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Evolution of directional hearing in moths via conversion of bat detection devices to asymmetric pressure gradient receivers

Andrew Reid^{a,1}, Thibaut Marin-Cudraz^{b,2}, James F.C. Windmill^a, Michael D. Greenfield^{b,1}

^a University of Strathclyde, Centre for Ultrasonic Engineering, Department of Electronic & Electrical Engineering, Glasgow G1 1XW, Scotland ^b Institut de recherche sur la biologie de l'insecte (IRBI), CNRS UMR 7261, Université François Rabelais de Tours, 37200 Tours, France

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Small animals typically localize sound sources by means of complex internal connections and baffles that effectively increase time or intensity differences between the 2 ears. But some miniature acoustic species achieve directional hearing without such devices, indicating that other mechanisms have evolved. Using 3D laser vibrometry to measure tympanum deflection, we show that female lesser waxmoths (*Achroia grisella*) can orient toward the 100-kHz male song because each ear functions independently as an asymmetric pressure gradient receiver that responds sharply to high-frequency sound arriving from an azimuth angle 30° contralateral to the animal's midline. We found that females presented with a song stimulus while running on a locomotion compensation sphere follow a trajectory 20° - 40° to the left or right of the stimulus heading but not directly toward it, movement consistent with the tympanum deflections and suggestive of a monaural mechanism of auditory tracking. Moreover, females losing their track typically regain it by auditory scanning – sudden, wide deviations in their heading – and females initially facing away from the stimulus quickly change their general heading toward it, orientation indicating superior ability to resolve the front-rear ambiguity in source location. X-ray CT scans of the moths did not reveal any internal coupling between the 2 ears, confirming for the first time that an acoustic insect can localize a sound source based solely on the distinct features of each ear.

acoustic communication | Lepidoptera | sound localization | ultrasound

Introduction

The localization of sound sources by small animals is a fundamental problem in bioacoustics (1). Where body size is diminutive and inter-ear distance is short, as is generally the case in acoustic insects and anurans, it is improbable that an animal can rely on inter-aural intensity difference (IID) or inter-aural time difference (ITD) mechanisms to resolve the azimuth angle towards the source (2). Some acoustic insects and anurans overcome their size constraints by means of multiple pathways along which sound arrives at both the external and internal surfaces of the tympanal membranes (3,4): because the separate pathways leading to the 2 surfaces of a tympanal membrane differ in length when the sound source is ipsilateral, a phase difference between the external and internal sound waves can arise for specific sound frequencies and yield vibration of the membrane. The inter-aural phase difference (IPD) between these external-internal phase differences, by generating a disparity in vibration between the 2 ears, may then serve as a reliable indicator of the azimuth angle toward the sound source. Curiously, certain small acoustic insects that do not possess the anatomical features permitting an IPD mechanism are nonetheless quite efficient at sound source localization. For example, in the tachinid fly *Ormia ochracea*, a parasitoid of singing male crickets, the 2 ears are separated by 500 μm and sound waves arrive only at the external surfaces of the tympanal membranes, but a specialized 'rocker-arm' apparatus connecting

the ears magnifies both the IID and ITD (5). These enhanced inter-aural differences, combined with pooling the responses of many individual receptor neurons, allow female *O. ochracea* to localize their hosts with a high degree of directional precision. Given the diversity of hearing organs that have evolved among insects (6,7), it is likely that yet other localization mechanisms exist. Thus, examining directional hearing in small acoustic species would be valuable in an evolutionary context, as well as in the development of bio-inspired technology for sound localization.

Whereas the majority of research on acoustic insects has focused on conspicuous singers among the Orthoptera and Cicadidae (8), and more recently on *Drosophila* spp. (9) owing to available genetic resources, most acoustic insects are to be found in the Lepidoptera. Approximately 55% of lepidopteran species have tympanal ears (10), hearing evolved independently between 10-12 times in the order (11), and sound signaling – using ultrasound frequencies – for mating communication is now known to occur in diverse species in several families, including those characterized by small body size (12). Because mates may need to be localized with the same precision as hosts, some degree of directional hearing is expected in moth species that communicate acoustically. Moreover, the apparent absence of inter-aural bridges and of tracheal anatomy conducive to IPD mechanisms in moths (13) strongly suggests that novel strategies for sound source localization operate in lepidopteran hearing.

Among acoustic Lepidoptera, the lesser waxmoth (*Achroia grisella*; Pyralidae: Galleriinae) was an ideal candidate for study-

Significance

In most acoustic animals directional hearing evolved alongside basic ear structure. Pyraloid moths differ because their ears generally function as simple bat detectors with little directional ability. Pyraloids that broadcast mating calls represent a yet more special case, as these species do localize sound but the ability evolved well after hearing and may be constrained by fundamental auditory features. Analyzing a species with male calling songs, we report a novel localization mechanism wherein the membrane structure of each ear affords sharp sensitivity to sound arriving from a distinct angle. Females can thereby track male calls but only via an indirect, curvilinear trajectory. Such inefficiency may characterize specialized perceptual traits that rely on general ones having already undergone extensive prior evolution.

