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Vibrometric studies of the middle ear of the bullfrog *Rana catesbeiana*

I. The extrastapes

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Summary

Laser vibrometry was used to measure the vibration velocity at different points on the ossicular apparatus of the bullfrog *Rana catesbeiana* in response to free-field sound. The ascending process of the extrastapes, neglected in most accounts of frog middle ear mechanics, supports a rocking motion of the extrastapes and is critical to the normal function of the ossicular apparatus. The articulation between extrastapes and the bony stapes shaft acts as a hinge, although movement at this hinge is usually small. The ratio of tympanic membrane to footplate vibration velocity is significantly greater in male frogs than in female frogs. Differences in this ratio between male and female frogs are probably mainly due to flexion between the extrastapes and stapes rather than to differences in the coupling between tympanic membrane and extrastapes. It is argued that flexibility in the ossicular system represents a protective mechanism in frogs, and functional analogies are drawn between the stapes/extrastapes system of frogs and the tri-ossicular system of mammals.

Key words: middle ear, bullfrog, *Rana catesbeiana*, lever ratio, stapes, ear evolution, hearing.

Introduction

When the tympanic membrane of the mammalian middle ear is inflected, the manubrium of the malleus is pushed inwards. As a result, the malleus and incus rotate, and the stapes, which is articulated with the incus, is pushed into the oval window (the entrance to the inner ear). Because of the difference in lever arm lengths between the malleus and incus, the stapes moves with a lower velocity than the tip of the manubrium. The velocity ratio of the middle ear is here defined as the velocity amplitude of the centre of the tympanic membrane divided by the velocity amplitude of the stapes footplate. A mechanical lever arrangement, resulting in a velocity ratio greater than one, is thought to be common to all tetrapods (Saunders et al., 2000). The velocity ratio contributes to the impedance-matching function of the middle ear (see, for example, Dallos, 1973).

Non-mammalian vertebrates lack a malleus and an incus. In typical frogs, such as the American bullfrog *Rana catesbeiana*, the vibrations of the tympanic membrane are conveyed to the oval window by means of the extrastapes and stapes (also known as the extracolumella and columella) (Fig. 1). The extrastapes, or pars externa plectri, is a cartilaginous element articulated distally with the inside of the tympanic membrane and proximally with the pars media plectri, the bony shaft of the stapes proper. The extrastapes is also attached to the skull by means of a strap-like cartilaginous process, the ascending process (processus ascendens plectri), which is articulated with the ventral side of the parotic crest (Wever, 1985). The pars media expands medially, where it is continuous with the thick, cartilaginous pars interna. This proximal portion of the stapes is known as the footplate and is contained within the rostral half of the oval window. Just ventrolateral to the pars interna, a ridge of the pars media articulates with the otic capsule (Bolt and Lombard, 1985; Jaslow et al., 1988; Hetherington, 1992). The caudal half of the oval window is occupied by the operculum, a cartilaginous or bony element unique to amphibians.

Jørgensen and Kanneworff (1998) used laser vibrometry to study the middle ear lever mechanism of the grass frog (*Rana temporaria*). The stapes footplate was found to rock about the articulation between the pars media and otic capsule (referred to henceforth as the ‘footplate axis’; Fig. 1), resulting in a phase difference of 180° between the tympanic membrane and footplate at low vibration frequencies. The vibrometrically measured velocity ratio of the grass frog was more than 10 dB higher than the value expected if the extrastapes and stapes were to vibrate as a stiff unit firmly tethered to the tympanic membrane. Jørgensen and Kanneworff (1998) argued that the discrepancy between vibrometric and anatomically predicted values could be accounted for by a drop in velocity amplitude between the tympanic membrane and extrastapes due to the

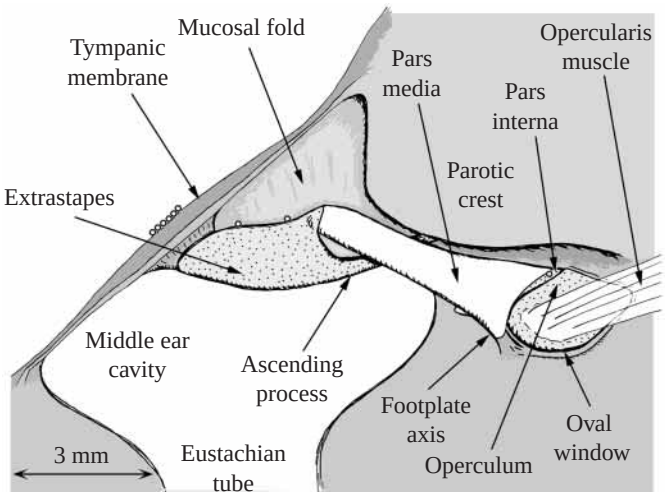

Fig. 1. Diagrammatic transverse section of the left middle ear of a female bullfrog, seen from the caudal direction: lateral is towards the left. Cartilage is stippled. The pars media and pars interna are collectively referred to as the stapes. The hinge-like articulation between the pars media and the otic capsule, along the ventral rim of the oval window, forms the 'footplate axis'. Reflective beads, representing points from which laser measurements were made, are represented by small circles on the tympanic membrane, extrastapes and stapes footplate. The tympanic membrane of a male frog would be greater in diameter and would have a large, fatty pad at its centre.

angle between them, together with bending of the extrastapes. Moffat and Capranica (1978), apparently by analogy with Manley's (1972a) model of the reptile and bird ossicular system, suggested that the extrastapes in frogs might be hinged at its connection to the pars media, which could also increase the velocity ratio, but Wilczynski and Capranica (1984) asserted that this articulation is stiff. It would appear that the motion of the extrastapes of frogs has never been directly investigated.

In the present study, the movement of the extrastapes was experimentally examined with regard to elucidating the function of this structure. Vibrometric measurements of the extrastapes shaft were made, apparently for the first time in frogs. These measurements allowed direct examination of the hypotheses that the vibration velocity ratio is increased by means of the connection between tympanic membrane and extrastapes, by bending of the extrastapes or by a hinge-like motion between the extrastapes and pars media. The role of the ascending process of the extrastapes, almost totally ignored in previous studies of middle ear function, was also considered.

Materials and methods

Laser vibrometric measurements were obtained from 12 male and six female bullfrogs *Rana catesbeiana* (Shaw, 1802), ranging from 151 to 308 g in body mass; snout–vent length (SVL) 121.7–145.1 mm. An additional 11 male and 11 female bullfrogs (mass 165–270 g, SVL 113.4–140.3 mm) were dissected but were not examined with laser vibrometry. These animals were obtained as corpses from other projects. Data from some of these frogs were also used as part of the companion study (Mason and Narins, 2002). All animals were purchased from commercial suppliers (Rana Ranch, Twin Falls, ID, USA; Carolina Biological, Burlington, NC, USA; W. A. Lemburger Co., Oshkosh, WI, USA).

Vibrometric measurements were made on the left ear only of each frog. The animal was anaesthetised with intramuscular injections of pentobarbital sodium solution (Nembutal; Abbott Laboratories) (50 mg ml^{-1}, 1.2 μl g^{-1} body mass) and ketamine (Ketaject; Phoenix Scientific) (100 mg ml^{-1}, 1.2 μl g^{-1} body mass). Smaller supplementary doses were given as necessary to maintain a state of areflexia. The mouth cavity was examined to ensure that the Eustachian tubes were free of mucus. Throughout surgery and the experimentation procedure, the frog was sprayed regularly with water to facilitate cutaneous respiration.

The surgical approach to the stapes footplate involved removing a small, square piece of skin from just rostral to the left suprascapular cartilage. Part of the m. depressor mandibulae was cut away, and the operculum was exposed by clearing away the connective tissue around the m. operculari. Some of the cranial nerves passing over the operculum were also usually removed, as was part of the jaw adductor musculature. Care was taken to avoid damage to the vena capitis lateralis, which crosses the stapes and lateral part of the operculum. The caudal part of the shelf formed by the prootic bone and parotic crest, which overlies much of the pars media and pars interna of the stapes, was shaved away with a sharp scalpel. By these means, the caudomedial part of the stapes footplate was exposed. Since the middle ear cavity extends medially only approximately half-way along the length of the pars media of the stapes, the cavity was not breached in this surgical approach.

