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## RESEARCH ARTICLE

# Sound-induced tympanal membrane motion in bushcrickets and its relationship to sensory output

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**SUMMARY**

**In the auditory system of bushcrickets, sound can reach the receptors *via* two different paths: (i) by acting on the outside of the tympana situated on both sides of each foreleg or (ii) through the acoustic trachea that opens at a spiracle on the thorax. While the spiracle is considered to be the main point of sound entry for higher audio and ultrasonic frequencies, the role of the tympana is still unclear. The tympana border the air-filled acoustic trachea as well as the fluid-filled haemolymph channel containing the receptor organs. To understand their role during sound transduction, the sound-induced neuronal response of the hearing organ was recorded in combination with measurement of tympanal membrane motion using laser-Doppler vibrometry. For far-field stimulation, the frequency of the most sensitive hearing (~16 kHz) matched the frequency of a pronounced maximum of tympanal membrane vibration. A second maximum of tympanum motion at lower frequencies (~7 kHz) was correlated with an increased nerve activity at higher intensities (>70 dB sound pressure level, SPL). These correlations support the hypothesis of functional coupling between tympanum motion and nerve activity. When sound stimuli were applied locally, through either the tympanum or the spiracle, significant differences between tympanum motion and nerve activity were found. These discrepancies show that tympanum motion and neuronal response are not coupled directly and that there is no linear relationship with the applied SPL. Taken together, these data verify a functional, albeit indirect, coupling of tympanum motion and sensory cell activity for one of the pronounced vibration maxima, which appears to represent a resonance frequency of the tympanum.**

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Key words: laser-Doppler vibrometer, *Mecopoda elongata*, katydid, tympanal nerve, spiracle, insect hearing.

## INTRODUCTION

In bushcrickets and crickets, the auditory organs are located distal from the tibio-femoral joint in the tibiae of all six legs. However, it is only in the forelegs that sensitive transduction of airborne sound occurs. Here, an acoustic trachea that opens on the thorax and two thin cuticle regions on the tibiae, called tympanal membranes, are believed to be responsible for the 50 dB sensitivity difference between the forelegs and the four other legs (Kalmring et al., 1994). The hearing organs are chordotonal organs and are located on top of the auditory trachea (dorsal wall) within a haemolymph-filled channel (Fig. 1). The chordotonal organ itself comprises units named scolopidia. A scolopidium is a multicellular unit, consisting of at least one bipolar sensory neuron that has a ciliary dendritic structure (Yack, 2004). This dendrite is surrounded by a scolopale cell, which is terminated at the top by a cap cell. The scolopidium acts as mechanoreceptor (type I) (Keil, 1997; Eberl, 1999) and it has been suggested that a stretching of the dendrite leads to the transduction of the sound-induced motion into an electrical signal in the sensory cell (French, 1988). The hearing organ in bushcrickets consists of three parts – the subgenual organ at the proximal end, the intermediary organ, and the crista acustica at the distal end (Schumacher, 1975; Rössler, 1992; Lin et al., 1993; Sickmann et al., 1997) (for a review, see Yager, 1999). In katydids, the crista acustica is tuned to a broad frequency spectrum from 5 to ≥70 kHz (Autrum, 1940; Oldfield, 1985; Lakes and Schikorski, 1990) (for a review, see Yack, 2004), whereas the subgenual and intermediary organ are tuned to low frequencies (<10 kHz) of airborne sound and vibration. The nerve fibres of all three organs form a bundle proximal to the tympanum comprising the tympanal nerve, which runs into the leg nerve (Bangert et al., 1998).

It is generally agreed that the opening in the thorax, termed the spiracle, is the main entrance for sound input (Lewis, 1974a; Lewis, 1974b; Nocke, 1975; Michelsen and Larsen, 1978; Seymour et al., 1978; Hill and Oldfield, 1981; Larsen, 1981; Heinrich et al., 1993; Shen, 1993; Kalmring and Jatho, 1994; Kalmring et al., 1995b; Hoffmann and Jatho, 1995). However, there is still disagreement about the transmission properties of the acoustic trachea. Nocke described the acoustic trachea as a tube-resonator system where the acoustic transmission and thus the tuning of the receptor cells depends on the length of the trachea (Nocke, 1975). Then again, the acoustic trachea has also frequently been described as an exponential horn, which amplifies sound over a broad frequency range above a certain cut-off frequency (Lewis, 1974a; Hill and Oldfield, 1981; Shen, 1993; Heinrich et al., 1993; Hoffmann and Jatho, 1995). This cut-off frequency is believed to characterize the high-pass sound amplification resulting from the shape of the acoustic trachea (e.g. Lewis, 1974a; Kalmring and Jatho, 1994).

At the level of the crista acustica, two tympanal membranes are located on the anterior and posterior side of the leg, respectively. The somas of the sensory cells are located behind the anterior

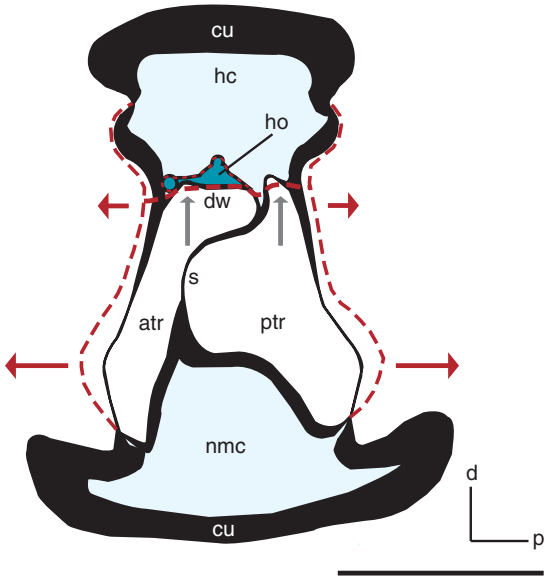


Fig. 1. Schematic cross-section of the right tibia foreleg at the level of the hearing organ. The dark grey arrows indicate a possible internal sound pressure acting on the dorsal wall and therefore on the bottom of the hearing organ (see also Bangert et al., 1998). Additionally, sound-induced tympanic membrane motion and a possible corresponding stretch-and-pull effect on the dorsal wall as well as the translocation of the hearing organ are indicated (red arrows and dashed line; movement is not drawn to scale). Black structures, cuticle; light blue structures, haemolymph-filled channels; white structures, air-filled tracheae. atr, anterior tracheae branch; cu, cuticle; d, dorsal; dw, dorsal wall; hc, haemolymph channel; ho, hearing organ (dark blue structure); nmc, nerve muscle channel; p, posterior; ptr, posterior tracheae branch; s, septum. Bar, 500 μm.

tympanum on top of the anterior tracheal branch. It has been shown that the mobility of the tympana is essential for receptor cell activity. Here, manipulation of tympanum mobility causes a considerable increase in the hearing threshold of up to ~30 dB in crickets (Nocke, 1972; Hill, 1974; Lewis, 1974b; Kleindienst et al., 1983) and up to ~32 dB in bushcrickets (Nocke, 1975).

