The Journal of Experimental Biology 213, 1876-1885

doi:10.1242/jeb.040196

Exceptional high-frequency hearing and matched vocalizations in Australian pygopod geckos

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Accepted 16 February 2010

SUMMARY

We describe exceptional high-frequency hearing and vocalizations in a genus of pygopod lizards (*Delma*) that is endemic to Australia. Pygopods are a legless subfamily of geckos and share their highly specialized hearing organ. Hearing and vocalizations of amniote vertebrates were previously thought to differ clearly in their frequency ranges according to their systematic grouping. The upper frequency limit would thus be lowest in chelonians and increasingly higher in crocodilians, lizards, birds and mammals. We report data from four *Delma* species (*D. desmosa*, *D. fraseri*, *D. haroldi*, *D. pax*) from the Pilbara region of Western Australia that were studied using recordings of auditory-nerve compound action potentials (CAP) under remote field conditions. Hearing limits and vocalization energy of *Delma* species extended to frequencies far above those reported for any other lizard group, 14 kHz and >20 kHz, respectively. Their remarkable high-frequency hearing derives from the basilar papilla, and forward masking of CAP responses suggests a unique division of labor between groups of sensory cells within the hearing organ. These data also indicate that rather than having only strictly group-specific frequency ranges, amniote vertebrate hearing is strongly influenced by species-specific physical and ecological constraints.

Key words: lizard hearing, audition, lizard vocalization, gecko, reptile.

INTRODUCTION

The hearing component of the vertebrate ear underwent its formative evolution later than the other great sense organs of the head (Manley, 2000). Within a relatively short time geologically, in the Triassic period, all major groups of amniotes independently developed an effective middle-ear impedance-matching device – a tympanic middle ear (Clack and Allin, 2004; Manley and Clack, 2004). Since these events were preceded by the separation of the lineages leading to the mammals, archosaurs, lepidosaurs and amphibians, the inner-ear hearing epithelia of these lineages each independently evolved a response to the increased auditory input (Manley, 2000). The final size of the auditory papillae of amniotes differs between modern mammals, birds, crocodilians and lizards, in that the longest are found in mammals, the next longest in birds and crocodilians, and lizards have the shortest papillae (Manley, 1971; Manley, 1973; Manley and Köppl, 1998).

Since the mammals were coincidentally developing a secondary jaw joint at the same time as a tympanic middle ear, redundant bones were incorporated into their middle ear resulting in three ossicles connecting the eardrum to the inner ear in mammals, rather than one as in lizards, birds and crocodilians. Although both middle-ear systems are effective impedance matchers, the first order type of lever system of mammalian middle ear performs somewhat better at high frequencies than the second-order system of non-mammals (Manley, 1990). This difference was thought to adequately explain why on average the upper frequency limit of mammals (>100 kHz) (Manley, 1973) far exceeds that of non-mammals. However, earlier indications also supported an influence of the inner ear on the high-frequency responses of the middle ear. Manley showed that in geckos, the columella-extracolumella system shows flexing at frequencies above about 4 kHz, resulting in poor transmission through the middle ear (Manley, 1972). However, the flexing was reduced by destroying the cochlear organ or by detaching the columella from the oval window. Thus, the rising impedance of the inner ear at frequencies that exceed the inner ear's upper limit reflects back on the middle ear and reduces its displacement amplitudes. The influence of the inner ear has since been demonstrated in a number of other cases, indicating that the upper frequency limit of amniotes is determined by a combination of inner- and middle-ear factors (Ruggero and Temchin, 2002). Within the non-mammals there are also well-known tendencies in high-frequency hearing limits, with birds generally known to respond to higher frequencies (up to ~12 kHz) than reptiles (4–6 kHz), the exception being turtles (1 kHz). Until recently, these differences have been regarded as well established.

The differences between non-mammals are not trivial to explain and the picture recently has become confused due to the discovery of middle-ear responses in some lizards to unexpectedly high frequencies (>8 kHz) (Christensen-Dalsgaard and Manley, 2005; Christensen-Dalsgaard and Manley, 2008). The results of the present study suggest that hearing limits are much less specific to systematic groups than previously thought and depend less on phylogenetic position than on species-specific physical (size) and ecological (temperature regime, lifestyle) constraints.

The lizards form an especially interesting group of land vertebrates with regard to hearing, because their basilar papilla shows family-, genus- and even species-specific morphology (Manley, 1990; Miller, 1992; Wever, 1978). The structural variety is remarkable and to some extent can be correlated to physiological response patterns (Manley, 2002). Some of the most complex lizard basilar papillae are found in the gecko family *Gekkonidae*. Here, uniquely among lizard families, the high-frequency area of the

papilla lies apically instead of basally (i.e. the tonotopic organization is reversed) (Manley and Köppl, 1998; Manley et al., 1999), and the high-frequency area is divided longitudinally by a hair-cell-free hiatus into two separated groups of sensory hair cells lying neurally and abneurally (Miller, 1992; Chiappe et al., 2007; Köppl and Authier, 1995). These two hair-cell groups are covered by different tectorial structures that make no contact across the hiatus, suggesting a segregation of function between them. Indeed, detailed anatomical study and modeling strongly indicated that the frequency response ranges of the two parallel-lying hair-cell groups would be different (Köppl and Authier, 1995; Authier and Manley, 1995).

Recently, it was shown that the inner, neurally-lying hair-cell group in *Gekko* is devoid of an afferent innervation (Chiappe et al., 2007). This has recently been confirmed for pygopod geckos (C. Köppl, personal communication), providing substantial evidence for a functional specialization between these two hair-cell groups. We have studied the hearing of pygopod geckos to further investigate these remarkable papillae and to help elucidate functional patterns. The close familial resemblance of their inner ear to that of other geckos has been known since the seminal work of Shute and Bellairs (Shute and Bellairs, 1953).

Superficially, pygopod geckos bear little resemblance to 'classical' geckos, because they lack limbs and can be highly elongated (small vestiges of hindlimbs remain astride the vent). This snake-like body form has evolved a great many times independently during the evolution of squamates (Brandley et al., 2008). Of the ~40 species of pygopods, 19 belong to the genus *Delma* and of these, 10 are found in Western Australia (Wilson and Swan, 2003). Pygopod systematics are very difficult because they are really different to the most suitable outgroup for rooting, the diplodactyline geckos. Jennings et al. conclude that there are two main sub-groups of Pygopods, one lineage that led first to *Lialis*, then *Pygopus* and three related genera (Jennings et al., 2003). The second lineage, with later speciation than the first lineage, led to the genus *Delma*. As the conservation status of *Delma* species cannot be assessed on the basis of the meager studies so far carried out on this group, they are considered endangered and are protected. For this reason, we carried out our study remotely in the field, using a small mobile laboratory and releasing animals as quickly as possible. Most recordings were carried out in the Pilbara region of Western Australia and both Cattle stations and National Parks provided access. We report data on the vocalizations and the hearing capacity of four species of *Delma*, *D. desmosa*, *D. fraseri*, *D. haroldi* and *D. pax*. Comparison with other geckos is provided by recordings from three less-derived pygopod species, the Common Scaly-Foot *Pygopus lepidopus*, the Hooded Scaly-Foot *Pygopus nigriceps* and Burton's Snake Lizard *Lialis burtonis*.

