RESEARCH ARTICLE

Temperature dependence of distortion-product otoacoustic emissions in tympanal organs of locusts

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SUMMARY

Distortion-product otoacoustic emissions (DPOAEs) in tympanal organs of insects are vulnerable to manipulations that interfere with the animal's physiological state. Starting at a medium temperature, we raised and lowered the locust's body temperature within the range of 12 to 35°C by changing the temperature of the surrounding air, while recording DPOAEs. These experimental manipulations resulted in reversible amplitude changes of the $2f_1-f_2$ emission, which were dependent on stimulus frequency and level. Using low f_2 frequencies of up to 10 kHz, a temperature increase (median +8–9°C) led to an upward shift of DPOAE amplitudes of approximately +10 dB, whereas a temperature decrease (median −7°C) was followed by a reduction of DPOAE amplitudes by 3 to 5 dB. Both effects were only present in the range of the low-level component of DPOAE growth functions below L2 levels (levels of the f_2 stimulus) of approximately 30 dB SPL. DPOAEs evoked by higher stimulus levels as well as measurements using higher stimulation frequencies above 10 kHz remained unaffected by any temperature shifts. The Arrhenius activation energy was calculated from the −10 dB SPL thresholds (representing the low-level component) of growth functions, which had been measured with 8 and 10 kHz as f_2 frequencies and amounted to up to ~34 and 41 kJ mol^{-1}, respectively. Such activation energy values provide a hint that the dynein-tubulin system within the scolopidial receptors could play an essential part in the DPOAE generation in tympanal organs.

INTRODUCTION

Upon simultaneous stimulation with two pure tones f_1 and f_2 ($f_1<f_2$), tympanal organs of insects such as locusts (Kössl and Boyan, 1998; Möckel et al., 2007), moths (e.g. Kössl and Coro, 2006; Kössl et al., 2007) and bushcrickets (Möckel et al., 2011) emit pronounced distortion-product otoacoustic emissions (DPOAEs) that resemble those measured in the ears of vertebrates (Kössl et al., 2008). They appear as additional spectral peaks at frequencies of $nf_1-(n-1)f_2$ and $nf_2-(n-1)f_1$. In insects as well as in vertebrates, the $2f_1-f_2$ emission is the most prominent one, as it is evoked by the lowest stimulus levels. The largest $2f_1-f_2$ amplitudes are reached at frequencies of high auditory sensitivity, and in moths and locusts, the $2f_1-f_2$ DPOAEs are elicited with stimuli levels near the species-specific auditory threshold (Kössl et al., 2008).

The $2f_1-f_2$ emission often shows non-monotonic growth behavior with increasing stimulus levels, and the slope of the corresponding growth function changes at intermediate stimulation levels. The two parts of the growth function are referred to as the low-level component and the high-level component. The low-level component is more strongly affected by changes in the animal's physiology induced by hypoxia (Kössl and Boyan, 1998) or the application of ethyl ether (Coro and Kössl, 2001; Kössl et al., 2007), suggesting a metabolically sensitive biological origin of the acoustic two-tone distortions. Evidence that the scolopidia, the auditory mechanoreceptor cells in tympanal organs, are involved in DPOAE generation comes from two types of experiments. In one, mechanical lesions in the

locust ear, which affected a specific group of scolopidia tuned to high sound frequencies above 12 kHz, deleted only those DPOAEs that were evoked by stimulus frequencies above 15 kHz (Möckel et al., 2007). In the other, the anatomical separation of the main site of sound input (the spiracle at the prothorax) and the site of its perception (the tympanal organ in the foreleg tibia) in bushcrickets permitted the local application of the insecticide pymetrozine. The compound appears to act selectively on scolopidia (Ausborn et al., 2005), causing a pronounced and irreversible decrease of DPOAE amplitudes (Möckel et al., 2011). The underlying cellular components, however, which may be involved in DPOAE generation in insects, have not been identified so far.

The tympanal organ of locusts consists of a peripheral ganglion, called Müller's organ, a large tympanal membrane, and an adjacent tracheal system. Müller's organ comprises approximately 80 scolopidia, each of them containing a bipolar sensory neuron that is coupled to the inside of the tympanal membrane *via* its accessory cells (Gray, 1960). Based on the exact attachment position of their dendrites and their frequency tuning, four (Michelsen, 1971; Römer, 1976) or three groups of neurons (Jacobs et al., 1999) have been distinguished. Those scolopidia that are attached to the thin part of the tympanal membrane at the so-called pyriform vesicle (named 'd-cells' or 'group II', respectively, by the authors mentioned above) are most sensitive to sound frequencies above 12 kHz. Those coupled to the thick part of the tympanal membrane are most sensitive to lower frequencies. Frequency analysis is determined by

the travelling-wave-like vibration pattern of the tympanal membrane. From their initiation at the outer rim of the tympanum, waves that are induced by sound frequencies above 12 kHz are attenuated as soon as they reach the pyriform vesicle, whereas those induced by lower sound frequencies continue to travel towards the thick membrane region (Windmill et al., 2005).

Hearing involves mechano-electrical transduction mechanisms. For locusts (Oldfield, 1988) and cicadas (Fonseca and Correia, 2007), sensory transduction has been shown to be sensitive to changes in body temperature. The sensitivity and the characteristic frequency to which the receptor is tuned both increase with increasing temperature. In both of these previous studies, these effects exceeded those expected from merely mechanical property changes of the auditory organ. Laser Doppler vibrometry measurements of the tympanal vibrations in cicadas in response to acoustical stimulation were independent of temperature within a biologically relevant range (18–35°C). The changes in the neuronal response characteristics owing to shifts in body temperature were therefore suggested to be caused by intrinsic properties of the receptor neurons.