Laser-Doppler vibrometer (LDV) measurements showed that the tympana act as 'hinged flaps' and the anterior and posterior tympana are phase coupled (Bangert et al., 1998; Kalmring et al., 2003; Nowotny et al., 2010). There is strong evidence that the outward movement of the tympana during a sound event results in a stretching of the dorsal wall (dorsal part of the hearing trachea) with the crista acustica on top of it. Additionally, negative pressure in the haemolymph channel, which contains the hearing organ, could be caused by a spreading of the channel. Both the outward movement and the negative pressure could influence the response of auditory receptor cells (Fig. 1). Thus, the tympana could be functionally coupled with sensory cell activity by acting as a bridge between airborne sound and crista acustica motion. A previous study by Bangert and colleagues presented preliminary findings of a correlation between tympanal membrane vibration and neuronal response in the species *Mygalopsis marki* and suggested that the tympana might also have an impedance-matching function (Bangert et al., 1998). However, it is difficult to gain information about the impedance of the system because of a lack of information about essential properties like mass, stiffness and damping of the tympanum.

Hence, the aim of this study was to compare in detail tympanal membrane motion and sensory cell responses through non-invasive measurements and to learn more about the role of the tympana in bushcricket hearing. If there is a correlation between tympanal membrane motion and the neuronal response, the tympanal vibration pattern could provide information about certain auditory characteristics, like the frequency hearing range or the threshold minima. We used successive LDV motion measurements of the anterior tympanum and nerve recordings to test whether tympanal nerve activity and tympanal vibration patterns in the tropical bushcricket *Mecopoda elongata* are functionally coupled. Furthermore, we reasoned that manipulations of the system, such as occlusion of the spiracle and local stimulation of the sound entry points, might provide evidence on the degree to which the tympanum motion is functionally coupled, directly or indirectly, to the sensory cell responses.

## MATERIALS AND METHODS

All experiments were performed using adult male and female bushcrickets ($N$=21) of the species *M. elongata* L. (Insecta, Orthoptera, Tettigoniidae). This species has the anatomical advantage of uncovered tympana. For this reason the tympana are accessible without further preparative manipulations. The animals were taken from our own breeding colony, where they were kept at a temperature of 25±1°C and a humidity of 60±10%. The animals were briefly anaesthetized with $CO_2$ and the midlegs and hindlegs as well as parts of the wings were removed. Afterwards, the animals were fixed, ventral surface down, on a T-shaped metal holder using rosin–beeswax (Fig. 2A). The tarsi of the forelegs were held using wax on metal rails on both sides in a natural position with ~140 deg between the femur and the tibia. In all experiments the right leg was used for data acquisition. To ensure free access to the spiracle for separate acoustic stimulation, a small part of the cuticle lip, which covers the spiracle, was removed. LDV measurements verified that this manipulation had no effect on sound input. The experiments were performed at a temperature of 22±2°C.

### LDV measurements

A LDV was used to measure sound-induced vibration of the bushcricket tympanum. The experimental setup consisted of a LDV scanning system (Polytec MSV-300 with a sensor head OFV-534, Waldbronn, Germany) attached to a microscope (Axio Examiner, Zeiss, Germany) *via* a microscope adapter (OFV-072, Polytec). Two different acoustic stimulus conditions were used: (i) a high frequency loudspeaker (D2904/7000, Scan Speaker, membrane diameter ~3.7 cm) with its central spine directed towards the spiracle from a distance of 30 cm (defined as far-field situation) and (ii) a half-inch probe microphone used as a loudspeaker (MK202, Microtech Gefell, Gefell, Germany, membrane diameter ~1.3 cm; termed a 'probe speaker' in the following description) positioned at a distance of 1 mm from the tympanum or inserted a short distance into the spiracle for local stimulation (distance ~0.2 mm). Because of the small size of the tympanum (~1.7 mm length) and the spiracle (~1.5 mm diameter), the loudspeaker was connected to a coupler that terminated in a pipette with a tip diameter of ≤1 mm. The pipette tip was filled with glass wool to reduce acoustic interference and could be positioned accurately in front of the tympanum or the spiracle. The signals (4–78 kHz) used for acoustic stimulation were produced by a function generator (NI 611x, Polytec) and amplified (RB-850, Rotel, North Reading, MA, USA, for far field; two custom-made amplifiers for probe speaker). In both arrangements a wideband signal (frequency sweep; implemented as 'periodic chirp' in the LDV software) was used. Constant sound pressure levels (SPLs) across the applied frequency range were ensured by

