

# The Auditory System of the Dipteran Parasitoid *Emblemasoma auditrix* (Sarcophagidae)

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## Abstract

Several taxa of insects evolved a tympanate ear at different body positions, whereby the ear is composed of common parts: a scolopidial sense organ, a tracheal air space, and a tympanal membrane. Here, we analyzed the anatomy and physiology of the ear at the ventral prothorax of the sarcophagid fly, *Emblemasoma auditrix* (Soper). We used micro-computed tomography to analyze the ear and its tracheal air space in relation to the body morphology. Both tympana are separated by a small cuticular bridge, face in the same frontal direction, and are backed by a single tracheal enlargement. This enlargement is connected to the anterior spiracles at the dorsofrontal thorax and is continuous with the tracheal network in the thorax and in the abdomen. Analyses of responses of auditory afferents and interneurons show that the ear is broadly tuned, with a sensitivity peak at 5 kHz. Single-cell recordings of auditory interneurons indicate a frequency- and intensity-dependent tuning, whereby some neurons react best to 9 kHz, the peak frequency of the host's calling song. The results are compared to the convergently evolved ear in Tachinidae (Diptera).

**Key words:** parasitoid fly, morphology, tympanate ear, single-cell physiology, micro-computed tomography

In insects, several taxa independently evolved auditory organs mainly for intraspecific communication, predator detection, and host location (Strauß and Lakes-Harlan 2014). Consequently, ear anatomy is of great diversity between taxa (Yager 1999, Yack 2004). Nevertheless, a typical tympanal insect ear consists of three basic structures, a tympanal membrane able to vibrate in response to sound pressure changes, a tracheal air space backing the tympanum and scolopidial sensory units to register the vibrations of the membrane.

Two taxa of Diptera are known to possess a tympanal ear, the Ormiini (Tachinidae) and Emblemasomatini (Sarcophagidae), both evolved independently (Lakes-Harlan et al. 1999, Robert et al. 1999). The ear of Diptera is located at the ventral prothorax and both tympanal membranes face forward (Lakes-Harlan and Heller 1992; Robert et al. 1992, 1996; Lakes-Harlan et al. 1999, 2007; Robert and Willi 2000). In the Ormiini, the ear is a relatively large bulging structure at the ventral prothorax, with one tracheal air space backing both membranes (Robert et al. 1994, Robert and Willi 2000, Lakes-Harlan et al. 2007). About 200 sensory units connect to each of the tympanal membranes. The tuning of the hearing

thresholds of the different species is adapted to their host's acoustic communication signals (*Homotrixia alleni* to *Sciarasaga quadrata*: Stumpner et al. 2007; *Emblemasoma* sp. to *Tibicen purinosus*: Farris et al. 2008; *Ormia ochracea* to various Grylline crickets emitting more continuous trills: Rosen et al. 2009; Review: Lakes-Harlan and Lehmann 2015). At least some auditory interneurons of *Therobia leonidei* and *H. alleni* are tuned to the frequency spectra of their hosts, and phasic interneurons might also be adapted to the temporal parameters of the host song (Stumpner and Lakes-Harlan 1996, Stumpner et al. 2007). In some cases, the auditory tuning is also discussed as potential predator avoidance mechanism although it is only documented for *O. ochracea* (Rosen et al. 2009).

In recent years, many behavioral experiments have been performed with a species of sarcophagid parasitoids, *Emblemasoma auditrix* (Soper). *E. auditrix* is a parasitoid of the cicada *Okanagana rimosa* (Say) and occurs in the Northeast of the United States and the Southeast of Canada during early summer. Gravid female *E. auditrix* acoustically locate the sound-producing males of the host cicada *O. rimosa* and infest them with a larva (Lakes-Harlan et al. 2000, Lakes-Harlan and Köhler 2003, Schniederkötter and Lakes-

Harlan 2004). The larva feeds inside the cicada and eventually kills it before emerging to pupate in the soil (Schniederkötter and Lakes-Harlan 2004). Other species of *Emblemasoma* have different host species, but all hosts are cicadas (Farris et al. 2008, Stucky 2015). In contrast to the behavior, the ear and characteristics of the auditory system of hearing sarcophagids are less well described. The ear is a flat inflation of the prosternum and faces the rear of the head (Robert et al. 1999). At both membranes, a scolopidial sense organ inserts with about 35 uniformly sized mononeuronic scolopidia, each with one sensory neuron (Lakes-Harlan et al. 1999). The tympanal ear of *Emblemasomatini* and *Orminii* represent an example of convergent evolution from a precursor structure, the vibration-sensitive chordotonal organ in nonhearing flies (Edgecomb et al. 1995, Lakes-Harlan et al. 1999) and possibly an advancement of the membranous prosternal organ in the tsetse fly *Glossina morsitans* (Tuck et al. 2009). Physiologically, the hearing system of *E. auditrix* seems to be adapted to its host calling song (CS; Lakes-Harlan and Lehmann 2015), but anatomical and physiological data at large are missing. Here, we analyze the auditory organ of the *E. auditrix* especially in respect to the tracheal system and present first results on the tuning of auditory afferents and suprathreshold responses of auditory interneurons.

## Materials and Methods

### Animals

Female *E. auditrix* were collected by phonotactic attraction to a broadcasted CS of the host. The CS of *O. rimosa* consists of chirps with 8–10 kHz peak frequency, about 6-ms duration and a repetition rate of about 83 chirps per second (Stölting et al. 2004). A recorded CS was stored on CD. A Discman (Sony D-131, Tokyo, Japan) was connected to an amplifier (Denon Power Amplifier DCA-450, Nettetal, Germany) and a piezo loudspeaker (HT-Horn, Conrad Electronic, Hirschau, Germany). The CS was broadcasted with 90 dB SPL (rel. 20  $\mu$ Pa) at 1-m distance, measured with a sound-level meter (Bruel & Kjael 2210, Bremen, Germany) and microphone (Bruel & Kjael 4133). Phonotactically attracted flies were captured at the loudspeaker in habitats around the University of Michigan Biological Station (UMBS) at Pellston, Michigan, and kept in small cages for up to 14 days under a photoperiod of 14:10 (LD) h (lights on at 07:00 a.m.) at 20–24°C. They were provided with water and sugar ad libitum. Flies were transported to Justus-Liebig-University Gießen, Germany, for anatomy and to the Georg-August University of Göttingen, Germany, for physiology.

