The Journal of Experimental Biology 212, 2204-2214
Published by The Company of Biologists 2009
doi:10.1242/jeb.027516

Function of the sexually dimorphic ear of the American bullfrog, *Rana catesbeiana*: brief review and new insight

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Accepted 27 April 2009

SUMMARY

The dimorphic ear of the bullfrog, *Rana catesbeiana*, has long been enigmatic. The male's tympanic membrane (TM) area approximates twice the area of the female's; however, similar size differences in the area of the columellar footplate were not observed between the sexes. Hence, the male's hearing is expected to be more sensitive than the female's but this is not the case. Asking what offsets the advantage of the large TM, we applied a series of experiments to the auditory system. Male and female audiograms based on stimulation with airborne sound and on both multi-unit responses from the brain and alternating cochlear potentials ('microphonics') showed equal sensitivity and a small difference in frequency response; at low frequencies the male was more sensitive than the female. Amputating the columella and stimulating the stump with mechanical vibration showed that for an equal microphonic response, the male's footplate vibrated with lower amplitude than the female's footplate. Mechanically stimulating the TM of the intact ear replicated this result, excluding the involvement of the mechanical lever. The TM of the male weighs five times the TM of the female, and artificial loading of the TM of either sex greatly reduced the ear's sensitivity. Hence, the male's excessive area ratio (TM to columellar footplate) is offset by the heavier cartilage cushion on the male's TM, damping the TM's response to sound. This is corroborated by experimentally artificially loading the TM. The product of area ratio and footplate vibration amplitude would result in similar stimulation of the inner ear in the two sexes.

Key words: American bullfrog, hearing, middle ear, *Rana catesbeiana*, sexual dimorphism, sexual diergism, tympanic membrane.

INTRODUCTION

'The frog … The croaking that is heard going on in the water is made by the male frogs, and is their call to the females at breeding time.'

Aristotle (384–322 BC)

The intersexual vocal communication of anurans was already described in the fourth century BC by Aristotle (Aristotle, 1984), who said that male frogs croak to attract the females, and in recent decades, this area has generated an assortment of investigations and reviews, such as Bogert (Bogert, 1960) and Schneider (Schneider, 1990). In particular, the structure and function of the anuran ear have attracted research and reviews (Capranica, 1978; Purgue and Narins, 2000; Mason, 2007). But despite recent advances (Mason and Narins, 2002a; Mason and Narins, 2002b; Mason et al., 2003; Werner, 2003), the riddle of sexually dimorphic (dually formed) and diergic [dually functioning (Rhodes and Rubin, 1999)] anuran ears has not yet been solved.

In Tetrapoda, hearing depends on the synergism of the external ear, tympanic membrane (TM), middle ear ossicles, inner ear, auditory nerve and brain (Manley, 1990). In Anura, the inner ear contains three hearing organs (Capranica, 1976; Purgue and Narins, 2000), and sound may be conducted to the inner ear by two routes in addition to direct TM stimulation. First, the opercular system has been claimed to conduct lower sound frequencies, ground vibrations or both (Wilczynski et al., 1987; Hetherington, 1988; Hetherington, 1994a). Second, the lungs may conduct lower sound frequencies to the inside of the TM (Ehret et al., 1990; Jørgensen et al., 1991). The present study concerns sound reception only *via* the tympanic route.

In several species of *Rana*, the TM is much larger in the male than in the female (Wright and Wright, 1949). Among these, the North American bullfrog *Rana catesbeiana* (Fig. 1) is the largest, and its auditory physiology and vocalisations have been studied (Strother, 1959; Capranica, 1965; Capranica, 1966; Capranica, 1976; Mason et al., 2003; Werner, 2003). Early efforts to relate the sexual difference in TM size to ear function in this species, assessed through single auditory unit activity in the eighth nerve, have failed [see p. 554 in Capranica (Capranica, 1976)]. Similarly, in *Rana clamitans*, another North American species with an enormous TM observed in the male (Wright and Wright, 1949), tests of hearing, as assessed by respiratory responses, detected no sexual differences (Kleerekoper and Sibabin, 1959). Recently, it was discovered that the male bullfrog's large TM serves to broadcast the call (Purgue, 1997). Nevertheless, given the principles of acoustics (Relkin, 1988) and precedents from other animals (Werner et al., 2008), one expects the difference in TM size to affect hearing.

Aiming to relate the ear's function to TM size, we applied a step-wise analysis, combining assorted experimental methods, to bullfrog ears [interim reports (Werner, 1979; Werner and Pylka, 1997)]. We

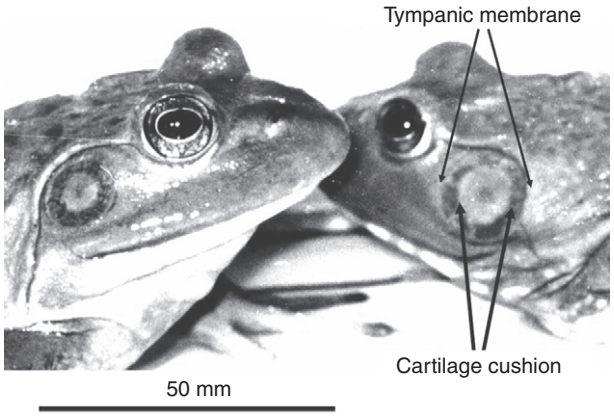

Fig. 1. Photograph of female (left) and male (right) live adult American bullfrogs (*Rana catesbeiana*), from the present study, showing the difference in the size of the tympanic membrane and its cartilage cushion, unlike the drawing in Purgue (Purgue, 1997). (Frogs are from the holding facility, Institut für Zoologie, Universität Bonn, Poppelsdorfer Schloss, July 1975.)

compared male and female ears in audiograms from two electrophysiological methodologies: (1) threshold audiograms based on evoked responses from the brain (Brzoska et al., 1977) were recorded at the Institut für Zoologie, Universität Bonn, Germany (Schneider, Walkowiak and Werner). (2) Isopotential audiograms based on the alternating potentials of the inner ear, commonly called cochlear microphonics (CM) (Strother, 1959) were obtained in the Auditory Research Laboratories, Princeton University, NJ, USA (Werner and Pylka). Finally, the source of any sexual differences found was sought by step-wise elimination of middle-ear components and experimental manipulation.

MATERIALS AND METHODS

Animals

Besides the ethical aspects, experiments using live frogs involve a number of problems, including sample homogeneity, the health of the experimental animals (McClelland et al., 1998) and nature conservation (Gibbs et al., 1971). The frogs used, *Rana catesbeiana* Shaw 1802, had been caught in Mexico about two weeks before being purchased by the Auditory Research Laboratories (Princeton, NJ, USA) from the Connecticut Valley Biological Supply CO. (Southampton, MA, USA). Following each purchase, some animals were sent to Bonn, Germany whereas others remained in Princeton, NJ, USA. Animal treatment accorded with all ethical requirements.

Physiological data were obtained from 65 adults: males measured 126–153 mm RA length (rostrum–anus length), mass 169–421 g; females, 118–158 mm RA length, mass, 124–382 g. We endeavoured to maximise the information derived from each individual (Gibbs et al., 1971).