A small square (approximately 0.1–0.25 mm^2) of reflective glass beads mounted on adhesive backing material (Polytec) was positioned on the centre of the left tympanic membrane. One or two glass beads, 40–60 μm in diameter, were positioned on the exposed caudomedial part of the footplate near its articulation with the operculum. Later dissection (following experimentation) showed that the beads were situated approximately three-quarters of the length of the footplate away from its rotatory axis, nearly always on the pars interna rather than the pars media. Beads were also placed on the parotic crest, an exposed region of the skull just rostral and dorsal to the stapes footplate. In some experiments, the extrastapes was later exposed by removing a small flap of tympanic membrane dorsal to its centre and reflection or excision of the connective tissue and blood vessels running between the extrastapes and the tympanic membrane. Reflective beads were placed in up to three positions on the dorsal surface of the extrastapes.

The anaesthetised frog was propped in what approximated an upright, sitting position by means of a piece of foam positioned under the pectoral girdle. The head was inclined, snout tilted slightly downwards, to an angle of approximately

20° below the horizontal to give access to the footplate from a vertical approach. In some experiments, a second piece of foam was positioned under the tip of the snout to support the head. The animal was placed on a vibration-isolated table (Backer-Loring Micro-g) within a double-walled, sound-attenuating chamber (IAC 1202-A). A single-point, He–Ne laser vibrometer sensor head (Polytec OFV-303) was positioned approximately 60 cm from the animal's head, and a binocular light microscope (Zeiss OP-1), its objective lens 22 cm above the head of the frog, was also resting on the table. The laser beam, if inclined at an angle of approximately 25° to the horizontal, could be aimed at the tympanic membrane. The velocities of movement of all other structures were recorded using a vertical beam. A prism was mounted in the microscope such that the beam, directed into the side-port of the barrel, could be reflected downwards onto the frog. The angle of the reflected laser beam deviated by up to 4° from the vertical according to the position of the laser point within the field of view of the microscope, but any effect of this deviation was neglected in the subsequent calculations for simplicity. The position of the laser point could be monitored through the microscope. A 10 cm diameter speaker (Analog and Digital Systems Inc. 300) was positioned in front of the frog with the centre of its cone 75 cm from the centre of the frog's interaural axis, at an azimuth of 30° to the midline and an elevation of 13°. The speaker was not resting on the same table as the frog. A probe microphone (Knowles Electronics, type EK-3033) was positioned approximately 2 mm from the centre of the tympanic membrane. The rest of the apparatus was located outside the sound-attenuating chamber.

Pure tones (2 s duration, from 180 Hz to 3 kHz in 30 Hz steps), were synthesised by a custom-designed program (Acoustic Analyzer 0.20β: author A. Purgue, 1999) running on an Apple Macintosh iMac computer. The output of the computer's 16-bit D/A board was amplified (Optimus MPA-50) and sent to the speaker; a returning signal from the amplifier was attenuated (Hewlett-Packard 350D or Agilent 355D) and sent back to the computer, where it was used as a reference signal for the phase measurements. The same software was also used to monitor and record the returning signal from either the probe microphone or the laser vibrometer. The sampling frequency was 44.1 kHz. The probe microphone was connected to a custom-made AC amplifier (J. Wang), the output from which was bandpass-filtered (Krohn-Hite 3323 active filter) with 100 Hz and 3500 Hz cut-off frequencies. The filter output was attenuated (Hewlett-Packard 350D or Agilent 355D and 355C array) and sent to the computer. The computer program automatically adjusted the sound pressure level (SPL) measured at the probe microphone at each successive frequency to 90 dB SPL. The sound pressure level produced, having been adjusted for the probe microphone's calibration curve, was flat ±3 dB from 180 to 3000 Hz at the position of measurement. To measure the harmonics produced by this experimental arrangement, a microphone (Brüel and Kjaer 4134) was positioned where the frog would be and was attached *via* a heterodyne analyser (Brüel and Kjaer 2010) to a fast Fourier transform (FFT) network analyser (Stanford Research SR770). For the velocity measurements, the probe microphone was removed. The laser sensor head was connected to a vibrometer controller processor (Polytec OFV-3001), which produced a voltage output proportional to the vibration velocity of the structure being measured. The output from the vibrometer controller was fed to the computer *via* the filter and the attenuator.

Comparisons of the tympanic membrane responses were made at the beginning and end of every experiment. In experiments in which the tympanic membrane was intact, the responses remained very consistent. Three consecutive measurement runs were usually made from each structure being examined, and each set was averaged. In experiments in which the tympanic membrane was perforated to give access to the extrastapes, its response often changed slowly over time as a result of drying. In these experiments, single measurement runs of each structure in turn were made, generally in the order tympanic membrane – extrastapes – footplate – parotic crest – tympanic membrane. Three sets of runs were obtained in this manner, and data were compared. Velocity ratios obtained in this way were very consistent despite small changes in absolute responses. The velocity amplitudes considered are peak values.

The response that could be measured from the stapes, following the surgical approach outlined earlier, was the vertical component of the movement of the footplate relative to the otic capsule, superimposed onto the response of the otic capsule itself (the 'background vibration'). The response of the parotic crest was taken to be representative of the response of the otic capsule. The velocity of the footplate within the oval window (V_{FPVa}) was calculated by subtracting the velocity of the parotic crest from the velocity of the footplate. The measured responses were both assumed to be pure sinusoids, and the movements of the stapes footplate were assumed not to affect the response of the parotic crest. In the calculation below, V_{FPV} is the vertical component of the velocity of the stapes footplate at frequency ω and time t; its amplitude is a mm s^{-1}. V_{PCV} is the vertical component of the velocity of the parotic crest at the same frequency, of amplitude b mm s^{-1}. θ_1 and θ_2 are the phase lags of these two structures, respectively, relative to the signal returning from the speaker. It can be shown that:

$$V_{FPV} - V_{PCV} = V_{FPVa} = c\sin(\omega t + \theta_3)\,, \tag{1}$$

where

$$\tan\theta_3 = \frac{a\sin\theta_1 - b\sin\theta_2}{a\cos\theta_1 - b\cos\theta_2} \tag{2}$$

and

$$c = \frac{a\sin\theta_1 - b\sin\theta_2}{\sin\theta_3}\,. \tag{3}$$

These equations were used to control for the vibration of the head when calculating velocity ratios involving the stapes footplate. Because of the ossicular lever, the response of the extrastapes was so much greater than that of the footplate

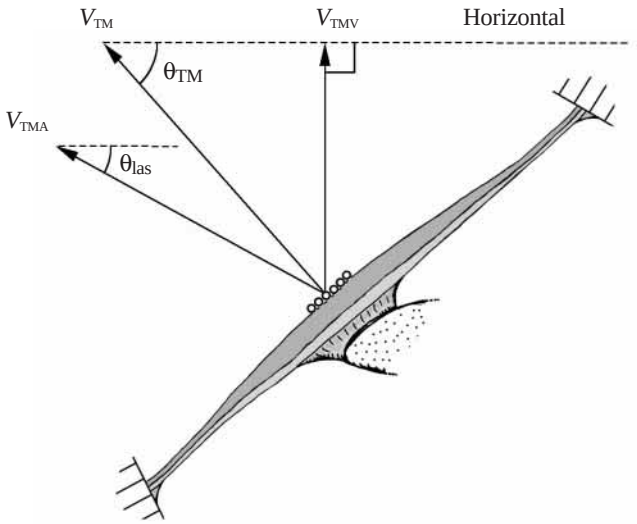

Fig. 2. Laser measurements of tympanic membrane vibration velocity. Two components of the tympanic membrane velocity were measured, the vertical component V_{TMV} and an angled component, V_{TMA}. These components, together with the angle θ_{las} between V_{TMA} and the horizontal, were used to calculate the true tympanic membrane velocity, V_{TM}. θ_{TM}, angle between V_{TM} and the horizontal. See text for details.

at most frequencies that this control was not considered necessary. The response of the tympanic membrane was found to be up to 50 dB greater than that of the skin just caudal to the membrane (data not shown); the skin velocity was therefore considered negligible, and no adjustments were made to the tympanic membrane response either.