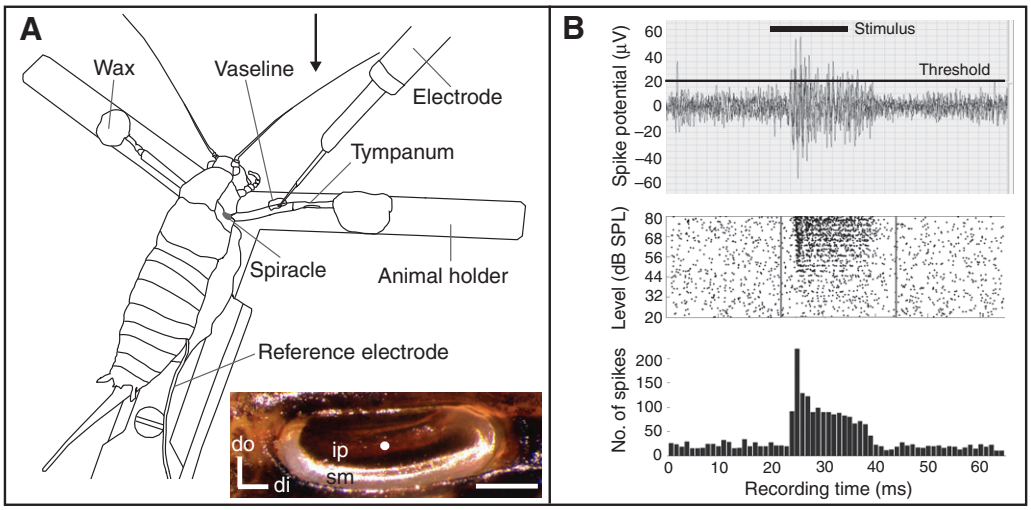


Fig. 2. Experimental set-up and electrophysiological data processing. (A) The animal is held by wax on a metal holder and the electrode for extracellular recording is placed in the right femur near the tympanal nerve. The reference electrode is inserted into the abdomen. The black arrow indicates the direction of the laser beam for tympanum membrane motion measurements. The inset shows an example of the anterior tympanum with the laser-Doppler vibrometry (LDV) measuring point indicated as a white dot. di, distal; do, dorsal; ip, inner plate; sm, surrounding membrane. Bar, 500 µm. (B) Example of the tympanal nerve response to a stimulus of 15 ms duration. Upper trace: spike activity in response to 19 kHz stimulation at 80 dB sound pressure level (SPL). The black line indicates the chosen threshold for spike discrimination. Middle trace: spike dot display in response to increasing SPLs of all trials (from 1 to 80 kHz, 3 kHz steps). Lower trace: poststimulus time histogram of spike activity for all frequency-level combinations used.

calibrating the far-field loudspeaker as well as the probe speaker with a condenser microphone (MK301, Microtech Gefell; membrane diameter ~7 mm; flat frequency response up to 80 kHz) positioned at the location of the animal (at distances of 30 cm and 1 mm to the loudspeaker, respectively). The calibration curves were used for frequency-dependent amplitude correction of the applied SPLs.

Because of the localization of the somata of the receptor cells on top of the anterior tracheal branch, the anterior tympanum was used to detect the pattern of membrane motion. Recordings were obtained at a sampling rate of 256 kHz and were averaged 10 times per measuring point. The recording of each of these measuring points took 16 ms. Data were fast Fourier transform (FFT) analysed at a resolution of 62.5 Hz. The coherence between the stimulus and measured motion had to be ≥90%, otherwise the data were not taken into consideration.

### Electrophysiological recordings

To determine neuronal responses at different SPLs, the evoked spike activity of the nerve fibres in the right front leg was measured using a steel needle electrode (Austerlitz Insect Pins® Minutiens, Slavkov u Brna, Czech Republic; diameter 0.2 mm). A small hole of ~1×1 mm maximum was made in the cuticle on the anterior site of the femur. The animal was then positioned under the microscope and the steel electrode was carefully inserted into the opening until it reached the leg nerve including the axons of the complex tibial organ (Fig. 2A), hereafter referred to as the tympanal nerve. It is assumed that the axons of the subgenual and intermediary organ influence tympanal nerve activity during low frequency airborne sound. The tympana cover all three hearing organs. Therefore, the tympanal vibration pattern was compared with the nerve response of all of these fibres. Only after a distinct acoustically induced response was detected by visual and auditory monitor was the cuticular window closed by Vaseline to prevent desiccation. Another steel electrode, used as a reference electrode, was inserted into the abdomen of the animal.

Pure-tone stimuli were generated by a data acquisition board (DAP 5216a board, Microstar Laboratories, Bellevue, WA, USA) and modified by a programmable attenuator (PA5, TDT PC1, Tucker-Davis Technology, Alachua, FL, USA) to produce different SPLs. The used amplifiers and loudspeakers were the same as those in the LDV setup described above. To determine auditory thresholds, pure-tone stimuli of 15 ms duration and a rise/fall time of 4 ms were used. Pure-tone stimuli within a wide range of frequencies (1–80 kHz, in 3 kHz steps) and levels (20–80 dB SPL, in 3 dB steps) were randomly presented at a repetition period of 500 ms. Each frequency–level combination was presented 5 times and the resulting responses were averaged. The recorded nerve activity was amplified 1000 times, band-pass filtered (0.04–3.0 kHz) and fed back into the DAP board. The typical recording time per loudspeaker stimulus condition was about 15 min.

### Experimental procedures

The following arrangements were used to measure tympanal nerve activity and the motion of the tympanum: (i) far field at a distance of 30 cm directed to the ipsilateral spiracle, (ii) probe speaker pointing to the tympanum and (iii) probe speaker inserted into the ipsilateral spiracle. Additionally, the ipsilateral spiracle could be closed in the first two arrangements (for detailed presentation of the experimental arrangements see also supplementary material Fig. S1). To simulate as close as possible the natural conditions, an approximately 5 mm long tube was fixed to the microphone, comparable to the entrance of the auditory trachea. The occlusion of the spiracle was conducted using small removable patches of sticky cellulose tissue. To evaluate the sound attenuation of the cellulose tissue, stimulus frequencies of 5–65 kHz (5 kHz steps) with 60 and 80 dB SPL were used, while the loudspeaker output was measured with and without a cellulose patch between the loudspeaker and the measuring microphone. The sound attenuation by the cellulose patch was frequency dependent. In the frequency

range of interest (1–40 kHz) the attenuation of the cellulose patch amounts to about 10–20 dB, starting with 10 dB attenuation at 1 kHz.

### Data analysis

The analysis of tympanal membrane motion was performed using PSV software (v 8.5, Polytec). Depending on the size of the anterior tympanum, the actual number of measuring points varied for the respective animals, with a mean of about 300 points per tympanum. From this array of points, a single measuring point in the centre of the tympanum (Fig. 2A, inset), which best represented the mean frequency characteristics of the whole tympanum motion, was chosen to evaluate the displacement amplitudes of the tympanal membrane motion. Although the tympanal membrane motion has distally and proximally different displacement amplitudes, as previous described (Nowotny et al., 2010), the ratio of vibration changes between the two conditions (e.g. open and closed spiracle) remains constant over the whole scanned membrane. For analysis of sound-evoked tympanal motion, only amplitudes ≥20 pm were taken into consideration.

Data acquisition and evaluation of neuronal responses of the tympanal nerve were done by custom-made software in MATLAB v 7.6.0 (The MathWorks, Inc., Natick, MA, USA). The software first high-pass filtered the measured signal with a cut-off frequency of 800 Hz to exclude possible evoked potentials of the summed tympanal nerve response. Subsequently, only distinct spikes with amplitudes above the noise level were used for further analysis (Fig. 2B). Spike waveform analysis (Lewicki, 1998; Abel and Kössl, 2009) was performed to exclude recording artifacts. The resulting multi-unit spike data are assumed to represent evoked activity of the whole tympanal nerve. The frequency- and SPL-dependent threshold curves of the tympanal nerve response were calculated using a threshold criterion of 30% of maximum nerve activity. For a comparison of the single measurements in one animal, the threshold curves were normalized to the maximal spike rate measured in this animal. From the resulting threshold curves, the threshold minimum and the bandwidth of the threshold curve 10 dB above this threshold minimum ($BW_{10dB}$), which indicates the tuning sharpness of threshold curves, were determined. The mean spike activity is illustrated in 5 kHz steps. To get this resolution the measured spike activities (stimulation: 3 kHz steps) were interpolated. Statistical evaluation was done by Jump 7.0 (SAS Institute Inc., Cary, NC, USA). Two non-parametrical tests, the Wilcoxon signed-ranks test (matched pairs test) and the Wilcoxon–Kruskal–Wallis test, were used, depending on whether the samples were paired or not. Averaged values of both the LDV measurements and the electrophysiological recordings are given as the median (Q2), supported by quartiles 1 and 3 (Q1, Q3).