After initially being considered to be purely linear mechanical systems, insect ears were shown, as mentioned above, to comprise non-linear sound processing. Such non-linearity and generation of sensitive DPOAEs were found to depend on intact scolopidia, to be vulnerable to changes concerning their physiology, and could potentially rely on each of the scolopidial cell types. The present study concerns two open questions: (1) which components of the intracellular machinery could contribute to mechanical non-linearity; and (2) is non-linear hearing in tympanal organs of locusts based on mechanical principles similar to those found in Johnston's organs of mosquitoes and flies? The suggested biological origin of acoustic two-tone distortions should involve metabolic processes, whose temperature-dependence would directly affect the DPOAE generation. A relationship between body temperature shifts and the rate of potential DPOAE changes, plotted into an Arrhenius graph and converted into the activation energy, would provide conclusions on possible mechanisms involved in non-linear hearing in tympanal organs. Such assumptions would allow a comparison with non-tympanic insect hearing organs such as those found in mosquitoes and flies, which have a much lower frequency range of operation.

MATERIALS AND METHODS
Animals and preparations
Adult locusts [*Locusta migratoria* (Linnaeus 1758), Insecta, Caelifera, Acrididae] were acquired from a commercial supplier (Terra Tropica, Friedrichsdorf, Germany) and kept in the laboratory at room temperature. Previous studies reported no obvious gender differences in the functional anatomy or in DPOAE generation (Kössl and Boyan, 1998; Möckel et al., 2007). We therefore used males and females ($N=13$) for the present study. After slight anesthesia with CO_2, legs and wings were removed, and the animals were laterally fixed with their thorax onto a metal holder using rosin-beeswax. The cuticular lid that partly covers the ear opening was removed under visual control. The temperature sensor probe of a digital thermometer (Voltcraft Digital Thermometer K101, Conrad Electronic, Hirschau, Germany) was placed under the dorsal abdomen cuticle *via* a small cuticle opening and was also fixed onto the metal holder. CO_2 was not re-applied after these preparations, and the animals remained awake during all measurements. Normal breathing movements and fast reflexes upon touching the antennae indicated recovery from anaesthesia. After the experiments, the animals were re-anesthetized and killed by decapitation.

Measurement of DPOAEs
Experimental setup and measuring procedures followed a previous report on DPOAEs in locusts (Möckel et al., 2007). A Microstar DAP 840 DSP card (Microstar Laboratories, Bellevue, WA, USA; sampling rate 200 kHz) that was controlled by custom software accomplished the generation of the two acoustic stimuli and the analysis of the incoming microphone signal. The two output channels were connected to two attenuators (TDT System3, Tucker Davis Technologies, Alachua, FL, USA), an amplifier, and two 1 inch microphones (Microtech Gefell, Gefell, Germany, Type 103.1) that served as loudspeakers. The acoustic signals were measured by an additional ½ inch microphone (Brüel & Kjær, Bremen, Germany, Type 4133) connected to the input channel of the DSP card by a preamplifier (Brüel and Kjær, Type 2660) and an amplifier (Brüel & Kjær, Type 2610). The adjacent channels for stimulation and recording were brought together in an acoustic coupler whose tip diameter fitted the size of the locust's ear opening. The animals, fixed on the metal holder, were placed in a soundproof chamber. The coupler was positioned rectangular to the tympanum at a distance of 0.2–1 mm. The remaining space between the coupler and the cuticle rim that surrounds the ear was sealed using Vaseline or toothpaste. The sound system was calibrated *in situ*, with the coupler placed in measuring position. The arrangement of animal and coupler was not affected by the manipulations during the course of the experiment, as only the surrounding air temperature was changed in order to shift the animal's body temperature.

During acoustical stimulation with two pure tones of different frequencies ($f_1 < f_2$), the level of the f_1 stimulus (L1) was chosen to be 10 dB above that of f_2 (L2), as this has been found to induce maximal DPOAE levels (Kössl and Boyan, 1998). The measured microphone signal was averaged 100 times before fast Fourier transform (FFT) analysis. Each presentation of a pair of pure-tone stimuli took 4.2 s for 100 averages. Accordingly, the measurement of a DPOAE audiogram and growth function took approximately 1.5–2 min, depending on the respective number of frequency/level steps.

Recordings of DPOAEs included the following procedures: DPOAE audiograms were obtained by evoking DPOAEs over a wide frequency range (1–30 kHz) while keeping a constant frequency separation and level of the two stimuli [f_2/f_1 ratio of 1.04, 1.08 and 1.15; L1/L2 60/50 dB sound pressure level (SPL)]. From these DPOAE audiograms, f_2 frequencies that elicited large $2f_1 - f_2$ emissions were chosen for further measurements. The respective f_1 frequency was adjusted for each f_2 frequency according to the optimum f_2/f_1 ratio that evoked the largest $2f_1 - f_2$ emissions. DPOAE growth functions were obtained by keeping both stimulus frequencies constant and increasing their levels in 2 dB steps, starting from 10/0 or 20/10 dB SPL and reaching up to 64/54 dB SPL. At the used stimulus levels, no setup-generated distortions were visible above the noise level. Background noise level was measured 100 Hz below the DPOAE frequency.

Measurements of DPOAE audiograms and growth functions were repeated at each consecutive body temperature step. The f_2/f_1 ratio for the chosen f_2 frequency was determined only once for each respective animal, and both stimulus frequencies, f_1 and f_2, were kept constant for all consecutive growth functions. Frequencies for f_2 were chosen to be 8 kHz ($N=11$), 10 kHz ($N=5$), 12 kHz ($N=5$) and 18 kHz ($N=7$).