MATERIALS AND METHODS

We used a mobile field laboratory to study the hearing of pygopods in remote locations in the Pilbara region of Western Australia. Animals were caught under license, mostly on roads and on private property. They were held in small terraria, generally studied within one or two days and released at the individual site of capture. Here, we describe measurements from the following individuals: four Burton's Snake Lizard *Lialis burtonis* (Gray 1834), one Common Scaly-Foot *Pygopus lepidopus* (LaCépède, 1804), one Hooded Scaly-Foot, *Pygopus nigriceps* (Fischer, 1882), four *Delma desmosa* (Maryan et al., 2007), three Fraser's Delma, *D. fraseri* (Gray, 1831), nine Neck-Barred Delma, *D. haroldi* (Storr, 1987) and seven Peace Delma, *D. pax* (Kluge, 1974). The first three are relatively large species (total length up to 70 cm, weighing >30 g). *Lialis* preys on other lizards. The latter four are small (maximum length ~30 cm, weight >4 g) and eat arthropods. In the field, pygopods were identified using Wilson and Swan (Wilson and Swan, 2003); using detailed photographs, these were confirmed at the Western Australian Museum (B. Maryan). The Western Australian subspecies of *D. fraseri* used here, *D. fraseri petersoni*, was elevated by Jennings et al. (Jennings et al., 2003) to species status as *D. petersoni*; this change was, however, not reflected in Wilson and Swan (Wilson and Swan, 2003).

Auditory-nerve fibers respond to appropriate tones by an increase in their discharge levels. The cochlear compound action potential (CAP) is considered to be the sum of synchronized discharges from all active afferent fibers that innervate the hearing organ. Following an increase in discharge, the activity of the neuron is depressed, leading to a time period during which the fiber's sensitivity is reduced. Recovery follows with an exponential time course (Harris and Dallos, 1979). This leads to the phenomenon known as forward masking, in which a fiber's response threshold is reduced as a result of exposure to a preceding sound. The same effect can be observed in the whole-nerve or CAP response. The magnitude and duration of the masking or response depression depends on several factors, especially the frequency content, level and duration of the preceding masking sound but also the time delay between the masker and the probe stimulus. In a study of chinchilla auditory-nerve fibers, Harris and Dallos showed that for a 100 ms pure-tone masker and a stimulus 20 dB above the respective neural threshold, 50% of the original response magnitude was reached after 6 ms (Harris and Dallos, 1979). We used a 3 ms delay between noise pulse and pure-tone probe pulse in order to avoid the use of high masker levels.

We recorded the CAP non-invasively in animals sedated by low doses of gas anaesthesia (0.7–1 vol.% Isoflurane, 500 ml min^{-1} free-flow over the nares). Animals were stabilized in their position using narrow strips of Plasticine™ attached to a steel plate. The plate was warmed or cooled by adding and changing the position of warm-water or ice containers. The temperature next to the animal's thorax was continuously monitored using a thermistor calibrated against a glass thermometer. All devices used were battery operated and the air pumps placed out of hearing range. Experiments were controlled using Labview™ (National Instruments, Austin, TX, USA) hardware (PCMCIA 6036E and BNC-2110 interface) and software on a laptop PC (Samsung, South Korea; X60plus). Shaped tone bursts (mostly max 75 dB SPL, 10 ms duration, 6 s^{-1}) were presented *via* an individually calibrated earphone system [calibrated using a low-noise G.R.A.S. 0.5″ microphone (40AF/26AK), Holte, Denmark] sealed with Vaseline™ over the ipsilateral ear canal. The rise and fall times of the tone bursts were 1 ms, except for 0.5 kHz, where they were 2 ms. A specially-shaped silver-wire loop electrode insulated except for the loop was placed in the buccal cavity near the round window of the ipsilateral inner ear and its position was optimized for maximum CAP amplitude. The response voltage to the sound onset was amplified (1000×; DAM-80; WPI, Sarasota, FL, USA, bandwidth 100 Hz to 3 kHz) and averaged (N=36 or N=42). A voltage of 1.0 μV was used as criterion for visual threshold CAP response (initial synchronized peak), being clearly above the noise (~0.3–0.4 μV), and this was derived for all frequencies in steps of 0.5 kHz. The CAP was measured as the voltage difference between the first negative peak and the positive peak immediately following. All responses were saved to disk for subsequent analysis. From these saved files, amplitude-level functions were calculated offline in order to check the visual thresholds noted during the experiments. To obtain objective thresholds from these data, a level two standard deviations (s.d.) above the respective noise level was defined. The

time window for the noise determination was the noise (difference between maximum and minimum noise) averaged over the last 2 ms for all presentations of a given probe tone. This criterion (noise + 2 s.d.) was comfortably above the prevailing noise but slightly below the subjective criterion of 1 μV used for rapid visual threshold checks. The slopes of the amplitude-level functions were compared between the different frequencies by creating a simple linear fit to the data in each case. The criterion 'noise + 2 s.d.' was read off the linear fit, if necessary extrapolated along the fit line. This criterion was only used as a quality control for visual thresholds.

To record the tuning of suppression by forward-masking stimuli, the tones were preceded by 1 kHz-bandwidth noise stimuli centered on the same frequencies used for the tones from which the CAP audiogram was derived. The masking stimuli were 30 ms in duration, rise–fall 3 ms and ended 3 ms before onset of the tone. The following probe tone was emitted at a level that generated a CAP of 1.4 μV. Suppression threshold was defined as the masker level that suppressed the CAP response to 1.0 μV (generating the same signal/noise ratio as the normal CAP threshold measurements).

Vocalizations were recorded from six animals while they were being removed from their holding container and handled. They belonged to the species *D. pax*, *D. haroldi* and *D. fraseri* (two animals each, more than one occasion per animal). Recordings were made using a digital recording device (iKEY plus™, GCI Technologies Corp., Edison, NJ, USA) recording at 44.1 kHz resolution. Analysis was carried out using Raven™ software (Cornell University Ornithology Laboratory, Ithaca, NY, USA). Recordings consisted of 34 vocalizations that were separated by silent pauses. Vocalizations that varied greatly in their internal structure over time were divided into different components and separately analyzed, giving 44 analyzed sound units. Analysis primarily concerned the spacing between the clicks, the damped oscillation rates of the clicks and the frequency-band separations in the spectra. To estimate sound levels, recordings were also made of calibrated tone signals under the same conditions.

Animal care and anesthesia were according to the guidelines of the University of Western Australia (license RA/3/100/330). The Western Australian Government Department DEC (CALM) kindly provided a license to catch the animals (SF005216).