Reserved for Publication Footnotes

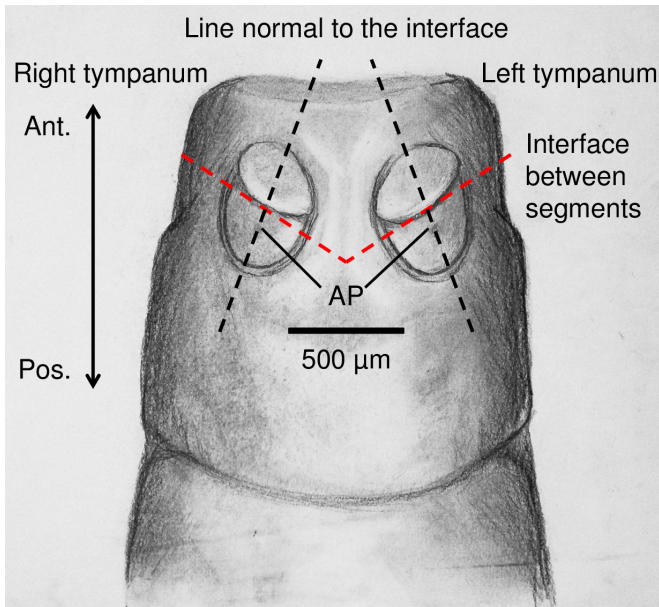


Fig. 1. Schematic diagram of ventral side of the first abdominal segment of *A. grisella* showing the general shape and position of the 2 tympana. Each tympanum is divided into a relatively thick anterior segment, the conjunctivum or counter-tympanum (55,56), and a membranous posterior segment where the peripheral neurons, collectively forming the scoloparium, are attached (AP). The interface between the segments is highlighted by a dashed red line, and the line passing through the attachment point and that is normal to the inter-segmental interface is shown. The average distance between the left and right attachment points in a female is approx. 600 μm , which would yield an interaural time difference (ITD) less than 1.75 μs for sound arriving from a lateral source. We estimated interaural intensity difference (IID) between the 2 tympana using the calculations for scattering of sound in air by a cylindrical object (57). We assume that the moth's abdomen is a cylinder of radius 0.6 mm and that the angular separation of the tympana is $\approx 30^\circ$ along the abdomen circumference. For a biologically relevant sound, male song whose dominant frequency ≈ 100 kHz, arriving from a lateral source, we calculate an SPL differential of 3.1 dB between the superposition of incident + scattered sound at the left and right tympana (Supporting Information S1). The actual differential is expected to be considerably less than 3.1 dB, as the ventral surface of the abdomen in the region of the tympana is flattened, thereby reducing the angular separation between axes normal to the body surface at the 2 locations.

ing alternative means by which small animals might achieve directional hearing (14). Adult body lengths of males and females average 8.5 and 11.5 mm, respectively, and the 2 tympana, situated ventrally on the 1st abdominal segment, face in nearly the same direction and are separated by $< 600 \mu\text{m}$ (Figure 1) (15). Thus, even when sound is of very high frequency and arrives from a lateral source, a standard IID mechanism would fail to generate a meaningful differential between the 2 ears (Figure 1; Supporting Information S1). Likewise, the ITD would be 1.75 μs , less than stochastic variation in neural transmission. Despite these anatomically imposed constraints, *A. grisella* display a level of directional hearing that affords the localization of a distant sound source. Male *A. grisella* remain stationary on the substrate and, via wing-fanning, produce a train of damped pulses of ultrasound (70-130 kHz; 80-100 pulses $\cdot\text{s}^{-1}$; pulse length 100-150 μs) that are attractive to females up to 1 m away (16). Receptive females will run in the general direction toward a singing male or a speaker broadcasting a synthetic song stimulus, and the many behavioral studies conducted on *A. grisella* showed that such localization is often accomplished within 20-30 s (17). Moreover, females experience little difficulty in turning to localize the sound source regardless of their initial orientation, implying that their directional hearing incorporates a mechanism for overcoming the

problem of front-rear ambiguity. *A. grisella* in flight respond to bat echolocation signals by dropping vertically, and when running on the substrate their response is a sudden cessation of movement (18).

As in other Pyralidae, each tympanum in *A. grisella* is divided into a thick anterior and a thin posterior segment (Figure 1) (19,20). Vibration of the 2 segments in response to sound is complex, and deflections are greater in the posterior segment. We hypothesized that directional hearing in this small moth relies on fine-scale aspects of vibration in the segmented tympana, and we employed 4 approaches to specify the ability to localize a sound source and to discern how features of tympanal vibration afford this ability. We began by analyzing the orientation of female *A. grisella* to a synthetic male song stimulus while moving on a locomotion compensation sphere. We then used 3-dimensional laser Doppler vibrometry to measure deflections of the tympanal membranes in response to sound broadcast from different azimuth angles. Via X-ray computer aided tomography (CT) scanning we searched for potential morphological connections or baffles between the left and right ears. Finally, we used the CT scans to construct a finite element model of the sound field around the moth's body, and we modelled this field for sound sources located at different azimuth angles.

Results and Discussion

74% of *A. grisella* females tested on the locomotion compensation sphere ran toward the virtual source of the synthetic song stimulus for at least 15 s (e.g. Fig. 2.a). Absolute angular deviations of travel headings with respect to the stimulus, whose heading was defined as 0° , taken over the previous 1 s of movement ranged from 21° - 38° for the initial 10 s of phonotaxis (average values of 17 females taken at 1-s intervals beginning at 1 s). Travel headings were evenly divided between the left and right sides of the 0° heading (sign test, $p > 0.25$) and had standard deviations ranging from 15° - 25° . Whereas the highest absolute angular deviation (38°) occurred at 1 s following the onset of phonotaxis, deviations did not decline significantly over the following 9 s (repeated measures ANOVA, $p = 0.07$; for 1-s intervals beginning at 2 s, $p = 0.30$). At 15 s, the absolute angular deviation was still $27^\circ \pm 24^\circ$. 6 moths began their movement with an 'instantaneous heading', measurement taken over the last 100 ms, $> 90^\circ$ with respect to the stimulus. But in 5 of these 6 insects the travel heading measured over the previous 1 s was already $< 45^\circ$ at 1 s. Running speed remained high throughout the trials ($5.7 \pm 1.6 \text{ cm}\cdot\text{s}^{-1}$, mean \pm sd), equivalent to 5-6 body lengths $\cdot\text{s}^{-1}$ as measured along the trajectory. 90% of the females tested made at least one circling movement of small radius ($< 3 \text{ cm}$), as well as angular deviations exceeding 90° , during their trials.

Females tested with a song stimulus that included a 3-s silent pause beginning at 8 s usually made angular deviations wider than 90° , and even circled completely, at the beginning of the silent pause, movement that had been observed less frequently during the last 3 s of the stimulus prior to the pause (sign test, $p < 0.01$; Fig. 2.b). By the end of the pause many females had ceased movement (13 of 25 insects), but they usually began reorienting and moving toward the stimulus when the sound continued and again made wide angular deviations during the initial 3 s, activity performed less frequently during the succeeding 3-s interval (sign test, $p < 0.01$). Females tested with a song stimulus broadcast from 2 sound sources, the first 8 s from a speaker at 0° and the second 8 s from a speaker at 135° , mostly turned clockwise (16 of 21 females), as opposed to counter-clockwise, during the initial 2 s of the broadcast from the second speaker and reoriented and moved toward it (Fig. 2.c). These reorientations were generally gradual and finished with a travel heading within 45° of the heading of the second speaker.