Shifting the body temperature of the animals
The animal's body temperature was shifted by changing the temperature of the surrounding air within the soundproof chamber.

A Peltier element (PKE 36H 021, Petron, Fürth, Germany) and additional heating and cooling pads were placed around the metal holder that carried the animal. The temperature sensor that had been placed under the abdomen cuticle recorded the resulting body temperature shift with a resolution of 0.1°C.

The procedure involved measurements with the animal's body temperature at medium temperatures (which correlated with the surrounding room temperature), and at body temperatures that were shifted up and down, compared with the preceding medium temperature, in the range between 12 and 35°C. DPOAEs were recorded at each consecutive body temperature step. The return to the medium temperature in between served as a control condition to the temperature shifts and made it possible to assess the stability of the preparation during the course of the experiment. DPOAE measurements were started as soon as the respective body temperature was stable for several minutes. As the return to the medium temperature was achieved by merely removing the heating and cooling devices, breaks between these respective temperature steps could take up to 30 min.

Data analysis

To evaluate temperature-induced changes in DPOAE amplitude and sensitivity, two approaches were chosen. First, after the measurement of $2f_1$–f_2 growth functions at different temperatures, the changes in DPOAE amplitudes at given L2 stimulus levels (Fig. 1A) were assessed by subtraction of the respective growth functions and displayed as DPOAE level shift (Fig. 1B). The background noise level in all measured growth functions had a median value of −23.8±6.1 dB SPL, and to keep a safe distance to the noise level, −15 dB SPL was used as a reference. DPOAE levels below −15 dB SPL were therefore rejected from further analyses. Second, from the two growth functions obtained at different temperatures, the L2 level that induced a DPOAE threshold level of e.g. −10 dB SPL was interpolated (Fig. 1C) and the two threshold values were subtracted (Fig. 1D).

In the DPOAE level shift data, positive difference values indicate increased DPOAE amplitudes at the respective body temperature in comparison to the medium temperature condition (Fig. 1B). The used growth functions were measured at f_2 stimulus frequencies of 8 and 18 kHz, and the level shifts were separately averaged and displayed as medians and the 25th and 75th percentiles (their range hence containing 50% of the values). For each 2 dB step of the L2 level, the values of both f_2 frequency groups were compared and tested for statistically significant differences, using a Wilcoxon rank sum test for unpaired samples (MATLAB R2007b, Statistics Toolbox, The MathWorks, Natick, MA, USA). The null hypothesis 'no difference' was rejected at P-values <0.05.

For the DPOAE threshold shifts, positive values indicate higher L2 levels, and negative values lower L2 levels, that were sufficient to evoke the emission threshold level. The temperature shift indicated the change of the animal's body temperature in relation to the initial medium temperature. For each DPOAE growth function measured at lowered or raised temperatures, a value of DPOAE threshold shift and temperature shift was obtained (Fig. 1D). All resulting threshold shifts for the respective f_2 stimulus frequencies of 8, 10, 12 and 18 kHz were plotted using linear regression curves (Regression Wizard, Sigma Plot 10.0, Systat Software, San Jose, CA, USA).

Using Arrhenius coordinates, the determined values for DPOAE thresholds were plotted against the body temperatures (converted into Kelvin) that had been measured during the experiments. DPOAE thresholds were chosen as opposed to the actual DPOAE amplitudes to achieve better comparability across all measured animals. By connecting data points for each individual animal, the slope m and hence the activation energy (E_A) were determined, using the following equation:

$$|E_A| = m\,(-R),\qquad(1)$$

where R is the gas constant ($8.314\,\mathrm{J\,mol^{-1}\,K^{-1}}$). We used the absolute value of the calculated activation energy, as the inclusion of DPOAE thresholds implicated an inverted relationship, that is, an increase of DPOAE amplitudes in the measured growth functions caused decreased DPOAE thresholds. The values were separately calculated for every animal and averaged. Three data sets (two for 8 kHz and one for 10 kHz) that were included in the threshold shifts displayed in Fig. 4 were excluded from the Arrhenius plot because they consisted of two measured temperature steps without intermediate temperature values.
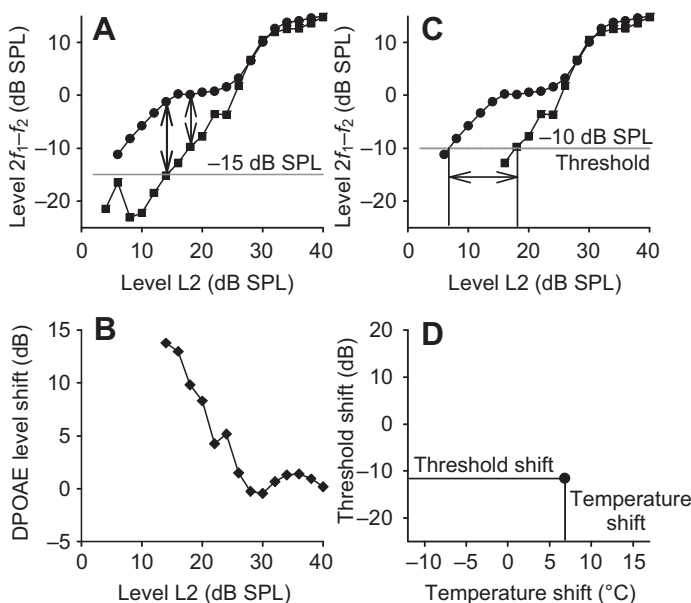