## RESULTS

### Far-field stimulation experiments

To assess the relationship between tympanum motion and tympanal nerve activity, we first measured the response of the tympanal nerve to different frequencies and SPLs. Under far-field conditions, the neuronal spike activity of the tympanal nerve of *M. elongata* was evoked by stimulus frequencies of 1–60 kHz at higher SPLs (>70 dB SPL; Fig. 3A). The threshold minimum of the tympanal nerve activity (e.g. Fig. 3A, black curve) was on average 16.0 kHz (*N*=10 animals, with quartiles Q1 of 14.5 kHz and Q3 of 18.0 kHz) at a threshold level of 35.0 dB SPL (Q1: 32.3 dB SPL, Q3: 40.5 dB SPL, *N*=10). The threshold curve was sharply tuned with a $BW_{10dB}$ of 10.5 kHz (Q1: 6.0 kHz, Q3: 15.3 kHz, *N*=10). All averaged values of the electrophysiological recordings of tympanal nerve activity are also summarized in Table 1.

To gain more insight regarding the hearing properties of *M. elongata* in general and the influence of the spiracle as the main sound input in particular, the effects of spiracle occlusion on nerve activity were investigated. For this purpose a small removable patch of sticky cellulose tissue was attached to the prothoracal segment at the position of the spiracle opening (sound attenuation of 10–20 dB between 1 and 40 kHz). Correspondingly, in the example given in Fig. 3A, the tympanal nerve threshold curve was elevated by 10–20 dB (red line) for frequencies ≥8 kHz. In comparison to the spiracle open condition, after occlusion the threshold minimum of the tympanal nerve activity was significantly (*P*=0.0368) shifted upwards to a level of 44.0 dB SPL (Q1: 37.7 dB SPL, Q3: 46.5 dB SPL, *N*=10). The frequency of the threshold minimum was 15.0 kHz (Q1: 13.5 kHz, Q3: 16.0 kHz, *N*=10) after occlusion.

Additionally, when the spiracle was closed, a slight decrease in the averaged threshold curves was measured up to a frequency of about 10 kHz (Fig. 3B). At higher frequencies (>10 kHz) the auditory sensitivity decreased between 10 and 15 kHz (arrow in Fig. 3B indicating the change) by about 10 dB with significant differences between the open and closed stimulus conditions (≥20 kHz). In the closed spiracle condition at higher frequencies (>40 kHz), neuronal activity did not reach the 30% threshold value for levels below 80 dB SPL (Fig. 3A). In such cases, a minimum difference between the open and closed condition was assessed by subtraction of the open threshold value from 80 dB SPL, which was the maximal SPL used. The actual difference is likely to be much greater.

At 80 dB SPL, tympanal membrane motion is evoked by frequencies between 5 and 60 kHz. Fig. 3C (black curve) shows the tympanal membrane vibration for the same animal as described in Fig. 3A. Within the range of stimulus frequencies used, two displacement maxima were apparent at 9.8 and 15.9 kHz with respective displacement amplitudes of 388 and 308 pm. In all but one animal, two maxima were present, on average at 7.2 kHz (Q1: 4.8 kHz, Q3: 7.6 kHz, amplitude 336.4 pm, *N*=20) and 15.9 kHz (Q1: 14.4 kHz, Q3:16.0 kHz, amplitude 244.7 pm, *N*=21). No significant differences (*P*=0.4085) between the tympanal nerve threshold minimum and the frequency of the second maximum of the tympanal membrane motion were found.

Subsequently, we also measured tympanal membrane motion with the spiracle closed. In this case, displacement amplitudes of the tympanum were slightly larger at lower stimulus frequencies (e.g. Fig. 3C, red curve). On average, the tympanal membrane vibration was slightly larger than in the open condition at frequencies up to 17 kHz (Fig. 3D) with significant changes at 7–10 kHz. Above 17 kHz (see arrow in Fig. 3D), the amplitude of the tympanum displacement was smaller than that for the open spiracle condition and from 30 to 50 kHz the amplitudes were significantly smaller in the closed condition. Above 60 kHz, the displacement amplitudes of the tympanum vibration were below the coherence criterion. The maxima of displacement amplitudes were not shifted significantly, amounting to 6.9 kHz (Q1: 4.8 kHz, Q3: 7.6 kHz, *N*=18; *P*=0.7002) and 15.4 kHz (Q1: 14.4 kHz, Q3: 16.0 kHz, *N*=18, *P*=0.8322). The results persist over the whole scanned tympanum and are also reversible. In summary, while the spiracle is closed, both tympanal nerve activity and tympanal membrane motion become more sensitive at lower frequencies up to 13 kHz (tympanal nerve response) and 17 kHz (tympanal membrane motion), respectively.

The measurements of the tympanal vibration were taken at a constant stimulus level of 80 dB SPL. To allow a direct comparison between LDV measurements of tympanal vibration and tympanal