The tympanic membrane of the bullfrog is inclined at an angle of approximately 50° to the horizontal plane in both sexes (M. J. Mason and P. M. Narins, personal observation). The experimental apparatus permitted the measurement of the component of membrane velocity at an angle (θ_{las}) of approximately 25° to the horizontal (Fig. 2). This component of velocity is here termed V_{TMA}. By deflecting the laser beam with the prism, the vertical component of tympanic membrane velocity, V_{TMV}, could also be measured. The two components V_{TMA} and V_{TMV} can be used to establish the true tympanic velocity, V_{TM}. V_{TM} is the maximum velocity of the centre of the tympanic membrane and is expected to be normal to the plane of the membrane. Let us call the angle between V_{TM} and the horizontal θ_{TM} (see Fig. 2). In this case, the angled laser will record a velocity:

$$V_{TMA} = V_{TM}\cos(\theta_{TM} - \theta_{las}) , \quad (4)$$

whereas the vertical laser, deflected through the microscope, will record a velocity:

$$V_{TMV} = V_{TM}\sin\theta_{TM} . \quad (5)$$

Solving for V_{TM}:

$$V_{TM} = \sqrt{\left(\frac{V_{TMA} - V_{TMV}\sin\theta_{las}}{\cos\theta_{las}}\right)^2 + V_{TMV}^2} . \quad (6)$$

Equation 6 was used to examine whether the tympanic membrane velocity component measured with an angled laser was a good measure of its true velocity.

Following each experiment, the frog was euthanized with an overdose of Nembutal or by double-pithing, and was then decapitated. The head was positioned under a light microscope (Wild, 12×–100× magnification) fitted with a grid eyepiece lens, such that the microscope image was of a caudal view of the left middle ear apparatus. Lightly pressing on the tympanic membrane resulted in a visible rocking motion of the stapes footplate. The estimated position of the footplate axis always corresponded with the articulation between the pars media and otic capsule, just ventrolateral to the footplate (see Fig. 1). Angles and lengths were measured from scale diagrams, including the angle θ_{bead} subtended between a line joining the bead position on the footplate to the footplate axis and the horizontal at the axis.

Results

The harmonics produced by the speaker were more than 30 dB below the primary tone at all frequencies other than at 480 Hz, where the second harmonic was 23.3 dB below the primary. Absorption of sound between the speaker and microphone required an unusually high voltage output to the speaker at this frequency to maintain 90 dB SPL at the eardrum: overdriving the speaker evidently resulted in some distortion. The table on which the frog was positioned also appeared to vibrate at this frequency, this vibrating the whole frog, as reflected by the unusually high velocity amplitude often measured at the parotic crest.

The responses of the tympanic membrane at different sound pressure levels (ranging from 70 to 100 dB SPL) were measured in five frogs, and the responses of the stapes footplate were also examined in one of these animals. No non-linearities were apparent in the tympanic membrane response at these levels. Although the stapes response was not examined at levels above 90 dB SPL, no consistent non-linearities were apparent at 90 dB SPL or below, and the velocity ratios measured at the different sound pressure levels were very similar.

Angle of the tympanic membrane velocity vector

Equation 6 was used to establish the true vibration velocity of the tympanic membrane (V_{TM}) before surgery in five male and three female frogs. At most frequencies below 2 kHz, the difference between V_{TM} and V_{TMA} was very small, generally less than 2 dB. Above 2 kHz, the difference often rose (to a maximum of approximately 6 dB), suggesting that the angle θ_{TM} was increasing, but the mean differences for both sexes were less than 3 dB at all frequencies. Measurements obtained from two animals after the stapes had been surgically exposed were similar. The results of these experiments suggest that the tympanic membrane velocity component V_{TMA} is a very good approximation to the true velocity V_{TM} at frequencies below 2 kHz and is a reasonable approximation at higher frequencies.

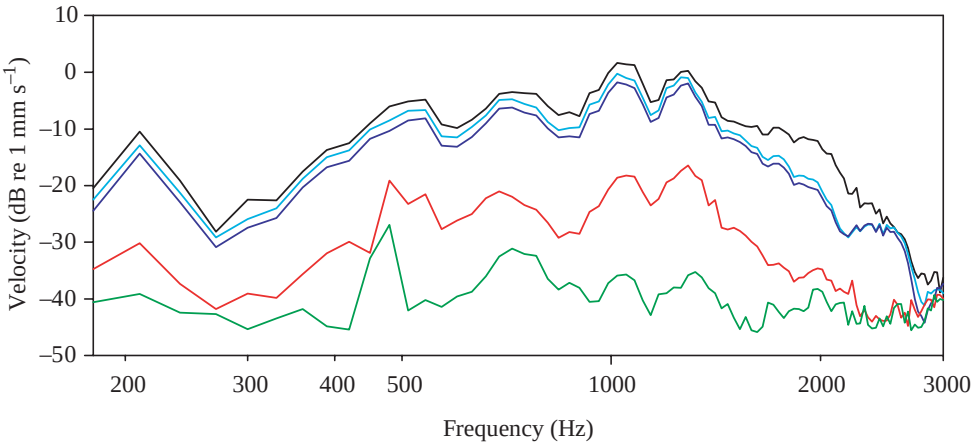

Fig. 3. Responses of the tympanic membrane (black), distal extrastapes (light blue), proximal extrastapes (dark blue), footplate (red) and parotic crest (green) in a male frog (SVL 131.8 mm). The response of the parotic crest was measured as a control for stapes footplate vibrations.

V_{TMA} was used as an estimate of V_{TM} in all further calculations.

The stapes response and the velocity ratio

Tympanic membrane, extrastapes and stapes responses from a representative male frog are depicted in Fig. 3. The responses measured from both the proximal and distal extrastapes positions (dark blue and light blue traces, respectively) are close to the response of the tympanic membrane (black trace) over a broad frequency range. The response of the stapes footplate (red trace) is smaller than that of the extrastapes, but is considerably greater in amplitude than that of the parotic crest (green trace) at most frequencies. At the lowest and highest frequencies, and between 450 and 480 Hz, the velocity amplitude of the parotic crest is high relative to that of the footplate. As a result of this, the measured footplate response does not follow the response of the extrastapes as faithfully at these frequencies. Such discrepancies can be greatly reduced or eliminated when the 'background' head response is controlled for (by the application of equation 1). Results from