Fig. 1. Illustration of analysis paradigms regarding temperature-dependent effects on distortion-product otoacoustic emission (DPOAE) growth functions in *Locusta migratoria*. (A,B) Calculation of DPOAE level shifts. (A) Growth functions measured at the initial medium temperature (squares) were subtracted from those obtained at a shifted body temperature (circles). Only DPOAE data points ≥−15 dB SPL (grey line), well above the noise level, were used for subtraction. (B) The subtraction curve represents the amplitude shift between these two DPOAE growth functions (indicated by arrows in A) plotted against the respective L2 stimulation level. (C,D) DPOAE threshold shifts in dependence on the temperature shift. (C) DPOAE thresholds (i.e. the L2 level that is sufficient to evoke an emission of a certain level, for example −10 dB SPL) were determined for both growth functions. The L2 level shift between these values ('threshold shift') is marked by an arrow. (D) The resulting data point is defined by the DPOAE threshold shift (y-axis) and the corresponding temperature shift (x-axis). Used stimuli f_1/f_2: 6.909/8 kHz. Body temperatures: 18.4°C (medium temperature; squares) and 25.2°C (temperature shift up; circles).

RESULTS

The temperature dependency of DPOAE amplitudes in the locust was investigated by measuring DPOAE audiograms and growth functions at different body temperatures (representative examples in Fig. 2). It is noteworthy that DPOAE audiograms obtained using very low stimulus levels of 30/20 dB SPL did not evoke emissions above the noise level (Fig. 2A, grey circles). Only when the body temperature of the animal was raised by ~9°C were DPOAEs evoked by low f_2 frequencies of up to ~9 kHz, and they reached levels of >20 dB above noise level for 7 and 8 kHz (Fig. 2A, black circles). When DPOAE audiograms were recorded at higher stimulus levels, such as 60/50 dB SPL, there were no obvious effects induced by different body temperatures (Fig. 2B).

DPOAE growth functions were measured at different body temperatures using f_2 frequencies of 8, 10, 12 and 18 kHz (representative examples in Fig. 2C–F). DPOAE amplitudes during control measurements at medium temperatures (grey lines) displayed only slight variations within a range of 3 dB, demonstrating a reversibility of any temperature-dependent effects, once the animal's body temperature had returned to its original value. DPOAEs emerged from the noise level at an L2 level of 10 to 20 dB SPL, depending on the respective f_2 frequency, and reached ~40 dB SPL at the highest L2 levels of 54 dB SPL. If the body temperature of the animal was decreased or increased, DPOAE amplitude modulations occurred only for those emissions that had been recorded with low f_2 frequencies up to 10 kHz. Also, such modulations concerned only the so-called low-level component of these growth functions, that is, those DPOAEs that were evoked by low L2 stimulus levels of up to 25–30 dB SPL (Fig. 2C,D). When the body temperature was lowered by ~6°C, the emissions were slightly reduced in amplitude by ~3–10 dB (Fig. 2C,D, black triangles). Raising the animal's body temperature by ~7°C caused an increase of DPOAE amplitudes by ~10–15 dB (Fig. 2C,D, black

circles). No such effects were observed for DPOAEs that were evoked by higher L2 levels (above 25–30 dB SPL). At higher stimulus levels, the emission levels remained the same over the entire applied temperature range. Such temperature- and L2-level-dependent effects, however, appeared only for emissions evoked by f_2 frequencies up to ~10 kHz. Amplitudes of DPOAEs evoked by higher f_2 frequencies did not differ when measured at the medium temperature or at shifted body temperatures (Fig. 2E,F).

DPOAE level shifts represent amplitude changes between DPOAE growth functions measured at different temperatures (see Materials and methods; Fig. 1A,B). Data obtained for f_2 frequencies of 8 kHz (black circles) and 18 kHz (grey circles) were separately averaged for decreased and increased body temperatures (Fig. 3A and 3B, respectively). The lack of data points for the lowest L2 levels comes from the fact that here often the DPOAE amplitude of one of the two measurements was below −15 dB SPL and too close to the noise level to allow a reliable subtraction of DPOAE levels obtained at two temperature conditions. Using f_2 frequencies of 8 kHz, a reduction of body temperature (temperature decreased by ~7°C in comparison to the medium temperature at the beginning; Fig. 3A) induced a small DPOAE amplitude shift of approximately −3 to −5 dB at L2 levels between 24 and 32 dB SPL. If 18 kHz was used as the f_2 frequency, no such shift in DPOAE amplitudes within a range of ±2 dB was recorded; this shift would represent the usual fluctuations between growth function measurements. An increase of body temperature (temperature increased by ~8–9°C; Fig. 3B) led to a strong increase of DPOAE amplitudes measured with 8 kHz as the f_2 frequency. This amplitude enhancement was found within the low-level component of the growth functions, for L2 levels between ~14 and 40 dB SPL. The largest average DPOAE level shift of ~9–12 dB is evident at L2 levels of 18 to 24 dB SPL. Level shifts of DPOAEs evoked by f_2 frequencies of 18 kHz decreased by only −2 to −3 dB at L2 levels of 36–40 dB SPL. When comparing one
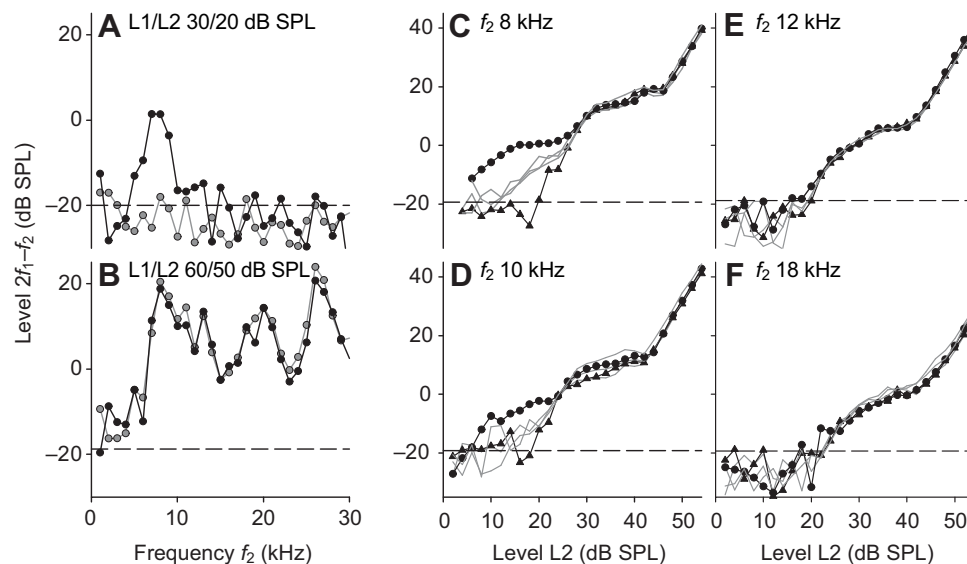