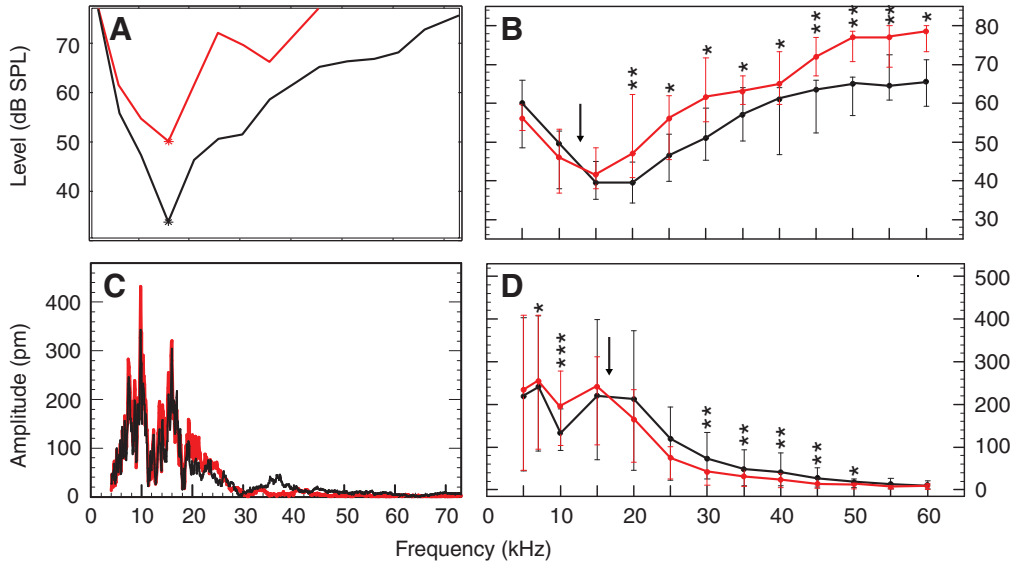


Fig. 3. Tympanal nerve activity and tympanal membrane vibration during far-field stimulation with open (black lines) and closed spiracle (red lines). (A) Examples of tympanal nerve threshold curves (threshold criterion: 30% of maximal activity; curves show the lowest threshold in 5 kHz steps) in one animal (Mec9). Asterisks indicate the threshold minima. (B) Mean thresholds (30% threshold criteria; *N*=10 animals). Asterisks indicate a significant difference between the open and closed spiracle condition (**P*<0.05, ***P*<0.01). The frequency at which the threshold starts to increase after closing the spiracle is indicated by a black arrow. (C) Example of tympanal displacement at a representative recording position in the centre of the tympanum membrane measured with LDV using a similar range of stimulation frequencies to that in A. Stimulus level: 80 dB SPL. (D) Mean displacement amplitude of the tympanal membrane motion at 80 dB SPL (*N*=18); **P*<0.05, ***P*<0.01, ****P*<0.001. The frequency at which the amplitude starts to decrease after closing the spiracle is indicated by a black arrow. The data are given in medians and the lower and upper error calculation in 25th and 75th percentiles, respectively. Significant differences of single frequencies are given in supplementary material Table S1.

nerve recordings, poststimulus time histogram (PSTH) data were obtained at the same stimulus level of 80 dB SPL. Apart from the use of cellulose tissue for spiracle closing, the experimental conditions were the same in the two stimulus situations with an open and closed spiracle and, thus, the differences between these responses can be used for a comparison of PSTHs. In some animals, neuronal spike activity decreased at frequencies higher than 34 kHz with the spiracle closed (PSTHs, Fig. 4A). In the averaged data set, this decrease was not statistically significant except at a frequency of 18 kHz (*P*=0.0488, *N*=10; Fig. 4B). In summary, occluding the spiracle leads to no significant differences of the spike rate at 80 dB SPL over the whole stimulus frequency range, whereas the tympanal membrane motion increases for frequencies up to 17 kHz (significantly between 7 and 10 kHz).

### Local stimulation through the spiracle or tympanum

To evaluate by which acoustic pathway the hearing organs receive their input, a probe speaker was either inserted into the spiracle or positioned close to the tympanum. By stimulating through the ipsilateral spiracle, neuronal activity was generally evoked by stimulus frequencies of about 1–60 kHz at a level of 80 dB SPL (Fig. 5A, red curve). On average, the minimum of the threshold curves was at a frequency of 16.0 kHz (Q1: 13.0 kHz, Q3: 20.5 kHz, *N*=14) with a threshold level of 40.9 dB SPL (Q1: 39.2 dB SPL, Q3: 47.8 dB SPL, *N*=14). The $BW_{10dB}$ was 20.0 kHz (Q1: 16.0 kHz, Q3: 25.3 kHz, *N*=13) indicating a broad tuning of the summed activity of the tympanal nerve (Fig. 5A).

When the probe speaker was inserted into the spiracle, large amplitudes of tympanal membrane motion were reached for frequencies between 5 and 50 kHz. In the example shown in Fig. 5B (red curve) the maximal displacement amplitude reached about 10 nm. On average, maximal amplitudes of 5.4 nm (Q1: 3.1 nm, Q3: 8.7 nm, *N*=16) were evoked by a stimulus frequency of 12.2 kHz (Q1: 11.3 kHz, Q3: 17.9 kHz, *N*=16). Compared with the far-field stimulation, the amplitudes were higher by a factor of about 18.

When the probe speaker was positioned at 1 mm distance from the tympanum, neuronal threshold was significantly elevated by about 18 dB (*P*<0.0001, *N*=10) in comparison to the spiracle stimulation. The most effective stimulation frequencies were between 1 and 40 kHz (supplementary material Fig. S2A). The frequency of the

Table 1. Measured averaged values given as the median (second quartile, Q2) and the first and third quartiles (Q1/Q3) of the electrophysiological recordings of the summed tympanal nerve spike activity in different stimulations

| Stimulation | *N* | Frequency of threshold minimum (kHz) | Threshold minimum (dB SPL) | *N* | $BW_{10dB}$ (kHz) |
|---|---|---|---|---|---|
| Far field, open spiracle | 10 | 16.0 (14.5/18.0) | 35.0 (32.3/40.5) | 10 | 10.5 (6.0/15.3) |
| Far field, closed spiracle | 10 | 15.0 (13.5/16.0) | 44.0 (37.7/46.5) | 10 | 12.0 (10.5/22.0) |
| Probe speaker pointed at tympanum, open spiracle | 10 | 16.0 (15.3/18.0) | 62.9 (60.7/66.0) | 10 | 16.0 (13.0/23.8) |
| Probe speaker pointed at tympanum, closed spiracle | 10 | 16.0 (14.3/16.8) | 66.5 (63.8/69.1) | 8 | 13.5 (8.5/24.5) |
| Probe speaker inserted into ipsilateral spiracle | 14 | 16.0 (13.0/20.5) | 40.9 (39.2/47.8) | 13 | 20.0 (16.0/25.3) |

*N*, number of tested animals; $BW_{10dB}$, frequency bandwidth 10 dB above the threshold minimum.