### Anatomy

#### Scanning Electron Microscopy

The anterior ventral part of the thorax was dehydrated via ascending alcohol series (50, 70, 90, 96 and 2\*100%, remaining in each concentration for at least 1h), followed by critical point drying (Balzers, CPD 030, Hudson, NH). The specimen was attached to tape on an aluminium tub and sputter coated with gold (BAL-TEC, SCD 050, Liechtenstein). The specimen was viewed and photographed under a scanning electron microscope (Leo/Zeiss 438 VP, Oberkochen, Germany) equipped with a CCD camera (1,024\*768 pixels).

#### Micro/Nano-Computed Tomography

To achieve an optimal voxel resolution of the internal thoracic structures without truncation artefacts of the computed tomographic (CT) images, flies ( $N = 3$ ) were freshly killed by freezing and

wrapped in Parafilm wax foil (Sigma Aldrich, Munich, Germany) and fixed on the tip of the micro-, respectively, nano-CT specimen holder. The holder was mounted on the rotation stage of the CT system. The flies were scanned using a SkyScan 1173 micro-CT System (Bruker MicroCT, Kontich, Belgium). The system is equipped with a high-energy X-ray tube (30–130 kVp), a CsI-Scintillator and a 2,240×2,240 flat panel X-ray detector. The best achievable voxel size of the system is 5.6- $\mu$ m isotropic voxel side length. For this study, the tube potential was set to 35 kVp with a tube current of 190  $\mu$ A and an X-ray exposure time of 1.6 s/frame. Flies were scanned over 240° with rotation steps of 0.25°. A fourfold frame averaging was performed aiming an image noise reduction.

For a higher resolution of the tympanalorgan in *E. auditrix*, the thorax was rescanned ( $N = 1$ ) in a nanofocussed CT-System (Nano-CT SkyScan 2011, Bruker MicroCT). The System is equipped with a nanofocussed transmission X-ray tube (Tohken, Japan) a Gd<sub>2</sub>O<sub>2</sub>S:Tb Scintillator, an image amplifier and a 1,280×1,024 pixel CCD detector. The best achievable voxel size of the system is 150-nm isotropic voxel side length. For this study, tube potential was set to 60 kVp tube voltage with a tube current of 140  $\mu$ A and an X-ray exposure time of 2 s/frame. The fly was scanned over 185.2° (=180° + fan opening angle) with rotation steps of 0.20°. A fourfold frame averaging was performed aiming an image noise reduction. Aiming to avoid truncation artifacts with an impaired image quality, we adjusted the geometric magnification of the nano-CT to the thoracic diameter of the flies. This resulted in a spatial resolution of 4- $\mu$ m isotropic voxel side length.

The reconstruction of cross-sectional images was performed using a Feldkamp cone beam algorithm (Feldkamp et al. 1984) with a symmetrical boxcar smoothing kernel. The resulting isotropic voxel side length was 6.05  $\mu$ m (SkyScan 1173), respectively 4  $\mu$ m (Nano-CT SkyScan 2011), for further information on the micro/nano-CT technique see Kampschulte et al. 2016.

Image postprocessing techniques ( $N = 1$ ) were used for the visualization of internal structures and external surfaces and comprised cross-sectional (2D) and volume compositing (3D) and maximum intensity projection image postprocessing techniques (Analyze 12.0, AnalyzeDirect Inc., Overland Park, KS). Furthermore, overlay techniques of outer morphology and segmented internal structures were used for a depiction of anatomical details. Therefore, soft tissue and aerated internal structures were segmented manually or based on gray-scale thresholds.

#### Biometric Measurements

A sample of ethanol-fixed specimens ( $N = 25$ ) was used for measurements of the width and the area of the tympanal membranes, the length and width of spiracles and the femur length of the left first leg. In addition, the distance between head and the probasisternum has been measured from lateral. All specimens were photographed with a calibrated ocular in a dissection microscope (Leica MS 5) and a microscope camera (Leica DFC 320, Wetzlar, Germany). The area of the tympanal membrane was outlined by hand (three times each) with a graphic pad (intuos<sub>3</sub>, PTZ-930, Wacom Co. Ltd., Krefeld, Germany). All measurements were done with the image editing application Fiji (Schindelin et al. 2012).

#### Histology

The dissected thorax was fixated with 4 % paraformaldehyde in phosphate buffer and embedded in Agar 100 epoxy resin (Plano GmbH, Wetzlar, Germany). These specimens were sectioned 5–14- $\mu$ m thick and counterstained with methylene blue. For further details see Mücke and Lakes-Harlan (1995).

## Physiology

All experiments took place in a setup for electrophysiological measurements in the laboratory at Georg-August-University of Göttingen, Germany, similar to that used for experiments on tachinid flies (Stumpner and Lakes-Harlan 1996, Stumpner et al. 2007). Animals were fixed with wax dorsal side up to a plastic holder. Thereafter, thoracic cuticle was removed dorsally, and the central nervous system was exposed. The large flight muscles were removed by tissue coagulation to a hot needle. Care was taken not to damage the thoracic abdominal ganglion (TAG) that was kept moist with saline (7.48 g NaCl, 1.39 g KH<sub>2</sub>PO<sub>4</sub>, 1 g Na<sub>2</sub>HPO<sub>4</sub>, 0.061 g CaCl<sub>2</sub>·2H<sub>2</sub>O, and 0.03 g MgCl<sub>2</sub>·6H<sub>2</sub>O dissolved in 1-liter distilled water).

Acoustic stimuli were computer-generated sine wave pulses of 50-ms duration with 2-ms rise and decay time. For threshold tests, 5-dB increments were used; for intensity scans, 50, 70, and 90 dB SPL were tested. For threshold tests, the frequency steps were 1 kHz for frequencies below 10 kHz, and 2 kHz for frequencies above 10 kHz; for intensity scans, the tested frequencies were 3, 5, 8, 12, 16, 26, and 34 kHz. In some cases, additional parameter combinations were tested (as described under Results). The programs for signal generation and data evaluation were custom written with the software LabView (National Instruments Corporation, Austin, TX). Signals were converted with a DAQ-board (AT-Mio 16E1, National Instruments Corporation) at 500-kHz sampling rate. Sound pressure level was measured with a sound-level meter (Bruel & Kjael 2210) and a calibrated Bruel & Kjael 4133 microphone, rel. 20 μPa. The microphone was placed at the position of the fly for measurements of sound intensity.

