). Here we show, however, that *O. tormota* can actively close its ETs, drastically reducing the volume of air behind the tympanic membranes (TMs). This volume reduction increases the ear's impedance and shifts the tuning of the middle ears toward high frequencies, with potential biological roles related to the attenuation produced at low frequencies, the sensitivity gain produced at high frequencies, and the physical obstruction of the ET.

## Results

We found that the frog *O. tormota* can actively close its ETs [Fig. 1A and B and supporting information (SI) Movie S1]. This discovery was facilitated by this frog's transparent TMs, which permitted noninvasive observation of the state of the ETs in the laboratory and in the field (Fig. 1C–E and Movie S2).

**Mechanism.** To determine the mechanism of ET closure, we performed fresh tissue dissections and electrical stimulation of the muscles surrounding the ET. The cartilaginous anterior horn of the hyoid attaches to the skull next to the caudal edge of the ET (Fig. 2; ref. 16). The distal end of the hyoid horn is broad, but it only attaches to the skull at its rostralmost pole, forming a hinge, which allows for pivoting in the coronal plane of the animal. The pivoting of the hyoid attachment to the skull causes the hyoid horn to bend in the form of an arch that advances over the ET lumen and closes it, resembling the mechanism by which the curtain in a focal plane shutter closes to block the passage of light.

Of all of the muscles that were stimulated electrically (see *Materials and Methods*), detectable pivoting of the hyoid horn over the skull was observed only during the stimulation of the submaxillary muscle and of the petrohyoid group. Stimulation of the submaxillary muscle with the petrohyoids and other buccal muscles ablated from the preparation excluded the possibility of crosstalk during stimulation and confirmed its effect on ET closure. Likewise, stimulation of the petrohyoids with the submaxillary and other muscles removed confirmed their effect on ET closure.

The closure movement is mainly produced by the submaxillary muscle (16), which forms the floor of the mouth and has a caudal extension that inserts into the anterior hyoid horn just proximal to its attachment to the skull (Fig. 2B, red). Contraction of this muscle closes the ET by causing the hyoid horn to pivot over its hinge with the skull and bend (Movie S3). This movement is enhanced by the petrohyoid muscles, which insert into the posterior horn of the hyoid and into the skull and lay just caudal to the hinge between the anterior hyoid horn and the skull. Contraction of the petrohyoid muscles causes them to shorten, thicken, and move rostrally, pushing the hyoid forward to promote ET closure (Movie S3). Only partial ET closure was obtained with electrical stimulation, possibly because the hyoid motion observed during active ET closure was lacking in these experiments, or because the bipolar electrode did not stimulate all of the muscle fibers.

Natural ET closure in *O. tormota* can be complete or partial. Complete closure of the ET was observed during vocalization, swallowing, and during sudden movements of the whole body. It was not observed at rest or in response to sound sweeps (2–40 kHz at 80 dB sound pressure level at the TM) or ultrasonic conspecific calls. Video analysis of eight natural vocalizations of

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closure on TM spectral response should be much reduced when the middle ear volume is large (see model description in *Materials and Methods*).

*O. tormota* exhibits three traits that are key to active ET closure: (i) the hinged hyoid horn–skull connection; (ii) the insertion of the submaxillary muscle into the hyoid horn is caudal to its hinged connection with the skull so that contraction causes the hyoid horn to pivot; and (iii) active neural control of the closure mechanism. ET closure has not been reported in other species, possibly because it is not readily visible in frogs with opaque eardrums, and because the anterior hyoid horn–skull connection has not been a target of comparative anatomical studies. Examination of North American *L. pipiens*, however, failed to reveal the presence of any of the key traits mentioned above. Middle ear tuning by ET closure is not, therefore, general to ranid frogs, and further studies are necessary to determine the pervasiveness of this trait.