RESULTS

The use of only battery-operated equipment in the evening hours provided excellent conditions for electrophysiological recordings of responses to sound in the field without a sound-attenuating chamber. The remote regions of study were completely free of human-induced noise and of interference from AC electrical systems. The recording noise level was 0.3–0.4 μV, which allowed easy use of a 1 μV visual threshold. Below 1 kHz, interference from cochlear microphonic (CM; the summed receptor potentials of hair cells) potentials made it difficult to measure CAPs and, because thresholds were high there, in most cases 1 kHz was deemed the lowest frequency at which a reliable CAP could be measured.

CAP patterns

The CAP of the auditory nerve in response to pure-tone bursts was a sharp, asymmetrical biphasic wave with latencies from 2.8 ms, with additional oscillations (Fig. 1A). CAP audiograms for *P. lepidopus* (N=1), *P. nigriceps* (N=1) and *L. burtonis* (N=3) had best frequencies between 2 kHz and 3 kHz, thresholds from 40 dB SPL to 50 dB SPL (Fig. 2A) and upper frequency limits (at 75 dB SPL) between 6 kHz and 7 kHz. Compared with these, *Delma* audiograms, while similar up to 5 kHz, showed responses to much higher frequencies (*D. desmosa*, N=4; *D. fraseri*, N=3; *D. haroldi*, N=9; *D. pax*, N=7; Fig. 2A–C). Although their best frequencies were between 2.5 kHz and 4.5 kHz, above 8 kHz they showed a small but remarkable second sensitivity area with upper frequency limits (at 75 dB SPL) between 12 kHz and 14 kHz.

CAP thresholds were temperature sensitive. In four cases (one *L. burtonis* tested between 18.5°C and 34°C), one *D. desmosa* (18–30°C), one *D. haroldi* (18–32°C) and one *D. pax* (19–34°C), CAP thresholds were measured at several temperature steps to establish temperature optima. The temperature shift was quantified using frequency thresholds at 65 dB SPL on the steep high-frequency flank of the CAP audiogram (Fig. 2D). The Q_{10} of shift of frequency was 0.0377 octaves °C^{-1} in *Lialis*, 0.039 octaves °C^{-1} in *D. desmosa*, 0.052 octaves °C^{-1} in *D. haroldi* and 0.0286 octaves °C^{-1} in *D. pax*. Because above 30°C the temperature shift became small, a temperature near this was used for normal recordings and measurements of CAP in different individuals of the same species were made within as narrow a range of temperatures as feasible (*D. desmosa* 28.8±0.58°C; *D. pax* 30.3±1.36°C; *D. haroldi* 29.8±1.26°C; *D. fraseri* 29.0±1.0°C).

The best CAP thresholds for criterion 1 μV for the four *Delma* species lay between 39 dB SPL and 44 dB SPL. The mean difference between this threshold criterion and a criterion 'noise level + 2 s.d.' (Fig. 1D) calculated from recorded amplitude-level (I/O) functions for *D. pax* was 3.22±2.51 dB and for *D. haroldi* was 3.25±1.61 dB, with the subjective visual thresholds being less sensitive. Using the objective criterion, the best CAP thresholds lay between 36 dB SPL and 41 dB SPL.

The amplitude-level functions for CAP amplitude varied as a function of frequency (Fig. 1A–C). These were always steepest at 8 kHz (Fig. 1C) and less steep both below and above this frequency. Latencies of CAP decreased with sound pressure level, on average from near 4.5 ms at threshold to ~2.8 ms at the highest levels used.

Tuning of forward masking

In order to examine whether the secondary sensitivity above 8 kHz was mediated by the same nerve fibers as below 8 kHz or not, forward-masking experiments were carried out (Fig. 3A). The suppression tuning curves caused by narrow-band (1 kHz bandwidth) forward masking of a probe tone were unimodal at low probe frequencies (below 3 kHz) and bimodal at probe frequencies above 3 kHz (Fig. 3B). For probe frequencies of 4 kHz and above, the best suppression frequency always lay between 5 kHz and 8 kHz. An additional local minimum was centered at a frequency exceeding 8 kHz. The ratio 'best suppression frequency to probe frequency' was clearly related to the probe tone frequency (linear regression, N=19, P<0.00001). The best suppression frequencies for probe tones below 6.4 kHz were higher than the frequency of the tone, those for probe tones above 6.4 kHz were lower.

Vocalizations

The vocalizations recorded from three *Delma* species (*D. desmosa*, *D. fraseri*, *D. pax*) were 'release calls' emitted while being held (a presumably stressful situation; Fig. 4A,B). The 34 vocalizations studied all consisted of a rapid series of clicks (Fig. 4C,D) and had series durations between 3.2 ms and 2460 ms (44% were less than 200 ms and 82% less than 1 s). The vocalizations were very broad-band, consisting of many harmonically-related frequency bands probably exceeding the Nyquist limit (22 kHz at a sample rate of 44.1 kHz; Fig. 4B).