Morphological methods

When anaesthetised, frogs were weighed and the RA length was recorded. After testing, the TM was measured. In some of the killed frogs, the ear was dissected and components of the middle ear were measured using callipers under a stereoscope. Approximate areas of the TM, stapedial footplate and operculum were calculated as if these structures were elliptical. Sometimes the middle-ear components were weighed. Some heads were used to display the middle-ear elements in transverse or frontal transaction.

General experimental methods

Males and females were always tested in alternation to preclude a season effect (Köppl et al., 1990). In Bonn, animals were immobilised by injecting 2% succamethonium chloride (Succinyl-Asta®) ~0.14 ml per 100 g of frog mass whereas in Princeton, animals were immobilised by injecting 20% ethyl carbamate (Urethane), in doses amounting, eventually, to 3.5 ml or more per 100 g. Some frogs were used for their other ear after a day or two. In all experiments, the animal was placed in a chamber that was electrically shielded, light proof and (in Bonn, partially) sound proof.

In nature, the bullfrog behaviourally thermoregulates during much of daytime, maintaining a body temperature (BT) ranging from 28°C to 31°C (Lillywhite, 1970; Lillywhite, 1971). Temperature effects on its physiology include shifting the frequency tuning of units in its amphibian papilla (Narins, 1995; Narins, 2001).

In order to select the experimental temperature for brain responses, we recorded such responses in July at BT=23–30°C. The most sensitive audiogram was obtained at BT=30°C. Because this animal died before the run was completed, the main work proceeded in July and early October at BT=24–27°C. In late October and November, the frogs tolerated less heat and experimentation was conducted at BT=21–23°C. For CM in August, the best sensitivity was at BT=27°C, so the main work proceeded at this BT and only these results are presented.

Experimental temperature was regulated by heating the room (in Bonn) and either similarly or by heating the animal with an electric blanket (in Princeton) (Werner, 2003). Ear temperature was not monitored directly. When rectal temperature reached 27°C under the electric blanket, buccal temperature approximated 25°C. The issue of body temperature is further discussed elsewhere (Werner, 2003).

During surgery and experimentation, the animal remained covered with moist cotton wool or a paper towel. In Bonn, the animal was seated in pseudonatural posture (limbs folded) unrestrained whereas in Princeton, the animal was held by Plasticine™ strips over all of its extremities with its head especially firmly held in place by Plasticine™.

Surgical methods

The surgical approach was dorsal [see fig. 1 in Werner (Werner, 2003)]. An incision through the cranial skin, along three sides of a quadrangle (~20×10 mm) released a skin flap hinging on its posterior border where a cutaneous artery entered the skin flap. Left intact, this artery indicated animal condition. A small area of skull was cleared of soft tissue and periost.

For recording auditory responses from the torus semicircularis of the mesencephalon (Potter, 1965a), a hole of ~3 mm diameter was drilled in the top of the skull over the middle of the median half of one optic lobe. This hole was centered in the frontoparietal bone between the (paired) crest along this bone and the adjacent, parallel, occipital artery, about two percent of the RA length anterior to the caudal end of the crest. The dura mater and most of the arachnoidea were teased open and a few drops of gelatin (12% in water) reduced brain movement and prevented drying.

For recording CM, the contact area of the frontoparietal and prootic bone was exposed, and a small hole was drilled in the prootic into the anterior semicircular canal (which parallels the caudolateral aspect of the branch of the superior spinalis vein) about 1 mm from the anterior ampulla [see fig. 1 in Werner (Werner, 2003)].

Equipment for brain responses

Threshold audiograms were plotted from the multi-unit activity of neurones in the torus semicircularis, in response to stimulation by aerial sound (free field). Neural activity was recorded using an electrode of 50 μm diameter tungsten wire, which was glass insulated except at the tip. The reference electrode was placed in the tissues of the incision and a grounding electrode was placed under the snout skin. The responses were amplified, band-pass filtered for 0.4–2.0 kHz or 0.4–6.0 kHz and displayed on a storage oscilloscope. The stimulus was a pure tone burst of 30 ms; rise and fall times, 5 ms each, were controlled by a pulse generator that modulated the output of a sine-wave generator. Tones at 20 frequencies between 0.1 kHz and 3.4 kHz were presented; frequency was monitored by a counter. Stimulus repetition rate of once per second was determined by a stimulator. Stimulus intensity was regulated by an amplifier and at each frequency was adjusted to the level yielding the weakest detectable neurone activity on the oscilloscope. The voltage input to the loudspeaker as seen on an oscilloscope was recorded as a measure of stimulus intensity. After the experiment, the voltage at each frequency was reproduced and calibrated for the corresponding sound pressure level (re. 20 μPa), using a condenser microphone where the ear had been and a measuring amplifier.

Equipment for CM

Isopotential audiograms were obtained for a standard response of 0.1 μV RMS (root mean square) of the CM, in response to a succession of continuous pure tones. Equipment and methodology, developed and detailed by Wever (Wever, 1978; Wever, 1985), were briefly as follows. The active electrode, a steel needle, was in the anterior semicircular canal; reference and ground electrodes were as for brain responses. The potentials were preamplified differentially (×10,000; 0.05–25 kHz) and led to a wave analyser, which acted as a selective millivolt meter, and to a monitoring oscilloscope. The same wave analyser generated the continuous pure tone stimuli; frequency was monitored by a counter. The electrical signals were led through an amplifier and two attenuators (one of these followed the amplifier; its attenuation was kept high to reduce the electrical noise) to a speaker outside the experimental chamber. The sound was led into the chamber by a tube, the replaceable end-piece of which was sealed over the frog's ear with cotton wool soaked with petroleum jelly. A probe tube from a condenser microphone opened within the sound tube close to the ear and served for the calibration of sound levels (re. 20 μPa).

In some experiments stimulation was mechanical; the tip of a probe driven by a piezoelectric crystal was applied to the ear and stimuli were applied at the same frequencies as with acoustic stimulation. The details of this technique are explained elsewhere (Wever and Werner, 1970; Wever, 1978; Werner, 2003).

Each experimental run comprised the presentation of pure tones at up to 25 frequencies between 0.05 kHz and 4.0 kHz. At each frequency, the sound level or vibratory amplitude that yielded the standard response of 0.1 μV was recorded or calculated on the assumption of a 1:1 input–output function. The actual voltages measured were almost always between 0.06 μV and 0.2 μV; as will be seen in the methodological tests section, the error expected from such calculation is up to ~2 dB.

Statistics and presentation

For each experiment, the data from each sex were averaged. Sexual diergism was analysed through *t*-tests comparing the thresholds (in dB) for each frequency separately. To prevent excess rejections of the null hypothesis of no difference between the sexes due to the number of comparisons made, rejection level (α) was adapted by Bonferroni multiple comparisons (Zar, 1999). Additionally, the significance of the differences between pairs of averaged audiograms was tested by a residual autocorrelation test. The test used (run test) checks whether the pattern of the two audiograms differs, by analysing the residuals created between the two audiograms. If the switches between successive residuals (from positive to negative or *vice versa*) are predictable (i.e. differ from random), the shape of the curves of the two audiograms is significantly different (Zar, 1999); see Werner et al. (Werner et al., 2008) for an example. We present audiograms on a linear (rather than logarithmic) frequency scale for a clearer view of the higher frequencies.