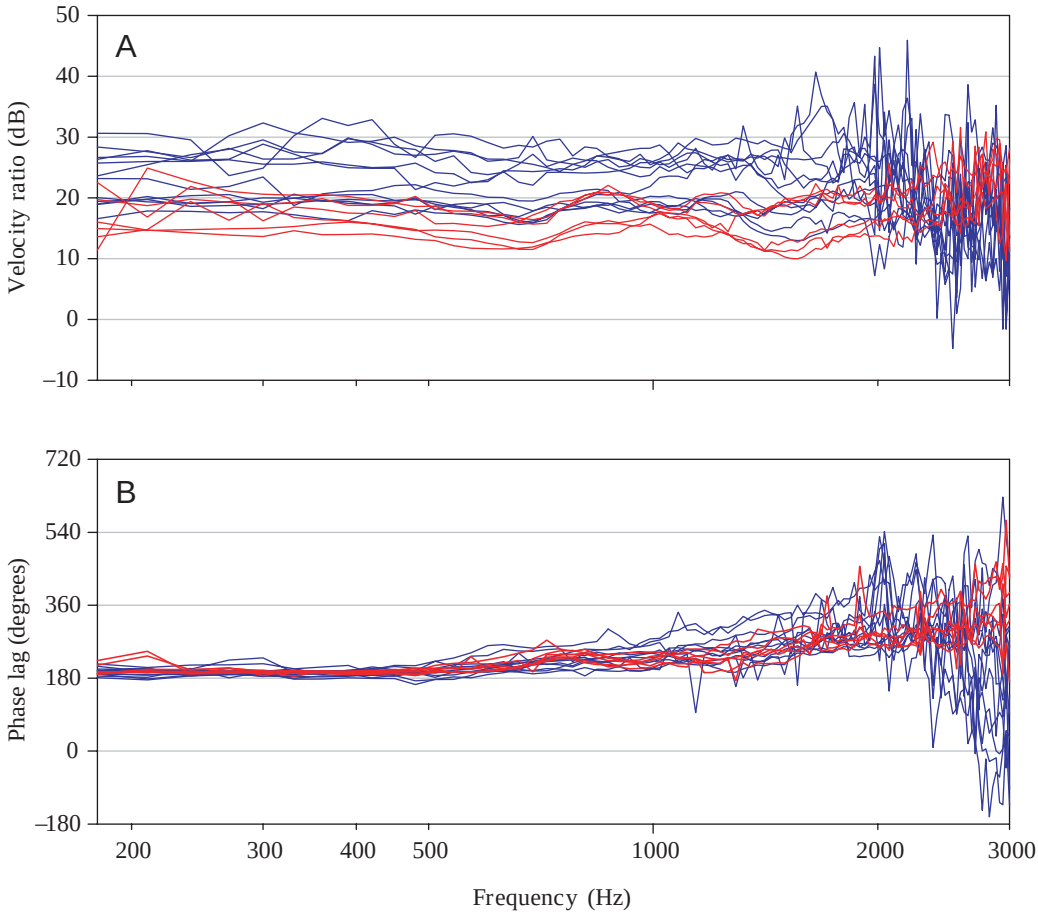

Fig. 4. (A) Tympanic membrane/stapes footplate velocity ratios in 12 male (blue) and six female (red) bullfrogs. The stapes footplate velocities used to calculate these ratios were adjusted for the responses of the parotic crest. Male values are generally greater than female values. (B) Phase lags between the tympanic membrane and stapes footplate, from the same experiments.

females were broadly similar, except that females lack the peak in tympanic membrane and stapes footplate responses at 200 Hz.

Velocity ratios, calculated as tympanic membrane velocity (V_{TM}) divided by the adjusted vertical component of stapes footplate velocity (V_{FPVa}), are presented in Fig. 4A for 12 male frogs (blue traces) and six female frogs (red traces). For frequencies below 2 kHz, the velocity ratios remain approximately flat. Data become noisy at frequencies higher than this. A mean value of V_{TM}/V_{FPVa} for frequencies below 2 kHz was calculated for each frog. In males, this value averages 24.3 dB (range 17.5–28.8 dB) and in females 17.0 dB (range 13.7–19.7 dB). The variances of these values (converted to absolute units) differ (F-test: F=12.94, d.f.=11.5, P=0.011) so a heteroscedastic t-test was used to examine the difference between (absolute) male and female values. The difference is statistically significant (t=3.84, d.f.=14, P=0.002). The phase lags between the tympanic membrane and footplate are approximately 180° at low frequencies (Fig. 4B), increasing to a mean of approximately 290° in females and 330° in males at 2 kHz.

The extrastapes

As a result of the perforation made in the tympanic membrane to expose the extrastapes, the response of the membrane always dropped, especially at low frequencies. However, the V_{TM}/V_{FPVa} ratios measured before and after this surgical procedure were found to be similar (generally within ±3 dB at frequencies under 2 kHz) in all but one frog (discussed below). This suggests that the middle ear system was not greatly affected by the surgery.

The vertical component of the velocity of the distal extrastapes (the lateral half of this structure) was measured in three female and seven male frogs. In most frogs, the velocity amplitude measured was within 5 dB of the tympanic membrane response at frequencies up to 2 kHz (Fig. 5A). At the lowest frequencies, the phase lag between the tympanic membrane and extrastapes is zero, increasing to an average of approximately 45° at 2 kHz (Fig. 5B). At frequencies above 2 kHz, the relative velocity amplitudes are more variable. This variability probably reflects a change in the velocity vector direction of the tympanic membrane, and possibly of the extrastapes, together with the low signal-to-noise ratio at the highest frequencies. There are no consistent differences between female and male frogs.

In one male, the mean velocity amplitude difference between the tympanic membrane and distal extrastapes, at frequencies up to 2 kHz, was 9.0 dB (Fig. 5A), considerably greater than in the other frogs tested. However, the mean V_{TM}/V_{FPVa} ratio of this frog over the same frequency range had risen by 5.8 dB after exposing the extrastapes, a much greater change than in any other frog, and its tympanic membrane was clearly drying.

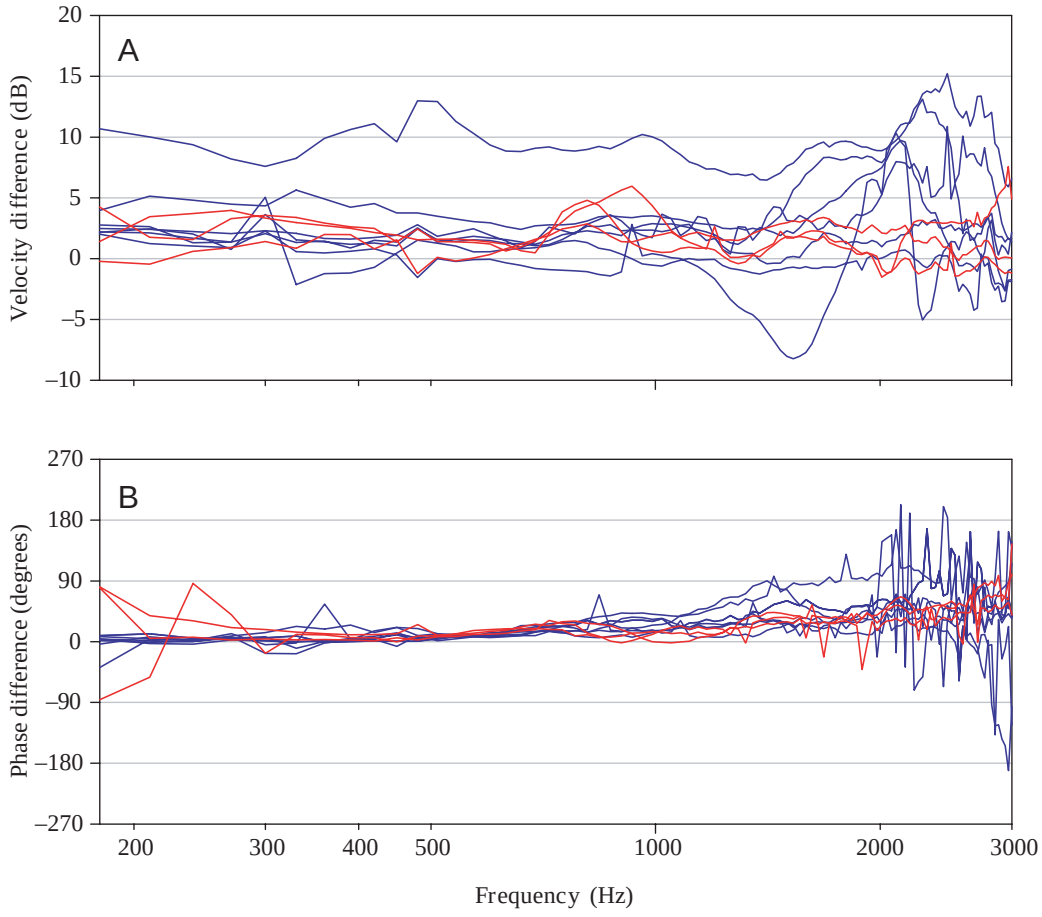

Fig. 5. (A) Velocity amplitude differences between the tympanic membrane and distal extrastapes in seven male (blue) and three female (red) frogs. (B) Phase lags between the tympanic membrane and distal extrastapes, from the same experiments.

It seems probable that the increase in lever ratio was because the extrastapes of this frog was becoming uncoupled from the tympanic membrane as a result of the drying. If so, the velocity difference between the membrane and extrastapes in the ear prior to extrastapes surgery would have been approximately 3 dB, in the middle of the normal range.

