Mechanical tuning of the moth ear: distortion-product otoacoustic emissions and tympanal vibrations.

short title: Mechanical tuning of the moth ear.

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Abstract

The mechanical tuning of the ear in the moth *E. pugione* was investigated by distortion-products otoacoustic emissions (DPOAE) and laser Doppler vibrometry (LDV). DPOAE-audiograms were assessed using a novel protocol that may be advantageous for noninvasive auditory studies in insects. To evoke DPOAE, two-tone stimuli within frequency and level ranges that generated a large matrix of values (960 frequency-level combinations) were used to examine the acoustic space in which the moth tympanum shows its best mechanical and acoustical responses. The DPOAE-tuning curve derived from the response matrix resembles that obtained previously by electrophysiology, is V-shaped, tuned to frequencies between 25 and 45 kHz with low $Q_{10\text{dB}}$ values of $1.21 \pm 0.26$. In addition, while using a comparable stimulation regime, mechanical distortion in the displacement of the moth’s tympanal membrane at the stigma was recorded with a laser Doppler vibrometer. The corresponding mechanical vibration audiograms were compared to DPOAE audiograms. Both types of audiograms have comparable shape but most of the mechanical response fields are shifted towards lower frequencies. We showed for the first time in moths that distortion-product otoacoustic emissions have a pronounced analogy in the vibration of the tympanic membrane where they may originate. Our work supports previous studies that point to the stigma (and the internally associated transduction machinery) as an important place of sound amplification in the moth ear, but also suggests a complex mechanical role for the rest of the transparent zone.

Keywords: audiogram, DPOAE, *Empyreuma pugione*, laser Doppler vibrometry, mechanical tuning, moth

List of Abbreviations:

- DP: distortion-products
- DPOAE: distortion-products otoacoustic emissions
- FFT: fast Fourier transform
- LVD: laser Doppler vibrometry
- DPLVD: distortion-products laser Doppler vibrometry
- SPL: sound pressure level
Introduction

Moths are known for having ears adapted to detect the ultrasonic echolocation calls of their bat predators to which they show a range of escape behaviors that substantially reduce the risk of predation (Roeder and Treat, 1957; Fullard, 1988; Pavey and Burwell, 1998; Miller and Surlykke, 2001). Therefore, the hearing sensitivity of most studied tympanate moths tends to reflect the characteristic frequencies and intensities of the local bat fauna (Fullard, 1987). During evolution, moths have responded to the selective pressure from bats with new evasive mechanisms, and these responses in turn put pressure on bats to improve their tactics (Fenton and Fullard, 1979; Goerlitz et al., 2010). There are moths however, capable to detect the bat’s echolocation calls without taking visible evasive actions (Acharya and Fenton, 1992; Conner and Corcoran, 2012). These moths are generally unpalatable after the accumulation of defensive compounds, frequently noxious by virtue of their capacity to irritate, damage, poison and/or drug potential predators (Hristov and Conner, 2005). In some of these species, the acoustic communication between moths is ecologically more relevant than bat-to-moth communication and accordingly their hearing capabilities correlate with the acoustic features of conspecific mating sounds (Coro and Pérez, 1993; Sanderford et al., 1998; Nakano et al., 2008). In either way, i.e. the bat-moth or the moth-moth acoustic interaction, it is the moth hearing what ultimately determines the insect’s ecological success. That is why evolution has the moth ear adapted to perceive certain frequencies best.

Based on the anatomy, moth ears are simple insect hearing organs, with only one to four sensory neurons per ear. In Noctuoidea, ears are found on the lateral surfaces of the metathorax and comprise a round or oval tympanal membrane, supported by a chitinous ring and backed by an enlarged tracheal air sac (Yack, 2004). Two sensory cell types, A1 and A2, are attached to the same point in the opaque zone of the tympanum, the stigma (Fig. 1A). Under pure tone stimulation both cell types exhibits identical tuning curves that differ by about 20 dB in sensitivity; A1 is the most sensitive receptor (Suga, 1961; Coro and Pérez, 1993). However, when describing moth hearing, the neural tuning curve of the A1 cell serves as the standard (Skals et al., 2005; Fullard et al., 2008; Lane et al., 2008; Nakano et al., 2008; Jackson et al., 2010). Neural audiograms indicate that moth ears are broadly tuned with low $Q_{10dB}$ (< 2) values and frequencies at minimum threshold between 20 to 50 kHz (Fullard, 1982; Surlykke, 1986). As a consequence of the parallel tuning curves for the two A-cells, moths are considered tone-deaf (Waters, 2003).