Fig. 2. Effects of a shifted body temperature on DPOAEs in *L. migratoria*. (A,B) DPOAE audiograms for f_2 frequencies between 1 and 30 kHz (f_2/f_1 ratio=1.15), measured in the same animal. Body temperatures: 16.6–16.8°C (grey circles) and 25.3–26.2°C (black circles). Stimulus levels: (A) 30/20 dB SPL; (B) 60/50 dB SPL. (C–F) DPOAE growth functions for different stimulus frequencies, measured in the same animal. Each series of recordings consisted of five growth functions, measured at: (1) initial medium temperature, (2) downward temperature shift, (3) medium temperature, (4) upward temperature shift and (5) medium temperature. Body temperatures: 24.3–25.2°C (temperature shift up, black circles), 12.1–12.5°C (temperature shift down, black triangles) and 18.3–18.7°C (medium temperature, grey lines). Stimuli: (C) 6.909/8 kHz, (D) 8.008/10 kHz, (E) 11.23/12 kHz and (F) 16.211/18 kHz. Horizontal dashed lines in all panels indicate the noise level expressed as means + 1 s.d.
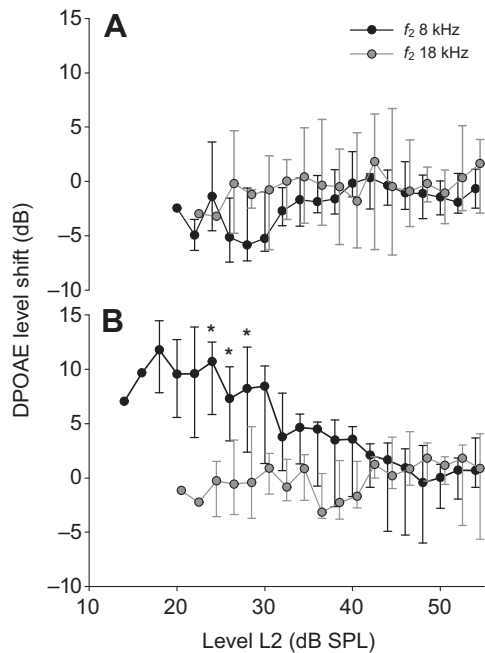
Fig. 3. Average DPOAE level shifts for f_2 frequencies of 8 kHz (black circles) and 18 kHz (grey circles). Given are DPOAE level changes at a shifted body temperature compared with those measured at the initial medium temperature, plotted against the respective L2 level (see also Fig. 1A,B). (A) Downward temperature shifts for f_2=8 kHz (N=11, −6.5±1.6°C) and f_2=18 kHz (N=7, −6.1±2.0°C). (B) Upward temperature shifts for f_2=8 kHz (N=11, 8.8±2.7°C) and f_2=18 kHz (N=7, 7.5±2.2°C). Data are expressed as medians and 25th and 75th percentiles. Asterisks indicate statistically significant differences between the two frequency groups (Wilcoxon test for unpaired samples, *P<0.05). The symbols for the 18 kHz data sets are graphically offset in the horizontal direction by 0.5 dB to provide better visibility compared with the 8 kHz values.

frequency group with the other, increased body temperatures caused statistically significant differences between DPOAE level shifts in an L2 level range between 24 and 28 dB SPL (P<0.05).

DPOAE threshold shifts represent the difference in threshold value (that is, L2 levels sufficient to evoke an emission of a certain level) obtained from two DPOAE growth functions. The shifts were assessed at two different threshold criteria to represent the low-level component (−10 dB SPL threshold; Fig. 4A,B) and the high-level component (20 dB SPL threshold; Fig. 4C,D) of the DPOAE growth functions. Such threshold shifts were set into relation to the shift in temperature at which the compared growth function had been measured (see Materials and methods; Fig. 1C,D). These temperature shifts spanned from approximately −11 to +16°C, based on the value of the original medium temperature during the first measurements. Emissions evoked by low f_2 frequencies such as 8 and 10 kHz and low L2 stimulus levels were highly temperature dependent (Fig. 4A). The −10 dB SPL thresholds increased with lowered body temperatures, and decreased with raised body temperatures. Threshold shifts amounted up to approximately +8–9 dB and −12 dB if the body temperature had been shifted by −10°C and +10°C, respectively, resulting in a threshold temperature shift of −1.10 dB °C^{-1} for 8 kHz and −1.07 dB °C^{-1} for 10 kHz (linear regression plots in Fig. 4A, black line for 8 kHz and grey line for 10 kHz, with R^2 values of 0.82 and 0.77, respectively). In contrast, emissions also evoked by low L2 stimulus levels, but with higher f_2 frequencies such as 12 and 18 kHz, did not depend on body