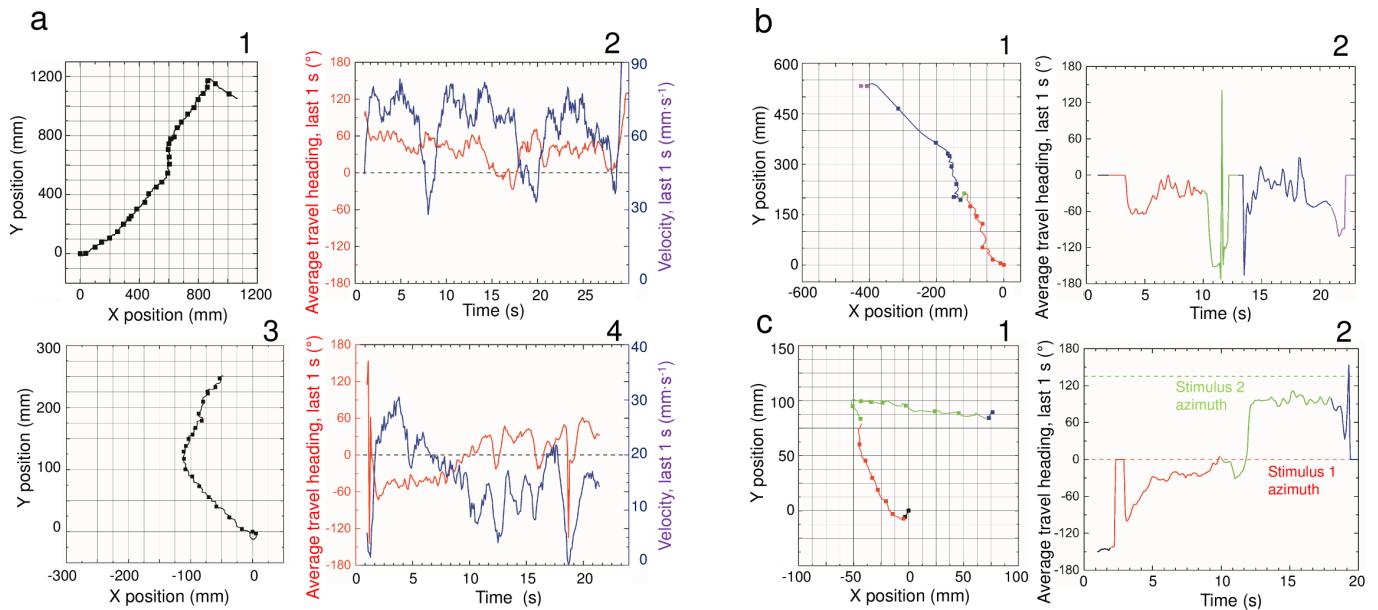


Fig. 2. a) Trajectories of 2 representative *A. grisella* females tested for orientation to a 30-s male song stimulus while running on a locomotion compensation sphere. Panels 1 and 3 show the females' x,y coordinates in a virtual plane over the course of their trials. The starting point is 0,0 and the sound source is located along the vector normal to the x-axis. Symbols along each of the trajectories indicate the female's position at successive 1-s intervals. Panels 2 and 4 show the females' average headings relative to the sound source over the preceding 1-s interval as measured every 100 ms (red line) and their velocities over that preceding 1-s interval (blue line). The female depicted in panels 1 and 2 retains a heading from 30° to 50° throughout her trial, whereas the female depicted in panels 3 and 4 follows a heading approximately +45° for the first 10 s and then switches to a heading approximately -30°. b) Trajectory of a representative female tested for orientation to a 19-s stimulus consisting of 8 s of song, 3 s of silence, and 8 s of song. Panel 1 shows the trajectory and panel 2 shows the average heading over the preceding 1 s, as in a) above. The female follows a heading approximately -30° during the first 8 s of song, circles during the 3-s silent pause and stops, and then resumes a heading from -20° to -40° during the second 8 s of song. c) Trajectory of a representative female tested for orientation to a 16-s stimulus consisting of 8 s of song from a speaker at 0° followed by 8 s of song from a speaker at 135°. Panel 1 shows the trajectory and panel 2 shows the average heading over the preceding 1-s interval, as in a) above; the average heading is shown relative to 0° for both parts of the trial. The female follows a heading approximately -30° with respect to the first speaker and then turns clockwise and follows a heading approximately 100°, equivalent to -35° with respect to the second speaker.

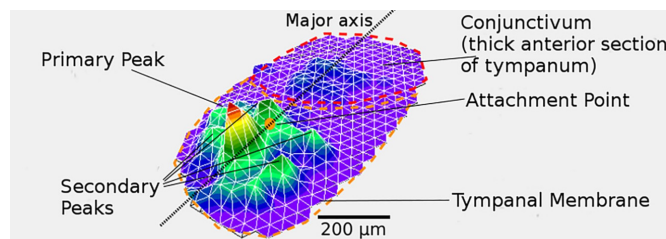


Fig. 3. Laser vibrometry measurement of deflection magnitude measured at 390 points in a representative tympanum (the tympanum presented is the insect's right); see text in Laser Vibrometry section of Methods for determination of deflection. The deflections are characterized by a sharp, primary peak and several smaller secondary peaks nearby. The sharp primary peak was found in the center of the posterior, membranous segment of the tympanum (region surrounded by dashed orange line). When the sound source was located along the major axis of the tympanum, the primary peak coincided with the neural attachment point (see Figure 1), but the peak moved slightly when sound arrived from other directions.

3-dimensional laser vibrometry testing demonstrated that *A. grisella* can localize high frequency sound because their segmented tympana are not oriented in parallel and each functions as a distinct pressure-gradient receiver. Measurements of the response of a tympanum to broadcasts of 100 kHz sound showed that the maximum deflection occurred at or close to the location in the center of the thin posterior segment where the peripheral neurons are attached (Figure 3). Membrane deflection at this attachment point is expected to influence the probability that an action potential(s) is transmitted by the peripheral neurons, and we measured the maximum deflection there for sound broadcast

at all azimuth angles. Responses to these broadcasts revealed a primary deflection peak in the right tympanum for a sound source located 30° to the left of the insect's midline (azimuth = -30°) as well as a secondary deflection peak for sound located at 150° azimuth. Similarly, the tests of the left tympanum revealed a primary deflection peak at +30° azimuth. The primary peak averaged 1.6 x the deflection magnitude of the secondary peak, and 2.8 x the deflection magnitude for all other speaker angles tested (Figure 4.a). Significantly, a comparable study of tympanum responses in a closely related species, *Galleria mellonella* (greater waxmoth; Pyralidae: Galleriinae), that perceives high frequency sound but has neither a male calling song nor female phonotaxis (12) did not reveal pronounced deflection peaks for any azimuth (Figure 4.b).