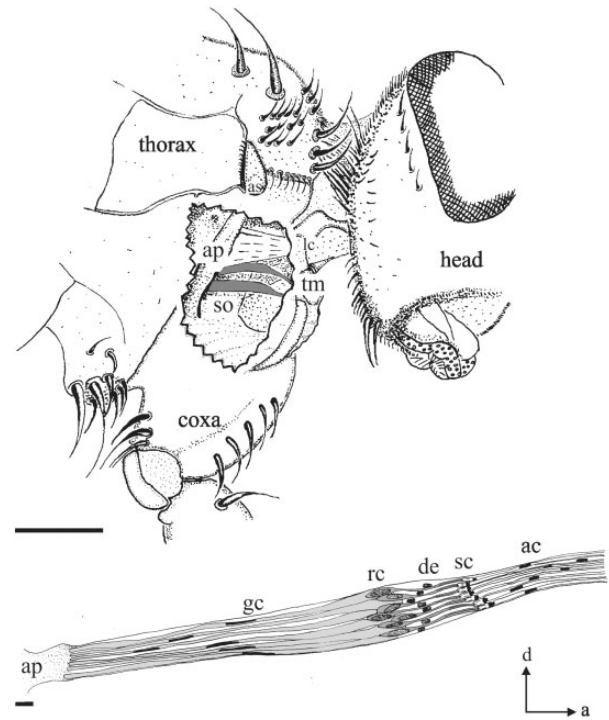
Summed action potentials of the sense organ were recorded by positioning a sharpened tungsten electrode within or close to the auditory nerve. The recordings were amplified 1,000× (custom-build amplifier), displayed on an oscilloscope and headphones, and stored on DAT tape (Sony PCR 204). For the determination of the threshold, the gliding length of the recording trace (time window 5 ms) at intensities below and above threshold was calculated (NEUROLAB, Hedwig and Knepper 1992). The threshold was defined as sound pressure level that elicited higher values in three out of five stimulus presentations. For single-cell recordings, a glass microelectrode (1.0/0.5 outer diameter/inner diameter) was inserted into the prothoracic neuromere of the TAG close to its midline. The recordings were amplified by an SED-05L amplifier (NPI-Instruments, Tamm, Germany). Data were recorded on computer with LabView software, and data analysis was done by custom-written scripts in LabView. Intracellular recordings proved to be difficult in *E. auditrix* (in comparison to tachinid flies) resulting in rather short recording times. Only in a few cases, neurons could also be marked with dye. In these cases, neurons were iontophoretically stained with 5% Lucifer-Yellow in 1 M lithium chloride by hyperpolarising currents with 0.3 nA. The marking was amplified by standard immunohistochemistry, using anti-Lucifer Yellow antisera (1:300, Molecular Probes; Stumpner et al. 2007).

Data analysis and statistics were done with Excel (Microsoft Inc., Redmont, WA) and GraphPad Prism 6.01 for Windows (GraphPad Software Inc., La Jolla, CA).

## Results

### Anatomy

The ear of *E. auditrix* is located at the ventro-anterior prothorax (Figs. 1 and 2). The two tympanal membranes face forward and are connected with the rigid prosternal bridge at the midline. Medioventrally, the probasisternum borders the

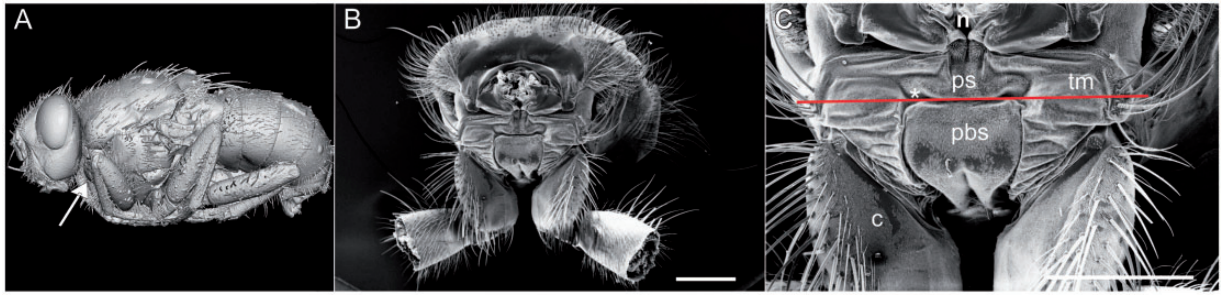


**Fig. 1.** Schematic drawings of the ear and the scolopidial organ. The ear is located behind the head and close to the forelegs coxae (the neck is stretched for visualization of the ear). The ear has been opened to allow a view into the air filled cavity, the prosternal air sac, with the bilaterally arranged sensory organs (so). The scolopidial sense organ is attached to the tympanal membrane (tm) and a cuticular apodeme (ap). Below, the cellular arrangement of the scolopidial organ (gc, glial cell; rc, receptor cell; de, dendrite; sc, scolopidia; ac, attachment cell). Upper scale bar = 1 mm, lower scale bar = 10 μm.

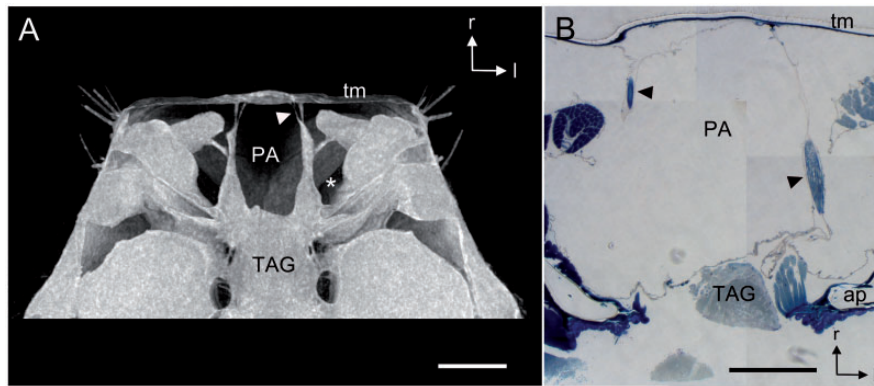
tympanal membranes. Ventrolaterally, the tympanal membranes adjoin the coxal membranes. The major part of the ear is overshadowed by the head with a mean gap width between head and probasisternum of 0.38 mm (SD 0.095,  $N = 23$ ). Interiorly, the tympanal membranes are backed by a single undivided tracheal space (Figs. 1 and 3), the prosternal air sac. Inside the ear, a pair of scolopidial sense organs span between the tympanal pit, a thickened cuticular part mediolateral on the membrane and the posterior prosternal apodeme at the back of the air chamber, flanking the thoracicoabdominal ganglion. The sensory organ consists of about 35 uniformly sized mononeuronic scolopidial units (Lakes-Harlan et al. 1999) each with one sensory cell (Figs. 1 and 3B).