Measurements were made from more than one position on the extrastapes in three female and six male frogs (Fig. 6). At frequencies below 2 kHz, the velocity amplitude of the distal extrastapes is greater than that of more proximal positions, typically by 1.2–5.0 dB (Fig. 6A). The phase lags between these positions are generally negligible, less than 10° in most frogs for frequencies up to 2 kHz (Fig. 6B). Data are noisy at the highest frequencies.

The ascending process

In two female and two male frogs, a small central portion of the ascending process of the extrastapes was excised. Access to this structure was afforded by means of the wide Eustachian tube opening into the buccal cavity. Following the excision, the stapes footplate response (Fig. 7; red trace) dropped substantially in all frogs, becoming almost indistinguishable from that of the parotic crest (representing background skull vibration; green trace) in the male animals. In the females, the stapes response was still clearly greater than that of the parotic crest after the process had been severed, but the mean velocity ratio (for frequencies from 180 to 1980 Hz inclusive) had increased in both animals by approximately 20 dB (Fig. 8). In subsequent dissections, it was verified that the ascending process was indeed severed in all cases and that the rest of the extrastapes was still intact and formed a complete connection between the tympanic membrane and pars media.

In the female frog of Fig. 8, measurements at two different points on the extrastapes were made before and after the ascending process had been severed (Fig. 8A,B). The velocity difference between tympanic membrane (black trace) and distal extrastapes (light blue trace) increased by approximately 5 dB at frequencies below approximately 1.5 kHz, the difference diminishing at higher frequencies. A more dramatic change was seen in the relative velocity amplitudes of the two extrastapes positions. The velocity difference between the distal and proximal positions was approximately 3 dB prior to ascending process surgery: after surgery, this difference increased to approximately 13 dB at low frequencies (300 Hz), dropping to approximately 7 dB at 2 kHz. A sharp drop in the response of the proximal bead (dark blue trace) between 700 and 800 Hz interrupted this general decline (Fig. 8B). This coincided with a rapid change in the phase lag between the distal and proximal extrastapes, from approximately 0° at frequencies of up to 650 Hz, through 180° at approximately 750 Hz, and then back to approximately 0° above approximately 1 kHz.

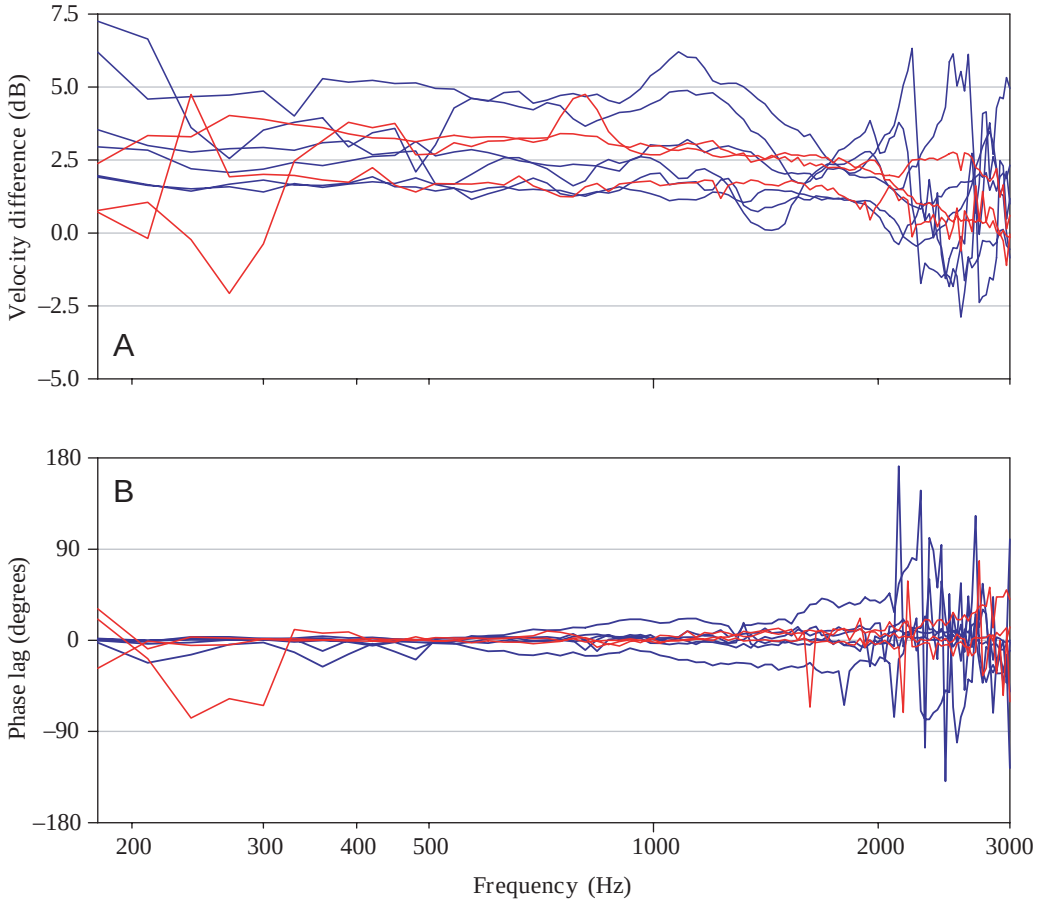

Fig. 6. (A) Velocity amplitude differences between the distal and proximal extrastapes positions in six male (blue) and three female (red) frogs. (B) Phase lags between the distal and proximal extrastapes, from the same experiment.

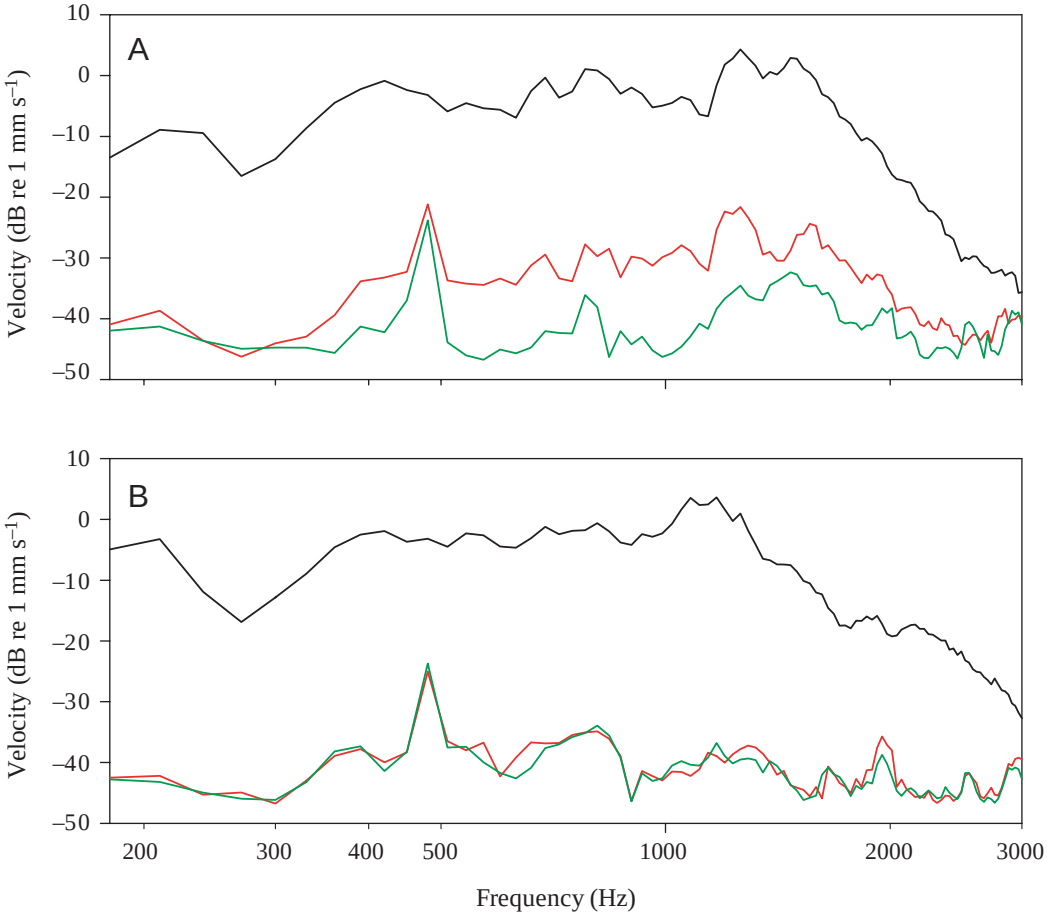

Fig. 7. (A) Responses of the tympanic membrane (black), stapes footplate (red) and parotic crest (green) of a male frog (SVL 131.1 mm) prior to surgery on the ascending process. (B) Responses of the same structures after the ascending process of the extrastapes had been severed. Note that the response measured from the footplate has fallen and now approximates that of the 'background' response of the parotic crest.