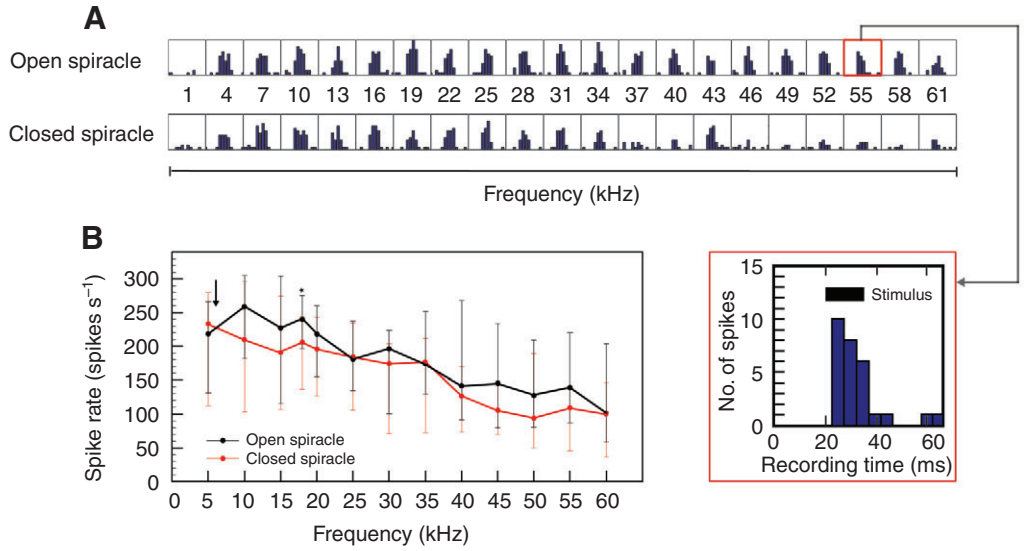


Fig. 4. Comparison of tympanal nerve activity at a stimulus level of 80 dB SPL with the spiracle open or closed. (A) Example of PSTHs for a stimulus frequency range 1–61 kHz in one animal (with enlarged view of PSTH in the red box). (B) Mean spike rates at a level of 80 dB SPL ($N$=10). The asterisk indicates a significant difference (*$P$<0.05) between the open and closed spiracle stimulation condition. The data are given in medians and the lower and upper error calculation in 25th and 75th percentile, respectively.

threshold minimum (Q1: 15.3 kHz, Q2: 16.0 kHz, Q3, 18.0 kHz, $N$=10) did not differ from that measured during local stimulation through the spiracle ($P$=0.7452). On average, the level of the threshold minimum was 62.9 dB SPL (Q1: 60.7 dB SPL, Q3: 66.0 dB SPL, $N$=10) and the $BW_{10dB}$ value was 16.0 kHz (Q1: 13.0 kHz, Q3: 23.8 kHz, $N$=10). Subsequently, we closed the spiracle during stimulation of the tympanum to ensure that sound could not have reached the hearing organ through the spiracle. No differences in frequency and level of the threshold minima or in the $BW_{10dB}$ value were detected (threshold minimum frequency: Q1: 14.3 kHz, Q2: 16.0 kHz, Q3: 16.8 kHz, $N$=10, $P$=0.3912; threshold minimum level: Q1: 63.8 dB SPL, Q2: 66.5 dB SPL, Q3: 69.1 dB SPL, $N$=10, $P$=0.0743; $BW_{10dB}$: Q1: 8.5 kHz, Q2: 13.5 kHz, Q3: 24.5 kHz, $N$=8, $P$=0.7538). Tympanal displacement amplitudes were evoked by frequencies from 4 to 78 kHz when using high stimulus levels of 80 dB SPL during local stimulation of the tympanum (supplementary material Fig. S2B). The largest displacement amplitudes of on average 402.9 pm (Q1: 306.8 pm, Q3: 445.2 pm, $N$=13) were found at 9.7 kHz (Q1: 9.4 kHz, Q3: 9.7 kHz, $N$=13).

Additionally, when the spiracle was closed while the tympanum was stimulated, the threshold minimum was increased by about 4.0 dB ($P$=0.0195, $N$=10) and slightly shifted towards lower frequencies. The maximum of tympanal displacement with the spiracle closed was not significantly changed in amplitude or frequency (Q1: 283.7 pm, Q2: 358.5 pm, Q3: 405.9 pm, $P$=0.4119; Q1: 9.4 kHz, Q2: 9.7 kHz, Q3: 9.7 kHz, $P$=1.0000; $N$=13).

### Comparison of local and far-field stimulation

There are pronounced differences when tympanal nerve activity and tympanal motion are compared for local and far-field stimulation. For example, at a stimulation frequency of 15 kHz and 80 dB SPL, spike activity for local spiracle and far-field stimulation was nearly equal (Fig. 5C), whereas the displacement amplitude of tympanum motion was significantly larger (by about a factor of 18, $P$<0.0001) at this frequency as well as over the whole stimulus frequency range used (Fig. 5D). In addition, the minimum threshold during far-field stimulation was at significantly lower levels compared with local stimulation through the spiracle ($P$=0.0280, $N$=14). Moreover, in local tympanal stimulation the maximum tympanal displacement amplitudes were comparable with amplitudes measured during far-field stimulation, whereas the neuronal spike rate was strongly reduced (supplementary material Fig. S2). Thus, there is a huge discrepancy between tympanal nerve activity and tympanal membrane vibration using local and far-field stimulation.

## DISCUSSION

### Hearing in *M. elongata*

The present study evaluated the relationship between tympanal membrane motion and sensory cell activity using LDV measurements and electrophysiological recordings in the bushcricket *M. elongata*. Neuronal activity can be evoked over a broad stimulus frequency range from ~2 to 60 kHz at higher SPLs (~80 dB SPL). This frequency range is comparable to the auditory range of other bushcricket species (for a review, see Rössler et al., 2006). Typically, the frequency of most sensitive hearing of certain bushcricket species as well as for *M. elongata* is between 10 and 30 kHz (Autrum, 1940; Hartbauer et al., 2010) (for reviews, see Yack, 2004; Rössler et al., 2006). Our neurophysiologically measured threshold curves had their minimum on average at ~16 kHz, comparable to previous results.

The tuning of the hearing organ in most bushcricket species matches the carrier frequency of their calling song (Nocke, 1975; Kalmring et al., 1995a; Kalmring et al., 1996; Stumpner, 1997). Nityananda showed for the power spectra of the calling song that there are only small species-specific differences within the genus *Mecopoda* (Nityananda, 2007). The calling songs cover frequencies from 2 to 70 kHz with two narrow spectral peaks between 7 and 8 kHz, and 12 and 18 kHz, and a broad spectral maximum extending from 22 to 70 kHz. Thus, the acoustic sensitivity of *M. elongata* seems to correspond well with the calling song characteristics of the genus *Mecopoda*.