The prosternal air sac and the connecting trachea have been studied further by means of micro-CT and digital reconstruction (Fig. 4A and B). The trachea supply the frontoventral tissues in the thorax and originate at the lateral anterior spiracles of the mesothoracic segment. The dorsoanterior part, the parenteric air sac, extends into the neck and forms the cervical trachea, which is the air supply of the head. Ventrally, the two leg tracheae originate at the prosternal air sac. Posteriorly, a pair of tracheae extends into the scutellar sac and the sternopleural sac of the thorax. The volume of the prosternal air sac and the trachea leading to the spiracles (red volume Fig. 4B; Supp Movie 1 [online only]) occupies about 4.1% of the whole thorax volume. Structures emerging from the prosternal air sac are traced just enough to document the position and dimensions. In summary, the prosternal air sac is an enlargement within the tracheal network for air supplies in various tissues.

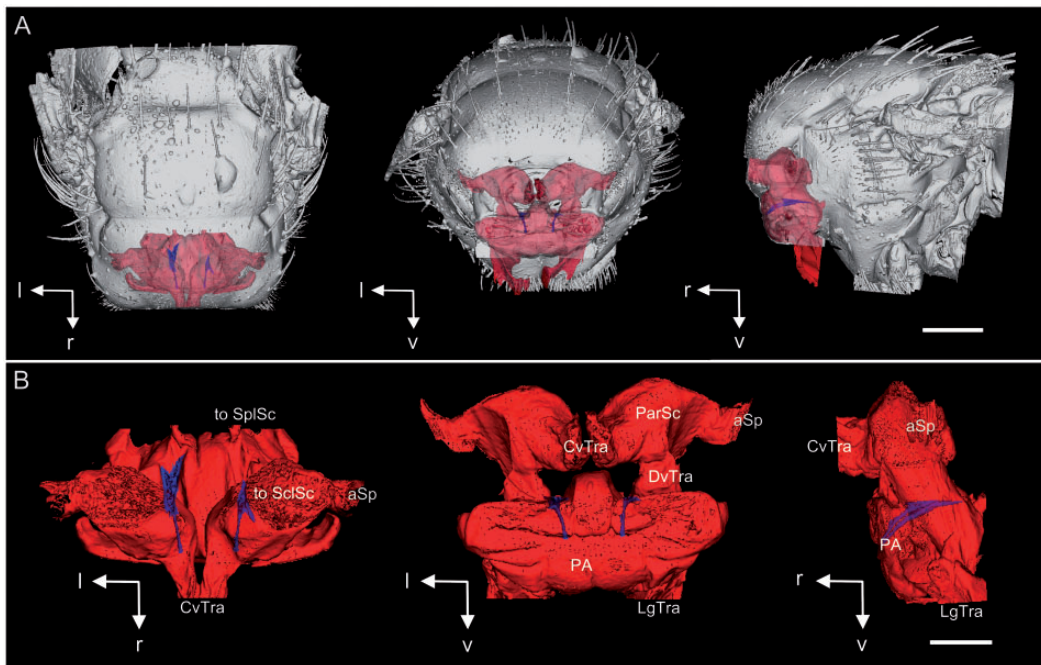




**Fig. 2.** Morphology of *E. auditrix* and the tympanate ear. (A) Lateral view of the whole reconstructed fly (volume composition from micro-CT) to demonstrate the location and size of the ear (arrow) in relation to body size. (B, C) Frontal view of the thorax and the tympanal membranes, the head was removed to reveal the whole ear (scanning electron micrographs). c, coxa; n, neck; pbs, probasisternum; ps, prosternum; tm, tympanal membrane; asterisk, tympanal pit. The red line in C indicates the approximate cutting level shown in Figure 3. The scale bars = 1 mm refer to the plane of the tympanum.



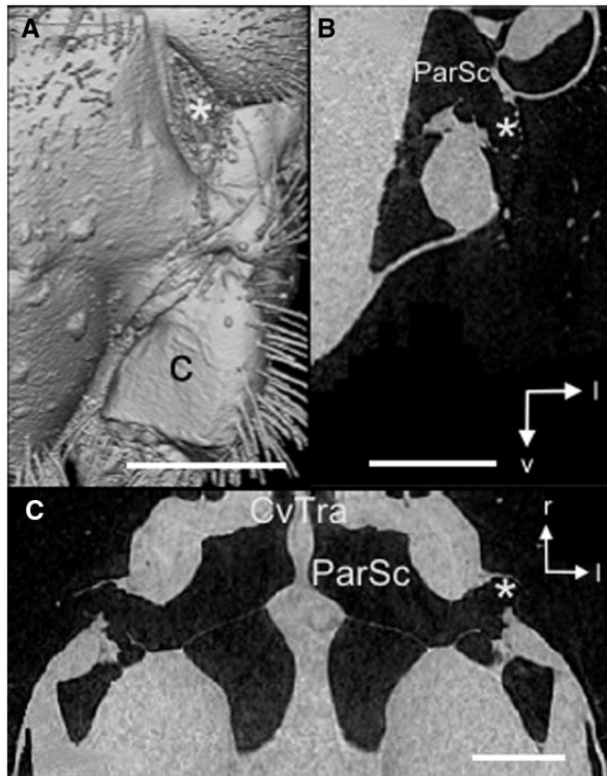
**Fig. 3.** Anatomy of the tympanate ear. (A) Maximum intensity projection of the anterior thorax from micro-CT (dorsal view). Note that the tympanal membranes (tm) are aligned in a plane and backed by the prosternal air sac (PA). One of the bilateral symmetric sensory organs is indicated by an arrow head. \*The connection of the prosternal air sac to the leg trachea. (B) Near horizontal section at level of the sensory organs (histological section counterstained with methylene blue). The bilateral sensory organs sectioned at different levels (arrowheads), showing the attachment to membrane (tm) and apodeme (ap). TAG, thoracoabdominal ganglion. Scale bar = 500  $\mu$ m.