RESULTS

Morphology

Middle-ear elements

A lateral opening in the inner ear capsule, the (external) 'oval window', houses two coupled elements: the footplate of the columella and posteriorly the operculum [see figs 2 and 3 in Werner (Werner, 2003)]. Bridging between the columellar footplate and the TM is the ossicular chain, comprising a proximal (medial) osseous unit and a distal (lateral) cartilaginous unit (Fig. 2). Herein we call these, respectively, columella and extracolumella. The two units are linked by a joint with a ventrally open angle between them. The extracolumella is proximally broad in the vertical plane; its dorsal head connects with the head of the columella; its ventral head continues as the ligamentous or cartilaginous ascending process that runs medio–dorsad to the ceiling of the middle ear cavity. Distally the extracolumella is attached to a cartilage cushion that occupies the centre of the inner face of the TM. Around this cushion the TM is thin. Its margin is spanned on the outer rim of the osseous annulus tympanicus. Thus, the functional morphology of the bullfrog ear deviates from conventional textbook descriptions, as already noted by Werner (Werner, 2001; Werner, 2003), Mason and Narins (Mason and Narins, 2002a; Mason and Narins, 2002b), Mason et al. (Mason et al., 2003) and Mason (Mason, 2007).

Hence, the mechanics of this system must be the opposite of that in amniotes (Wever and Werner, 1970; Pickles, 1988). In the bullfrog, when the TM moves outwards and pulls the extracolumella, which is restrained by the ascending process and rotates on it, its dorsal head moves inwards, pushing the columella towards the oval window (Jørgensen and Kanneworff, 1998; Werner, 2001; Werner, 2003). Vibrometry has confirmed a 180 deg. phase difference between the movements of the TM and the footplate (Mason and Narins, 2002a).

Sexual dimorphism and allometry

The sexual difference in the bullfrog TM develops gradually during ontogeny (Mason et al., 2003). Here we present size data of middle-ear components of adult specimens from this study. The data are segregated by sex, and the differences between the sexes were tested by two-tailed *t*-tests (for unequal variances). Table 1 contains the frequently taken measurements and Table 2 details supplementary data based on small samples. The sexual dimorphism in TM area is not parallelled in other middle-ear elements. The size (area, mass) of the cartilage cushion on the medial side of the TM is the exception, although we have few data.

Methodological tests

A convincing comparison of male and female audiograms depends on the accuracy of the methods used. Several methodological aspects have been discussed elsewhere (Werner, 1972; Werner, 1976;

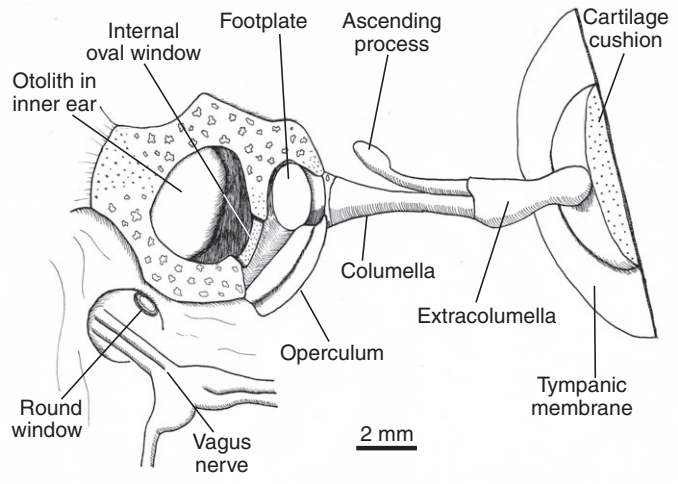

Fig. 2. The middle ear of *Rana catesbeiana,* right side, as seen in a frontally bisected head of an adult male, viewed from above (dissection of specimen F-984 on 15 October 1975).

Wever, 1978; Wever, 1985). Two crucial issues were addressed as follows.

Electrode placement

(1) In the brain. Reputedly, when the recording electrode is in the centre of the auditory region of the torus semicircularis, its more precise location has little effect on the audiogram (Potter, 1965a; Brzoska et al., 1977; Pettigrew et al., 1981). Additionally, we performed two runs on each of four males and four females; between the two runs the active electrode location was shifted by 200–1300 µm in any plane. The resulting thresholds differed by 0–10 dB (rarely more) for single frequencies or groups of adjacent frequencies. This exceeds the variation that is common between repeated runs at the same site but the characteristics of the curve usually persisted. Consequently we endeavoured to place the electrode centrally. (2) In the inner ear. In frogs the CM cannot be recorded from the round window membrane, which is inside the vagus nerve canal (Fig. 2). Potentials from the semicircular canal system have about a tenth of the amplitude of those from within the sacculus (E. G. Wever, unpublished). But because electrode placement in the sacculus risks damage to sensory structures and results will vary with proximity to these structures, we placed the electrode in the anterior semicircular canal, near the ampulla.

Linearity of CM (intensity functions)

Because it was sometimes necessary to calculate the standard response from lower or higher actual response levels, we needed to ascertain the slope of the input–output (intensity) function. We obtained these functions from three frogs at a few selected frequencies (including those of maximum sensitivity). Sound pressure was increased by 5 dB steps, and each response level was recorded, until the slope levelled off. The results showed that the error expected from such calculation is <2 dB.

Responses from the brain

We obtained 18 threshold audiograms from males (Fig. 3). The frequency of greatest sensitivity was modally (*N*=7) 1.4 kHz, otherwise mostly 1.2 kHz or 0.7–1.0 kHz. The common sensitivity peak (1.2–1.4 kHz) had a threshold sensitivity ranging from 15 kHz to 42 dB SPL (sound pressure level), commonly (*N*=8) 20–26 dB. In individual audiograms, a secondary sensitive peak occurred at 0.7–0.9 kHz and another one often occurred around 0.2–0.3 kHz.

Females yielded 15 audiograms. The best frequency was again modally 1.4 kHz (*N*=8) but was sometimes 1.6–1.8 kHz or 0.9–1.2 kHz. Threshold sensitivity at the 1.4–1.8 kHz peak ranged from 22 dB to 55 dB but commonly (*N*=7) was 22–31 dB. Individual audiograms with the sensitive peak at 1.4 kHz often contained a secondary sensitive peak at 1.8 kHz and another around 0.7–0.9 kHz.

Thus, the sexes did not differ in greatest sensitivity but differed a little in frequency response (Fig. 3). At the lower frequencies, males were more sensitive than females whereas at the higher frequencies females were more sensitive. This difference was statistically significant at 0.2 kHz (t_{23}=3.974; *P*=0.001) and 3.0 kHz (t_{13}=4.031; *P*=0.001).