At 100 kHz the wavelength of sound in air is 3.4 mm, less than 6 x the average dimension of an *A. grisella* tympanum. Consequently, a pressure gradient will occur between 2 points on the tympanum when sound waves arrive from a source situated along the line through those points (21). The gradient increases as the 2 points are separated more widely, and maximum gradients will thus occur for sound arriving along the major axis of the tympanum, which is roughly aligned with the longitudinal axis of the body (Fig. 1). Because the neural attachment point is situated along or close to the major axis and higher pressure gradients will result in greater membrane deflections, sound arriving along the major axis will elicit maximum deflection of the attachment point. It is likely that this general feature of tympanum geometry is further modified and enhanced by material properties of the membrane that yield greater deflection for sound arriving from a distinct azimuth at each ear. For example, when sound arrives at the right (left) tympanum from -30° (+30°), wavefronts will be parallel to the boundary between the thick anterior and thin

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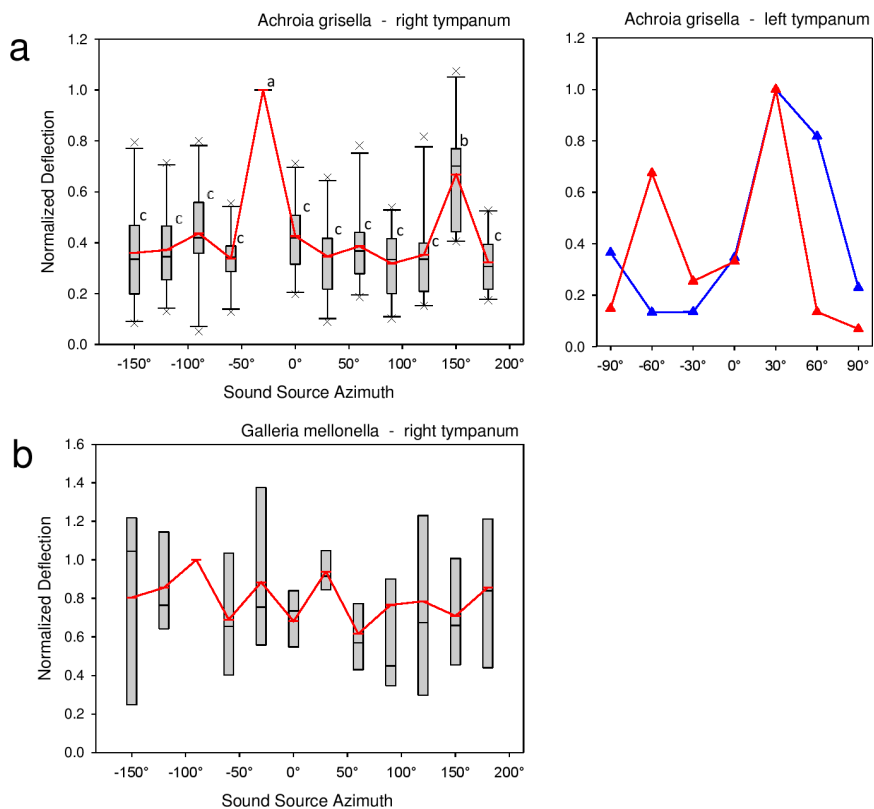


Fig. 4. a) Left panel: Normalized deflection magnitude at the attachment point in the right tympanum of 10 *Achroia grisella* females for sound arriving from different azimuth angles. Angles are measured clockwise around the vector normal to the dorsal surface of the moth, with the body midline defined as 0°. Absolute deflection magnitudes were highest at an azimuth = -30° in 9 of 10 females; the remaining female's maximum deflection occurred at +150°. We therefore normalized each female's deflection magnitudes by setting her value at -30° to 1.0 and adjusting her other values accordingly. Box plots show median (black), mean (red), 25%-75% range (box), 10%-90% range (whiskers), and outliers for each azimuth angle. Azimuths not sharing a letter marking the box are significantly different; Repeated measures ANOVA (SigmaStat 3.5) with Holm-Sidak post-hoc test for pairwise differences, $p < 0.001$. Right panel: Normalized deflection magnitude in the left tympanum of 2 *A. grisella* females (red and blue plots). Absolute deflection magnitudes were highest at +30° in both females, and we therefore set their values at this azimuth to 1.0. b) Normalized deflection magnitude at the attachment point in the right tympanum of 8 *Galleria mellonella* females. The highest deflection magnitude averaged across all 8 females occurred at -90°, and we therefore set each female's value at this azimuth to 1.0. Normalized deflections do not differ between azimuths; Repeated measures ANOVA, $p = 0.625$.

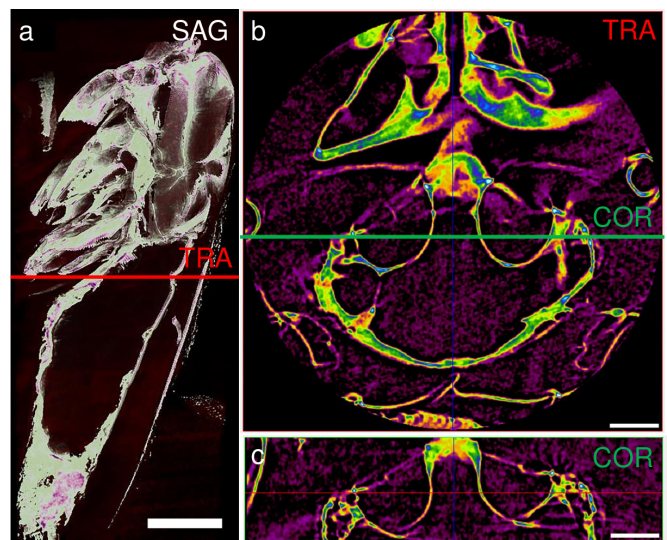


Fig. 5. X-Ray CT scans of a female moth, focusing on the region between the 2 tympana. a) Sagittal scan of entire body. TRA indicates transverse plane through the tympana on the first abdominal segment; scale bar is 1 mm. b) Transverse scan of plane indicated by TRA in scan a. Yellow indicates a sclerotized region; tympanal cavities are clearly shown (TY) and no internal sclerotized connection between the cavities is evident. COR indicates coronal plane through the tympana; scale bar is 200 μm . c) Coronal scan of plane indicated by COR in scan b. Again, no internal sclerotized connection is evident; scale bar is 200 μm .

posterior segments (Fig. 1) and may thereby generate maximum excitation at the attachment point, in the center of the posterior segment.