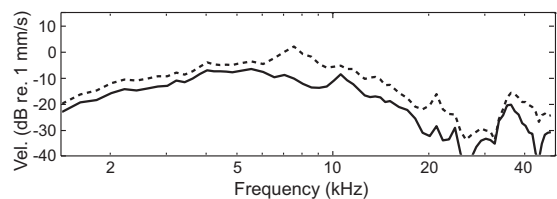
Most calls of *O. tormota* abound in energy >10 kHz, and these frogs communicate in an environment that is dominated by broadband noise with intensity increasing exponentially at low frequencies (21, 22). ET closure produces attenuation <10 kHz and gain above it, and thus one might expect the frogs to close their ETs during conspecific calling to filter out stream noise and better detect calls. We did not observe such behavior, possibly because: (i) filtering of waterfall noise for high-frequency communication is not a role of ET closure; (ii) the noise levels produced by the stream during the period of field work were not high enough to cause ET closure; and (iii) ET closure might be restricted to male–female vocal interactions, which were not monitored and are part of the reproductive behavior in this species (23). In addition, ET closure might be mechanically dependent on hyoid movement because of insertion of both the submaxillary and the petrohyoid muscles into the hyoid. Calling, which involves extensive hyoid movement, was always accompanied by complete ET closure in the field. Males of *O. tormota* frequently produce a narrow-pulsed, low-level, low-frequency (2–6 kHz) call [staccato call (23)] with long and variable duration ( $3.9 \pm 1.2$  s; mean  $\pm$  SD;  $n = 8$  individuals) and very reduced duty cycle ( $0.11 \pm 0.03$ ; measured as proportion of time >10% of the pulse's peak amplitude;  $n = 8$ ), i.e., sound is produced only one tenth of the time. It is conceivable that ET closure may facilitate conspecific detection during pulsed vocalizations.

The behavioral contexts in which ET closure is observed also suggest other nonexclusive potential roles: (i) the attenuation of TM vibration produced <10 kHz may protect the inner ear from the energy contained in the lower harmonics of the frog's own call or from increased buccal air pressure during calling (15); and (ii) the physical occlusion of the ET during swallowing might be important in protecting the very thin TM from injury by live arthropod prey items.

Our study revealed that the middle ear of the frog *O. tormota* can transfer ultrasound to the inner ear and that such transfer can be modulated by an active mechanism of ET closure, which was previously uncharacterized in vertebrates. We characterized the anatomical specializations underlying ET closure, the closure mechanism, and the effects of ET closure on hearing, and we documented the occurrence of ET closure in nature, during communication. Further insight into the biological role and evolution of ET closure in *O. tormota* will require a more complete understanding of its communication and mating behaviors, as well as a comparison with closely related species.

## Materials and Methods

**Subjects.** Adult males of *O. tormota* were captured in Huangshan, Anhui province, China, in May 2006, May 2007, and April 2008, and were transported to the Institute of Biophysics, Beijing, China, for initial experiments and observations. Four animals were further transported to the University of California, Los Angeles (UCLA), where the reported measurements were



**Fig. 6.** Vibration velocity amplitude at two points on the tympanic membrane (TM) of *O. tormota* with the ear canal open. Amplitudes on single reflective beads placed at the center of the TM (solid line) or at the midpoint between the center and the periphery of the membrane in the ventrocaudal direction (dashed line). Both lines are averages of five measurements each.

obtained. All experiments were conducted following the UCLA Animal Research Committee protocol no. 1994-086-42.

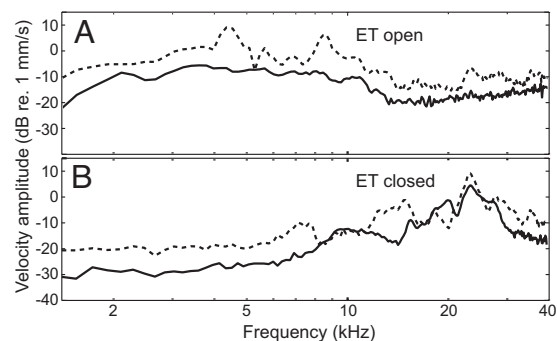
**Monitoring the Eustachian Tube.** A transparent container allowed videotaping of the state of the ET through the transparent TM with the mouth illuminated from below by a cold light source. Direct observations were made by physically restraining the awake animal and holding its mouth open for < 1 min, which produced no apparent signs of chronic stress.

**Doppler Laser Vibrometry.** The vibration velocity of the TM in response to acoustic stimulation was measured at an angle normal to the tympanic membrane by using a single-point Doppler laser vibrometer (Polytec; model HLV-1000). A single retroreflective hemispheric glass bead (diameter of 30–50  $\mu$ m; 3M Scotchlite) was placed on the TM to increase its reflectivity and maximize measurement precision.

The reflective bead was placed in the center of the TM in *L. pipiens*, but in *O. tormota* it had to be placed at half the distance between the TM center and its margin, in the ventrocaudal direction, because the TM center is not visible through the ear canal aperture. We examined the effect of bead placement by exposing the entire TM by surgical removal of the integument that partially covers the ear canal. Relative to the central position, the TM's vibration range in the periphery is unaltered, but the amplitude is higher, particularly around 7 kHz (Fig. 6). The bead placement in *O. tormota* might have given rise to higher vibration amplitudes than those observed in *L. pipiens*.