**Fig. 4.** Digital reconstruction of the anterior part of the tracheal space and micro-CT sections. (A) Digital volume composition (from micro-CT) of the thorax (gray) with tracheal space (red) and sensory organ (blue). Head and legs are removed digitally. Scale bar = 1 mm. (B) Digital volume composition of tracheal space with the sensory organs (blue). aSp, anterior spiracle; CvTra, cervical trachea; LgTra, leg trachea; ParSc, parenteric air sac; PA, prosternal air sac; ScSc, scutellar sac; SplSc, sternopleural sac. Scale bar = 0.5 mm.

**Table 1.** Biometric measurements of female *E. auditrix* (done on alcohol preserved specimen).

Parameter	No. of values	Mean (SD)
Femur length (mm)	24	2.6310 (0.1663)
Ear width (mm)	25	1.9800 (0.1299)
Area left tympanum (mm <sup>2</sup> )	24	0.31 (0.05)
Area right tympanum (mm <sup>2</sup> )	24	0.32 (0.06)
Spiracle length left (mm)	25	0.59 (0.08)
Spiracle length right (mm)	25	0.57 (0.08)



**Fig. 5.** Structure of the anterior spiracle. (A) Digital surface reconstruction of the spiracle (\*) and the neighboring cuticle c, coxa. (B, C) micro-CT sections showing the spiracle entrance (\*) into the parenteric air sac (ParSc). (B) Transversal section, scale bar = 200  $\mu\text{m}$ . (C) Horizontal section showing the bilateral symmetry of the trachea, spiracles and the direct connection to the cervical trachea (CvTra), scale bar = 500  $\mu\text{m}$ .

The anterior spiracle is located laterally on the mesothoracic segment and left and right spiracles do not differ in size (paired *t*-test,  $P = 0.0919$ ,  $t = 1.756$ ,  $df = 24$ ,  $N = 25$ ; Table 1). It is oval shaped and covered with long interlaced setae; the valve structures could not be detected in the scans (Fig. 5).

The ear width correlates positively with the femur length (Fig. 6; Pearson correlation  $r^2 = 0.3889$ ,  $P = 0.0011$ ,  $N = 24$ ), as well as the spiracle length correlating to the femur length (Pearson correlation, left:  $r^2 = 0.3632$ ,  $P = 0.0018$ ; right:  $r^2 = 0.3416$ ,  $P = 0.0027$ ,  $N = 24$ ). The tympanal membranes are slightly asymmetric with an approximately 4 % larger area of the right tympanum (paired *t*-test  $P \leq 0.0171$ ,  $t = 2.569$ ,  $df = 23$ ,  $N = 24$ ; Table 1).

### Physiology

Responses of auditory sensory cells were extracellularly recorded with a tungsten electrode from the tympanal nerve ( $n = 4$  animals).

The hearing sensitivity is broadly tuned with a threshold of about 65 dB SPL at 5 kHz and another sensitivity peak with 68 dB SPL at 14 kHz (Fig. 7).

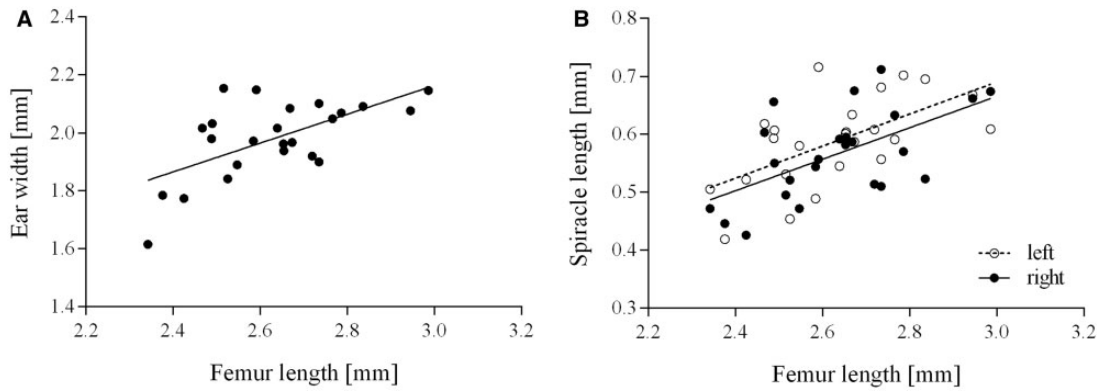
Individual auditory interneurons have been recorded in the ganglion near the prothoracic neuromere. These recordings proved to be quite difficult in *E. auditrix*, and most neurons could only be tested for a short time. Thus, it was not possible to fully characterize the neurons physiologically and anatomically, as many other invertebrate neurons, including auditory neurons from tachinid flies (Stumpner and Lakes-Harlan 1996, Stumpner et al. 2007). Nevertheless, some important characteristics of auditory interneurons could be extracted from the recordings ( $n = 10$ ). Most tests have been made with a series of sound pulses of constant intensity and different frequencies. The resulting iso-intensity curves show that most recorded interneurons are rather broadly tuned in a high-intensity range and more narrowly tuned at lower sound pressure levels (Fig. 8A and B). A narrow tuning is typically seen in the frequency range from 5 to 10 kHz. The reaction of some neurons also peak at around 9 kHz, the peak frequency of the host CS. The neurons differ largely in their spike rate, from a few action potentials per 50 ms stimulus to more than 20 spikes. Five neurons could at least partly be anatomically characterized. An ascending neuron with striking response to the peak frequency of the hosts CS is depicted in Figure 8C and D. The neuron responded strongly to 9 kHz at 90 dB SPL, whereas other frequencies and other sound pressure levels were only weakly responded to. The anatomy (Fig. 8D) shows dense neuronal arborizations on both sides of the CNS, especially in the median ventral association centre (mVAC) of all thoracic neuromeres (the position of the soma has not been unequivocally identified, but is likely in the T3 neuromere). Other ascending interneurons have their soma in different thoracic and abdominal neuromeres and project into the ventrolateral protocerebrum (data not shown). The neurons respond either phasically or tonically to auditory stimuli, and most neurons have monotonic intensity–response curves (Fig. 9). These intensity–response curves for two tested frequencies show different types of neurons: some react more strongly to 9 kHz (Fig. 9A), others have a lower threshold at 5 kHz (Fig. 9B and D), and one neuron showing similar responses at both frequencies (Fig. 9C).