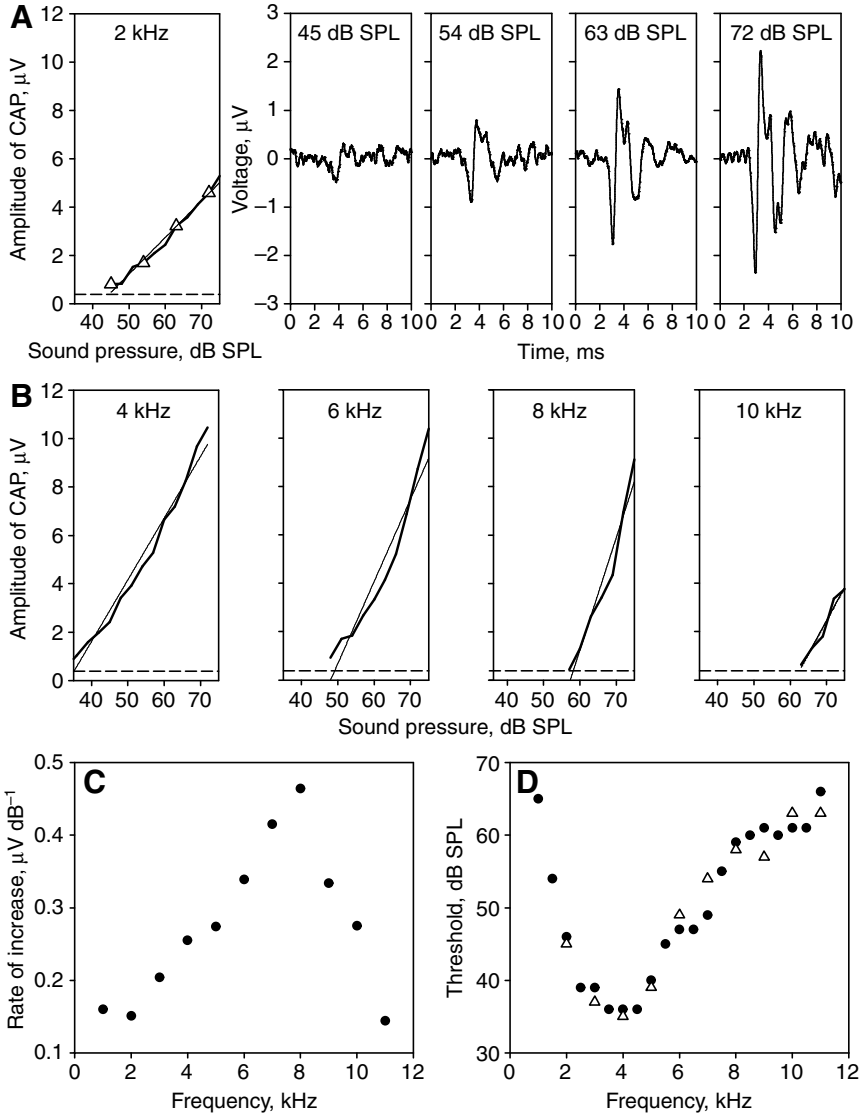

Fig. 1. Compound action potential (CAP) waveforms and amplitude-level functions in one individual of *Delma haroldi*. (A) Amplitude-level function for a 2 kHz tone. The thin line is a linear regression fit to the data. The broken line shows the criterion level 'noise + 2 s.d.'. The triangles indicate the four attenuation levels corresponding to the averaged CAP waveforms shown to the right. At the highest sound pressure shown, a small cochlear microphonic component is visible and has a shorter latency than the CAP. (B) Amplitude-level functions for four additional frequencies. Line codes as in A. (C) The slope of the amplitude-level functions as a function of frequency. (D) The CAP thresholds for this individual according to two criteria: the dots represent visually derived thresholds, the triangles correspond to the 'noise + 2 s.d.' criterion for the available frequencies.

Depending on the inter-click intervals in each series, these vocalizations were perceived as 'noisy' or more 'tonal' squeaks by the human ear, the transition occurring at click intervals of approximately 1 ms. Faster click rates were tonal. Inter-click intervals varied from 0.343 ms to 2.2 ms, 69% were less than 1 ms. As expected, there was a very clear dependence of the spectral structure of the vocalization on the click rate (Fig. 4B–D), with the harmonic band distance determined by the click rate (e.g. an inter-click interval of 0.5 ms generated harmonics separated by 2 kHz). The concentration of sound energy in a smaller number of harmonically related, more widely spaced frequency bands produced the impression of tonality rather than noise.

The sound pressure differences between neighboring harmonics in call spectra was often very small (<1 dB). Most (83%) of the strongest components lay between 6 kHz and 9 kHz, their sound pressures were between 45 dB SPL and 80 dB SPL and 52% were between 55 dB SPL and 65 dB SPL. The strongest frequency component was highly significantly related to the duration of the first oscillation in the individual clicks (linear regression, N=36, P=0.00001) but less significantly to the second or third oscillations (both P=0.018). Thus, the structure of the spectrum is determined both by the click rate (band separation) and the click oscillation periods (strongest component). The clicks showed a high degree of damping (see Fig. 4C,D).

Averaging the sound pressures at all frequencies over all spectra, it is clear that, on average, the sound pressure rises from the lowest frequencies up to a maximum near 7 kHz at a rate of 4 dB kHz^{-1}, decreasing steadily above that at a rate of 2.5 dB kHz^{-1} to about 16 kHz, where it essentially levels off. There were, however, differences between the species, with *D. fraseri*, the largest of the *Delma* species used, having the lowest spectral pattern and *D. haroldi* the highest (Fig. 4E). When compared with their species' audiograms, this pattern ensured good audibility for the lizards over a broad range of frequencies (Fig. 4F).

DISCUSSION

CAP frequency limits and thresholds

Our CAP data for the larger *P. lepidopus*, *P. nigriceps* and *L. burtonis* (Fig. 2A) revealed audiogram shapes that resemble neurophysiological data on the gecko *Gekko gecko* (Manley et al., 1999; Eatock et al., 1981) (Fig. 5) and to some extent CAP and

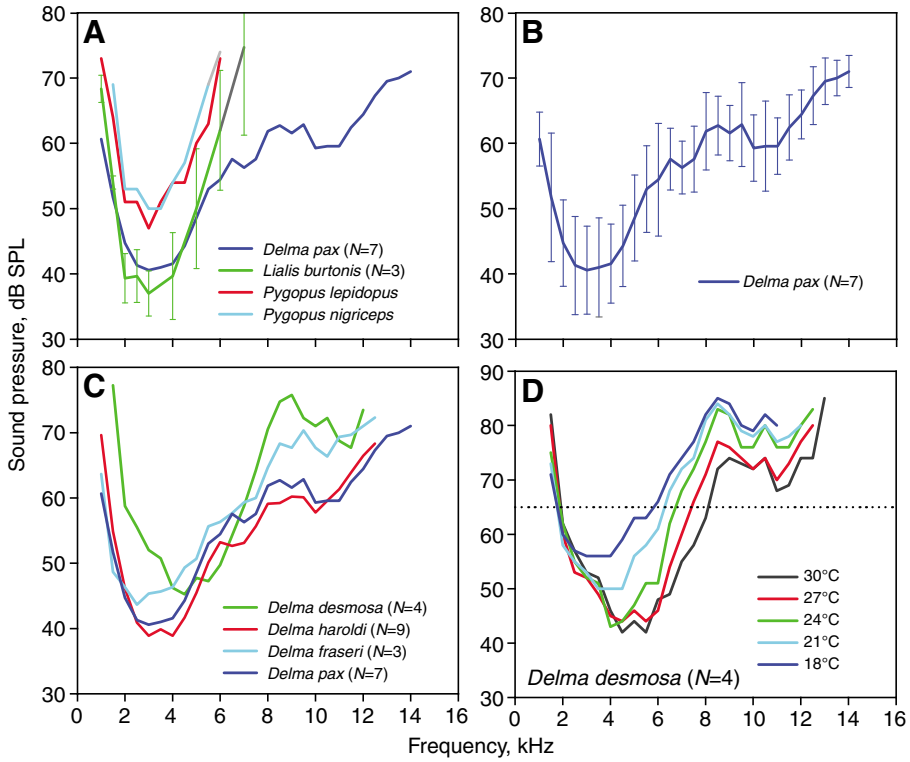

Fig. 2. Compound action potential (CAP) audiograms of pygopod geckos. (A) A comparison of CAP audiograms from three less-derived species, Burton's Snake Lizard *Lialis burtonis* (green curve, *N*=3, ±s.d.), the Common Scaly-Foot, *Pygopus lepidopus* (red curve) and the Western Hooded Scaly-Foot *Pygopus nigriceps* (light blue curve) with the mean CAP audiogram for *Delma pax* (dark blue curve, *N*=7). (B) Means and standard deviations for the CAP audiograms of seven *D. pax*. (C) A comparison of mean audiogram data from the four *Delma* species, *Delma desmosa* (green curve, *N*=4), *Delma haroldi* (red curve, *N*=9), *Delma fraseri* (light blue curve, *N*=3) and *D. pax* (dark blue curve, *N*=7). To avoid confusion, standard deviations are not shown but were comparable with those in B. (D) CAP audiograms from one individual *D. desmosa* measured at five temperatures, each 3°C apart and color-coded, to illustrate the shift in frequency sensitivity. The frequency shift was quantified using the 65 dB SPL level on the high-frequency flank (dotted horizontal line).

auditory brainstem response (ABR) data from this and other geckos (Brittan-Powell et al., 2008; Johnstone and Werner, 2001; Werner et al., 2008). Compared with these other groups of geckos, our CAP data from the *Delma* species show substantially improved high-frequency thresholds, with upper limits at 75 dB SPL of 12 kHz to 14 kHz. Such a high hearing limit has never been observed in any reptile and this kind of secondary sensitivity peak is unknown among amniotes except in some bats (e.g. Bohn et al., 2006). The fact that the audiograms of non-*Delma* pygopods – which were collected interleaved between the *Delma* audiograms – did not show the high-frequency extension was an empirical control for the possibility that any technical problems that may have been responsible for a spurious extra sensitivity area.