The typical procedure to construct the moth neural audiogram in Noctuoidea includes the removal of legs, wings and scales, the dissection of the thorax and the exposure of the auditory nerve, which is then hooked with an extracellular electrode (Roeder, 1966). Sounds are played to the subject at a particular frequency and increased in intensity until the threshold criteria set by the experimenter is met, and the
process is repeated for sounds spanning the animal’s entire range of audible frequencies (Jackson et al., 2010). The validity of the neurophysiology studies of moth hearing rests on the assumption that the dissection and recording procedures do not affect the biomechanics of the ear. A recent study using laser Doppler vibrometry (LDV) showed that there were no consistent differences in tympanal velocity or displacement when moths were intact or prepared for neural recordings for sound levels close to neural threshold, indicating that neurophysiological studies provide good estimates of what intact moths hear at threshold (ter Hofstede et al., 2011). Only a stimulation with low frequencies at high intensities exhibits changes in tympanal mechanics between intact and dissected moths.

In principle, hearing involves a chain of mechanical events for capture of acoustic energy, and the frequency response. Each of these events can be used to construct objective audiograms or auditory threshold curves. The measurement of tympanal membrane vibration by means of LDV, for example, allows unveiling the biophysical characteristics of the moth tympanum (Schiotlen et al., 1981; Windmill et al., 2006; Windmill et al., 2007). By measuring the amplitude of the mechanical response of the eardrum to different frequencies it is possible to construct LDV- audiograms that coincide with the neural audiogram if membrane displacement at the stigma is taken into account, as has been recently shown for the moth *Noctua pronuba* (ter Hosftede et al., 2011).

Another type of measurable mechanical sensitivity of the moth ear are distortion-product otoacoustic emissions (DPOAE). Upon sound stimulation with two pure tones of frequencies f1 and f2, moth ears generate distinct DPOAE of which the one at 2f1-f2 is the most prominent (Coro and Kössl, 1998; Kössl et al., 2007). The emitted additional acoustic energy can be recorded with a microphone placed close to the tympanic membrane. By determining the stimulus level that suffices to evoke a 2f1-f2 DPOAE of certain amplitude for different f1 and f2 frequencies, it is possible to calculate the DPOAE-audiogram of the ear, which has not been constructed for the moth so far. Such calculations predict the frequency-specific sensitivity of the nonlinear mechanics of the tympanal organ that could reveal an active neuronal processing of low amplitude sounds (Kössl et al., 2008). In fact, the so called DP-gram, which differs from the DPOAE-audiogram in that it analyses the DP level obtained with equal-level stimuli (i.e. L1 = 60 dB SPL, L2 = 50 dB SPL) of different frequency (i.e. f1 = f2 / 1.09, 5 < f2 < 65 kHz), has been shown to resemble the inverted neuronal threshold of the ear in the moth species *Empyreuma pugione* and *Ascalapha odorata* (Coro and Kössl, 1998).

The construction of distortion-product audiograms with both techniques, OAE and LDV measurements, will help to clarify whether DPOAE in moths have their direct origin in mechanical intermodulations arising in the vibration pattern of the tympanum, as previously proposed for other insects (Kössl and Boyan, 1998a; Kössl and Boyan, 1998b; Möckel et al., 2011). If that is the case, the DPOAE tuning curve should overlap, in the frequency acoustic space, with the distortion-product laser Doppler
vibrometry (DPLDV) tuning curve. Therefore, the moth DPOAE tuning curve may also shed light onto
the mechanical vibratory response of the moth ear, at least at the place of the stigma in the opaque zone
of the tympanum, where the largest vibrations have been found to occur (Windmill et al., 2007).

In principle, only few studies have used DPOAE (Coro and Kössl, 1998; Coro and Kössl, 2001; Kössl
and Coro 2006; Kössl et al., 2007) and LDV measurements (Schiolten et al., 1981; Windmill et al.,
2006; Windmill et al., 2007; ter Hofstede et al., 2011) to study moth audition and therefore both
techniques open possibilities to investigate auditory mechanics in detail. First, we will examine whether
acoustically measurable distortion products can also be detected as movements on the tympanal
membrane, more specifically on the region of the stigma. Subsequent, both techniques are used to
create detailed audiograms, which are then compared in their frequency and level domain.

Materials and Methods

Animals and experimental setup

Recordings were obtained from 9 moths (five males and four females) of *Empyreuma pugione* Linnaeus
(Noctuoidea, Erebidiae, Arctiinae); one of which was only studied with LDV. The advantage of using
this species is that, for comparison purposes, the neural audiogram of its tympanic organ and the power
spectrum of the species mating sounds have already been described by Coro and Perez (1993). In
addition, for DPOAE studies, best f2/f1 ratios and appropriate levels of f1 and f2 tones have been
established (Coro and Kössl, 1998; Kössl and Coro, 2006).