### Discussion

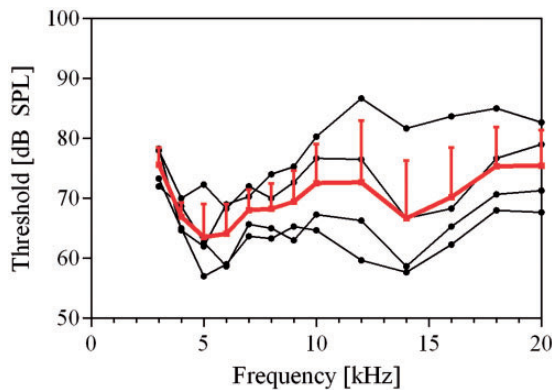
The parasitoid fly *E. auditrix* uses its auditory system to locate the male cicada *Okanagana rimosa* by its CS. Although aspects of the behavior and the evolutionary novelty of the hearing sense have already been described (Köhler and Lakes-Harlan 2001, Lakes-Harlan and Köhler 2003, Schniederkötter and Lakes-Harlan 2004, de Vries and Lakes-Harlan 2005, Tron et al. 2015); here, the anatomy of the ear and the auditory physiology are discussed in order to understand the properties of the hearing sense of *E. auditrix*.

### Anatomy

The ear of *E. auditrix* is located at the ventral prothorax directly below the cervical sclerites and the two tympanal membranes face forward (Lakes-Harlan et al. 1999), as it is the case for all known tympanate Diptera (Lakes-Harlan and Heller 1992, Edgecomb et al. 1995, Lakes-Harlan et al. 2007, Tuck et al. 2009). The tympanal membranes have an average area of 0.32 mm<sup>2</sup> (mean of left and right tympana) and are within the range of other flies, like *O. ochracea* (0.29 mm<sup>2</sup>, Robert et al. 1994 or *G. morsitans* (0.53 mm<sup>2</sup>, Tuck et al. 2009). In *E. auditrix*, we found a positive correlation of the



**Fig. 6.** Allometric data of *E. auditrax*; correlation of body size and the size of the tympanate ear. (A) Correlation of ear width to femur length with linear regression (Pearson correlation:  $r^2 = 0.3889$ ,  $P = 0.0011$ ; best fit slope =  $0.4976 \pm 0.1330$ , equation,  $Y = 0.4976 * X + 0.6710$ ). (B) Correlation of spiracle length to femur length with linear regression (Pearson correlation:  $r^2_{\text{left}} = 0.3632$ ,  $P = 0.0018$ ,  $r^2_{\text{right}} = 0.3416$ ,  $P = 0.0027$ ; best fit slope left =  $0.2769 \pm 0.07816$ , equation,  $Y = 0.2769 * X + 0.1403$ , best fit slope right =  $0.2718 \pm 0.08044$ , equation,  $Y = 0.2718 * X + 0.1496$ ).



**Fig. 7.** Hearing threshold of *E. auditrax*. Sound evoked responses of the tympanal nerve had been recorded extracellularly and the threshold has been determined in four females of *E. auditrax* (thin black lines). Mean and SD in red.

size of the ear to body size, but it is unknown whether the size influences physiological properties of the auditory system. In the Australian bushcricket *Requena verticalis*, the hearing threshold for the intraspecific call's carrier frequency correlates positively with body size (Bailey 1998). Interspecific the picture is somewhat diverse, as a comparative study with 17 katydid species found no correlation of body size and hearing sensitivity for high frequencies, tested with bat signals (Römer et al. 2008), whereas a study with 44 noctuid moth species found a correlation between size and threshold (Surlykke et al. 1999). The asymmetry of the ear in respect to the size of the tympanal membranes is described here for the first time. Typically, the right ear is larger by 4%. Because the asymmetry in *E. auditrax* has been found in the preserved specimens, so far no functional tests have been made and it will be interesting to analyze physiologically whether sensitivity or tuning is different between left and right ear and whether the asymmetry might influence orientation behavior. An asymmetry in an auditory system has also been reported for the spiracle of a bush cricket with about 8% difference (Bailey and Yang 2002). However, this asymmetry had no influence on the auditory behavior. Furthermore, it should be considered that the overlap of the head and the narrow distance (0.37 mm) to the tympani may result in head-related transfer functions comparable to the vertebrate hearing system (Blauert 1996). It may seem unlikely considered the wavelength of 3.8 cm at the carrier frequency of the attractive signal. But in laser-Doppler vibrometric measurements,

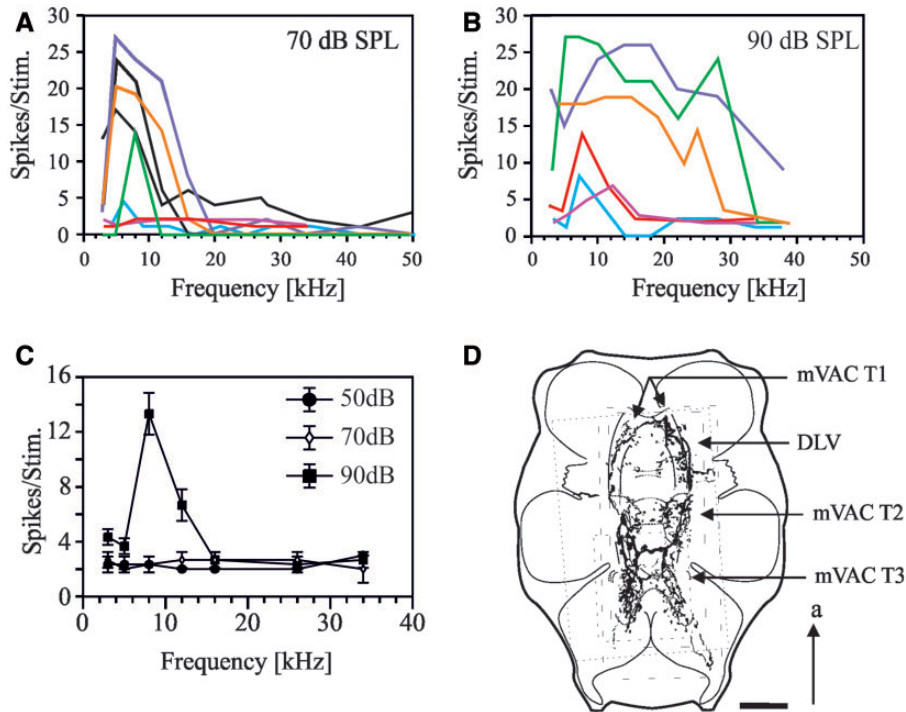
a massive change in direction-dependent resonance of the tympanum can be seen if the head is removed (RLH unpublished data A).