In Fig. 5, we compare *Delma* CAP thresholds (shifted down 20 dB to approximate putative single-neural thresholds) with single-unit data from the Tokay gecko *G. gecko* (Eatock et al., 1981), a barn owl *Tyto alba* (Köppl, 1997) and behavioral audiograms for the Grasshopper sparrow *Ammodramus savannarum* (Lohr et al., 2006)

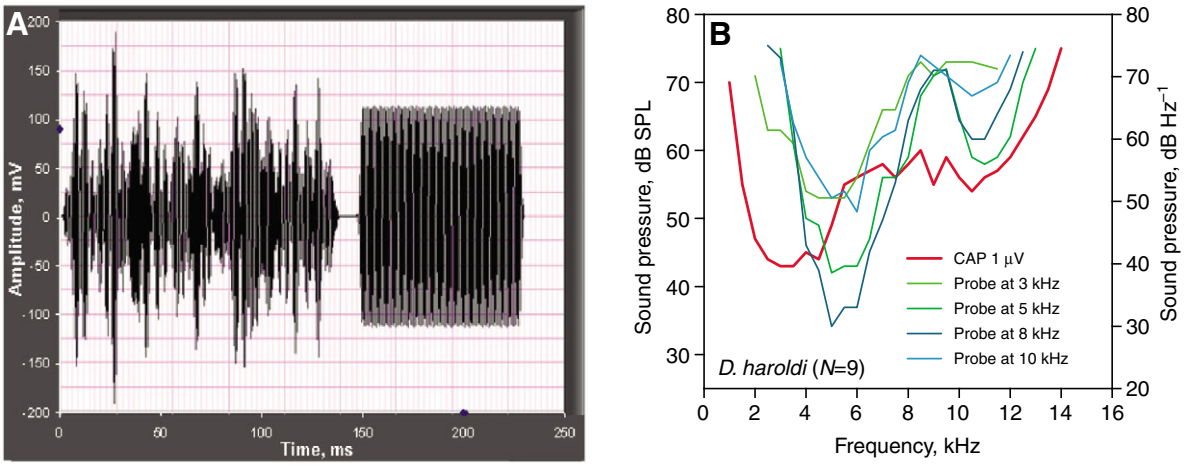

Fig. 3. Forward-masking effects in *Delma*. (A) A screen dump of the forward-masking paradigm. A narrow-band noise burst (center frequency 3 kHz, bandwidth 1 kHz, 50 ms duration, 3 ms rise and fall times) is followed after a 3 ms delay by a tone burst (3 kHz, 30 ms duration, 1 ms ramps). (B) Compound action potential (CAP) audiogram of one *Delma haroldi* (red curve, left axis) and forward-masking threshold curves from this individual. Four suppression contours are shown color-coded for probe tones of 3, 5, 8 and 10 kHz (right axis). Suppression was *via* 1 kHz-wide narrow-band noise centered at each frequency in 0.5 kHz steps. For probe tones above 3–4 kHz, the suppression contours were always asymmetrically bi-lobed.

and the canary *Serinus canaria* (Dooling et al., 1971). It can be seen that while *Delma* has poor low-frequency thresholds, above 10 kHz their thresholds are equivalent to the highest-frequency avian audiograms, even in specialized birds. These data also make clear that even within a single sub-family of lizards, remarkable differences in CAP sensitivity patterns can exist. The intriguing