The specimens were collected as last instars larvae in Havana City in June 2011 and kept in the
laboratory under the same temperature, humidity and photoperiod conditions as those of the
environment. The larvae were fed with fresh leaves of *Nerium oleander*, their foodplant, until they
reached the pupal stage. Pupae were transported to Frankfurt am Main, where adults finally hatched.
The adult moths were fed with 5% sugar water. Animals were used for experiments more than 48 h
after hatching, to make sure that the moths produce DPOAE (Coro and Kössl, 1998). To gain direct
access to the tympanal membrane with either the acoustic coupler for DPOAE or the laser beam for
LDV recordings, the bulla amplificatrix, a structure that externally covers the tympanal organ of
arctiinae moths (Fig. 1A), and the wings, were both left intact but reallocated with plasticine. The moth
body was tethered with plasticine dorsal side up to a cork platform atop a metal post that allowed free
rotation of the preparation. Only one ear was examined per animal. Before each experiment, the ears
were checked for scales, which were removed with a one-eyelash brush to avoid the undesired extra
loading. No anesthetics were used in the experiments. All experiments were carried out inside
acoustically isolated chambers at room temperature (23°C - 26°C) and relative humidity of 50%–65%.

Distortion-products otoacoustic emissions
To record DPOAE, an acoustic coupler was positioned 0.5–1 mm from the tympanal membrane in an open sound system configuration. The coupler has an overall tip diameter similar to that of the tympanal membrane and consisted of two adjacent conical tubes for stimulation and recording. The tympanal organ was stimulated simultaneously with two pure tones of different frequencies, $f_1<f_2$, to obtain DPOAE. One coupler channel was connected to a 1/4 inch microphone (Brüel & Kjaer 4135, Nærum, Denmark) and a measuring amplifier (Brüel & Kjaer 2670), and two 1 inch microphone capsules (Microtech Gefell MK 103.1, Gefell, Germany) served as speakers connected to the other coupler channel. The sound system was calibrated in situ for constant sound pressure level (SPL) at the microphone membrane using white noise, which was generated by addition of sinusoids of constant phase relation. The microphone’s sensitivity was calibrated with a Brüel & Kjaer sound-level calibrator (4235, calibration at 1 kHz, 94 dB SPL). Sound pressure levels applied in the experiments are expressed in dB SPL (rms dB re. 20 µPa). An M-Audio Audiophile 192 PCI sound card with a sampling rate of 192 kHz was used for the generation of the acoustic stimuli and for recording the microphone signal. Stimulation and data acquisition were controlled using software written in MatLab R2009. The stimulus duration for each pair of pure-tones was 42.7 ms corresponding to a stimulus length of 8192 points and a time period of 1/192 kHz. The recorded microphone signal was analyzed (rectangular window) with Fast Fourier Transform (FFT) and a frequency resolution of 23.4 Hz.

For each tympanum, the acoustic coupler was carefully positioned with a micro-manipulator to attain calibration curves that showed frequency responses between 10 and 90 kHz. Following calibration, the stimulation protocol consisted in the random presentation of pairs of tones in which the $f_2$ frequency was varied between 10 and 88 kHz in 2 kHz steps, and the $f_2$ level was varied between 30 and 78 dB SPL in 2 dB steps. The frequency of the $f_1$ tone was calculated using a constant $f_2/f_1$ ratio of 1.09, and the level of the $f_1$ tone ($L_1$) was maintained 10 dB higher than that of the $f_2$ tone ($L_1=L_2+10$ dB), following Coro and Kössl (1998). As a result of the stimulation protocol, a matrix of 960 frequency-level combinations (40 frequency x 24 level values) was sampled for the DPOAE data. For each stimulus combination in the matrix, the power spectrum of the microphone signal was calculated and the level values of the $f_1$ and $f_2$ tones, the $2f_1-f_2$ DP, and the background noise floor, were measured (Fig. 1B). Background noise was calculated by averaging 20 data points in the spectrum (frequency range of 468 Hz), at both sides and 40 Hz from the $2f_1-f_2$ DP frequency. Each stimulation protocol lasted for about 15 minutes and was repeated two times to look for signs of deterioration in the responses. Averaged differences in $2f_1-f_2$ DP-level (frequencies and levels pooled together) were below 2 dB between the two matrices in each of the eight moths studied. Within the acoustic space given by the stimulus combinations, the DPOAE response fields were calculated and represented in the form of color coded DPOAE level maps. DP-grams (the change of the DP level across frequencies for a constant level) and growth functions (the change of DP level across levels for a constant frequency)
were analyzed for each DPOAE response field. The setup did not produce two-tone distortions within the used level and frequency ranges as tested by pointing the coupler against a hard wooden surface as reflecting surface.