The trajectories of females on the sphere, which are centered around +30° and -30° with respect to the synthetic song stimulus, are congruent with our laser vibrometry measurements and description of the tympana as 2 distinct pressure gradient receivers. Interestingly, our data are not consistent with the standard model of tracking binaural cues (22,23) in which an animal attends to information received at both ears and turns toward the one more strongly stimulated. *A. grisella* females do not regularly zig-zag to the left and right of a 0° heading, and while following a heading of +30° (or -30°) they do not generally veer farther to the right (or to the left), as would be predicted by the standard model. Whereas females quickly orient in the general direction of the song stimulus during the initial 1 s of a trial, they do not continue to improve their alignment toward a 0° heading afterward. Rather, *A. grisella* seem to 'lock onto' the stimulus with either the left or the right ear, and then run forward while pursuing a heading of approximately +30° or -30°, respectively, and ignoring the other ear. The moths do waver from a precise heading, of course, which may have several consequences. Small departures from +30° or -30° might be corrected by random turning or a sequential comparison algorithm, a mechanism that *A. grisella* are known to possess (24). Larger departures can result in locking onto the stimulus with the other ear (e.g. Fig. 2.a) or in losing the stimulus entirely and then regaining it via wide deviations or circling, a form of auditory scanning (e.g. Fig. 2.b). Tracking of monaural cues – attending only to information received at a single ear – may function in *A. grisella* because the primary deflection peak is sharp (Fig. 4a) and likely evokes a relatively high excitation level in comparison with excitation at other headings, as would be experienced by the opposite ear. Moreover, the moths have 2 different opportunities to lock onto the stimulus, one with the left ear and one with the right, and each would yield phonotaxis toward the sound source (Fig. 2.a). Our model of monaural tracking predicts that off of the sphere a female would follow a curved trajectory in approaching

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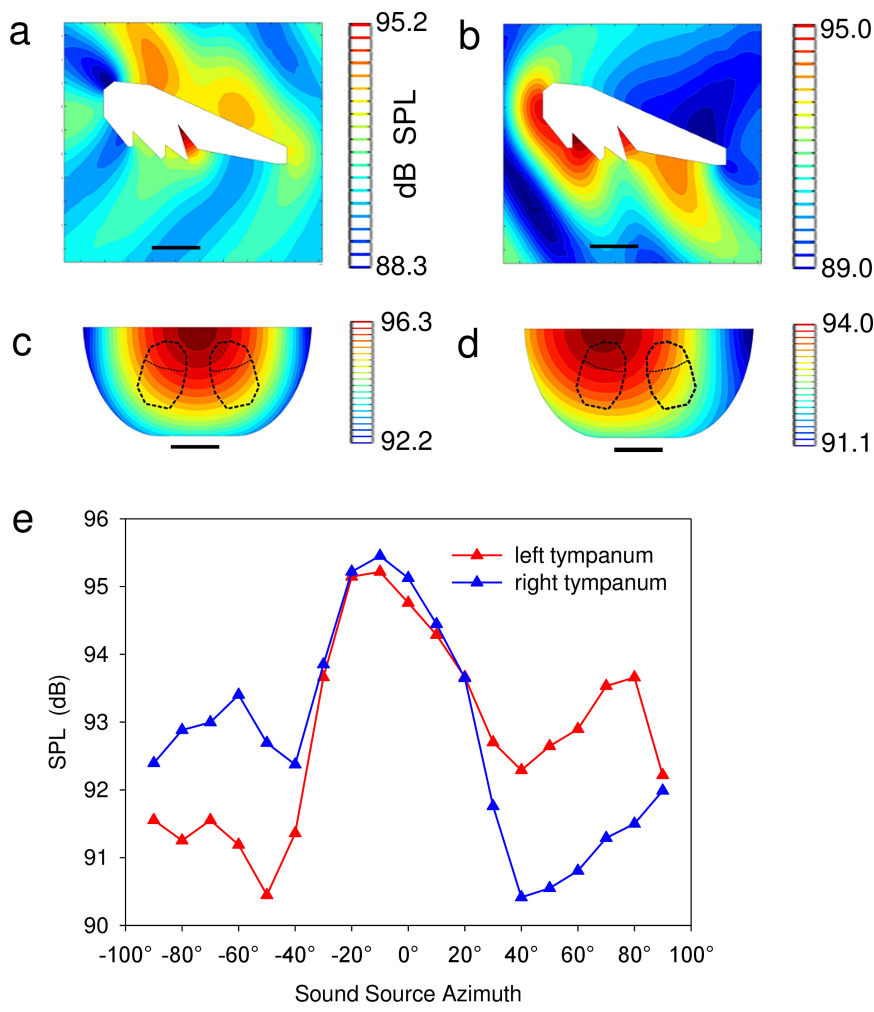


Fig. 6. Sound pressure level (SPL) around a simple model of a female moth as simulated by COMSOL MultiPhysics. a) Sagittal section, with anterior to the left, showing predicted SPLs for a 94 dB (measured at air domain boundary 10 cm from the insect) sound source situated directly in front of the moth. SPLs are 6-7 dB higher in the cleft between the abdomen and thorax, the location of the 2 tympana; scale bar is 1 mm. b) Predicted SPLs for the same sound source located directly behind the moth. The SPL in the cleft between the abdomen and the thorax is now 2-3 dB lower than when the sound source was in front of the insect; scale bar is 1 mm. c) Predicted SPLs across the ventral surface of the 1st abdominal segment for a sound source directly in front of the insect; approximate locations of the tympana are indicated by dashed lines; anterior is at the top. SPL is equivalent across both tympana and reaches a maximum of 96.3 dB; scale bar is 500 μ m. d) Predicted SPLs for a sound source at -30° azimuth with respect to the dorsal view of the insect (+30° in the ventral view shown). SPL is notably higher across the contralateral tympanum (ranging from 93.9-94.8 dB) than across the ipsilateral one (ranging from 93.9-92.1 dB); scale bar is 500 μ m. e) Predicted SPLs at the neural attachment points in the left tympanum (red) and the right tympanum (blue) for a sound source situated at azimuths ranging from -90° to +90°.