temperature (Fig. 4B). A temperature dependence of DPOAE thresholds was also absent when higher L2 stimulus levels were used, in recordings with 8 and 10 kHz (Fig. 4C) as well as with 12 and 18 kHz (Fig. 4D). DPOAE threshold shifts were small and scattered close to 0 dB across the entire temperature shift range, with threshold shift values of −0.16 to 0.02 dB °C^{-1} for all f_2 frequencies (linear regression plots, Fig. 4B–D). Apart from outliers, most data points (87.2–100%) in all of the eight data groups lay within a ±5 dB interval centred close to the corresponding linear regression plots.

As reported above, only emissions evoked by low L2 stimulus levels and low f_2 frequencies of 8 and 10 kHz showed a dependence on temperature. Based on these data sets, the logarithms of the L2 values for −10 dB SPL DPOAE thresholds, representing the low-level component of the DPOAE growth functions, were plotted against the inverse body temperatures (T, converted into Kelvin), using Arrhenius coordinates (Fig. 5). Data points for each individual animal were connected and are shown as grey lines. The slopes of the resulting curves were not linear, but displayed a break point at medium temperatures. For measurements using an f_2 frequency of 8 kHz (Fig. 5A, N=9 animals), median values for these break points amounted to 20.3±1.7°C, ranging from 18.4 to 23.1°C. For measurements using an f_2 frequency of 10 kHz (Fig. 5B; N=4 animals), median values amounted to 18.9±0.7°C, ranging from 18.4 to 20.1°C. The level-dependent variation between the individual animals occurred as consequence of different sensitivities in each measured tympanal organ. The individual graphs, however, had comparable slopes for the temperature range both below and above the break point. Based on these slopes, the activation energy was calculated for each temperature range and animal. For measurements using f_2 frequencies of 8 and 10 kHz, the average activation energy values in the warm temperature range (above 20.3 and 18.9°C; 1000/T below ~3.40 and 3.42) amounted to 34.01±12.24 and 41.08±13.58 kJ mol^{-1}, respectively. The corresponding values for the cold temperature range amounted to lower activation energy values of 12.74±5.03 and 2.64±4.78 kJ mol^{-1}, respectively.

DISCUSSION

DPOAEs are vulnerable to manipulations that interfere with the animal's physiology, such as changing its body temperature. In the present study, a change of body temperature resulted in level- and frequency-dependent effects on the $2f_1$–f_2 emission, which were reversible, once the animal's body temperature was back to its original medium value. Increasing the locust's body temperatures led to enhanced amplitudes of DPOAEs, which had been measured with low stimulation levels (below ~30–40 dB SPL L2 level) and using low f_2 frequencies of up to 10 kHz. The high-level component remained unchanged, as well as those emissions that were evoked by higher stimulation frequencies (e.g. 12 and 18 kHz).

The low-level component of the DPOAE growth function in vertebrates (e.g. Lukashkin et al., 2002) and in insects (e.g. Kössl et al., 2007) was found to be physiologically much more vulnerable than the high-level component, which could lead to the suggestion of two L2-level-dependent mechanisms for DPOAE generation. For mammals, Lukashkin et al. (Lukashkin et al., 2002), however, provided evidence that both components are evoked by a single source, a non-linear amplifier with saturating input/output characteristics. Mammalian cochlear amplification of basilar membrane vibration is largest at low levels (Dallos and Fakler, 2002), and the impact of active amplification on the basilar membrane vibration reaches saturation if the input levels are increased beyond ~40–60 dB SPL. If the gain of the cochlear amplifier is changed *via* physiological manipulations, this would
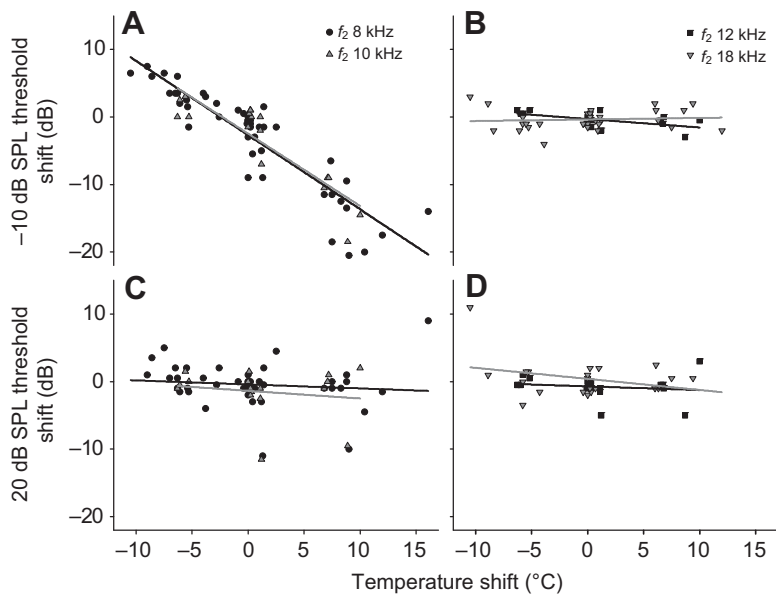
Fig. 4. Temperature-dependent shift of DPOAE threshold (see also Fig. 1C,D). Threshold levels were chosen to represent the low-level component (–10 dB SPL threshold; A,B) and the high-level component (20 dB SPL threshold; C,D) of the DPOAE growth functions. (A,C) f_2 frequencies of 8 kHz (47 measurements from 11 animals; black circles) and 10 kHz (17 measurements from five animals; grey triangles up). (B,D) f_2 frequencies of 12 kHz (17 measurements from five animals; black squares) and 18 kHz (30 measurements from seven animals; grey triangles down). Linear regression lines are fitted to each set of data. R^2 values are as follows: –10 dB SPL threshold: 8 kHz, 0.817 (A, black line); 10 kHz, 0.771 (A, grey line); 12 kHz, 0.301 (B, black line) and 18 kHz, 0.008 (B, grey line); 20 dB SPL threshold: 8 kHz, 0.012 (C, black line); 10 kHz, 0.027 (C, grey line); 12 kHz, 0.022 (D, black line) and 18 kHz, 0.096 (D, grey line).