*Laser Doppler vibrometry*

Tympanal sound-induced vibration pattern were measured by a microscanning Laser-Doppler-Vibrometer Scanning system (MSV-300 with a sensor head OFV-534, Polytec, Waldbronn, Germany). The scanning system was attached to a customary microscope (Axio Examiner, Zeiss, Göttingen, Germany) using a microscope adapter (OFV-072, Polytec). The resulting laser beam was positioned under video control (VCT-101, Polytec). This allowed the laser spot (~1 µm diameter) to be positioned with an accuracy of ~1 µm. The laser spot position was monitored by way of a live video camera connected to the vibrometer’s controlling computer. In this way it was ensured that the tympanic membrane was perpendicular to the laser beam and that measurements were always taken on the stigma, the point of attachment of the sensory neurons to the tympanal membrane. Even when the laser system covered a limited area for scanning, it did not affect our measurements as recordings were made in the single-point acquisition mode. Vibrations could be measured without mechanical contact or loading the tympanum with reflective beads. All vibration analyses included the simultaneous recording of the vibration velocity of the tympanal membrane and measurement of stimulus SPL (Fig. 1C, D), for which a 1/4 inch microphone (Brüel & Kjaer 4135) was positioned 5 mm from the moth’s tympanum, with its diaphragm perpendicular to the direction of sound propagation. In this configuration, in contrast to the DPOAE measurement, the microphone is directed away from the tympanal membrane, towards the speaker. This arrangement of course prevented capture of tympanic DPOAEs and was taken as a control of the existence of intermodulations in the acoustic stimuli, which may have arisen within the speakers or due to multiple reflections in the equipment of the setup. To minimize acoustic reflections in the vibrometer setup the microscope was covered by acoustic insulation foam. Arrangement details of the setup including relevant distances and angles are given in Fig. 2. The vibration of the stigma was recorded using the laser Doppler vibrometer’s single-point acquisition setting whilst the tympanal organ was acoustically stimulated by two tones. Distortion products were measured in tympanal velocity level, converted to tympanal displacement level, and then to dB relative to 100 pm following ter Hofstede et al. (2011) (Fig. 1E, F). Laser and microphone signals were sampled in 16.4 s temporal windows (4194304 samples at a rate of 256 kilo-samples per second). The analysis of membrane velocity and SPL was carried out with a combination of Polytec v.7.4 and custom made MatLab R2009 software. The magnitude-squared coherence between the vibrometer and microphone signals was computed to assess the quality of the data set. Data was considered of sufficient quality when coherence exceeded 90%.
Vibrations of the moth ear were examined in response to acoustic stimuli similar to those used to obtain DPOAE. Two pure tones of different frequencies, $f_1 < f_2$, were simultaneously presented. The frequency of the $f_1$ tone was calculated using a constant $f_2/f_1$ ratio of 1.09, and the level of the $f_1$ tone was maintained 10 dB higher than that of the $f_2$ tone. Two ScanSpeak Revelator R2904 speakers ($\pm 5$ dB flat frequency response between 10 - 90 kHz) were positioned 30 cm from the animal to deliver the acoustic stimuli. For the relevant frequency range (10–88 kHz), the animal was in the far field of the sound source. Direction of sound propagation was at 45° respect to the tympanic membrane. The sound system was calibrated in situ. For each experiment, the acoustic system was carefully positioned to attain calibration curves with frequency responses between 10 and 90 kHz at levels above 85 dB SPL. The 1/4 inch reference microphone (Brüel & Kjaer 4135) has a linear response in the measured frequency and amplitude ranges. The microphone’s sensitivity was calibrated with a Brüel & Kjaer sound-level calibrator (4235, calibration at 1 kHz, 94 dB SPL). A RME Fireface USB sound card with a sampling rate of 192 kHz was used for the generation of the acoustic stimuli. Stimulation was controlled using software written in MatLab. The stimulus duration for each pair of pure-tones was 171 ms corresponding to a stimulus length of 32768 points and a time period of 1/192 kHz. Following calibration, the stimulation protocol consisted in the random presentation of pairs of tones of constant level in which the $f_2$ frequency was changed between 10 and 88 kHz in 2 kHz steps. The two tone levels $L_1/L_2$ were varied between 40/30 and 85/75 dB SPL in 5 dB steps. The acoustic stimulation and the vibrometer’s recording were manually triggered. As a result of the stimulation protocol, a matrix of 360 frequency-level combinations (40 frequencies x 9 level values) was sampled for tympanal vibrations.

The resulting vibrometer’s stereo recording, including the tympanal vibration signal in one channel and the microphone reference signal in the other, was exported as two ASCII files and analyzed in MatLab R2009 software. Fast Fourier Transform (FFT) with a rectangular window and a FFT length of 65536 points was used to produce amplitude spectra with a resolution of 3.9 Hz for both the vibration and the reference signals in each combination of the stimulation matrix. The power spectrum of both the vibrometer and the microphone signal was calculated and the level values of the $f_1$ and $f_2$ tones, the $2f_1-f_2$ DP, and the background noise floor, were measured. Background noise for both recordings was calculated by averaging 20 level measurements (frequency range of 78 Hz) in the power spectrum, at both sides and 40 Hz from the $2f_1-f_2$ DP frequency. Each stimulation protocol lasted for about 20 minutes. As a control for the experiment, the stimulation with the protocol’s first used $L_1/L_2$ level combination was repeated at the end. Averaged differences in $2f_1-f_2$ DP-level (frequencies pooled together) were below 2 dB in each of the nine moths studied.