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a male, as a +30° (-30°) heading with respect to the male would be represented by an absolute heading that shifts continuously toward the left (right). In fact, curved trajectories are regularly observed in females approaching males in a laboratory arena (17).

Our X-ray CT scanning eliminated the possibility that some of the moth's localization of a sound source could be attributed to an IPD device or a rocker-arm bridging the 2 ears. These scans revealed no sign that a trachea leads to the air cavity behind the tympanal membrane, allowing sound waves to arrive at its interior surface. Similarly, they revealed no internal sclerotized connections between the two ears that could magnify the IID or ITD (Figure 5). Moreover, laser vibrometry measurements showed that tympanum deflections were equivalent for sound arriving at -90° and +90° (Fig. 4a), implying that simple cross-body transmission of sound was not generating an informative IID. The equivalence of deflections generated by sound sources at -90° and +90° also clearly refutes the possibility that the moth's body scatters high frequency sound arriving laterally and thereby affords a meaningful IID for localization (cf. Figure 1; Supporting Information S1).

The rapidity with which *A. grisella* females found the general direction of a playback stimulus at its onset demonstrates reliable resolution of the front-rear ambiguity in directional hearing (25). Unlike phonotaxis toward the stimulus, these rotational movements do appear to be initiated by comparison of information received at the 2 ears: At the end of the first part of the 2-speaker trials most females faced in the general direction of the

sound stimulus at 0° (e.g. Fig. 2.c). Consequently, their right ears were normally more closely aligned than their left ears with the sound stimulus at 135°, which was broadcast from behind them in the second part of the trial. Thus, their right ears would have been more strongly stimulated in the second part, and binaural comparison would have led them to rotate toward the right and eventually face in the general direction of the 135° speaker. Our data largely agree with this prediction, as most females turned clockwise (toward the right) to reorient, and the 5 females who turned counter-clockwise had all been strongly oriented toward the left of the 0° speaker. We suggest that binaural comparison operated in this context because the secondary deflection peak at -150° or +150°, being much less pronounced than the primary deflection peak (Fig. 4a), did not invoke a high enough level of relative excitation to release the monaural tracking process. The difference between the primary and secondary peaks may arise because the *A. grisella* tympanum itself acts as an asymmetric rocker-arm device that amplifies deflections at the neural attachment point in the center of the posterior segment when sound waves arrive along the major axis from the front but much less so when sound arrives from the rear.

Finite element analysis of the sound field around the moth indicated a localized elevation in sound pressure level in the immediate vicinity of the tympana when sound waves arrive from a source within 30° of the moth's midline. The region is approximately 1 mm in diameter and features sound pressure levels 6-7 dB above the rest of the sound field (Figure 6). It forms by

681 diffraction in the ventral cleft between the thorax and abdomen,
682 and it may increase general auditory sensitivity in *A. grisella*.
683 Because the pressure in the region of the contralateral tympanum
684 is 1-2 dB higher than on the ipsilateral side when sound arrives
685 from 30° to one side of the midline, the diffraction effect may
686 also improve directional hearing beyond the level afforded by the
687 distinct geometry and orientations of the tympana. At the same
688 time, finite element analysis also demonstrates that diffraction
689 cannot be the main factor responsible for directional hearing and
690 generating tympanum deflection peaks for sound at azimuths of
691 -30° and +30°: Our analysis predicts very similar sound pressure
692 levels at the neural attachment point in a tympanum for sound
693 sources at azimuths of -90° and +90° (Figure 6.e), and the small
694 differences (≈ 2 dB) seen between the contralateral and ipsilateral
695 tympana for azimuths from 30° to 80° and from -30° to -80° would
696 not account for the magnitude (≈ 9 dB) and specific position
697 (azimuth $\approx 30^\circ$) of the primary deflection peak (Figure 4.a).

698 *A. grisella* obviously localize sound sources, but the means
699 by which they do so is less direct than in other acoustic insects
700 (26,27). Their putative monaural tracking is suggestive of a line-
701 following robot (28,29), albeit a rather inefficient one. They
702 follow a curvilinear path toward a sound stimulus, their trajectory
703 is often interrupted by lateral excursions in order to regain a
704 signal, and any prompt arrival at the stimulus or a live male is
705 largely due to their running speed and maneuverability. Given
706 the importance of efficient mate finding, one must question why
707 this protocol persists. A likely answer may be found in studying
708 the timeline of evolution of acoustic communication in the Lep-
709 idoptera (30). All but one of the 10-12 independent origins of
710 hearing in the Lepidoptera occurred since 65 MA before present,
711 the currently accepted date for the appearance of echolocation
712 in bats (12,31). This timing, taken in conjunction with ecological
713 patterns, has led to the inference that tympanal organs and
714 hearing ability in Lepidoptera evolved as a response to hunt-
715 ing by insectivorous bats: In 3 major moth clades, Noctuoidea,
716 Geometridae, and Pyraloidea, each including $> 20,000$ species,
717 hearing is ubiquitous except in species found in geographical
718 regions lacking bats or that are active during seasons or times of
719 day when bats are not (32). In these latter moth species tympanal
720 organs and hearing ability are reduced or absent, presumably as
721 a secondary loss (33). The hearing that had evolved in moths is
722 simple but highly effective for avoiding and escaping predatory
723 bats. Moths have 1-4 neurons per tympanum and minimum tuning
724 over a broad frequency range which extends well above 100 kHz
725 in some pyraloids (34,35). While negative phonotaxis away from
726 bat echolocation signals does occur in large-bodied noctuoids
727 (36-38) and geometrids (39), whose relatively sensitive ears are
728 situated laterally and afford an IID as high as 15 dB (40,41),
729 directional responses as such may be negligible or absent in the
730 pyraloids (18). This latter group, being generally smaller, are not
731 superior fliers and have relatively high hearing thresholds (42,43),
732 features that would render the ability to localize a bat relatively
733 unimportant. Unlike noctuoids (44), a pyraloid moth may not
734 detect a bat until it is close, and any defensive flight maneuvers
735 might then be too slow to exploit information on the location of
736 the bat. Consequently, just diving toward the ground if in flight
737 and becoming immobile – and silent – if running on the substrate
738 (45-47) appear to be their main defenses that rely on hearing.