### Far-field experiments

For far-field stimulation (4–78 kHz), comparable with the natural hearing situation of bushcrickets, the tympanal nerve response is

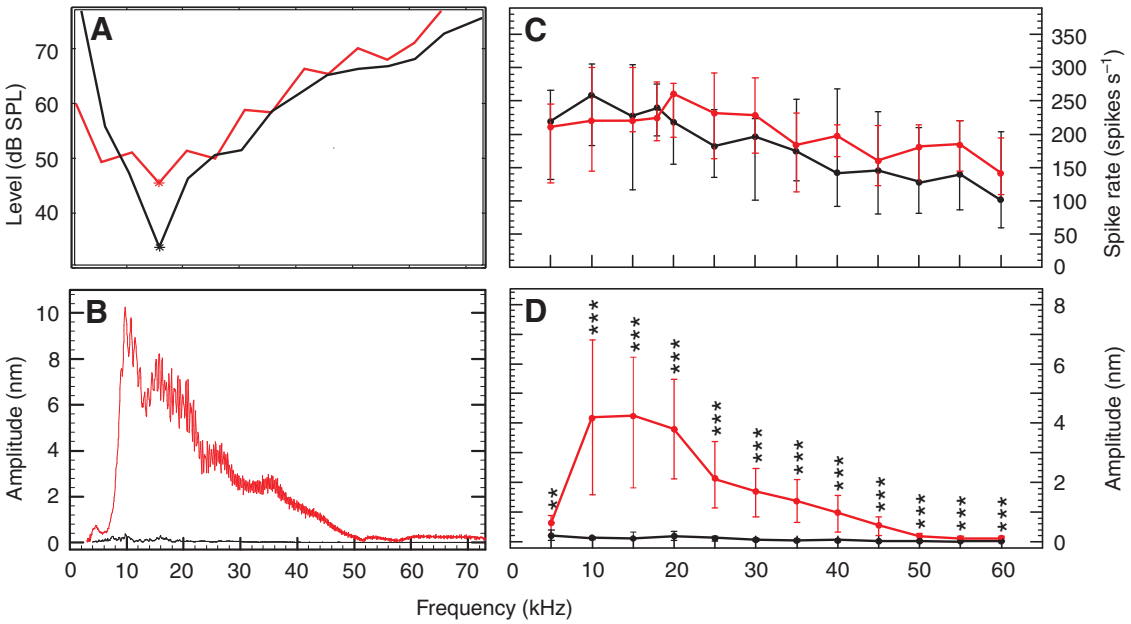


Fig. 5. Tympanal nerve activity and tympanal membrane vibration during far-field stimulation (black lines) and during local stimulation through the ipsilateral spiracle (red lines). (A) Tympanal nerve threshold curves (threshold criterion: 30% of maximal activity; curves show the lowest threshold in 5 kHz steps) in one animal (Mec9). Asterisks indicate the threshold minima. (B) Example of displacement amplitudes of tympanal membrane motion evoked at 80 dB SPL. (C) Mean tympanal nerve activity (far field, $N$=10; spiracle, $N$=14) and (D) mean displacement amplitude of the tympanal membrane motion (far field, $N$=21; spiracle, $N$=16) at a stimulus level of 80 dB SPL. The asterisks indicate a significant difference (**$P$<0.01, ***$P$<0.001) between the two experimental conditions. The data are given in medians and the lower and upper error calculation in 25th and 75th percentile, respectively. Significant differences of single frequencies are given in supplementary material Table S1.

characterized by a single threshold minimum at ~16 kHz. The tympanal membrane vibration, however, had two amplitude maxima at 7 and 16 kHz. The source of these two amplitude maxima is so far unclear. On the one hand the maxima could be the result of a resonance of the tympanal membrane itself and on the other hand they could be a product of the anatomical and physical properties of the trachea [for detailed discussion, see Nowotny et al. (Nowotny et al., 2010)]. The fact that the amplitude maxima did not change in frequency after the leg (including the acoustic trachea) had been removed suggests a mechanical resonance of the tympanum.

The discrepancy of tympanal nerve sensitivity *versus* tympanal membrane vibration at 7 kHz is surprising, because the tympana are known to transmit lower frequencies particularly well below the cut-off frequency of the acoustic trachea (Lewis, 1974a; Lewis, 1974b; Nocke, 1975; Michelsen and Larsen, 1978; Seymour et al., 1978). The acoustic trachea of other bushcrickets have a cut-off frequency in the range between 3 kHz (*Gampsocleis gratiosa*) and 7 kHz (*Mygalopsis marki*) (Kalmring and Jatho, 1994; Michelsen et al., 1994; Römer and Bailey, 1998). In our experiments, closing of the spiracle indicated that the cut-off frequency of the acoustic trachea was above 7 kHz with the strongest sound amplification for frequencies above ~13 kHz. This is seen mostly in the far-field stimulation when nerve responses did not decrease up to a frequency of ~13 kHz, although the spiracle had been closed (Fig. 3B, marked by the arrow). Therefore, the sensory cells were expected to receive lower frequencies from the tympanum, in particular frequencies that correspond with the first tympanal vibration maximum at 7 kHz. The discrepancy between tympanal vibration and tympanal nerve response could be explained by the fact that the sensory cells are not directly attached to the tympanal membrane. Additionally, the sensory cells could have their own high-pass filter. A correlation of the first displacement maximum of tympanal membrane motion and spike response was detected at least at higher intensities (80 dB SPL), which supports a functional coupling at lower frequencies.

Closing the spiracle reduces the neuronal response above a certain cut-off frequency, as found in previous studies (Lewis, 1974a; Lewis, 1974b; Nocke, 1975; Seymour et al., 1978; Shen, 1993; Heinrich et al., 1993; Kalmring et al., 1993; Kalmring and Jatho, 1994; Bailey, 1998; Römer and Bailey, 1998). This could also be verified for *M. elongata*, as well as a slight increase in the sensitivity of the tympanal nerve response at low frequencies (<12 kHz) after closing. Because of altered acoustic characteristics from a one-side open into a two-sides closed system, the increased sensitivity could result from a shift of the threshold minimum to lower frequencies, as the occlusion of the spiracle reduces the entry of sound energy as well as changing the boundary conditions of the whole tube system, as claimed by several earlier studies (Nocke, 1975; Kalmring and Jatho, 1994; Bailey, 1998). The actual difference of the threshold level between the open and closed spiracle condition is likely to be much greater. Differences between the attenuation profile of the cellulose tissue and the auditory response characteristics of both the membrane and the sensory cells might be caused by sound amplification through the acoustic trachea. The amplitude of tympanal membrane vibration in the closed spiracle situation increased for frequencies up to about 17 kHz. This finding is surprising, as both maxima of tympanal membrane motion (at 7 and 16 kHz) were increased, whereas the tympanal nerve threshold was already elevated at the frequency of the second displacement maximum. In contrast, a previous study found a good correspondence in the relative changes between the velocity amplitude of tympanal vibration and the tympanal nerve responses (Bangert et al., 1998) after occlusion of the spiracle. Moreover, these authors found that tympanal motion and nerve response decreased at lower frequencies (>5 kHz) in the tested species, *Mygalopsis marki*.