Data analysis
The analysis of the DPOAE- and the DPLDV-audiograms was performed with MatLab R2009 scripts. The DPOAE-audiogram contour was defined at 25\% of maximum DP level, the DPLDV-audiogram contour at 5\% of maximum mechanical DP level. If more than one independent response area was detected within the frequency-level matrix, only that with the largest contour was taken as the valid audiogram. From the individual audiogram contour, the minimum threshold (MT) and the frequency at minimum threshold (characteristic frequency, ChF) were determined. $Q_{10\,\text{dB}}$ was computed as the ChF divided by the curve’s bandwidth measured 10 dB above MT. Best frequency and best level (BF, BL: respectively, stimulus frequency and level that evoked the maximum DP level) and the arithmetic mean of the frequencies at +10dB above minimum threshold were determined within the frequency-level space of the audiogram. Statistical analysis of these parameters included the calculation of the mean and standard deviation (s.d.). Average DPOAE and DPLDV response areas were calculated on the matrix resulting from averaging the DP-level that characterized each individual in each frequency-level combination.

**Results**

The DPOAE-audiogram

The nonlinear mechanical response of the moth tympanum was measured in response to pairs of pure tones of different frequencies ($f_1=f_2/1.09$) and levels ($L_1=L_2+10$dB), and evaluated by means of the level of the dominant $2f_1-f_2$ distortion product. The representation of the $2f_1-f_2$ DP level in the form of a color coded map in the frequency-level acoustic space is herein called DPOAE-audiogram. As previous studies have shown (Coro and Kössl, 1998; Kössl and Coro, 2006), the $2f_1-f_2$ distortion product in this moth can be measured above background noise in broad frequency and intensity ranges, as shown in the average audiogram for the species represented in Fig. 3A. In the average audiogram as well as in every ear examined a lower stimulus level evoked significant levels of $2f_1-f_2$ DP at a smaller frequency range than higher levels. In consequence, the DPOAE-audiogram has a V-shape. From the contour of each individual audiogram, parameters that characterize mechanical auditory capabilities in the moth were calculated, such as characteristic frequency, minimum threshold, best frequency, best level, $Q_{10\,\text{dB}}$, and bandwidth.

Characteristic frequencies of individual DPOAE-audiograms in *E. pugione* were on average at $34.00 \pm 7.01$ kHz (n=8), a value very close to the characteristic frequency reported for the neurophysiological threshold curve of this species (35 kHz, termed “best frequency” in Coro and Pérez, 1993). Still, the frequency range of individual minimum threshold is plateau shaped and covers frequencies between 25-45 kHz. Average minimum threshold was at $L_2 = 44.35 \pm 5.18$ dB SPL. DPOAE-audiograms are broadly tuned with low $Q_{10\,\text{dB}}$ values of $1.21 \pm 0.26$. Best frequency was $40.25 \pm 10.17$ kHz and best intensity $L_2 = 60.25 \pm 5.06$ dB SPL. Audiograms were asymmetrical with a high-frequency tail, which
represents a more gradual loss in mechanical sensitivity above absolute threshold (Fig. 3B). At +10 dB above threshold, DPOAE that were above the noise level were detected for f2 stimuli in the frequency range from 26.11 ± 6.53 kHz to 55.89 ± 11.93 kHz, and at +25 dB, from 18.71 ± 1.60 kHz to 65.57 ± 7.23 kHz.

DP-grams and growth functions were analyzed within each DPOAE-audiogram: DP-grams at +5, +10 and +15 dB above threshold, and growth functions at 0, -10 and +10 kHz around characteristic frequency (Fig. 4A). DP-grams are characterized by broader bandwidth and higher DP levels at higher L2 levels (Fig. 4C). At the highest L2 values tested however, DP levels can decrease, as a consequence of the occurrence of a notch in growth functions (see below).

The shape of growth functions (Fig. 4B) is similar for different stimulus frequencies and most of them are characterized by two distinct components. The presence of a prominent notch (15.4 ± 6.0 dB depth; n=24 growth functions) allows the definition of a low- and a high level component: L2 values that evoke a DPOAE just above noise (~15 dB SPL) up to L2 values just before the notch define the range of the low-level component. The high-level component extends from the latter value up to the highest L2 used. The slope of the growth of the low-level component is 2.1 ± 0.5 dB/dB (n=24 growth functions; range 1.2–3.2). In the non-monotonic growth functions, the notch appears at a level of the f2 stimulus between 58 and 74 dB above threshold (Fig. 4B), and the minimum difference between the level of the 2f1–f2 DPOAE and that of the f2 stimulus was 16.5 ± 6.5 dB (range: 5.7–32.4 dB). This minimum difference was obtained for stimulus levels below the appearance of the notch.