influence the low-level component much more strongly than the high-level component, even if there is a single source of non-linearity (see Lukashkin et al., 2002). This explanation is supported by findings in a notodontid moth that has tympanal organs with only one auditory receptor neuron (Kössl et al., 2007). DPOAE growth functions from such hearing organs are also characterized by low-

Fig. 5. Arrhenius plot of DPOAE thresholds for a threshold criterion of –10 dB SPL. This criterion was chosen to represent the low-level component of the growth functions. Plotted are the logarithms of these L2 threshold values against the inverted temperature (T, in Kelvin). Data points of each individual animal are connected (grey lines). Linear regression plots were fitted to the warm and the cold temperature ranges, and are shown in black. (A) DPOAE thresholds determined from growth functions measured for f_2=8 kHz (N=9 animals). R^2 values for the linear regression plots: warm temperature range, 0.332; cold temperature range, 0.151. (B) DPOAE thresholds determined from growth functions measured for f_2=10 kHz (N=4 animals). R^2 values for the linear regression plots: warm temperature range, 0.669; cold temperature range, 0.055.

and high-level components that have a different emission phase and are separated by pronounced notches. In addition, the moth's DPOAE growth functions are strongly vulnerable to physiological manipulation, all of this apparently being contributed by only one scolopidium. As shown in the present study, changing the physiological state of the locust by enhancing its body temperature led to increased DPOAE amplitudes for low-level stimulation, whereas high-level DPOAEs were temperature insensitive. Level-dependent temperature effects have also been reported for vertebrates. In the amphibian papilla of leopard frogs (Meenderink and van Dijk, 2006) and in rats [for temperatures below 33°C (Khvoles et al., 1998)], lowered body temperatures led to decreased DPOAE amplitudes induced by low stimulus levels.
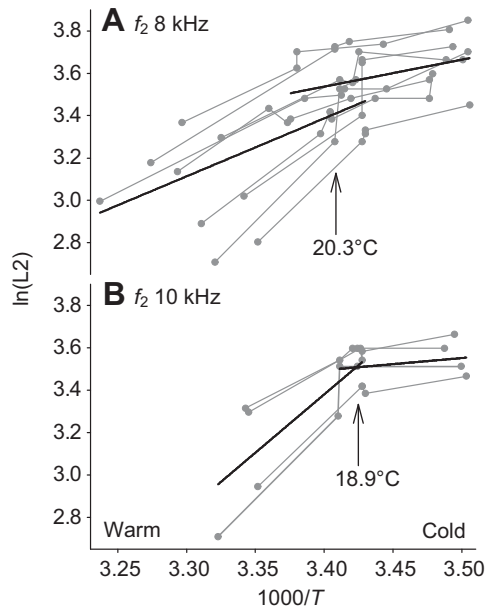
Temperature dependence within the auditory pathway of insects has been reported for locusts (Oldfield, 1988) and cicadas (Fonseca and Correia, 2007). Both of these studies indicated that the magnitude of the effects regarding frequency tuning and sensitivity exceeded those one would expect from changes in the passive mechanical properties of the tympanum. Laser vibrometry measurements of tympanum vibrations in the cicada revealed that temperature changes between 18 and 35°C had no impact on the tympanal response to acoustic stimulation (Fonseca and Correia, 2007). The temperature shifts in the present study ranged between 12 and 35°C and were comparable to those used by Fonseca and Correia. The temperature effects on DPOAEs in locusts are therefore most probably not caused by a mere temperature-dependent change in the mechanical properties of the tympanum. Also, one would expect that any shifts in the vibration amplitude of the tympanum would have caused DPOAE amplitude changes at all L2 levels, and therefore would not only affect the low-level component of the DPOAE growth function, as was the case in our study. Our findings are more likely caused by frequency-selective transduction mechanisms based upon intrinsic properties within the scolopidia.