These differences do not necessarily derive from a difference in ear function, let alone middle-ear function; they could arise more centrally. The function of the ear itself can be analysed using CM, as reported below.

Responses of the intact inner ear to aerial stimulation

When stimulating the ear by airborne sound and recording CM audiograms, likewise some individual variation occurred. In males (*N*=9), the best frequency was modally 1.4 kHz (*N*=5). Sensitivity at 1.0–1.4 kHz ranged from 14 dB to 53 dB but commonly (*N*=5) was 32–42 dB. Males with the best frequency at 1.4 kHz commonly had secondary sensitivity peaks at 0.8 kHz and 0.2–0.3 kHz.

In females (*N*=11), the best frequency was modally 1.4–1.6 kHz (*N*=5; in one audiogram the peak extended to 1.8 kHz). Sensitivity at the best frequency ranged from 30 dB to 54 dB but commonly

Table 1. Commonly measured parameters of the bullfrog middle ear

	RA (mm)	Mass (g)	TM area (mm^2)	Footplate area (mm^2)	Area ratio	Operculum area (mm^2)
Males						
Mean	136.7	233.4	161.8	3.44	47.6	4.84
s.d.	10.4	55.2	43.2	0.57	12.0	1.11
Range	115–155	140.7–311.1	101.1–260.0	2.4–4.32	26.2–66.6	3.03–6.86
N	20	19	20	20	20	19
Females						
Mean	133.2	195.2	87.0	3.3	26.6	4.85
s.d.	9.7	47.5	18.9	0.34	6.5	0.98
Range	118–146.5	123.8–275.5	60.8–127.1	2.79–3.9	18.3–36.7	2.86–6.1
N	15	13	15	15	15	11
P (*t*-test)	0.313	0.051	<0.001	0.409	<0.001	0.980

'Area ratio' is the ratio of the total tympanic membrane (TM) area to the columellar footplate area. *P* is the significance of the difference between the male and female samples, by two-tailed *t*-test for unequal variances. RA, rostrum–anus length.

Table 2. Occasionally measured parameters of the bullfrog middle ear

	RA (mm)	TM Mass (mg)	Extracolumella Length (mm)	Extracolumella Width, (prox.), (mm)	Extracolumella Mass (mg)	Extracolumella Lever ratio	Columella Length (mm)	Columella Mass (mg)	Footplate Mass (mg)	Operculum Area (mm^2)	Operculum Mass (mg)	Oval window Area (mm^2)
Males												
Mean	144.2	88.8	3.5	1.3	2.7	2.84	5.6	6.0	1.4	5.44	4.3	3.54
s.d.	7.5	43.6	0.4	0.4	0.6	1.31	0.5	1.9	0.5	1.32	1.5	0.29
Range	134–153	58.0–119.7	3.0–3.9	0.9–1.7	2.0–3.1	1.88–4.33	5.1–6.3	4.5–8.2	1.0–2.0	3.5–6.94	3.3–6.0	3.34–3.75
N	5	2	5	3	3	3	5	3	3	5	3	2
Females												
Mean	132.6	18.7	3.6	1.4	3.6	3.14	5.1	6.5	2.1	5.03	3.6	3.63
s.d.	10.8	n.a.	0.7	n.a.	n.a.	n.a.	0.7	n.a.	n.a.	0.94	n.a.	n.a.
Range	118–146.5	n.a.	2.8–4.4	n.a.	n.a.	n.a.	4.5–6.0	n.a.	n.a.	4.0–6.1	n.a.	n.a.
N	7	1	5	1	1	1	4	1	1	5	1	1
All												
Mean	137.4	65.5	3.6	1.32	2.9	2.92	5.4	6.1	1.6	5.24	4.1	3.57
s.d.	10.9	50.9	0.54	0.33	0.7	1.08	0.6	1.7	0.6	1.1	1.24	0.21
Range	118–153	18.7–119.7	2.85–4.4	0.9–1.7	2.0–3.6	1.88–4.33	4.5–6.3	4.5–8.2	1.0–2.1	3.5–6.86	3.3–6.0	3.34–3.75
N	12	3	10	4	4	4	9	4	4	10	4	3

'Lever ratio' is the ratio of extracolumella length to extracolumella vertical width at its proximal end. *P* is the significance of the difference between the male and female samples, by two-tailed *t*-test for unequal variances. No differences were statistically significant, and the samples are pooled at the bottom of the table. TM, tympanic membrane, RA, rostrum–anus length.

(*N*=5) was 35–44 dB. Individually, secondary sensitivity peaks occurred anywhere within 0.3–1.0 kHz.

By this method, as in the previous method, the sexes did not differ significantly in best frequency (t_{19}=1.129; *P*=0.273). Fig. 4 compares the means of male and female audiograms, obtained from those individuals that were also used in the subsequent experiments described below. There was some difference in frequency response: in the range of 0.2–1.6 kHz, the males were more sensitive in 6 out of 12 test frequencies, and in the range 1.8–4.0 kHz, the females were more sensitive than the males in 6 out of 9 test frequencies. Testing sexual differences at each frequency, using a *t*-test, none were significant (always *P*>0.09, after correction for multiple comparisons). By the run test, the sexes differed significantly (*P*<0.01).

Apparently with CM, as with brain responses, male and female audiograms do not differ in overall sensitivity but differ a little in frequency response, and this situation occurs already in the ear. Next, we tested whether the difference arose in the inner or middle ear or both, by applying mechanical vibratory stimulation as follows.

The appearance of sexual diergism

The inner ear was first stimulated by mechanical vibration as close as possible to the oval window, bypassing the middle ear, in the same males and females used in the preceding test. This was done by bisecting the columella (Fig. 5) and removing the distal fragment with the extracolumella and the excised TM. The needle tip of the vibrator was lined up with the axis of the osseous columella and brought into stable contact, and the vibrator was activated. The

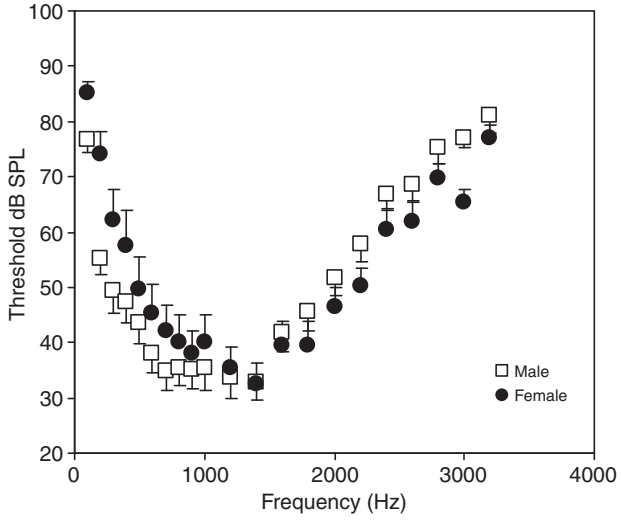

Fig. 3. Mean audiograms from brain responses (to aerial stimulation) of bullfrog males (*N*=15 but above 2.4 kHz not all were tested) and females (*N*=11 but above 2.8 kHz not all were tested). Bars show the standard error. SPL, sound pressure level.