740 In contrast to hearing, acoustic communication in moths is
741 rare and only occurs among isolated species and genera in the 3
742 major clades, a pattern that reflects an origin of sound signalling
743 via a sensory bias mechanism (45,48). In many cases the acoustic
744 communication is restricted to close-range courtship (49) wherein
745 directional hearing would not be critical, but in species such as
746 *A. grisella* that transmit sound signals over longer distances the
747 evolution of accurate directional ability would be expected. Con-
748 ceivably, this development could have been achieved via evolution

of an entirely new, highly efficient mechanism; e.g., the rocker-
arm device in parasitoid *Ormia* flies. But unlike *Ormia*, whose
acoustic perception of host crickets had probably evolved de
novo, *Achroia* already had an ancient system for perceiving sound.
Thus, it apparently followed a different evolutionary trajectory
and refitted old equipment – a very basic bat detector – for a new
task, localizing a mate.

Despite the overall similarity between *Achroia* ears and those
of related moth species wherein the ears still serve primarily as
bat detectors, some aspects of the directional hearing mechanism
in *A. grisella* may be highly derived characters. The differential
deflection of the neural attachment point in response to sound
sources at different azimuth angles around the tympanum, partic-
ularly for sources in front vs. behind, depends on specific
vibration properties of the 2 segments of the tympanal membrane.
That such differential deflection is not observed in the closely
related species *G. mellonella* (Fig 4.b) reinforces the notion that
the directional hearing in *A. grisella* is a derived trait which
coevolved with its long-distance acoustic communication. Even
so, the special vibration modes and deflections of its tympana
must operate within the confines of the basic pyraloid moth ear,
whose simplicity can be a constraint. For example, the primary
deflection peak of tympanal vibration is rather sharp, exhibiting
changes in magnitude of 0.4 dB / ° azimuth, and one might expect
the females to use this information for accurately following a $\pm 30^\circ$
heading toward the song stimulus (27). But with only 3 peripheral
neurons per tympanum (15,50), *A. grisella* would not be able to
benefit from pooling the responses of a population of neurons,
and its heading precision remains limited (Fig. 2) as a result of
intrinsic transmission variation within individual neurons.

Our findings are consistent with the general premise that
when specific trait B depends on the existence of general trait A,
and A is already present and functional, the evolution of B may
be constrained to forms that are less than optimal with respect
to what is known to be possible. That is, peaks and valleys in
the adaptive landscape may restrain evolutionary trajectories to
certain routes (51,52), thereby ruling out various possibilities for
some species. Further study of communication in diverse acoustic
species should reveal the extent to which this premise may be
applicable to directional ability and basic hearing.

Materials and Methods

Moths and Phonotaxis

A. grisella used in the experiments came from a laboratory colony
established from moths collected in the vicinity of honeybee colonies in
Florida in 2003. We reared the moths on a standard diet (16) and kept them in
an environmental chamber maintained at 25° and a 12:12 L:D photoperiod.
Because *A. grisella* females normally become unreceptive following a single
mating, we only used virgin females in our tests. All females were between
1-3 days old at the time of their tests, which were conducted during the first
4 h of the photoperiodic night.

We measured sound localization by analyzing phonotactic responses of
females to a male song stimulus while running on a locomotion compensa-
tion sphere (Syntech LC300). The stimulus was a song recorded from a single
male individual in the laboratory colony who exhibited acoustic features that
were average for the population. We broadcast the stimulus from a high-
frequency speaker (Avisoft Scanspeak, driven by an Avisoft Ultrasound Gate
Player 216M digital: analogue converter/amplifier) attached to a moveable
arm. This apparatus allowed us to position the speaker at the same elevation
as the test female situated on the top of the sphere while orienting it directly
at her but from a variable location behind the sphere. In each trial the
stimulus was adjusted to 80 dB SPL (peak measurement; 0 dB = 20 μ Pa) at
the female's location, 15 cm distant from the speaker (cf. ref. 16). Stimulus
amplitude was re-calibrated at the beginning of each test day.

The operation of the locomotion compensation sphere retained the test
female on the top of the sphere throughout the trial. To ensure that she
remained at this location and did not fly off the sphere, we removed the
distal 2/3 of her wings on the day prior to testing. Females were immobilized
by brief cooling before wing removal. We did not observe any adverse effects
of wing removal on the female's running or phonotaxis, which was similar
to that seen in laboratory arenas (17) and in their natural milieu adjacent to
honeybee colonies (53).

We tested 23 females in 30-s trials that analyzed responses to the song
stimulus broadcast from a location at 0° azimuth, directly behind the frame

817 holding the sphere. In each trial we obtained the female's trajectory as
 818 reconstructed with Syntech TrackSphere software from the series of her x,y
 819 coordinates in virtual space sampled at 100-ms intervals. We used these data
 820 to determine 1) a running average of the insect's azimuth heading relative to
 821 the stimulus taken over the previous 1 s, 2) the running velocity, taken over
 822 the previous 1 s, along the trajectory, and 3) the initial heading at the start
 823 of the trial. In a second locomotion experiment we tested 26 females in 19-s
 824 trials that comprised 8 s of the song stimulus, 3 s of silence, and a second 8
 825 s of song. And in a third locomotion experiment using 2 speakers, we tested
 826 another 26 females in 16-s trials in which the song stimulus was broadcast
 827 from a speaker at 0° for the initial 8 s and then from a speaker at 135° for
 828 the next 8 s.