The DPLDV-audiogram

In each individual moth the protocol to obtain a DPOAE-audiogram (Fig. 5A, B) was followed by a laser Doppler vibrometry study of the stigma (Fig. 5C, D), the attachment site of the auditory receptors to the tympanic membrane. The mechanical response of the stigma was measured during stimulation with pairs of pure tones of different frequencies (f1=f2/1.09) and levels (L1=L2+10dB). The stimulation matrix covered a f2 frequency range of 10-88 kHz in 2 kHz steps, comparable to the acoustic DPOAE measurements. F2 levels (L2) between 30-75 SPL were used. The L2 step size was 5 dB and therefore slightly larger than in the acoustic measurement. Mechanical 2f1-f2 distortion in the vibration of the stigma point on the tympanum was evaluated from spectra calculated for the mechanical response to each of the frequency-level combinations (Fig. 5D) and the 2f1-f2 response area was plotted with color coded amplitudes in the DPLDV-audiogram (Fig. 5C). No distortion-products were present in the sound recordings obtained with the microphone directed towards the speakers (see Methods), indicating that it was the tympanum, and not the speakers that produced the distortion (see Fig. 1C, D).
The DPLDV-audiogram also is V-shaped similar to the DPOAE-audiogram, with lower stimulus levels evoking significant levels of 2f1-f2 DP at a smaller frequency range than higher levels (Fig. 6). Characteristic frequencies of individual DPLDV-audiograms are on average at 24.00 ± 4.47 kHz (N=9) and in comparison to neuronal threshold curves (35 kHz, Coro and Pérez, 1993) are shifted to lower frequencies. Average threshold of individual audiograms was at L2 = 53.94 ± 5.55 dB SPL. Best frequency was 23.33 ± 6.32 kHz and best intensity was always found at the maximum level of L2 = 75 dB SPL. At +10 dB above threshold, distortion-products that were above the noise level were detected for stimuli in the f2 frequency range from 18.20 ± 4.98 kHz to 35.90 ± 7.37 kHz (N=9).

Absolute membrane displacement at the 2f1-f2 distortion-product frequency varied considerably within and between individuals. At the characteristic f2 frequency and at minimum threshold, the 2f1-f2 membrane vibration was on average 47.74 (s.d. = 43.83) pm (N=9). At best frequency and best intensity the average 2f1-f2 vibration was 2473.74 (s.d. = 3040.73) pm. The average noise of the system was 6.90 ± 3.12 pm.

Comparison between the DPOAE- and the DPLDV-audiograms

In each of the eight moths used for audiogram comparisons, the frequency-level acoustic space covered by the DPOAE- and the DPLDV-audiograms overlapped, thus suggesting that the stigma in the opaque zone of the tympanum plays an important role in DPOAE generation. Two main types of relationships were found between the DPOAE- and the DPLDV-audiograms. In five moths, the DPLDV-audiogram area overlapped in less than 70% with the frequencies generating acoustic distortions, and the difference in characteristic frequency of both audiograms was larger than 10 kHz (Fig. 7A). In the other three moths, more than the 70% of the DPLDV-audiogram was contained within the acoustic space generating 2f1-f2 DP, and the respective characteristic frequencies of both audiograms were closer than 5 kHz (Fig. 7B).
**Discussion**

This is the first study to show in moths that the occurrence of distortion-products in otoacoustic emissions correlates with a membrane vibration. In insects, DPOAE have been shown to occur in the ears of locusts (Kössl and Boyan, 1998a, Kössl and Boyan, 1998b), moths (Coro and Kössl, 1998; Kössl et al., 2007) and bushcrickets (Möckel et al., 2011). Distortion-products in the motion of tympanal organs measured by laser Doppler vibrometry, however, have not been found so far in any insect species. Our study therefore, supports previous data indicating that sound processing by tympanal organs involves mechanical nonlinearities (Kössl et al., 2008).

*Mechanical distortion-products at the moth ear: vibration and otoacoustic emissions*

Among non-vertebrates, moths are known to have simple hearing organs regarding both anatomy and physiology. In tone-deaf Noctuoidea, ears comprise a tympanal membrane and two sensory cells, A1 and A2, internally attached to the stigma at the center of the opaque zone of the tympanum (Yack, 2004). Neither the conjunctivum membrane nor the transparent zone, but the opaque zone of the tympanic membrane, and specifically the stigma, has been identified to vibrate significantly in response to sound (Windmill et al., 2007). If distortion-product otoacoustic emissions are generated by distortion-products emerging in the vibratory pattern of the moth tympanum, then the latter should be found in the vibration of the stigma when stimulated with two simultaneous tones. Moreover, the DPOAE- and the DPLDV-audiogram should roughly overlap in the frequency acoustic space. We have proven both in this study (see Fig. 5, 7).