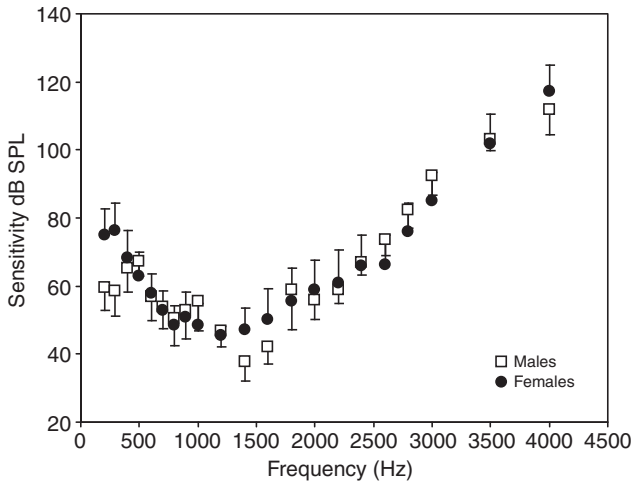

Fig. 4. Mean cochlear microphonics (CM) isopotential audiograms (from inner-ear responses to aerial stimulation) of bullfrog males (*N*=5) and females (*N*=5). These are the same individuals tested with vibratory stimulation (Figs 6 and 7). Bars show the standard error. SPL, sound pressure level.

control to this experiment is provided by the normal aerial audiograms of the same frogs (Fig. 4), with hardly a difference between the sexes. Unlike in this control, among the audiograms from direct vibrational stimulation of the columella stump (Fig. 6), the males were more sensitive than the females at 16 of the 20 test frequencies (validated by applying a run test to the means, $P<0.05$). Hence, for generating a CM response equalling that observed in the female, the male's ear required a much smaller vibration amplitude of the footplate than the female's. Because with aerial stimulation, sexually equal CM responses were elicited by sexually equal sound pressure at the TM, the question arises, how this sexual diergism arose.

The sexual diergism derives from the TM

Did the sexual difference in footplate amplitude derive from the ossicular chain with its mechanical leverage (Jørgensen and Kanneworff, 1998; Werner, 2003; Mason, 2007), from the TM with its sexually differing size or from both? To answer this, the intact ear was mechanically stimulated by applying a plastic cone topping the vibrator, from the outside to the centre of the TM (Fig. 5). This procedure neutralised the effects of TM size and middle-ear mass by the vastly overriding mechanical impedance of the vibrator but it should reveal differences in the function of the ossicular chain. At all frequencies but one, the males were more sensitive (required a smaller amplitude) than the females (Fig. 7); the significance was validated by a run test ($P<0.05$). Hence, the sexual difference between the audiograms obtained with vibratory stimulation of the columella stump seemed to derive largely from the differing TM (rather than leverage in the ossicular chain). In practice this experiment was performed on the same frogs before experiments involving amputation of the columella for stimulation at the columellar stump.

The audiograms from vibratory stimulation of the columellar stump (Fig. 6) were more sensitive, in their most sensitive range, than those from vibratory stimulation at the TM centre (Fig. 7). The difference approximated 20 dB in both sexes. This mechanical leverage is discussed elsewhere (Werner, 2003).

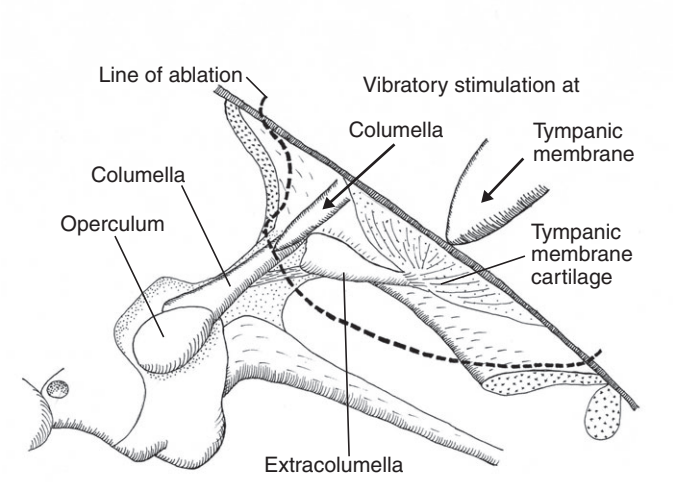

Fig. 5. Scheme of a transverse section through the head of a bullfrog at the level of the ear, right side, viewed from behind, as in fig. 3 in Werner (Werner, 2003), showing the two modes of vibratory stimulation (shown by thicker arrows): stimulation at the centre of the tympanic membrane (TM), applying a plastic cone topping the vibrator, and stimulation of the columellar stump by a needle topping the vibrator. The broken line indicates the extent of surgical resection that enabled access to the columella. Modified from Werner (Werner, 2003).

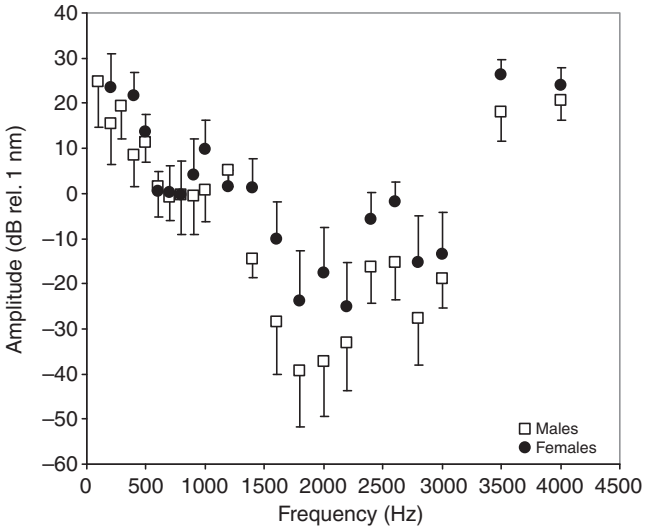

Fig. 6. Mean cochlear microphonics (CM) isopotential audiograms obtained with vibratory stimulation at the oval window (*via* the columellar stump), of the same individuals as in Figs 4 and 7. The site of the active electrode has not been changed. Bars show the standard error.