Upon pressing inwards on the tympanic membrane during dissections, the extrastapes tip moves inwards and downwards, sliding on the internal surface of the tympanic membrane to which it is only loosely attached. The ascending process bends to accommodate this, and there is a small amount of flexion between the extrastapes and pars media in the region of their articulation. Severing the ascending process increases the flexion at the extrastapes/pars media articulation while greatly reducing the movement of the pars media and footplate. These observations are entirely consistent with the experimental data, although the amplitude of movement in response to manually applied pressure is obviously many orders of magnitude greater than that in response to airborne sound.

Discussion

The phase difference of 180° between the tympanic membrane and stapes footplate suggests that, at low frequencies at least, the stapes undergoes a rocking motion. This agrees with the conclusions of other studies (Bolt and Lombard, 1985; Jørgensen and Kanneworff, 1998; Mason and Narins, 2002), which identify what is here termed the footplate axis as the point of rotation (see Fig. 1). The anatomy of the middle ear apparatus of frogs suggests that the stapes rocks about this axis in an approximately vertical plane (Bolt and Lombard, 1985). Ossicular movement in this plane only was examined in this study. Modes of ossicular vibration probably vary, especially at higher frequencies, and the position of the footplate axis may also be frequency-dependent. Some of the measurements in the calculations to follow depend on the location of this axis, which could only be established in dissections by applying pressures far exceeding those produced by airborne sound. Although the experimental data do seem to be consistent with observations made during dissections, these caveats must be borne in mind in the following discussion.

If the stapes and extrastapes together form a stiff unit vibrating around the footplate axis, and if the tip of the extrastapes has a velocity equal to that of the tympanic membrane, the measured velocity ratio V_{TM}/V_{FPVa} can be predicted from the following equation:

$$\frac{V_{TM}}{V_{FPVa}} = \frac{l_{ES}}{l_{bead}\cos\theta_{bead}}, \quad (7)$$

where l_{ES} is the distance from the extrastapes tip to footplate axis and l_{bead} is the distance from the reflective bead on the footplate (the point of measurement) to the footplate axis. If an imaginary line, perpendicular to the footplate axis, is extended between the bead and the axis, θ_{bead} is the angle between this line and the horizontal. Using anatomical data obtained from those frogs for which vibrometric values of V_{TM}/V_{FPVa} were available (12 males, six females), no differences were found in

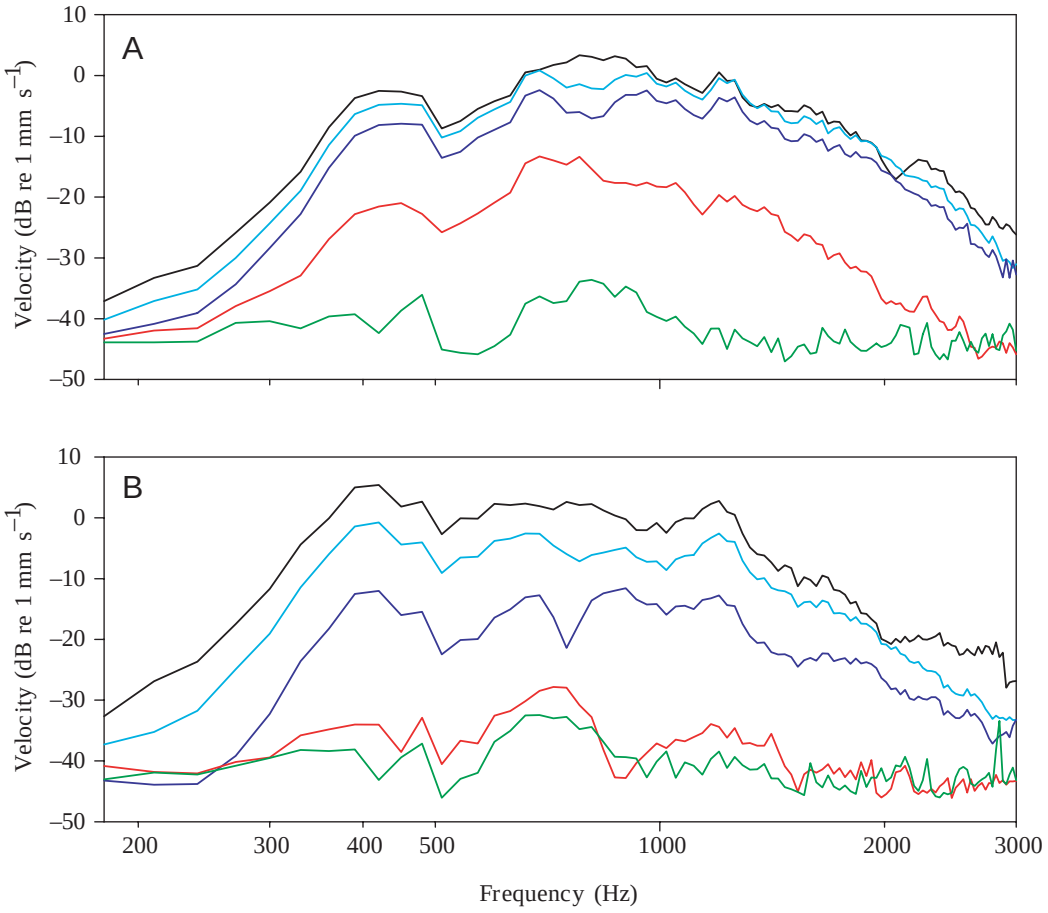

Fig. 8. (A) Responses of the left tympanic membrane (black), distal extrastapes (light blue), proximal extrastapes (dark blue), footplate (red) and parotic crest (green) of a female frog (SVL 133.9 mm) with the ascending process intact. (B) Responses of the same structures after severing the ascending process.

the ratio l_{ES}/l_{bead} between males and females (*t*-test: t=0.813, d.f.=16, P=0.428). However, the value of θ_{bead} does differ significantly between male and female frogs (t=2.97, d.f.=16, P=0.009), taking a mean value of 57° in males (range 50–67°) and 48° in females (range 38–56°). Applying data from individual frogs to equation 7, the predicted value of V_{TM}/V_{FPVa} in males was 17.2 dB (range 15.0–19.3 dB) and in females 14.8 dB (range 11.5–16.7 dB). This represents a significant difference between the sexes (*t*-test, absolute values used: t=2.92, d.f.=16, P=0.010). In all but four frogs, however, measured ratios are somewhat higher than predicted values (Fig. 9). The discrepancy is most exaggerated in some of the male frogs, in which the difference may reach 12 dB. These differences between predicted and measured values, especially in males, seem too large to be explained by errors in the anatomical measurements. Jørgensen and Kanneworff (1998) found a similar discrepancy between predicted and measured values in *Rana temporaria*.

Phase changes measured in the present study suggest that the coupling between the tympanic membrane and extrastapes is not perfectly stiff, and this is supported by anatomical observations (Jørgensen and Kanneworff, 1998; present study). However, the velocity drop between the tympanic membrane and distal extrastapes position at frequencies below 2 kHz is usually small, averaging approximately 2.5 dB. Since the apparatus used in the present study examines only the vertical component of the velocity of the extrastapes at each position, and since the distal extrastapes bead could not be placed on the very tip of this structure, the difference between tympanic membrane velocity and extrastapes tip velocity will be smaller than the measured value. The contribution of tympanic membrane–extrastapes coupling to the overall V_{TM}/V_{FPVa} ratio would appear to be very small.

