

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

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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 two ears. However, 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 computer-aided tomography (CT) scans of the moths did not reveal any internal coupling between the two ears, confirming that an acoustic insect can localize a sound source based solely on the distinct features of each ear.

acoustic communication | Lepidoptera | sound localization | ultrasound | insect ears

The localization of sound sources by small animals is a fundamental problem in bioacoustics (1). Where body size is diminutive and interear distance is short, as is generally the case in acoustic insects and anurans, it is improbable that an animal can rely on interaural intensity difference (IID) or interaural time difference (ITD) mechanisms to resolve the azimuth angle toward 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 two 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 interaural phase difference (IPD) between these external–internal phase differences, by generating a disparity in vibration between the two 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 two ears are separated by 500 μm; 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 interaural 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 other localization mechanisms exist. Thus, examining directional hearing in small acoustic species would be valuable in an evolutionary context as well as 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, *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 interaural bridges and 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 studying

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. Those pyraloid moths that broadcast mating calls represent a yet more special case, as these species 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 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.

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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 two tympana, situated ventrally on the first abdominal segment, face in nearly the same direction and are separated by $<600\ \mu\text{m}$ (Fig. 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 two ears (Fig. 1 and *SI Text* and Fig. S1). Likewise, the ITD would be $1.75\ \mu\text{s}$, 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·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 segment and a thin posterior segment (Fig. 1) (19, 20). Vibration of the two 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 used four approaches to specify the ability to localize a sound source and 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 3D 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 modeled this field for sound sources located at different azimuth angles.

Results and Discussion

Seventy-four percent 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 (Fig. 2A). Absolute angular deviations of travel headings with respect to the stimulus, with heading that was defined as 0°, taken over the previous 1 s of movement ranged from 21° to 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 SDs ranging from 15° to 25°. Whereas the highest absolute angular deviation (38°) occurred at 1 s after the onset of phonotaxis, deviations did not decline significantly over the next 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 $27^\circ \pm 24^\circ$. Six moths began their movement with an “instantaneous heading” (measurement taken over the last 100 ms) $>90^\circ$ with respect to the stimulus. However, in five of these six 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 five to six body lengths per second as measured along the trajectory; 90% of the females tested made at least one circling move-

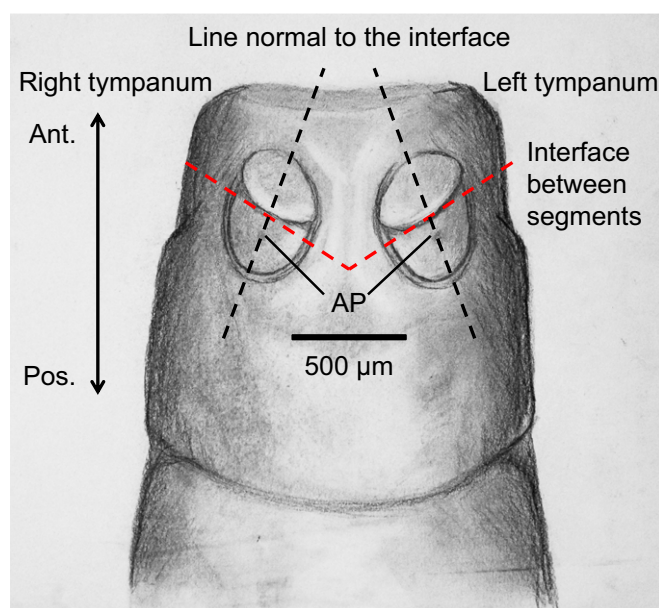


Fig. 1. Schematic diagram of the ventral side of the first abdominal segment of *A. grisella* showing the general shape and position of the two tympana. Each tympanum is divided into a relatively thick anterior segment, the conjunctivum or countertympanum (55, 56), and a membranous posterior segment 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 that is normal to the intersegmental interface is shown. The average distance between the left and right attachment points in a female is $\sim 600\ \mu\text{m}$, which would yield an ITD less than $1.75\ \mu\text{s}$ for sound arriving from a lateral source. We estimated IID between the two 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 of 0.6 mm and that the angular separation of the tympana is $\sim 30^\circ$ along the abdomen circumference. For a biologically relevant sound, male song with a dominant frequency of $\sim 100\ \text{kHz}$, arriving from a lateral source, we calculate an SPL differential of 3.1 dB between the superposition of incident and scattered sound at the left and right tympana (*SI Text*). The actual differential is expected to be considerably less than 3.1 dB, because 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 two locations.

ment 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 before the pause (sign test, $P < 0.01$) (Fig. 2B). 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 two 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 counterclockwise during the initial 2 s of the broadcast from the second speaker, reoriented, and moved toward it (Fig. 2C). These reorientations were generally gradual and finished with a travel heading within 45° of the heading of the second speaker.