Effects of loading the middle ear

Finally, we hypothesized that the somewhat lower frequency response of the male's ear with its larger TM would be due to the lower resonating frequency that would characterize a larger (Table 1) and, especially, heavier (Table 2) TM. We tested this hypothesis by manipulating the mass through additional loading. A medium-sized TM of a male weighed ~40 mg more than a medium-sized TM of a female (Table 2). Therefore, we prepared metal weights of 20, 40 and 81.2 mg. In experiments with a loaded TM, a weight was attached by a speck of stopcock grease to the centre of the TM from outside. All weights were much smaller than the central thickened

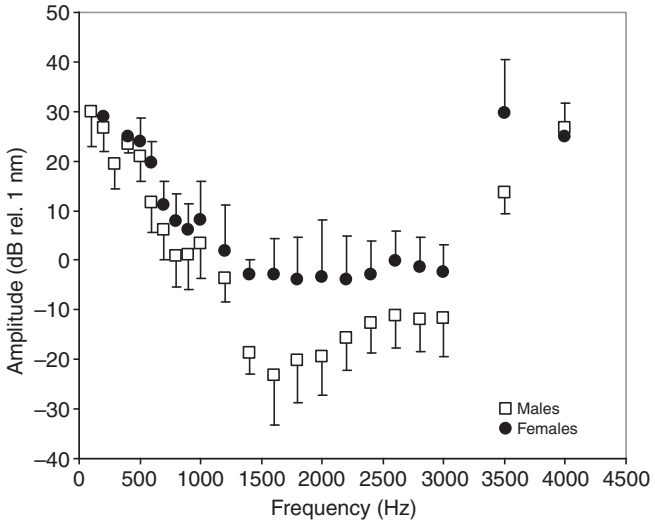

Fig. 7. Mean cochlear microphonics (CM) isopotential audiograms obtained with vibratory stimulation at the tympanic membrane, of the same individuals as in Figs 4 and 6, at the same site of the active electrode.

zone of the TM and hence interfered little with its sound reception and mobility.

We obtained 12 audiograms by aerial stimulation of variously loaded intact TMs as follows: 20 mg, 1 male, 3 females; 40 mg, 4 males, 2 females; 81.2 mg, 2 females. As a control, each run with load was both preceded and followed by a load-less run. These two control audiograms were always almost identical; therefore, the ears were not damaged by the loading.

The effects of loading are exemplified from one frog in Fig. 8. In 11 of the 12 experiments, loading (with 20, 40 or 81.2 mg) caused two changes. First, some lowering of the frequency response, sometimes with improvement of the sensitivity at 0.1–0.2 kHz. Second, reducing the sensitivity, especially in the frequency ranges 0.4–0.8 kHz and 1.6–3.0 kHz. Specifically, with a load of 20 mg, results were irregular; the most consistent sensitivity losses were: at 0.8 kHz, a loss of sensitivity by ≤22 dB; at 1.8–2.6 kHz, losses of ≤25 dB. With a 40 mg load, at 0.07–0.1 kHz there was no effect; at 0.2 kHz, a gain of ~10 dB; at 0.4–0.8 kHz, losses of ≤34 dB; at 0.9–1.6 kHz, losses were only 2–14 dB; at 1.8–3.0 kHz, losses of ≤38 dB and at higher frequencies the results varied. In both frogs tested with 81.2 mg, there was an ~10 dB gain at both 0.1 kHz and 0.2 kHz; responses up to 1.0 kHz resembled those with a 40 mg load but at higher frequencies the loss tended to be greater than with 40 mg, often being 35–40 dB.

The variation in these results may reflect both the normal individual variation and variation in the placement of the loads, despite efforts to place these centrally. No sexual difference was found, perhaps due to these variations and the small number of experiments. But both effects were clear. First, loading lowered the frequency response, making the bullfrog ear more ‘masculine’. Apparently low-frequency bullfrog ears owe this attribute largely to the low resonating frequency of a heavy TM, be it the large TM of a male or one experimentally loaded. Second, loading reduced the ear’s sensitivity, i.e. in response to a given areal sound pressure, the loaded TM vibrated at reduced amplitude and velocity, supporting the above interpretation.

The effects of loading *R. catesbeiana* ears accord in principle with the experience from loading human middle ears (Nishihara et al., 1993).

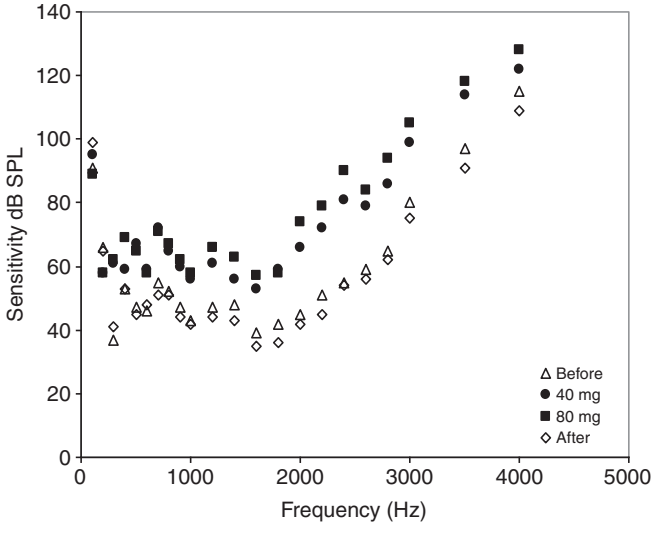

Fig. 8. An example of an experiment with loading the bullfrog tympanic membrane. Four cochlear microphonics (CM) isopotential audiograms from inner-ear responses to aerial stimulation were successively obtained from one individual, female F-994 [128 mm rostrum–anus length (RA)]. Open symbols show the normal control runs before and after the runs with loaded tympanic membrane, as further explained in the text. SPL, sound pressure level.

DISCUSSION

Allometry of sexual dimorphism

The sexual size difference in the bullfrog’s TM develops gradually with age. Body growth is accompanied by positive allometric growth of the TM, and the regression slope of TM width over body size is steeper for males than for females (Iwasawa, 1968; Boatright-Horowitz and Megela-Simmons, 1995). Hetherington extended the investigation to the internal middle-ear parts but without detailing the sexes [see fig. 21.4–21.5 in Hetherington (Hetherington, 1992)]. He noted that with growth, the TM increases and the operculum decreases relative to the columellar footplate. Mason et al. presented the allometric regressions of TM, stapedial footplate and operculum areas over RA and showed that these produce an area ratio (TM-to-footplate) that increases during ontogeny (Mason et al., 2003).

Likewise, we lack sufficient data to test whether the ossicular chain also showed significant sexual dimorphism. From Table 2, any such dimorphism would be minor. But a recent study of the osseous columella of the bullfrog found that, parallelling the dimorphism in TM area, the columella of males is significantly more robust: its width to length ratio averages 0.22 compared with 0.17 in females. This may relate to sustaining and transmitting force from the TM (Chipman, 1996; Werner et al., 1997; Werner, 2003).

Although the dimorphism in the size of the TM cartilage cushion is obvious to the unaided eye [see fig. 2 in Mason et al. (Mason et al., 2003)] (Fig. 1), in the current study, we present the first evidence for the sexual difference in TM mass, although derived only from two males and one female (Table 2). Mason (Mason, 2007) attributed this information to Purgue (Purgue, 1997), Mason et al. (Mason et al., 2003) and Werner (Werner, 2003) but none of these previous studies had expressly referred to this issue. Moreover, in Purgue’s drawing [see fig. 1A,D in Purgue (Purgue, 1997)], the cartilage in the female is the same size as the cartilage in the male despite her smaller TM.