In human and other vertebrates, experiments with LDV techniques found that vibrations inducing DPOAE could be measured on the eardrum (Dalhoff et al., 2007). Recordable human eardrum displacements causing the 2f1–f2 DPOAE were about 5.6 pm when the two tones applied were 65 dB SPL and f1=4.6 kHz and f2=5.5 kHz. This DPOAE displacement magnitude is consistent with that expected from DPOAE vibration measured at the DPOAE place on the basilar membrane of laboratory animals (Dalhoff et al., 2007). Our results place moth ears atop other animals concerning the relative level of DPLDV. In our study, displacements causing 2f1–f2 DPOAE corresponding to the characteristic frequency and L1= 75, L2 = 65 dB SPL were about 298.10 ± 574.18 pm (N=8), or 53 times larger than those recorded in humans at 65 dB SPL. Relatively large values in the level of distortion-products in the vibration of the moth ear are in agreement with high levels of acoustic DPOAE in these insects. The smallest differences in level between L2 and L(2f1-f2) are about 30 dB in locusts (Kössl and Boyan, 1998a; Kössl and Boyan, 1998b) and 34 dB in bushcrickets (Möckel et al., 2011), but only 17 dB in moths (range: 5 - 32 dB) (Kössl et al., 2007; this study). In fact, in locusts it seems to be difficult to record mechanical 2f1-f2 distortions in the motion of the tympanum (Moir et al., 2011).
Even when our data demonstrate mechanical distortion-products in the vibration pattern of the stigma, a good correspondence and large overlap of acoustic and mechanical audiograms is seen only in a minority of experimental animals (Fig. 7B). In the majority, these distortions, which are limited in frequency and level to the acoustic space represented by the DPLDV-audiogram, do occur close to or even below the low frequency region of the DPOAE-audiogram (see Fig. 7A). A reason for the DPDLV-audiogram shift towards lower frequencies in some specimens remains to be found. It seems possible that in some specimens a deterioration of the preparation could have taken place that primarily affects the LDV measurements that were done after the DPOAE recordings. However, OAE and LDV control measurements recorded at the end of each experiment do not indicate, respectively, substantial shifts in otoacoustic emission or displacement amplitudes. It also could be the case that the OAE-relevant vibration pattern of the moth tympanum in species like *E. pugione* significantly involves the area outside of the stigma. Significant vibration in response to sound at the transparent zone of the moth tympanum have been shown in *Achroia grisella* (Rodríguez et al., 2005) and already obtained as preliminary results in *E. pugione*, in which non-stigma regions even vibrate better at high frequencies (E.C. Mora et al., unpublished).

Mechanical nonlinearities during sound amplification can explain mechanical and otoacoustic distortion-products in insect tympanal organs. Such nonlinearities disagrees with former studies describing insect ears like linear systems (Michelsen, 1971a; Michelsen, 1971b; Sueur et al., 2006). It was previously thought that all insect tympanal organs functioned passively through the mechanical motion of the membrane (Yager, 1999). There is currently compelling evidence to indicate that sensitive insect ears like those in the moth employ nonlinear mechanical sound processing which generates distortion-product otoacoustic emissions (Kössl et al., 2008). Unresolved however, is whether nonlinear sound amplification in the insect is due to active or passive cellular mechanisms. Cumulative evidence in moths, locusts and bushcrickets points to active cellular mechanisms to be partly responsible for the generation of DPOAE in tympanal organs in specific frequency ranges, with two main lines of evidence including: 1. experimental manipulations that affect the physiological state of the animal, such as the application of certain anesthetics or hypoxic substances or cooling, also affect the insect's DPOAE (Kössl and Boyan, 1998a, Kössl and Boyan, 1998b; Kössl et al., 2007; Kössl et al., 2008; Möckel et al., 2011; Möckel et al., 2012); and 2. electrical or mechanical manipulation of sensory neurons causes changes in DPOAE-amplitude (Möckel et al., 2007). Both lines of evidence suggest that the mechanosensitive neurones, which attach to the tympanum in moths and the locust but not in the bushcrickets, are the most likely candidate for the production of DPOAEs in insects. So far, unfortunately, spontaneous otoacoustic emissions, which offer the most convincing proof of active sound generation in non-mammalian hearing organs (Manley, 2001), have not been observed in
tympanal organs. However in scolopidial hearing organs of mosquitoes and fruit flies spontaneous antennae movement has been associated with active hearing characteristics (Göpfert et al., 2005).