In addition to determining neuronal threshold curves, spike responses at 80 dB SPL were compared with the tympanal vibration pattern induced by the same sound level. Under far-field stimulation, neuronal activity was slightly higher at frequencies of about 10 and 18 kHz and decreased with increasing stimulus frequency (Fig. 4B). These spike rate maxima correspond approximately to the first and second displacement maximum of tympanal membrane motion, while the frequency shift could be due to the low frequency resolution of the neurophysiological recordings. When the spiracle was closed, no difference in the neuronal response occurred (Fig. 4B, marked by an arrow), whereas the tympanal vibration amplitude increased for frequencies up to 17 kHz (significantly between 7 and 10 kHz). Hence, we could show that the most sensitive auditory frequency corresponds to the frequency of the second tympanal vibration maximum, while a manipulation leads to a more pronounced difference between tympanum vibration and neuronal response.

### Functional coupling of tympanal membrane vibration and neuronal response

By local sound application at the tympanum *versus* through the spiracle, the contribution of both acoustic pathways to sensory cell and tympanal vibration responses was assessed in accordance with the techniques used by Michelsen and colleagues (Michelsen et al., 1994). When the tympanal nerve activity was evoked by stimulation through the ipsilateral spiracle, the neuronal responses had lower thresholds, and at high levels (80 dB SPL) neuronal activity was significantly increased in comparison to stimulation through the tympanum. This is in accordance with the finding that the ipsilateral spiracle is the main input point of acoustic energy.

During local acoustic stimulation of the tympanum the tympanal nerve response was restricted to lower frequencies (<25 kHz) and higher SPLs (>60 dB SPL). This confirms that the tympanum preferentially transmits low frequencies to the receptor organ, as already described in previous studies (Lewis, 1974a; Lewis, 1974b; Nocke, 1975; Michelsen and Larsen, 1978; Seymour et al., 1978; Bangert et al., 1998).

It has been claimed that the tympana may have an impedance-matching function by acting as a bridge between low density air in the tracheal channel and denser haemolymph in a channel containing the receptor cells (Bangert et al., 1998). However, it is hard to measure and calculate the transformation gain of the tympanum or its mechanical impedance because of a lack of information about the system properties like mass, stiffness and damping of the tympanum. Moreover, the number of measurements of tympanum motion and the related nerve activity are limited and have provided only preliminary results up to now (Bangert et al., 1998). For this reason, we focused on the question of a functional coupling between tympanum motion and nerve activity. Additionally, we measured and compared in detail the sound-induced tympanal membrane motion and neuronal response during certain manipulations to specify the characteristics of the coupling.

The match between the second tympanum vibration maximum (~16 kHz) and a pronounced tympanal nerve threshold minimum as well as the correspondence between the first and second frequency peaks of tympanal membrane motion and spike activity at high SPLs (80 dB SPL) during far-field stimulation argues in favour of a mechanical influence of the tympanal membrane motion. Moreover, three other findings suggest that the tympanum, in addition to its pressure-releasing action, has another important role: (i) the occurrence of a receptor cell response at lower frequencies (<30 kHz) during local stimulation of the tympanum, (ii) the increased $BW_{10dB}$ value (broadened frequency response) during stimulation through the spiracle in comparison to the far-field stimulation and (iii) the lower threshold minimum at 16 kHz during far-field stimulation in comparison to local spiracle stimulation. The first point emphasizes that the tympana transmit sound at low frequencies and high SPLs to the sensory organ. An increased $BW_{10dB}$ value of local spiracle stimulation as well as the lowered threshold minimum at 16 kHz during far-field stimulation suggest that under this condition the tympanum properties and its 16 kHz resonance are not having an influence on organ motion and neuronal activation. These auditory characteristics highlight the role of the tympanal membranes and their necessity for sensitive sound transduction, especially at lower frequencies and, in particular, at its resonance frequency. This would imply that the resonance frequency of the tympanum, as predicted by Bangert and colleagues (Bangert et al., 1998), determines the resonance properties of the sensory epithelium, which was also found by Lomas and colleagues (Lomas et al., 2011).

Despite the agreement of the resonance frequency of the tympanum and the threshold minimum of the neuronal response, the tympana do not seem to transmit sound directly or linearly relative to the induced SPL. The indirect coupling becomes obvious by comparing displacement amplitudes of tympanal membrane vibration and the spike rate of the tympanal nerve response. On the one hand, local stimulation of the tympanum results in displacement amplitudes that are comparable to those during far-field stimulation, whereas the spike rate is strongly reduced. On the other hand, local stimulation through the spiracle evokes significantly higher tympanal displacement amplitudes in comparison to the far-field situation but does not lead to more sensitive neuronal thresholds or increased spike rates during the 80 dB SPL stimulation. This is true except at lower stimulus frequencies (<10 kHz), where a decreased neuronal threshold could be caused by direct mechanical contact of the probe speaker with the internal tracheal structures, which could have led to increased neuronal responses of the subgenual and intermediate organs activated by low-frequency substrate-borne vibration. Above 10 kHz, the otherwise equal neuronal responses between far-field and local spiracle stimulation could be due to a saturation of auditory receptor potentials, but a statement about a saturating influence to the auditory receptor potential can only be made following intracellular recording of the sensory cells. Measurements of single receptor cell responses, however, revealed no saturation up to the maximally used SPL of about 70 dB SPL (Oldfield, 1984). For a final explanation of the transmission properties of the tympana further explorations are required.

To summarize, the responses found during far-field and local stimulation imply that the tympana are functionally coupled to sensory cell activity by transmitting low frequencies (<30 kHz) and resonant oscillations (16 kHz) to the hearing organ. As manipulation of the system, like closing the spiracle or local stimulation of the sound entry points, leads to considerable differences between the responses of tympanum motion and tympanal nerve fibre responses, it is apparent that tympanum motion and the sensory cell responses are not coupled directly and that there is no linear relationship to the applied SPL. Tympanal membrane motion and sensory cell activity seem to be coupled indirectly and this coupling is maximal at the resonance frequencies of the tympanum.

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experiments comply with the current laws of the country in which they were made. The authors declare that they have no conflict of interest.

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