A change in the ambient temperature brought about frequency-selective effects within the auditory pathway for both locusts and cicadas. Oldfield (Oldfield, 1988) reported that the temperature-dependent shift in characteristic frequency was larger in receptors tuned to frequencies below 10 kHz than in receptors tuned to higher frequencies. In cicadas, intracellular recordings from interneurons having two sensitivity maxima (3–8 and 14–24 kHz, respectively)

displayed temperature-dependent effects only at the low frequency band (Fonseca and Correia, 2007). An increase in temperature was followed by upward shifts of the characteristic frequency and by enhanced sensitivity. The same frequency-dependent results were found in auditory nerve recordings, where there is a shift of tuning and an increase in sensitivity during a temperature rise only at low frequencies. The authors of both studies suggested that a temperature dependence of possible electrical tuning properties of individual scolopidial receptors could be responsible for the effect, comparable to findings on electrical tuning in non-mammalian hair cells (Fettiplace, 1987). In hair cells, electrical tuning is based on membrane potential resonances produced by the density and kinetics of ion channels, such as that of large conductance potassium channels (e.g. Bai et al., 2011), and is limited to low frequencies. The temperature sensitivity of such ion channels (Crawford et al., 1989) might explain the frequency selectivity of our observations, assuming that: (1) electrical resonances were present in insect sensory neurons and (2) would act at frequencies up to 10 kHz. So far, however, there is no experimental evidence for either of these assumptions.

Warren et al. (Warren et al., 2010) recently reported a temperature dependence concerning spontaneous mechanical oscillations of the Johnston's organ in mosquitoes. Spontaneous oscillations are caused by active mechanisms, selectively amplifying the antennal hearing organ's response to low-level stimuli (Göpfert and Robert, 2001). Scolopidia are the mechanoreceptors of both the non-tympanic Johnston's organ of mosquitoes and the locust tympanal organ. However, the external anatomy of the two organs is quite different because the former is specialized to detect the particle velocity, and the latter the sound pressure component, of incoming sound. Particle velocity attenuates rapidly with increasing frequency, which leads to very different frequency ranges of operation, i.e. several hundred Hz for mosquitoes [although certain species have been found to detect frequencies of up to 2 kHz (Cator et al., 2009)] *versus* frequencies in the ultrasonic range in locusts, which is comparable to the frequency range of the sound-pressure-sensitive ears of vertebrates [for an overview, see Yack (Yack, 2004)]. After transferring their data on temperature dependency into an Arrhenius plot, Warren et al. (Warren et al., 2010) also found a break point in their function, at 17°C, which is comparable to our findings of break points of ~20°C (for growth functions using 8 kHz for f_2) and ~19°C (for growth functions using 10 kHz for f_2). Based on the slope of these curves, the activation energy was determined to be 30 and 43 kJ mol^{-1} for the warm and cold temperature range, respectively. For the warm temperature range, this activation energy is comparable to values of ~34 and ~41 kJ mol^{-1} for f_2 frequencies of 8 and 10 kHz, respectively. The corresponding activation energies for the cold temperature range, however, were ~13 and ~3 kJ mol^{-1}, respectively, and are significantly lower than those calculated for mosquitoes. The reasons for this difference remain unclear. The locust's activation energy concerning the warm temperature range, however, is in good accordance to that found for mosquitoes by Warren et al. (Warren et al., 2010). Taking into account the activation energy values for dynein and myosin given in the literature (median values of 31 and 66 kJ mol^{-1}, respectively), Warren et al. concluded that the generation of spontaneous mechanical oscillations of the Johnston's organ relies on an intact dynein-tubulin system within the sensory cilium, which is also emphasized by its vulnerability to colchicine applications. Despite the differences in external anatomy and frequency range described above, the temperature dependence of the kinetics of the non-linear mechanical active processes (sound processing) in these non-tympanic and tympanic insect hearing organs are surprisingly similar. The activation energy values for the underlying mechanisms in both species are comparable, with the implication that the mechanical non-linearity is linked to the mechanoreceptor type. Furthermore, as now found in locusts, the underlying mechanism is not restricted to low frequency ranges of several hundred Hz, which is typical of mosquito hearing, but seems to be capable of operating at much higher frequencies of up to 10 kHz.

Studies on *Drosophila* mutants regarding the power gain provided by motile mechanosensory neurons (which are amplifying the mechanical input of the ear by adding mechanical energy) determined that proteins within the mechanoelectrical transduction apparatus could possibly carry out such processes (Göpfert et al., 2005). Dynein motors could play such an amplifying role during mechanoelectrical transduction. The dendrite of scolopidial sensory neurons elongates distally into a modified cilium which is surrounded by the scolopale space, whose cavity holds a receptor lymph with high K$^+$ concentration, comparable to the vertebrate scala media [for an overview, see Yack (Yack, 2004)]. Within the cilium, nine microtubule doublets form a circular arrangement. The links between adjacent microtubule doublets may well be dynein, which is distributed along the length of the cilium, except in the ciliary dilation region. One has to keep in mind, though, that the activation energy calculations based on Arrhenius plots could be problematic if the examined processes occurred in complicated systems such as hearing organs (James, 1959). The data that led to the calculated activation energy were derived from the output of an intact and complex organ. The kinetics as well as the distribution of dynein within the cilium, however, hint that molecules of this particular protein family could be the basis for the observed non-linear processes – spontaneous flagellum oscillations of the Johnston's organ and DPOAE generation in tympanal organs. As the present study aimed to compare the mechanical properties and activation energy values for both types of hearing organs, we followed, as close as possible, the paradigm for the Arrhenius plot derived activation energy used by Warren et al. (Warren et al., 2010). An application of colchicine, as performed for the mosquito, and its potential effects on DPOAEs could corroborate this assumption. Yet in the locust, such an experimental treatment is problematic for anatomical reasons. Müller's organ (which comprises the scolopidia) sits on the inner surface of the tympanal membrane and is surrounded by air-filled chambers. The substance would have to be applied either directly onto the tympanal membrane or topically to the surface of the thorax. Besides the uncertainty about whether the substance reached the organ, both approaches would affect the adjacent structures and their mechanical properties to such an extent that would make the interpretation of the outcome of the experiments ambiguous.

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