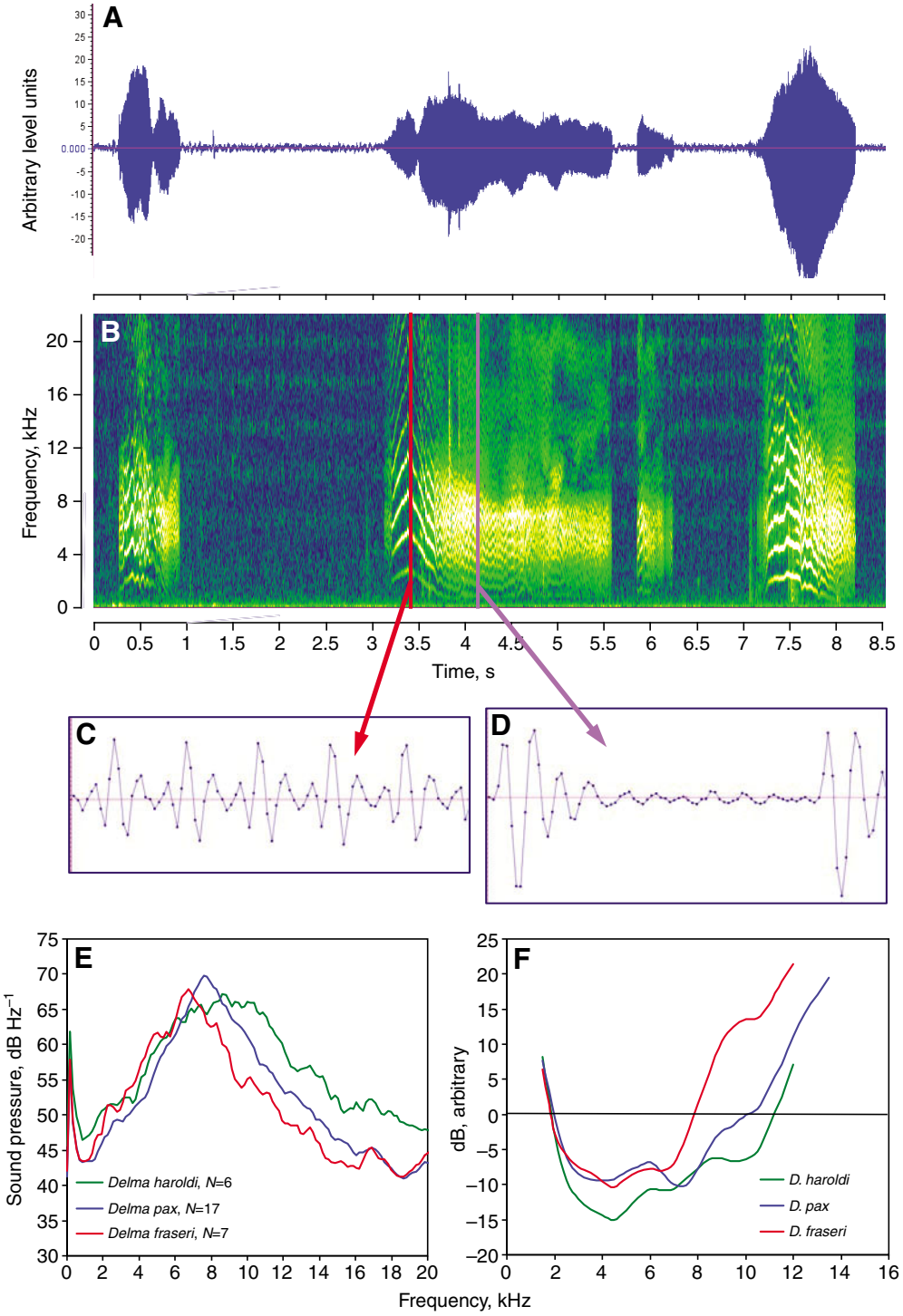

Fig. 4. Vocalizations of *Delma*. (A) Oscillogram of a series of vocalizations of one *Delma fraseri*, total duration 8.6 s. The ordinate is in arbitrary sound-level units. (B) Spectrogram of the vocalizations shown in A (Raven software, Hann window, 256 samples, 248 Hz filter bandwidth; 50% overlap, 128 hop size; DFT size 256 samples, 172 Hz grid spacing). (C) Two millisecond section of the vocalization in C at 3.4 s (red line in B), showing a rapid series of heavily damped clicks associated with widely spaced frequency bands in the spectrum. Time scale as in D. (D) Two millisecond section of the vocalization in C at 4.2 s (purple line in B), showing more widely spaced clicks associated with narrow frequency bands in the spectrum. (E) Mean sound pressure levels in all frequencies of the spectra of *Delma* vocalizations. *Delma haroldi* (*N*=6 vocalizations, green line), *Delma pax* (*N*=17, blue line) and *Delma fraseri* (*N*=7, red line). (F) Differences between mean sound levels in the vocalizations and mean compound action potential thresholds in the same species as in E. All parts of the curves below 0 dB should be audible to the animals. Color code as in E.

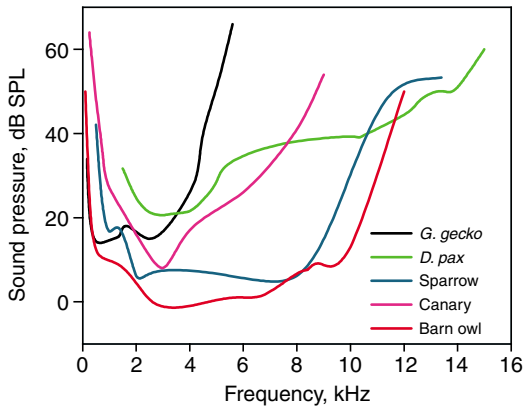

Fig. 5. (A) A comparison of hearing thresholds between species, a 'normal' gecko, *Gecko gecko* (auditory-nerve thresholds, black curve), the pygopod *Delma pax* [green curve, compound action potential (CAP) corrected down 20 dB, see text], the canary *Serinus canarius* [pink curve, behavioral data from Dooling et al. (Dooling et al., 1971)], the grasshopper sparrow *Ammodramus savannarum*, [blue curve, behavioral data from Lohr et al. (Lohr et al., 2006)] and the barn owl *Tyto alba* [red curve, behavioral thresholds from Dyson et al. (Dyson et al., 1998) and shifted up 20 dB to correct for the amplification provided by the facial mask].

difference between the *Delma* group and other pygopods and geckos is not easy to explain (see below).

When comparing upper frequency limits, the animals' body temperatures also need to be considered. In both lizards and birds, frequency responses are temperature-sensitive, preferred frequency shifting up with rising temperature. This was first demonstrated for geckos by a study of CAP thresholds in the gecko *Coleonyx variegatus* (Campbell, 1969). In birds, this effect is very large (>0.07 octaves °C^{-1}) (for a review, see Manley, 1990). The optimal temperature for the best CAP sensitivity in *Delma* was 30°C whereas birds have body temperatures from 37°C to 40°C. Considering this, it is remarkable how high the upper frequency limit of *Delma* actually is. Within the lizard families, the magnitude of the temperature effect on frequency responses correlates with papillar structure, being large in species having a continuous tectorial membrane over the high-frequency hair cells (e.g. varanids, teeids) and small in species that have either no tectorial membrane (e.g. iguanids, agamids) or a chain of tectorial sallets (e.g. skinks, geckos) (Manley, 1997). The magnitude of the temperature effect in our data on *Delma* can be compared directly with temperature-induced shifts in frequency for other auditory phenomena in geckos with a similar papillar structure (0.03–0.035 octaves °C^{-1}) (Manley, 1997) There is reasonable agreement with our data on the mean shift in the CAP upper frequency limit at 65 dB SPL (0.039 octaves °C^{-1}). Also, the temperature effect is smaller near the optimum temperature (here ~30°C) in both sets of data.

One final consideration with regard to the upper-frequency limit concerns the middle ear. As noted in the Introduction, the second-order lever system employed in non-mammalian middle ears lends itself to bending of the extracolumella when the inner-ear impedance rises at high frequencies (Manley, 1972). Wever discussed the middle ear of geckos, showing that at its best frequencies it is a very effective impedance-matching device (Wever, 1978). Unfortunately, the only pygopod middle ear Wever studied was that of a very immature *L. burtonis* (Wever, 1978). In pygopods that were killed for anatomical study, we examined the middle ears and found them unremarkable as compared with those of other adult geckos (Manley et al., 2007). There is therefore no indication of any particular anatomical specialization of the middle ear for high-frequency hearing.

It is remarkable that compared with the snakes, which have a very similar body form and, during their evolution from ancestral lizards, have totally lost all hearing above 1 kHz (Manley, 1990), pygopods have not taken the same path. This needs qualification, however. Most pygopods have retained an excellent middle ear, which snakes have strongly reduced. This reduction was most likely due to ancestral snakes ingesting prey whose size exceeded the snakes' normal gape. The resulting necessity of disarticulating the jaws was presumably incompatible with maintaining a normal eardrum. There are, however, pygopods, most belonging to the genus *Aprasia*, which have no external ear opening and presumably therefore (like snakes) have lost some hearing sensitivity. There is even a subspecies of *Delma concinna* that has lost its external ear canal. Unfortunately, there is no data on the hearing abilities of *D. concinna* or of *Aprasia* species.