The load of the reflective bead on the thin TM did not produce a significant shift in the TM's vibration spectrum, because the addition of a second bead adjacent to the first produced a modest attenuation of  $\approx 1.7$  dB that was fairly constant over the spectrum. The addition of two more beads produced further attenuation and shifted the peak toward low frequencies. Measurements obtained without any reflective bead on the TM confirmed the spectral ranges and the effect of ET closure obtained by using reflective beads (Fig. 7).

As an additional control, we measured the vibration of 97 points over the exposed TM using a scanning laser Doppler vibrometer (Polytec PSV-300) without reflective beads. Such measurements showed a heterogeneous distribution of vibration amplitude over the surface of the membrane that might



**Fig. 7.** Vibration velocity measured directly from the TM of *O. tormota* without reflective beads. (A) Eustachian tube open. (B) Eustachian tube closed. The ear canal was surgically opened to expose the TM completely, and the laser was aimed at the center of the membrane (solid line) or at the midpoint of the radius of the membrane in the ventrocaudal direction (dashed line).

explain the differences in vibration spectra observed between peripheral and central points.

Besides the TM, we also measured the vibration velocity spectrum of the stapes footplate to verify whether the high-frequency vibrations recorded at the TM were transmitted to the inner ear. In frogs, the stapes protrudes through the middle ear cavity to reach the oval window. Following Mason and Narins (24), a dorsal incision was made to expose the stapes footplate of an anesthetized animal (see below) without opening the middle ear cavity, and the vibration response of the stapes footplate to acoustic stimulation was measured.

**Setup.** The frog was anesthetized by immersion in 0.3% ethyl 3-aminobenzoate methanesulfonate (MS-222; Sigma) for 5 min, and a reflective bead was positioned on the TM under a dissecting microscope. The animal was positioned on a custom-made foam base that supported the body in a natural position while leaving the bottom of the mouth freely suspended. A reference microphone (GRAS 40BE, 0.2–97 kHz  $\pm$  2 dB) was positioned 1 cm above the midpoint of the intertympanic axis. Acoustic stimuli were broadcast from a broadband loudspeaker (1–95 kHz; Tucker-Davis Technologies; ES-1) placed 10 cm from the reference microphone. For stimulus frequencies  $<$ 1.4 kHz, the ES-1 speaker was replaced by an 8- $\Omega$  loudspeaker (Versa-Tronics DOB100R).

**Manipulations of the Eustachian Tube and Mouth.** Video monitoring of the ET in unrestrained, unanesthetized frogs in the laboratory showed that at rest, the ET remains open most of the time. Vibrometric measurements were, therefore, obtained with the ET open to mimic the resting condition. To quantify the effect of ET closure on the spectral response of the TM, we carried out two treatments. The first one involved placing a convex base below the floor of the mouth and collapsing the mouth with gentle pressure on the head of the frog. In the second treatment, we glued a 1-mm-thick disk of modeling clay to the roof of the mouth, across the medial opening of the ET to occlude it. The two treatments produced equivalent results and we used collapsing of the floor of the mouth throughout the study because it was the faster method.

**Calibration and Recording.** Stimulus generation, calibration, and recording were performed with the custom-written software VibroToolbox 0.9.1b (<http://vibrotoolbox.sf.net>). Stimuli were synthesized as a series of pure tone bursts (5-ms rise-fall time, 2-ms stabilization period, 20 cycles measurement duration) of ascending frequency separated by 2 ms of silence. The amplitude of each tone in each stimulus was calibrated in an iterative process that allowed for an error of  $\pm$ 0.5 dB up to 97 kHz. Other sources of bias in the data were compensated numerically post hoc: (i) the reference microphone was placed above the center of the head, not above the TM; and (ii) the microphone's protective grid biases its response by up to 4.5 dB over the frequency range used in the present study. In addition, diffraction by the head of the frog should have gradually increased sound pressure at the TM with frequency (25). The effect should have been negligible at low frequencies, but it should have produced a gain of 1 dB at 10,981 Hz, and it would have reached 6 dB at 109,810 Hz [wavelength-radius product ( $ka$ ) = 1 and 10, respectively, with *O. tormota* head radius = 5 mm].