827 Laser Vibrometry

828 We used laser vibrometry to measure *in vivo* tympanum deflections
 829 in response to high frequency sound. Female *A. grisella* were immobilized
 830 by cooling and then mounted, ventral side up, on a block of resin and
 831 beeswax. The moth's legs were removed and the thorax was lifted so that
 832 the (abdominal) tympana were fully exposed. We fixed the mounting block
 833 with the moth on a stable, rotating stage beneath a scanning 3-dimensional
 834 laser vibrometer (Polytec PSV MSA100-3D) and then precisely positioned
 835 the mounting such that the vibrometer focused on one of the moth's
 836 tympana. The results presented here represent the out of plane motion of
 837 the membrane only. A 100 kHz (pure tone sine wave) sound stimulus was
 838 generated from an Agilent 33220A 20 MHz Function Waveform Generator,
 839 passed through a Brandenburg 475R Photomultiplier Power Supply, and
 840 broadcast from a custom-built wide band air-coupled electrostatic transducer
 841 (54) situated 30 cm to the side of the moth. Previous studies demonstrated
 842 that *A. grisella* females move toward pure tone pulses generated from a wide
 843 range of ultrasound frequencies (14) and that pulsed or continuous pure tone
 844 sound at these frequencies elicits a certain level of tympanum response; this
 845 response only varies ± 2 dB for stimuli of a fixed intensity ranging from 80
 846 kHz to 100 kHz and ± 6 dB for 40 kHz to 100 kHz (20). We chose 100 kHz
 847 for our sound stimulus because it is the dominant frequency in male song
 848 among the *A. grisella* population tested, and it is also the frequency that
 849 elicits maximum tympanum responses (20). The stimulus was calibrated to
 850 86 dB SPL with the aid of a precision microphone (Bruel and Kjaer 4138)
 851 placed 1 cm behind the moth and oriented directly toward the broadcasting
 852 transducer. This stimulus and its orientation were designed to represent a
 853 nearby male's song as perceived by a female moving on a horizontal surface.
 854 We tested the right tympanum in each of 10 females with 12 broadcasts
 855 of the stimulus presented in 30° azimuth increments from -150° to +180°
 856 relative to the moth's midline. After each broadcast we rotated the stage
 857 with the mounting block and moth to the next azimuth, confirmed the
 858 position of the transducer relative to the moth, identified the midline of the
 859 moth using the crosshairs in the microscope of the vibrometer, and made
 860 final adjustments to the rotating stage relative to the midline. We used the
 861 microscope to identify the exact position of the neuronal attachment point
 862 in the tympanum for each azimuth increment.

863 Deflections were measured as the vertical distance between the highest
 864 and lowest displacement of a given point on the tympanum while the sound
 865 stimulus was broadcast. This procedure was conducted for 390 points on
 866 the tympanum, each point being measured 25 times. Data analysis was
 867 performed by Polytec v9.2 software, which sampled the vibrometer signals
 868 at 512 kHz with a sample time of 128 ms. An FFT was performed with
 869 a rectangular window and 25600 FFT lines giving a frequency resolution
 870 of 7.8125 Hz. Although our sound stimulus was a pure tone, an FFT was
 871 advantageous as it allowed us to use the vibrometer software's automatic
 872 generation of the linear frequency spectrum and corresponding coherence
 873 and cross-correlation figures. The complex average of the velocity, \bar{v} , was
 874 calculated as the average of the real (*Re*) and imaginary (*Im*) components
 875 (representing the magnitude and the phase of oscillation, respectively) of
 876 each measurement, V_n :

$$877 \bar{v} = \frac{1}{N} \left(\sum_{n=1}^N \text{Re}(V_n) + i \sum_{n=1}^N \text{Im}(V_n) \right)$$

878 The velocity was then converted into a displacement in post-processing.
 879 We focused specifically on measuring deflection at the point where the
 880 peripheral neurons are attached to the tympanal membrane.

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881 We tested the left tympanum in 2 additional moths with 7 broadcasts at
 882 azimuths from -90° to +90° and thereby compared the stimulus orientation
 883 angle generating maximum deflection in the left and right tympana. All
 884 12 moths remained alive throughout the series of measurements, which
 885 typically lasted 3 h. However, many moths would not survive longer testing,
 886 and this constraint precluded reliable measurements of the left and right
 887 tympana in the same individual. For each moth measurements began at 0°
 888 azimuth and were incremented clockwise. Thus, the stimulus eliciting the
 889 primary deflection peak (Figure 4) was the last one tested in measurements
 890 of the right ear but the second one tested in measurements of the left ear.

891 We obtained *G. mellonella* larvae from Blades Biological Supplies, Eden-
 892 bridge, U.K. Rearing was similar to that for *A. grisella*, and we followed
 893 the same laser vibrometry protocol to measure tympanum deflections in the
 894 right tympanum of 8 females except that the sound stimulus was 60 kHz, the
 895 frequency to which *G. mellonella* are maximally sensitive (42). The tympana
 896 of *G. mellonella* are approximately twice the size of those in *A. grisella*,
 897 implying that a pressure gradient mechanism in *G. mellonella*, should it
 898 exist, would function at much lower frequencies than in *A. grisella*, further
 899 justifying the stimulus we tested.

900 X-Ray CT Scanning

901 X-Ray Computer Tomography (CT) scans were performed using a Bruker
 902 Skyscan 1172. A moth was mounted on a block of dental wax with its dorsal
 903 side up and encased in a 5 mm diam. plastic tube. The tube was then placed
 904 vertically, with the moth's head upwards, in the imaging chamber of the
 905 scanner. A voltage of 80 kV was applied to the X-Ray source, which was
 906 positioned 85.79 mm from the center of rotation of the subject. No filter
 907 was applied to the X-Ray source, and images were generated with 2664 x
 908 4000 pixels at a resolution of 3.58 µm per pixel.

909 A total of 2400 images were taken in 0.3° increments around one
 910 hemisphere of the moth; an average of 4 frames was taken at each rotation
 911 increment. The images were collected and a volumetric reconstruction of
 912 the moth body was generated by Bruker CTvol software. These images were
 913 generated by calculating the rate of attenuation of the X-Ray through the
 914 moth's body. The threshold for this attenuation signal was set manually in
 915 order to reveal only sclerotized structures and ignore soft tissue of the moth.
 916 3 moths were scanned in this way, 2 of which were prepared by saturation in
 917 ethanol for 8 h followed by drying for 8 h. The scans were then performed the
 918 following day. The remaining moth was scanned immediately post-mortem
 919 with no additional preparation.

919 Sound Field Modeling

920 COMSOL MultiPhysics was used to simulate the 3-dimensional sound
 921 field around the moth's body. These simulations allowed us to estimate
 922 sound pressure levels around an intact moth assuming a normal posture,
 923 particularly in the region immediately adjacent to the tympana. A floating
 924 point mesh of the thorax and abdomen was generated by the X-Ray CT
 925 scanner and used as a template in SolidWorks for a simple geometrical
 926 representation of the moth body. A principal feature of this representation
 927 is the cleft between the thorax and abdomen, which is 500 µm wide at the
 928 ventral surface and extends 1500 µm back into the moth's body. The complete
 929 model comprised the thorax and abdomen fixed in a spherical air domain 10
 930 mm in radius. As in the laser vibrometry measurements, the sound stimulus
 931 was a pure tone of 100 kHz presented parallel to the tympanum surface,
 932 with a variable azimuth angle around the axis normal to this surface. The location
 933 of the simulated sound source was modified in 10° azimuth increments, with
 934 0° representing a sound source directly ahead of the insect and on its midline.
 935 We set the stimulus amplitude to 94 dB SPL at the boundary of the air
 936 domain.

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