Sexual diergism of hearing in the bullfrog: the frequency domain

Sexual diergism in frog ears has been addressed before, with emphasis on the frequency domain. A difference in TM size, whether age dependent or sexual (then often accompanying dimorphism in body size, males being smaller), has often been invoked to explain differences in frequency response (Loftus-Hills, 1973; Wilczynski et al., 1984; Ryan, 1988a). The mechanism behind such correlations has been investigated by diverse methodologies (Majeau-Chargois and McDanall Whitehead, 1971; Chung et al., 1978; Chung et al., 1981; Pettigrew et al., 1981; Hetherington, 1992; Boatright-Horowitz and Megela-Simmons, 1995). At another level, Moffat and Capranica (Moffat and Capranica, 1978) suggested that due to the size effect on TM resonance, the middle ear may act as a first frequency filter, as suggested by Manley for lizards (Manley, 1972).

However, in most of these previous studies, the function of the middle ear was not unambiguously separated from possible participation or effects of the inner ear (including its mechanically loading the middle ear). Only Pettigrew et al. (Pettigrew et al., 1981) conclusively demonstrated an independent role of the middle ear but their study did not extend to sexual dimorphism in ear size.

Our results, from both types of potentials, found a small sexual difference in *R. catesbeiana* ears, with the best frequency of males at 1.0–1.4 kHz but at 1.4–1.6 kHz in females, in line with the trend indicated by previous studies (Shofner and Feng, 1981; Hetherington, 1994b). Our observation of lesser sensitivity peaks at 0.7–0.9 kHz and (at least in males) 0.2–0.3 kHz accords with the presence of three populations of auditory neurones in the *R. catesbeiana* ear, with best frequencies around 0.3 kHz, 0.6–0.7 kHz and 1.3–1.4 kHz (Frishkopf and Goldstein, 1963; Potter, 1965b; Frishkopf et al., 1968; Feng et al., 1975). The fact that in each of these populations the neuronal tuning curves vary widely in sensitivity and frequency helps to explain the inconspicuousness of the corresponding peaks in our averaged audiograms.

Sexual diergism of hearing in the bullfrog: the sensitivity domain

The marked sexual dimorphism of the TM has attracted some specific investigations of *R. catesbeiana*. In earlier reports on CM audiograms of *R. catesbeiana*, the higher-frequency peak was less sensitive than the lower-frequency sensitive region (Strother, 1959; Strother, 1962; Capranica et al., 1966). Wever also presented a bullfrog CM audiogram in which the low frequencies (0.2–0.4 kHz) were up to 10 dB more sensitive than the high frequencies (1.5–2 kHz) (Wever, 1985). This variation between our results is difficult to evaluate because these reports lacked details of the frogs, the season and the experimental temperature.

Initially, single unit studies failed to find sex or age sensitivity differences in *R. catesbeiana* (Frishkopf et al., 1968) or some other anurans [R. R. Capranica and A. J. M. Moffat, unpublished, in p. 554 of Capranica (Capranica, 1976)]. Presumably in order to discern sexual differences, a prohibitively large number of units would be required from the basilar papilla of any one ear. But the numbers that are usually available averaged only 6–11 per total ear (Frishkopf and Goldstein, 1963; Feng et al., 1975).

Mason reviewed the most recent morphometric and vibrometric comparisons of male and female bullfrog ears but reported no full explanation for the similarity in auditory sensitivity in the face of morphological differences (Mason, 2007).

Our application of several methodologies to the same ears enables a fresh exploration of the dimorphism and diergism of the *R. catesbeiana* ear. We found that whereas with airborne sound stimulation equally applied to both sexes, the auditory responses of the sexes are similar, when applying vibratory stimulation the male ear requires a much smaller vibratory amplitude. Hence, in the male a given sound pressure causes a smaller vibrational amplitude than in the female. This seems to be due to attenuation by the heavy mass of its TM cartilage, as corroborated by the experiments with artificial loading of the TM. In stimulation of the inner ear, the smaller vibration amplitude of the male's footplate presumably compensates for the greater pressure that is due to the greater area ratio, perhaps further augmented by the size factor proposed by Werner et al. (Werner et al., 2008).

Comparative functional aspects

Some authors have addressed the correlations of auditory function with body size and TM size. Saunders and Johnstone have suggested that larger anurans possess better auditory sensitivity (with lower high-frequency cutoff) because of better impedance matching (Saunders and Johnstone, 1972). Capranica [see p. 554 in Capranica (Capranica, 1976)] argued that impedance matching is governed by the areal ratio and not by absolute TM size, and the functional proportion must be maintained in evolution, despite changes in body size. Hetherington too noted that tympana of larger anurans are more sensitive to sound than those of smaller species (Hetherington, 1992). He hypothesised that whereas the tympanic ear is important in larger animals, it becomes less effective as body size decreases, to the extent that some small species lack a TM. This morphological and functional scenario recurs in ontogeny, as in froglets the opercular system develops first and the tympanic system develops later, perhaps towards sexual maturity (Smirnov and Vorobyeva, 1988). Then, in ontogeny, positive allometry of the TM enlarges the area ratio during growth [see fig. 5 in Vorobyeva and Smirnov (Vorobyeva and Smirnov, 1987)]. Hetherington's hypothesis may explain why sexual dimorphism of TM size apparently evolved mainly in the larger species of frogs, as is the situation among North American *Rana* (Wright and Wright, 1949). Altogether, the recent conclusion of Werner et al. (Werner et al., 2008) that the function of the TM is affected by its absolute size, may help to explain the phenomena discussed.

The system that we have confirmed in *R. catesbeiana*, involving an enlarged TM in the male with related improvement of low-frequency hearing, differs from that which seems to be most common among anurans: a larger female possessing a larger TM in absolute terms and lower-frequency sensitivity in the audiograms (Loftus-Hills, 1973). However, no sexual diergism of TM motion occurs in *Rana esculenta* ears, a species lacking sexual TM dimorphism (Anson et al., 1985).

Behavioural, ecological and evolutionary aspects

The evolution of sexually dimorphic and diergic tympana was conventionally considered to depend on communicatory factors of ecological significance. Hypothetically, five potential factors come to mind, which are not all mutually exclusive.

(1) Visual signal. Conceivably the enlarged male TM could, in addition to any acoustic role, serve as an optical signal identifying the sex. Three lines of circumstantial evidence could support this hypothesis; if the dimorphic species were more diurnal than others, if their TMs were coloured more conspicuously than in others or if they were less vocal. We have no such supporting data for the bullfrog. However, in those North American *Rana* species in which the TM is dimorphic, the colouration of the throat also differs between the sexes (Wright and Wright, 1949; Stebbins, 1985). Similarly, a role as a visual signal has been discussed for the seasonal papilla on the male TM of the African ranid *Petropedetes parkeri* (Narins et al., 2001).

(2) Improving auditory sensitivity. In *R. catesbeiana*, the male's ear is more sensitive than the female's ear in the frequency range of the amphibian papilla. In this species, the amphibian papilla hears (among other things) the low-frequency component of the male's calls (Capranica, 1976). So, could this improved sensitivity constitute the selective advantage driving the evolution of dimorphic *Rana* ears? As already mentioned, the dimorphic species tend to be large. In principle, larger species are expected to be more widely spaced (Wynne-Edwards, 1962). Making the voice more intense to bridge distances would be energetically expensive (Prestwich et al., 1989) and could attract predators. Thus, the improved sensitivity might serve social functions, including spacing and territorial relationships. Unfortunately we lack comparative data on social structure among dimorphic *versus* other *Rana* species to evaluate this hypothesis.