CAP amplitudes and amplitude-level functions

The maximal CAP amplitude observed in any pygopod was 11 μV. As the number of nerve fibers in *Delma* is presumably maximally 1000 [cf. geckos in Miller and Beck (Miller and Beck, 1988)], a large CAP is not to be expected. The maximal CAP amplitude in birds, for example, with up to 32,000 auditory afferent fibers can be 200 μV (Köppl and Gleich, 2007). Differences seen in the slopes of the CAP amplitude-level functions for *Delma* species can be explained if each auditory-nerve fiber has a deep low-frequency sensitivity lobe and a secondary lobe most sensitive above 8 kHz. In fact, the single-neural tuning curves would presumably resemble narrower versions (as collected using pure tones rather than 1 kHz-bandwidth noise) of the suppression tuning curves such as those shown in Fig. 3B. The poor sensitivity of the CAP curve at 8 kHz suggests that all the nerve fibers with best frequencies below 8 kHz have similar, poor thresholds at 8 kHz, as can be seen for the suppression curves in Fig. 3. Thus, any increase in level at 8 kHz would lead to fast recruitment of nerve fibers and lead to the steep increase in the amplitude-level function (Fig. 1C). For a frequency well below or above 8 kHz, however, the different nerve fibers would be expected to have more different thresholds, and this would result in lower CAP amplitude-level function slopes.

CAP recordings and a comparison with other studies

The evoked electrical response of the auditory nerve (CAP) is a well-established and non-destructive technique for determining audiograms [see discussion in Köppl and Gleich (Köppl and Gleich, 2007)]. Studies of hearing of the related gecko *G. gecko* (Eatock et al., 1981) demonstrate that auditory-nerve fibers respond optimally to sound onsets with one or two strongly time-locked action potentials. Their own croak-like call, containing a fast series of onsets, produced very strong responses (Manley, 1990). It is likely that the same is true of the clicks in calls of *Delma*, which is compatible with the rapid early oscillations shown in CAP recordings to higher tone levels.

CAP recording has been used in the study of lizard hearing since 1969 (Campbell, 1969). There has been, however, a regrettable inconsistency in the protocols used, making it difficult or even impossible to compare published auditory sensitivities across animal groups. In some cases, rise times of the stimuli were so short (<0.1 ms) as to preclude a useful frequency selectivity (e.g. Turner and Shepard, 1986) and in other cases thresholds differed

dramatically for the same species in different studies, even from the same authors. Werner et al.'s (Werner et al., 2008) CAP results from *Gehyra variegata* (best threshold 64 dB SPL) and *Hemidactylus frenatus* (72 dB SPL) exceed both the earlier measurements of Johnstone and Werner (Johnstone and Werner, 2001) for *Gehyra variegata* and comparable studies of the small gecko *Coleonyx wislizenii* (Campbell, 1969) by a remarkable 50 dB. In their CAP measurements Werner et al. used an initial sound pressure of 110 dB SPL, a level that is sufficient to cause at least temporary threshold shifts (Werner et al., 2008). These authors show no original CAP trace data and they and Werner et al. (Werner et al., 1998) do not mention possible contamination by CM at low frequencies or indicate which threshold voltage was used. Werner et al. comment on the fact that their threshold data do not agree with single-neural recordings, which show best thresholds above 1 kHz (Werner et al., 2008). It is possible that the better thresholds below 1 kHz are due to contamination by CM, which almost always has a best sensitivity below 1 kHz (Wever, 1978). The data are thus not easily compared with our own. Their CAP threshold data for both large and small geckos shows a sharp dip at exactly 10 kHz in all species, which may be explained by a calibration error, as natural dips would be expected to vary, especially in frequency, with animal size. Due to these very significant differences in techniques and protocols we will not attempt a detailed comparison with other gecko CAP data, which are in any case not internally consistent (Campbell, 1969; Johnstone and Werner, 2001; Werner et al., 1988; Werner et al., 2008).

In addition to such technical differences between studies, as noted above, the size of the auditory nerve (that affects CAP amplitudes) differs greatly, especially between mammals and birds on the one hand (>3000 to >30,000 auditory-nerve fibers) and lizards on the other hand (most <1500). Although CAP thresholds clearly are higher than single-neural or behavioral audiograms (Köppl and Gleich, 2007), it is not clear how much higher they are and to what extent the difference is group- and/or frequency-specific.

An estimate of the difference between behavioral/single-neural thresholds and CAP thresholds within pygopod geckos can be obtained from data from related species. The threshold for suppression of a spontaneous otoacoustic emission (SOAE) in *D. haroldi* at 6 kHz (Manley et al., 2007) was at least 20 dB better than the CAP threshold we describe here. SOAE suppression thresholds in the skink *Tiliqua rugosa* are clearly well matched to single-neural thresholds (Köppl and Manley, 1994). The most useful threshold comparison is between single-neural thresholds in the related *G. gecko* (Manley et al., 1999; Eatock et al., 1981) and gross recordings from the brain (ABR) of the same species (Brittan-Powell et al., 2008). The single-neural data are on average 20 dB more sensitive than gross recordings. In view of the above data, we used a 20 dB adjustment to compare pygopod CAP thresholds with single-neural and behavioral thresholds in other species (see below, Fig. 5).

Forward masking of CAP

The thresholds of suppression caused by a forward masker are not only sensitive to the frequencies in the masker but also to its duration and the delay between masker and probe tone (Harris and Dallos, 1979). Thus, we concentrate here not on the absolute masking thresholds but on the shape of the suppression curves. The CAP data suggest that the most sensitive fibers and/or the densest innervation of the papilla would be near 6.4 kHz, because a regression line relating the ratio of best suppressor frequency to probe-tone frequency crossed the zero axis at 6.4 kHz.

It is evident that for single auditory-nerve neurons of best frequencies above about 3 kHz, the tuning curves are bi-lobed. The bi-lobed shape of suppression tuning curves for probe tones both below and above 8 kHz implies that responses to tones in both frequency regions were mediated by the same nerve fibers. It does not, however, necessarily imply that these responses are mediated by the same hair cells. The suppression-tuning characteristics show clearly that the remarkable high-frequency responses in the *Delma* species originate in the auditory papilla.

Possible anatomical substrate of high-frequency hearing

While there is no obvious explanation for the extraordinary hearing ability of these animals, it is known that the gecko–pygopod-type hearing organ is the most complex of all lizard papilla types (Fig. 6) (for a review, see Manley, 1990). Based on anatomical features such as hair-cell bundle and tectorial characteristics of the different hair-cell populations, an earlier mathematical model of the *Gekko* papilla suggested that the neural hair-cell population responds to much higher frequencies (roughly double) than the other, parallel-lying, abneural hair cells (Authier and Manley, 1995). Puzzlingly, recordings from the auditory nerve of *Gekko*, while confirming some