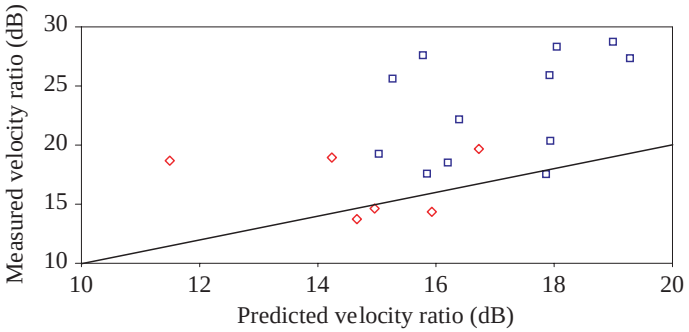

Fig. 9. A comparison of tympanic membrane/stapes footplate velocity ratios predicted from anatomical measurements if the ossicular apparatus were stiff with those measured experimentally in 12 male (blue squares) and six female (red diamonds) bullfrogs. The line has a gradient of 1, which would be expected if measured values were equal to predicted values. Note that most values fall above the line.

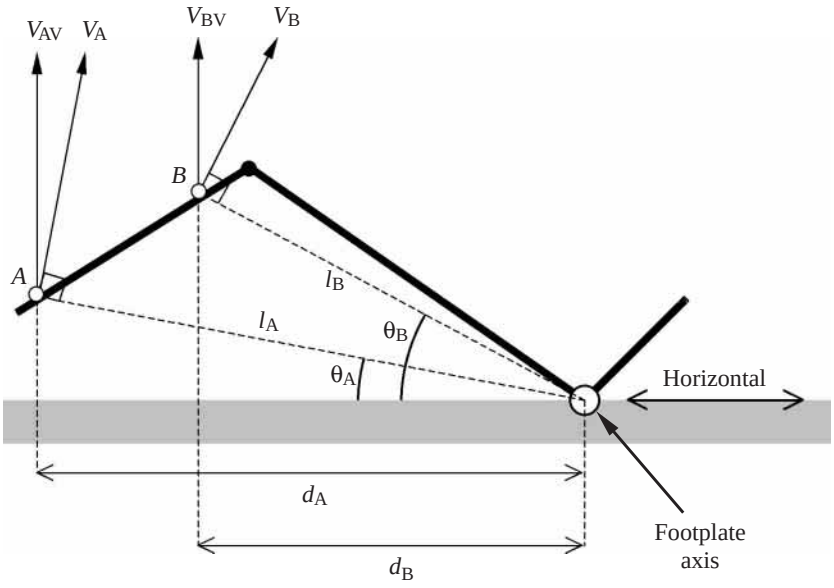

Fig. 10. Diagrammatic representation of the extrastapes and stapes of the bullfrog (see Fig. 11 for labelling of anatomical structures). Measurements of the vertical components of the velocity are made from two positions, *A* and *B*, on the extrastapes. The vertical velocity measurements V_{AV} and V_{BV}, at points *A* and *B*, respectively, together with the horizontal distance between the beads, d_A–d_B, are used to calculate the horizontal position of the apparent axis of rotation (see text for details). In the case illustrated, the axis of rotation of the extrastapes coincides with the footplate axis. l_A, distance between *A* and the footplate axis; l_B, distance between *B* and the footplate axis; V_A, velocity at *A*; V_B, velocity at *B*; θ_A, θ_B, angles between l_A and l_B, respectively, and the horizontal.

Experimental measurements show that points on the extrastapes move in phase across a broad frequency range and that the amplitude difference between these points varies little at frequencies below 2 kHz. This suggests that the main body of the extrastapes is stiff. To examine the motion of the extrastapes in more detail, consider a case in which the velocity amplitude is measured from two positions (Fig. 10). The difference between the amplitudes measured at these positions allows the calculation of an 'extrastapes axis', about which these points appear to be vibrating. Let us call the first and more distal position *A*, and the velocity at this position V_A. The vertical component of V_A measured by the laser is V_{AV}. The line between *A* and the extrastapes axis makes an angle θ_A with the horizontal, and the distance between *A* and this axis is l_A. Corresponding values for the second, more proximal, position *B* are given the subscript B. In Fig. 10, the extrastapes axis is assumed to be coincident with the footplate axis, but this need not necessarily be so. From the above definitions:

$$\frac{V_{AV}/\cos\theta_A}{V_{BV}/\cos\theta_B} = \frac{l_A}{l_B} . \quad (8)$$

Since $\cos\theta_A = d_A/l_A$, where d_A is the horizontal distance between *A* and the axis, it follows that:

$$V_{AV}/V_{BV} = d_A d_B . \quad (9)$$

The horizontal distance between the two measurement positions, d_A–d_B, was measured under the microscope after the experiments. From this value and the measured ratio V_{AV}/V_{BV}, the horizontal (but not the vertical) position of the extrastapes axis can be calculated.

For the female frog of Fig. 8, d_A–d_B was 0.9 mm. The velocity ratio V_{AV}/V_{BV} was relatively constant from 400 to 1000 Hz with the ascending process intact (Fig. 8A) and, within this frequency range, the mean value of V_{AV}/V_{BV} was 1.48 (3.4 dB). The horizontal distance between the extrastapes/pars media articulation and the distal extrastapes measurement position (point *A*) was between 1.1 and 1.3 mm; the footplate axis was at 6.3 mm distance from point *A*. If the extrastapes and stapes vibrate as a stiff unit, the extrastapes axis should coincide with the footplate axis (Fig. 10) and its horizontal position would coincide with the dashed line in Fig. 11A. However, using the values above, the horizontal distance d_A from the distal extrastapes bead to the extrastapes axis is calculated to be 2.8 mm. This falls between the extrastapes/pars media articulation and the footplate axis, but closer to the former: on the dashed line in Fig. 11B. The small changes in relative velocity amplitudes with frequency have little effect on the results of the calculation. Similar results were obtained in all six male and three female frogs for which this calculation was performed. In the males, the position of the extrastapes axis varied from 29 to 62% of the distance between the extrastapes/pars media articulation and the footplate axis, and in the females the range was 31–62%. These percentages do not differ significantly (*t*-test: t=0.34, d.f.=7, P=0.744), although the velocity ratios vary substantially among frogs.

When the ascending process was severed in the female frog of Fig. 8, the velocity difference between the proximal and distal extrastapes increased dramatically, especially at low frequencies (Fig. 8B). Excluding frequencies from 700 to 800 Hz, where a pronounced dip was seen, the mean velocity ratio V_{AV}/V_{BV} was 3.46 (10.8 dB). Using the same anatomical measurements, the horizontal position of the extrastapes axis is now calculated to be 1.3 mm from the distal extrastapes position. This corresponds with the position of the articulation between the extrastapes and pars media, falling on the dashed line shown in Fig. 11C.

Wever (1985) mentions that the ascending process 'probably adds stability to the tympanic membrane and protects it against undue forces', but does not go into any further detail. Moffat and Capranica (1978) refer to this structure as the 'plectral

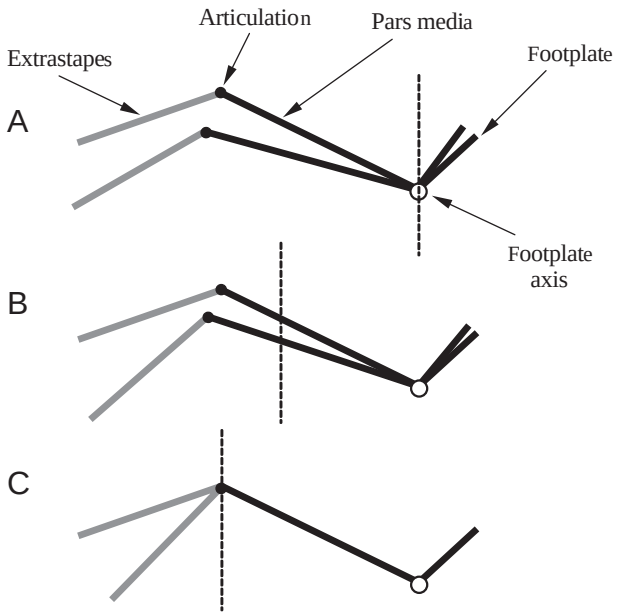

Fig. 11. Diagrammatic representation of the ossicular apparatus of the bullfrog showing hypothetical modes of motion. The predicted horizontal position of the 'extrastapes axis' is indicated in each case with a dashed line. In A, the extrastapes and pars media are stiffly connected and all rotation is about the footplate axis. In B, there is both rotation around the footplate axis and relative movement between the pars media and extrastapes. The extrastapes axis is predicted to lie somewhere between the footplate axis and the extrastapes/pars media articulation. This is the case in the intact frog ear. In C, movement is entirely restricted to rotation at the extrastapes/pars media articulation. There is no movement of the footplate. The middle ear approaches this condition when the ascending process (not shown) is severed. Amplitudes of motion are greatly exaggerated for clarity.