(3) Tuning the ear to a desirable frequency. Frogs communicate vocally (Schneider, 1990) and many, including *R. catesbeiana*, possess rich vocal repertoires (Capranica, 1968; Hoff and Moss, 1974). However, their auditory spectrum is relatively narrow (Wever, 1985). Voice and auditory frequencies should match and

differ between taxa. This is so for environmental and communicatory reasons (Hödl, 1977; Ryan, 1988b), coupled with the fact that acoustic advertisement is energetically expensive.

Indeed, anuran voice frequencies differ between syntopic breeders (Hödl, 1977) or between related populations (Joermann et al., 1988; Egiasarian and Schneider, 1990), and audiograms match voice frequencies in comparisons among species (Loftus-Hills, 1973) and within species (Brzoska et al., 1977; Schneichel and Schneider, 1988) or even covary among populations within a species (Capranica et al., 1973; McClelland et al., 1998). Other reports show that frogs recognise calls of the same species or population, although the acoustical identification cues are not always defined by frequency alone (Ryan, 1983; Nevo and Capranica, 1985). The ability of neurones in the torus semicircularis to identify calls is also not merely based on frequency (Diekamp and Schneider, 1988).

The matched frequencies of voice and of hearing often show a (negative) correlation to body size, so that the match may result from the parallel effects of size on resonance frequency in both organs (Hetherington, 1992). Nevertheless, in *Acris crepitans* variation in call frequency peak matches that in best auditory frequency, irrespective of body size, (Ryan and Wilczynski, 1988).

Hetherington pointed out that the bullfrog male TM's sensitivity to 0.2 kHz would improve the perception of the mating calls of other males (Hetherington, 1994b). But possibly the frequency matching occurs between adult *R. catesbeiana* males and females; females seem to have lower dominant frequencies in at least territorial, release, warning and distress calls (Capranica, 1968). Moreover, generally larger conspecific individuals have lower call frequencies, as reviewed by Martin (Martin, 1972), and in *R. catesbeiana* females reach 200 mm RA whereas males reach 180 mm RA. Presumably the larger male TM is tuned relatively more to the female voice and *vice versa*. Apparently the species of *Rana* with sexually dimorphic TMs become reproductively mature only after the dimorphism has become obvious (Martof, 1956; Hedeen, 1972). An inverse correlation of voice frequency with TM size is conspicuous among African *Bufo* (Tandy and Keith, 1972).

(4) Tuning the ear away from harmful sound. In birds and mammals, the middle-ear muscles apparently protect the ear from the individual's own vocalisations, as reviewed by Saunders et al. (Saunders et al., 2000). A similar arrangement occurs in vocalising lizards and geckos (Wever, 1978). According to Wever (see p. 65 in Wever, 1985), the comparable structure in frogs functions even more effectively. Nevertheless, the same end could conceivably be served by a permanent mismatch between an individual's voice and ear. But the voices of the species of *Rana* with sexually dimorphic TM are no louder than those of other anurans (Gerhardt, 1975) and their ears are no more sensitive than those with sexually isomorphic tympana (Wever, 1985).

(5) In *R. catesbeiana*, most of the call energy is actually radiated through the TM that functions as a post-glottal filter, depending on its resonating characteristics (Purgue, 1997). Thus, the vocal and auditory frequencies can be matched directly by the TM. Vocalisation, rather than audition, may be the main selective advantage driving the size increase of the male TM (Mason, 2007). Then, the increased area ratio could have an undesirable side effect of over-matching of impedance, when excessive input pressure foils correct impedance matching and sound transfer becomes suboptimal (Capranica, 1976; Haughton, 2002). The over-matching is probably even greater than that apparent from the sheer area ratio, if the recent proposal that a larger TM should be relatively more mobile (Werner et al., 2008) applies. We suggest that the over-matching is prevented by the heavy central cushion of the male TM, weighing about five times that of the female TM, and significantly reducing the amplitude and velocity of TM vibration in response to areal sound (as shown in experiments with artificially loaded TMs). Presumably the product of area ratio and velocity (or amplitude) is similar in the two sexes and similarly stimulates the inner ear. There is no reason to suspect a sexual difference in the inner ear or to doubt the conventional views of middle-ear mechanics (Mason et al., 2003).

Conclusions

The sexual differences in the ear of the American bullfrog, *R. catesbeiana*, go beyond TM area. In adults, the mean TM area is 162 mm^2 in males (averaging 137 mm RA length) but 87 mm^2 in females (averaging 133 mm RA length). This sexual size dimorphism in the TM develops gradually with the ontogenetic growth of the frog.

This sexual size dimorphism in the TM may be accompanied by subtle differences in other middle-ear components; at least, the male's columella is more robust.

In *R. catesbeiana*, the electrophysiological isopotential audiograms based on CM closely resemble the audiograms based on multi-unit responses from the brain.

In *R. catesbeiana*, the audiograms (based on either potential) show little sexual diergism and only in the frequency domain. The male's audiogram is more sensitive than the female's at the lower sound frequencies but less sensitive at the higher frequencies.

This sexual diergism derives from the sexual dimorphism in size and mass of the TM.

The male's enlarged TM, unaccompanied by similar enlargement of the columellar footplate, creates an excessively greater area ratio. This seems to be offset by the heavy cartilage cushion on the male's TM that reduces the TM's vibration in response to sound. Consequently the product of area ratio and footplate vibration amplitude would result in similar stimulation in the two sexes.

LIST OF ABBREVIATIONS

BT	body temperature (rectal, unless specified)
CM	cochlear microphonics, i.e. alternating potentials (frequency following) of the inner ear
RA	rostrum–anus length (Werner, 1971)
SPL	sound pressure level
TM	tympanic membrane

The experiments were made while Y.L.W. was a DAAD-supported visitor in the Institut für Zoologie der Universität Bonn, and Visiting Fellow in the Department of Psychology, Princeton University (1975); he remains indebted to Jerry Palin and the late E. Glen Wever for manifold support. The report was drafted while he was a guest scientist at the Institut für Zoologie der Technischen Universität München (Garching) under the auspices of the Sonderforschungsbereich 204 (Gehör) (1997); and he thanks Geoff Manley and the whole institute for a supportive environment. M.S. gratefully acknowledges the hospitality of Katia Tielbörger and the Abteilung Vegetationsökologie, Institut für Botanik, Universität Tübingen. We thank Hobart Smith for suggesting the term 'diergism' (on 1 November 1977), Nurit Sharet for redrawing Figs 2 and 5, and Belinda McClelland and Jim Saunders for thoughtful comments on earlier drafts. Dedicated to the memory of E. Glen Wever, 16 October 1902 – 4 September 1991, pioneer, teacher and friend.

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