Whether active or passive, cellular mechanisms amplifying sounds in moths would need to deal with high frequency oscillations of up to 65 kHz in *E. pugione* (present study) or up to 95 kHz in *Ptilodon cucullina* (Notodontidae) (Kössl et al., 2007). In mammals, there is strong evidence that prestin-based somatic motility of outer hair cells is an important component of the cochlear amplifier responsible for high-frequency sounds (Ashmore, 2008). In non-mammalian vertebrates, the stereovilli are responsible for amplifying mainly low-frequency sounds (Manley et al., 2001; Manley, 2001). In tympanal insects no rapid ciliary or somatic amplification mechanisms have been demonstrated (see Kössl et al., 2007), but in the antennal organs of *Drosophila* (Göpfert and Robert, 2001; Göpfert and Robert, 2003) and of male mosquitoes (Jackson and Robert 2006) an auditory amplifier in the lower frequency range (< 1 kHz) seems to be based on a ciliary mechanism. If in the moth, as in vertebrates, a nonlinear amplifier is located within the receptor cells, then the moth ear could become an excellent model to study high frequency sound amplification at the single cell level.

**The distortion-products mechanical audiograms to characterize moth audition**

Moth audition is one crucial factor affecting the survival of the prey in the bat-moth model of predator-prey acoustic interaction. Since the elegant studies of Roeder and Treat in the '60s (Roeder and Treat, 1957; Roeder, 1964) to recent refined works (Goerlitz et al., 2010, Corcoran et al., 2011), plentiful evidence reinforce the value of moth hearing in the bat-moth evolutionary acoustic arms race. As indicator for moth hearing in evolutionary scenarios, however, so far only the neural auditory threshold curve of the most sensitive receptor, the A1 cell has been used (Roeder, 1964; Skals et al., 2005; Goerlitz et al., 2010; Jackson et al., 2010). Alternative mechanical hearing audiograms may develop into valuable tools when analyzing moth audition thus avoiding the extreme invasive nature of auditory electrophysiology, but the implicated techniques and approaches still need validation.

Objective auditory threshold curves in insects have been determined by noninvasive DPOAE-measurements (Coro and Kössl, 1998; Kössl and Boyan, 1998b; present study), comparable to the procedures applied in vertebrates (Gaskill and Brown, 1990; Kössl 1992; Manley and Köppl, 1993; Whitehead et al., 1996). In the insect species however, so far evidence for a correlation between DPOAE measurements and the electrophysiology have been obtained by visual inspection after superimposing DPOAE threshold curves or DP-grams with neuronal threshold curves obtained in different individual insects by different research laboratories (Coro and Kössl, 1998; Kössl et al., 2008). To value DPOAE for the study of insect audition, further studies should address the auditory capabilities of these animals by using standardized stimulation matrices which make it possible to
derive and compare DPOAE-audiograms, DP-grams and growth functions at different sound frequencies.

Distortion-products in the vibration pattern of the stigma at the moth tympanum and the DPLDV-audiogram have been presented in this study for the first time, however their origin is still unclear. Previously, it was demonstrated in the moth *Noctua pronuba* that neurophysiological threshold sensitivity is explained by the stigma vibration if displacement and not velocity is taken into account (ter Hofstede et al., 2011). Applying the results in *N. pronuba* (Windmill et al., 2007, ter Hofstede et al., 2011) to the ear of *E. pugione* we constructed the DPLDV-audiogram to find out that there was a shift in its frequency range if compared with the electrophysiology threshold curve reported for this species (Coro and Pérez, 1993). This lack of "perfect" match between the two threshold curves (Fig. 7) may shed light onto the mechanical processes and the role of the stigma for sound transduction in the moth. In species such as *N. pronuba, Agrotis exclamationis* and *Xestia triangulum* (Noctuoidea, Noctuidae) it is expected that better matches will be found between the DPLDV- and the electrophysiology audiograms, since all along the frequency range evoking auditory responses it is the stigma the point that both shows the biggest deflections and determines the neurophysiological response threshold (Windmill et al., 2007; ter Hofstede et al., 2011). In characterizing moth audition, however, it is important to keep in mind that not every mechanical event implicates a neuronal correlate. For example, the dynamic tuning in resonant frequency that takes place in the mechanical vibration response of the stigma in *N. pronuba* when stimulated with high intensity sounds (Windmill et al., 2006) does not seem to affect the neuronal response of the A1 auditory receptor (Asi et al., 2009). There is no doubt that the tympanum of the moth keeps revealing that it is not a simple system.

**Acknowledgements**

We thank students at Havana University for their help with collecting ad keeping animals. We also thank two anonymous reviewers for their helpful comments.

**Funding**

This work was supported by the Institute Partnership Stipendium from the Alexander von Humboldt Foundation to the Research Groups of ECM and MK. We also thank the support by the DFG: NO 841 / 1-1.
References