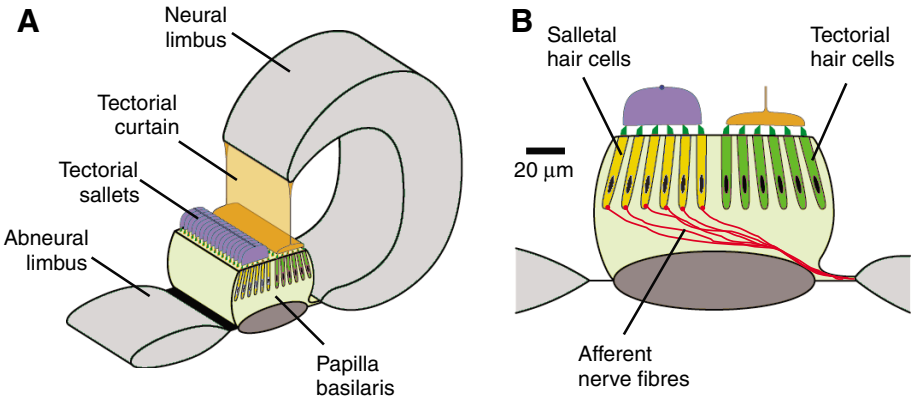

Fig. 6. A schematic diagram of the geckonid papilla. (A) A thick three-dimensional cross-section cut out of the doubly bi-directional, high-frequency region of the papilla, with the neural side on the right. This illustrates that the tectorial structure over the neural half of the papilla is connected to a tectorial curtain hanging down from the over-arching limbic 'lip', whereas the chain-of-pearls-like sallets have no connections to the limbus (shown in gray). (B) A schematic cross-section of the papilla and its tectorial structures. Only the salletal hair cells, here shown in yellow, are innervated by afferent nerve fibers (red). Drawing courtesy of A. J. Hudspeth and M. Gelfand.

predictions of the model, gave no indication of the presence of two populations defined by their frequency ranges. This puzzle was partly resolved by the finding that the neural population of hair cells totally lack an afferent innervation (Chiappe et al., 2007) (Fig. 6B). That nerve fibers in *Delma* contact hair cells in both neural and abneural areas and thus perhaps transmit data on different best frequencies is made very unlikely by the discovery that in their papillae, neural filaments stain just as in *Gekko*, implying that neural hair cells in these species, too, are not afferently innervated (C. Köppl, personal communication). It is possible that abneural hair cell themselves are alone responsible for responses to all frequencies, having bi-lobed tuning curves, but this would leave the neural hair cells without a sensory function. Perhaps hair cells of the neural hair-cell group do respond to higher frequencies than the salletal hair cells, as predicted by the model, and interact micro-mechanically with the adjacent hair cells of the abneural area, indirectly inducing responses of these hair cells to frequencies above 8 kHz. An indirect influence would be compatible with the substantially higher thresholds of responses to frequencies above 8 kHz. A similar suggestion was recently made for the responses of auditory receptor cells in Johnson's organ of the antennae of mosquitoes, for which a response forcing was envisaged, with some receptors responding to twice the best frequency of the hearing organ but amplifying the response of other receptors to lower frequencies (Jackson et al., 2009). It is an intriguing possibility worth pursuing that in *Delma*, a vertebrate ear has also realized the same twice-frequency forcing mechanism.

Chiappe et al. suggested that in *Gekko*, neural hair cells act as amplifiers for abneural hair cells, analogous to the outer hair cells of mammals (Chiappe et al., 2007). Although *Gekko* is not more sensitive than the skink *T. rugosa*, which has no separated hair-cell populations (Köppl, 1988), it is possible that in skinks, amplification phenomena are spread throughout all hair cells, rather than being concentrated in a dedicated hair-cell population as might be the case in geckos.

Our own unpublished SEM studies of *Delma* papillae show that compared with most geckos the higher-frequency hair-cell areas have been enlarged at the cost of low-frequency areas. Further understanding of the function of these papillae must await further comparative studies of structure and innervation in *Delma*. The present findings underscore the striking diversity of lizard hearing organs (Manley, 2002), now including a profound extension of their high-frequency hearing range.

Vocalizations

Weber and Werner briefly reported spectra of release calls of *Delma tincta* (a close relative of *D. pax*) to consist of rapid, rate-modulated clicks (their 'pulses') that produce broad-band, harmonically-structured squeaks whose frequencies exceeded the upper limit of their apparatus (16 kHz) (Weber and Werner, 1977). They also reported vocalization data for Burton's Snake Lizard *L. burtonis* with an upper limit of 12 kHz. *Lialis* calls recorded in the course of this study exhibited peak sound levels at 3.5 kHz but no vocalization energy above about 11 kHz, sometimes with little energy above 6 kHz. A lower upper limit of vocalization frequencies would be expected from the fact that *Lialis* is a larger animal than any *Delma*. We noted no vocalizations from the *Pygopus* species but these have been reported (Greer, 1989).

The strongest frequency components in the squeaks recorded here from *Delma* lay mostly between 6 kHz and 9 kHz, this presumably being determined by the acoustics of the sound-producing apparatus and buccal cavity. At present, it is not known how these clicks are produced, although it has long been known that the related *G. gecko* possesses vocal chords (Paulsen, 1967). In general, vocalizations often contain frequencies higher than can be perceived by the species (Konishi, 1969); thus, it need not be assumed that these animals hear all components of their vocalizations. There is as yet no published evidence that pygopods communicate (Greer, 1989) and no evidence for the production of such frequencies by their prey items. However, it is very interesting to note that in our data, the mean increase in sound pressure in the vocalizations up to 8 kHz (~4 dB kHz^{-1}) matches remarkably well the mean decrease in sensitivity of the audiograms (~4.5 dB kHz^{-1}) over this frequency range. Some individual vocalizations were biased towards high frequencies, with most sound energy between 6 kHz and 13 kHz, implying that these high frequencies are indeed perceived.

While the differences on the low-frequency side of the energy distributions in the vocalizations of different species are small, the high-frequency flanks are shifted almost 2 kHz with respect to one another (Fig. 4E). On average, the vocalizations of *D. haroldi* are the higher and more broad-band in frequency, then *D. pax*, then *D. fraseri*, their middle frequencies being *D. haroldi* 8.8 kHz, *D. pax* 7.8 kHz and *D. fraseri* 6.7 kHz. Our estimates of vocalization sound pressures and of hearing audiograms suggest that at close range, *Delma* species can hear their own call components up to a frequency of at least 12 kHz. Thus, they are indeed capable of picking up most of the call energy that would be entirely lost on other groups of lizards. One potential predator, *L. burtonis*, is not able to hear call components much above 6 kHz. We disagree with the second part of the statement of Weber and Werner that 'The sound energy at 6–10 kHz is certainly wasted on the ear of *Lialis* and this is presumably also true for most of the energy in the *Delma* vocalization' (Weber and Werner, 1977). Our data indicate that *Delma* can in fact hear most of the energy in its calls.

LIST OF ABBREVIATIONS

CAP	compound action potential
CM	cochlear microphonic
SOAE	spontaneous otoacoustic emissions

ACKNOWLEDGEMENTS

We thank Otto Gleich and Christine Köppl for comments on an earlier version of the manuscript and Brad Maryan (W.A. Museum), Don Robertson (University of W. Australia), Peter Kendrick (D.E.C. of W.A.), the owners of Anna Plains, DeGrey, Mt. Florance and Pardoo cattle stations, and National-Park rangers, especially Mark True at *Peron N.P.*, Peter Morris at Karijini N.P. and Scott Godley at Millstream-Chichester N.P. for their help, generous hospitality and unreserved support. Arne Laucht prepared the CAP and forward-masking Labview routines. Supported by the Deutsche Forschungsgemeinschaft (DFG; MA 871/10).

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