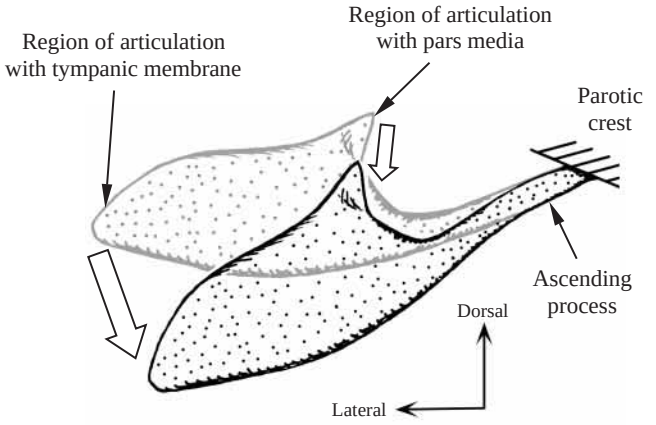

Fig. 12. Diagrammatic representation of the movement of the left extrastapes when the ascending process is intact. For clarity, surrounding middle ear structures are not shown. When the tympanic membrane is inflected inwards, the distal tip of the extrastapes moves ventromedially, sliding on its loose connection with the internal surface of the membrane. The thin ascending process, connected to the parotic crest dorsomedially, bends. As a result of the bending at the ascending process, the vibration velocity at the region of the extrastapes articulating with the pars media has a strong vertical component. This is necessary for the pars media and footplate to be set into motion. Movement is greatly exaggerated for clarity.

ligament', which implies a role as a tether. The results of the present study suggest that the ascending process is critical to the normal functioning of the ossicular apparatus of the frog. While most of the extrastapes is sturdy but slightly flattened in the transverse vertical plane, the ascending process is thin and flattened in the horizontal plane. This morphology favours bending at the ascending process when the extrastapes is exposed to forces acting in the vertical plane. Bending at the ascending process translates inward motion of the tympanic membrane into roughly vertical motion at the extrastapes/pars media articulation (Fig. 12), resulting in rotation of the pars media and footplate about the footplate axis. The calculated horizontal position of the extrastapes axis, close to the centre of the ascending process, would be consistent with this interpretation. Some flexibility at the articulation between the extrastapes and pars media is required to accommodate this mode of vibration. Severing the ascending process changes the motion of the extrastapes such that it now pivots on its flexible articulation with the pars media (Fig. 11C). The vertical component of motion at the articulation is much reduced, and the velocities of the pars media and footplate are greatly decreased. This general picture is complicated by the appearance of frequency-dependent vibration modes when the ascending process is severed. Between 700 and 800 Hz, the data suggest that the extrastapes rotates about a point just lateral to the proximal measurement position (which was close to the articulation with the pars media in this frog), resulting in a 'see-saw' type motion of the extrastapes over this narrow frequency range.

Although the velocity ratio V_{TM}/V_{FPVa} differs significantly between male and female bullfrogs, the above calculations suggest that the motion of the extrastapes is actually similar in both sexes. The high velocity ratios measured in some male frogs (Fig. 9) could be due to differences in the coupling between the extrastapes and pars media: increased flexibility at the articulation would result in a smaller movement of the pars media and footplate for a given displacement of the extrastapes. Increased flexibility in the ascending process might have a similar effect. Alternatively, increased impedance at the stapes footplate would result in greater flexion and a higher lever ratio even if the ossicular structures were identical in the two sexes. Frequency-dependent changes in inner ear impedance have been thought to affect the measured values of middle ear lever ratios in other vertebrates (Manley, 1972b; Gyo et al., 1987; Gummer et al., 1989), although no sex differences were noted. A higher impedance in the ear of the male bullfrog could reflect an anatomical difference in the inner ears between the sexes, a stiffer articulation between the stapes and oval window or perhaps an effect of the operculum, which is coupled to the stapes footplate (Mason and Narins, 2002).

When under water, or when accidentally touched, the

externally located tympanic membrane of a frog will be subject to a static pressure inflexion much greater than that induced by airborne vibrations. Under these circumstances, the ascending process will buckle, and the joint between the extrastapes and pars media will flex. This is observed during dissections when pressure is exerted on the outside of the tympanic membrane. As a result, the displacement of the pars media and footplate will be smaller than with a stiff ossicular system, helping to prevent large static pressure changes from being transmitted to the inner ear. Conversely, when the tympanic membrane is forced outwards during middle ear air pressure increases associated with breathing or vocalising (Hetherington and Lombard, 1983; Narins, 1992; Purgue, 1997), the stapes footplate would tend to be pushed into the oval window (Purgue and Narins, 2000). A large outward displacement of the extrastapes may be resisted by tension in the ascending process, reducing the displacement of the pars media and pars interna.

This system could act in conjunction with a newly proposed function of the opercularis system (Mason and Narins, 2002), whereby contraction of the opercularis muscle during breathing (and presumably also vocalisation) may restrain the operculum and stapes footplate and resist their being pushed into the oval window. In dissections, pulling on the opercularis muscle can be seen to pull both the stapes and operculum outwards (Mason and Narins, 2002). The tip of the extrastapes is attached only loosely to the tympanic membrane and it slides ventrally, perhaps explaining why manipulating this muscle does not have a measurable effect on tympanic membrane vibration (Hetherington, 1994). The loose connection between the extrastapes and tympanic membrane might represent another mechanism to restrict ossicular movement in response to high-amplitude tympanic displacement. The likely cost of such flexibility would be a reduction in transmission efficiency at high frequencies (Manley, 1972a).

It is interesting to note that reptiles and birds both have a cartilaginous extrastapes, and there is evidence for flexibility in their ossicular systems (Manley, 1972b,c; Norberg, 1978; Rosowski et al., 1985; Saunders, 1985; Saunders et al., 2000). Although mammalian ossicles are largely ossified, the majority of mammalian species examined have synovial articulations both between the malleus and incus and between the incus and stapes (see Mason, 1999). With the small fluctuations in pressure associated with normal sound transmission, mammalian ossicular articulations are often seen as being effectively rigid, although relative movement between the ossicles may increase at high frequencies (Møller, 1963; Guinan and Peake, 1967; Gyo et al., 1987). However, much greater relative motion is seen with increased pressures, when the flexible articulations between the ossicles help to decouple the excessively displaced tympanic membrane from the stapes footplate, thus protecting the inner ear (Cancura, 1980; Marquet, 1981; Hüttenbrink, 1988). The flexibility of the extrastapes relative to the pars media in frogs may have an analogous function.

Norberg (1978) functionally equates the extrastapes of the owl to the (fused) malleus and incus of mammals. It is suggested in the present study that the extrastapes and stapes of frogs are functionally two ‘ossicles’, rather than one, and that the middle ear mechanics of ‘columellar’ and tri-ossicular ears may be more similar than is commonly assumed.

In summary, the following conclusions are reached: (i) relative movement between the tympanic membrane and extrastapes, and bending of the extrastapes itself, makes only a small contribution to the discrepancy between anatomically predicted and experimentally measured velocity ratios; (ii) most of this discrepancy, especially in male frogs, probably arises as a result of flexion between the extrastapes and pars media; (iii) the ascending process of the extrastapes plays a vital role in limiting the amount of flexion at this articulation and in controlling the movement of the extrastapes; and (iv) flexibility within the middle ear apparatus of the bullfrog probably acts as a protective device.

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