**Muscle Stimulation.** With the frog anesthetized, skin was surgically removed from the roof of the mouth to expose the caudal half of the ET and the local muscles that attach to the skull, hyoid, lower jaw, and shoulders. Muscles were stimulated individually or in groups with bipolar electrodes (Teflon-coated silver wire 0.003-inch bare diameter; A-M Systems). Stimuli were 100-ms-long balanced pulses with a constant current of 100  $\mu$ A given individually or in trains of up to eight pulses separated by 100-ms intervals. Stimuli were delivered by a stimulator (World Precision Instruments; A310 Accupulser) connected to a stimulus isolation unit (WPI A360D-C). The following muscles were stimulated: m. submaxillaris, m. petrohyoidei, m. sternocleidomastoideus, m. levator anguli scapulae, m. temporalis, m. pterygoideus, and m. masseter (16).

**Cavity Volumes.** To determine the volume of the middle ear cavity, the anesthetized frog was positioned with the ventral side facing up and the mouth open. A micropipette (Eppendorf Reference 10  $\mu$ l) was used to transfer 0.5- $\mu$ l water aliquots to the middle ear cavity through the ET opening until the

fluid filled the ET and the water surface changed from concave to flat or convex. Such process produced a rough estimate of the total volume. The cavity was then drained with absorbent paper and a single aliquot was transferred containing the full estimated cavity volume. This full volume measurement was repeated, varying the aliquot volume in steps of 0.1  $\mu$ l to find the volume that best filled the middle ear producing a flat surface at the proximal opening of the ET. The whole process was conducted under a dissection microscope to clearly visualize the shape of the water surface and to be able to discard trials in which any air bubbles were present. Mouth cavity volume was measured in freshly euthanized frogs, ventral side up, mouth closed, with a 3-mm-diameter hole exposing the floor of the mouth through a ventral incision to allow micropipette access to the buccal air volume. Buccal volume was determined with the same procedure used for the middle ear cavity, but accumulating 20- $\mu$ l aliquots for the initial volume estimate by using a micropipette of 1-ml maximum capacity and then varying the full volume aliquot in steps of 1  $\mu$ l.

**Model of Middle Ear Impedance with the ET Open or Closed.** When the ET is open, the air space behind the TM approximates a Helmholtz resonator, with the minute middle ear volume acting as an acoustic mass in a tube with an open termination connected to a larger volume—the mouth cavity (Eq. 1; ref. 11).

$$Z_{\text{CavETOpen}} = j\omega\rho l/A_{\text{ET}} + 1/(j\omega V_{\text{CavMouth}}/\rho c^2). \quad [1]$$

$Z_{\text{CavETOpen}}$  is the impedance of the middle ear plus the mouth,  $j$  is the imaginary number,  $\omega$  is the angular frequency given by  $\omega = 2\pi f$  where  $f$  is frequency,  $\rho$  is air density,  $l$  is the length of the ET,  $A_{\text{ET}}$  is the cross-sectional area of the ET,  $V_{\text{CavMouth}}$  is the volume of the mouth, and  $c$  is the speed of sound.

With the ET closed, the middle ear cavity is separated from the mouth, and it acts as a compliance-dominated closed tube (Eq. 2).

$$Z_{\text{CavETClosed}} = 1/(j\omega V_{\text{CavET}}/\rho c^2). \quad [2]$$

The impedance of the TM can be approximated by a model dominated by compliance at low frequencies, mass at high frequencies, and presenting damping (Eqs. 3–6).

$$Z_{\text{TM}} = R_{\text{TM}} + j\omega L_{\text{TM}} + 1/(j\omega C_{\text{TM}}), \quad [3]$$

$$C_{\text{TM}} = V_{\text{TM}}/\rho c^2, \quad [4]$$

$$L_{\text{TM}} = 1/((2\pi f_{\text{TM}})^2 C_{\text{TM}}), \quad [5]$$

and

$$R_{\text{TM}} = 2\pi f_{\text{TM}} L_{\text{TM}}/2. \quad [6]$$

$V_{\text{TM}}$  is the air volume equivalent of the TM and  $f_{\text{TM}}$  is the mass-compliance resonance frequency of the TM, assumed to be the peak TM vibration frequency with the ET open (7,000 Hz for *O. tormota* and 1,300 Hz for *L. pipiens*).

The impedance of the ear was obtained by adding the impedance of the TM to that of the air cavity with the ET closed or open. The ratio between the modeled impedances of the ear with the ET closed or open should match the ratio between the TM vibration velocities measured by laser Doppler vibrometry with the ET closed or open under constant amplitude stimulation. That allows for a direct comparison of the model prediction against the observed data (Fig. 4). This simple model successfully predicts the observed frequency shift in the TM vibration spectrum with sensitivity gain at high frequencies when the ET is closed.

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