For the tachinid ear, it has been shown that the ear functions as mechanically coupled pressure receiver (Robert et al. 1998), and that the spiracle seems not to function as direction filter for sound waves, like in bush crickets and crickets (Römer 2014). In *E. auditrax*, the bilateral symmetrical spiracles can be closed by a valve-like structure, although the valve is not obvious from the CT scans. The spiracles seem not to have a function in auditory information processing, like enhancing directionality, because blocking of one spiracle does not alter the phonotaxis paths in *E. auditrax* (N.T. and R.L.H, unpublished observation B). This lack of function is somewhat surprising as sound waves might enter the tracheal space through the spiracles. Further studies will reveal the role of the spiracle in respiration and perhaps audition.

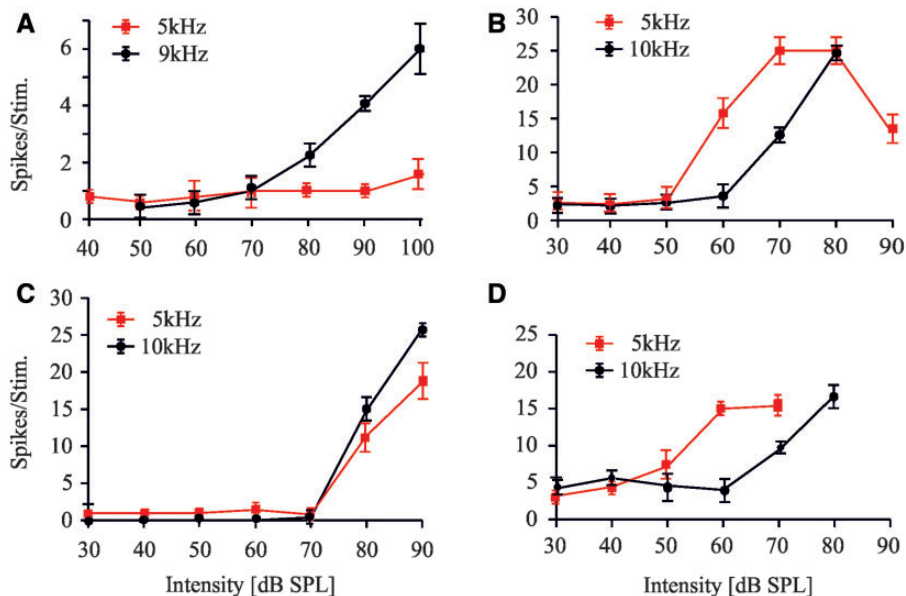
The tympanal membranes are backed by the undivided prosternal air sac, which originates from the anterior thoracic spiracles. This air sac supplies the frontoventral tissues in the prothorax and continues further into the cervical trachea, the frontal leg trachea, the sternopleural sac and the scutellar sac. Therefore, the auditory air chamber is continuous with the rest of the tracheal space. The prosternal air sac is also found in flies without a tympanal ear (Edgecomb et al. 1995, Lakes-Harlan et al. 1999) and interestingly the volume occupied of the air sac and the directly connected trachea (4.1 % of total thorax volume) is not bigger than in an unidentified species of nonhearing *Sarcophagidae* used in pilot micro-CT scans (5% of total thorax volume; N.T., unpublished data C). A prominent enlarged prosternal air sac is a characteristic of the tympanate tachinids, like *Th. leonidei* (Lakes-Harlan and Heller 1992), *O. ochracea* (Robert et al. 1994), and *H. alleni* (Lakes-Harlan et al. 2007). Nevertheless, not all Dipterans possess a prosternal air sac, in Acalyptratae, as *Eurostaso lidaginis* (Edgecomb et al. 1995), *Phormia regina* (Lakes-Harlan et al. 2007), and *Drosophila* (Demerec 1994), for example, the dorsoventral trachea are connected via a transversal trachea instead (Edgecomb et al. 1995).

The sensory organ extends in the frontal air sac, like in nonhearing Diptera. Nonhearing Diptera possess a prosternal chordotonal organ, the precursor organ to the hearing sense organ, which functions as a vibration sensor (Edgecomb et al. 1995, Lakes-Harlan et al. 1999, Lakes-Harlan et al. 2007, Stölting et al. 2007). The tsetse fly *G. morsitans* also possesses a prosternal membrane and is, therefore, considered as a possible intermediate form (Tuck et al. 2009). In number and arrangement, the sensory cells in *E. auditrax*





**Fig. 8.** Physiology and anatomy of auditory interneurons. (A, B) Iso-intensity response curves of eight different auditory neurons in eight different animals (same color refers to the same neuron; black, two curves recorded only for 70 dB SPL in two animals). The curves indicate narrow tuning at low sound pressure level and a broader tuning at higher sound pressure level. Each stimulus was presented only once. (C, D) Physiology and arborization in the TAG of an ascending interneuron. (C) Iso-intensity response curves. The neuron (also red in A, B) responded strongly only to stimuli with 9–12 kHz carrier frequency at a sound pressure level of 90 dB SPL (mean, SD, data from three measurements each). (D) Structure of the neuron as revealed from Lucifer Yellow stainings during the recording in the thoracic abdominal ganglion (overlay of three histological sections immunolabeled with anti-Lucifer Yellow). Note the dense arborizations on both sides of the TAG, especially in the median ventral association center (mVAC) of the respective thoracic neuromere; the precise soma position is unknown, but likely in the T3 neuromere; the ascending axon is not shown; DLV, dorsolateral tract that contains the main axon of the neuron; a, anterior; scale = 100  $\mu$ m.



**Fig. 9.** Intensity response curves of four auditory interneurons. Neurons have been tested with 5 kHz (best hearing frequency) and 9 or 10 kHz stimuli, respectively (peak range of the host calling song). The neuron reacts preferentially to 9 kHz (A) two neurons in which 5 kHz has a lower threshold than 10 kHz (B), (D) and a neuron with identical curves for both frequencies (C). Mean, SD, data from three measurements each.

resemble those in nonhearing flies (Lakes-Harlan et al. 1999). By contrast, hearing Tachinidae have much higher numbers of sensory units (Robert et al. 1996, Lakes-Harlan et al. 2007), and the sensory organ is attached to the ceiling of the prosternal air sac by a

veil-like, continuous thin membrane (Robert et al. 1994, Lakes-Harlan et al. 2007). Tachinids are more opportunistic in their host preference and are attracted by a range of cricket or bush cricket CSs (for review, Lehmann 2003), whereas *E. auditrix* usually is

attracted to just one species CS (Lakes-Harlan et al. 2000, de Vries and Lakes-Harlan 2005) and another unidentified *Emblemasoma* species (Farris et al. 2008). This behavioral difference could be reflected in the higher number of sensory units and a broader tuning, which is found in tachinids (Stumpner and Lakes-Harlan 1996, Oshinsky and Hoy 2002, Stumpner et al. 2007) may be supported by a more elaborate structure.

The connection of the air sac with the spiracles and the tracheal system of the body raise the question, how respiration might influence the hearing physiology. So far, we have no indications about the influences, but it has been shown in orthopterans, that respiration changes the auditory responses (Meyer and Elsner 1995, Meyer and Hedwig 1995).

### Physiology

Data on the physiology of the auditory system of parasitoid flies are limited. Most data are available from species of tachinids, in contrast to the group of Emblemasomatini. The peripheral auditory system of both taxa is clearly convergently evolved (Lakes-Harlan et al. 1999), including a similar central projection of the sensory cells. It remains to be shown whether the central auditory system of hearing flies also contains homologous elements. Here, we first discuss the peripheral hearing threshold, and second, data on the auditory interneurons of *E. auditrix*.

### Hearing Threshold

Previously, a hearing threshold of *E. auditrix* has been published in which the summated response from ascending interneurons has been evaluated (Lakes-Harlan et al. 1999). In the present study, we have been able to record from the primary sensory neurons. The threshold is in the same range as that from the interneurons although the minimum in the low frequency range is less pronounced. The seemingly less sensitivity of the afferents might be explained by difficulties in recording from thin receptor units. The recordings from the interneurons indicate that the afferent system is somewhat more sensitive than reflected in this threshold. Whether any of the about 35 sensory cells are differently tuned, like the afferents in *O. ochracea* (Oshinsky and Hoy 2002) remains to be shown.

The adaptive value of the 5 kHz minimum is not known because no respective signal has been found up to now (Lakes-Harlan et al. 2014). Other fly species have a matching of the hearing threshold curve to the host CS (Lakes-Harlan and Lehmann 2015). Interestingly, *E. auditrix* seems to be specifically adapted for one host species, *O. rimosa* (Lakes-Harlan et al. 2000). Other species of the genus seem to have a broader range of hosts (Farris et al. 2008) and therefore can be expected to be less specifically tuned. In respect to tuning, one has to keep in mind that the CS of many cicada species peak in about the same range. Hence, the species specificity is more likely to be coded in the temporal pattern of the CS as shown in corresponding behavioral experiments with *E. auditrix* (Lakes-Harlan et al. 2000, Köhler and Lakes-Harlan 2001). Nevertheless, at least some auditory interneurons are tuned to the frequency peak of the CS, indicating that afferent tuning might not be as important as tuning of higher neuronal networks.

### Auditory Interneurons

The recordings from auditory interneurons have been relatively difficult in comparison to those from tachinid flies. Therefore, only a few auditory interneurons could be characterized ( $n=10$ ) although anatomical identification is somewhat incomplete (even with

amplification of the tracer Lucifer Yellow by immunohistochemistry). Nevertheless, anatomical data allow to draw some conclusions of the auditory information processing in *E. auditrix*. Two ascending interneurons have been found with axonal projection in the ventrolateral protocerebrum (data not shown) and broad frequency tuning. At least one interneuron might represent a homologous neuron to the described AN1 in *Th. leonidei* (Stumpner and Lakes-Harlan 1996), but more data are needed. Other interneurons have distributed neuronal arborisations on both sides of the TAG and within the mVAC of all thoracic neuromeres. Such neurons could integrate the information from both ears and trigger respective behavioral responses. The bilateral dense arborizations might also indicate a processing of directional information, like in the omega neuron of crickets and bushcrickets (Rheinlaender and Römer 1980, Schildberger and Hörner 1988, Pollack 2000). These recorded neurons were tuned to the peak frequency of the CS in contrast to the previously mentioned ascending interneurons and might represent neurons directly involved in host detection and distance determination. This tuning of auditory interneurons suggests a filter mechanism in the neuronal network.

Several interneurons could be characterized by their response to different frequencies at fixed sound pressure levels (with or without anatomical identification). These interneurons mostly showed a narrower frequency tuning at lower intensity (70 dB SPL) than at higher intensity (90 dB SPL). So far no clear indication for a discrimination of narrow frequency bands has been found, perhaps except for the carrier frequency of the CS. Corresponding to the intensity dependency of the interneuronal response an intensity-dependent change in phonotactic behavior had been reported (Köhler and Lakes-Harlan 2001).

Taken the discussion about the importance of detection of temporal parameters, auditory interneurons need to be characterized for their response patterns. Our data indicate that the interneurons in the auditory system of *E. auditrix* have a variety of response patterns, from phasic to tonic response characteristics. Phasic (on and off), phasic-tonic, and tonic neurons also exist in *Th. leonidei* (Stumpner and Lakes-Harlan 1996) and in *H. alleni* (Stumpner et al. 2007). Such neurons may well transmit the temporal parameters of the hosts CSs in the brain for species recognition. It will be interesting although difficult to analyze the network in parasitoids with one or multiple hosts and to unravel their recognition mechanisms.

### Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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