3D laser vibrometry testing showed 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

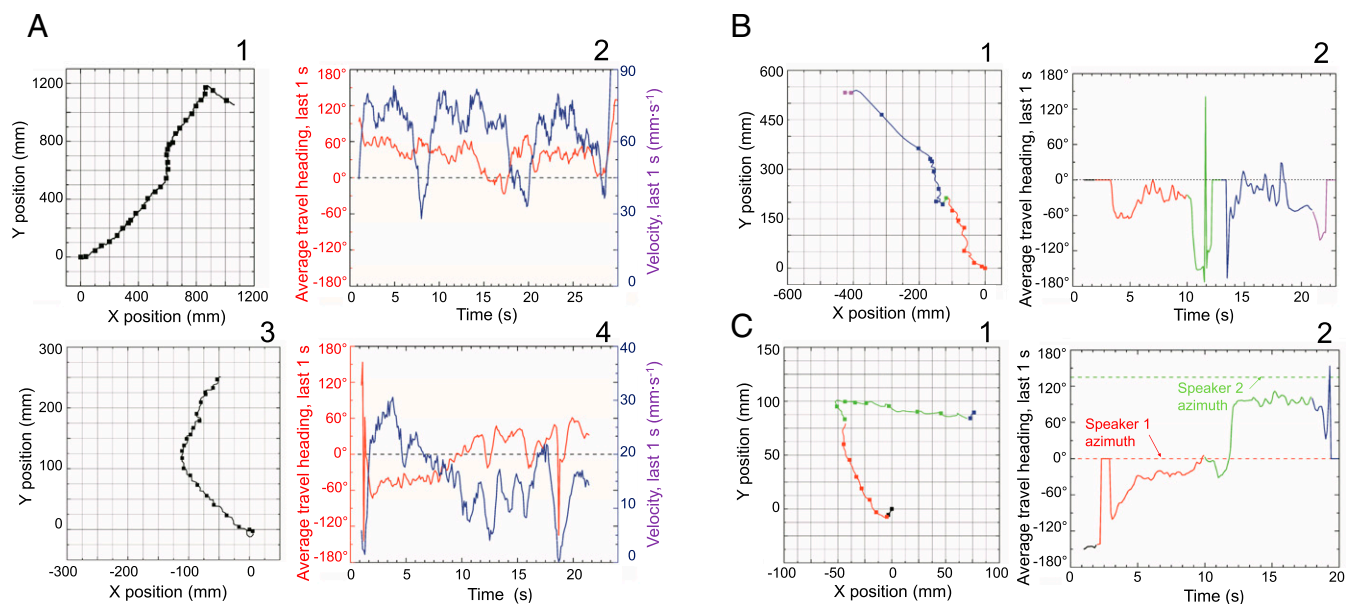


Fig. 2. (A) Trajectories of two representative *A. grisella* females tested for orientation to a 30-s male song stimulus while running on a locomotion compensation sphere. A, 1 and 3 show the females' x, y coordinates in a virtual plane over the course of their trials. The starting point is 0, 0, and 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. A, 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 lines) and their velocities over that preceding 1-s interval (blue lines). The female depicted in A, 1 and 2 retains a heading from 30° to 50° throughout her trial, whereas the female depicted in A, 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. B, 1 shows the trajectory, and B, 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°. C, 1 shows the trajectory, and C, 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 (red trace) and then, turns clockwise and follows a heading ~100°, equivalent to -35° with respect to the second speaker (green trace).

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 (Fig. 3). Membrane deflection at this attachment point is expected to influence the probability that an action potential 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 times the deflection magnitude of the secondary peak and 2.8 times the deflection magnitude for all other speaker angles tested (Fig. 4A). 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 (Fig. 4B).

At 100 kHz, the wavelength of sound in air is 3.4 mm, less than six times the average dimension of an *A. grisella* tympanum. Consequently, a pressure gradient will occur between two points on the tympanum when sound waves arrive from a source situated along the line through those points (21). The gradient increases as the two 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°), wave fronts will be parallel to the boundary between the thick anterior and thin posterior segments (Fig. 1) and may thereby generate maximum excitation at the attachment point in the center of the posterior segment.

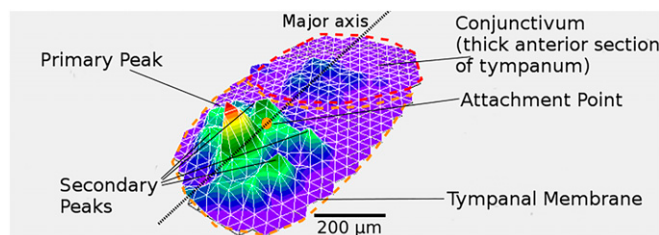


Fig. 3. Laser vibrometry measurement of deflection magnitude measured at 390 points in a representative tympanum (the tympanum presented is the insect's right); *Materials and Methods*, *Laser Vibrometry* discusses determination of deflection. The deflections are characterized by a sharp, primary peak 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 (Fig. 1), but the peak moved slightly when sound arrived from other directions.

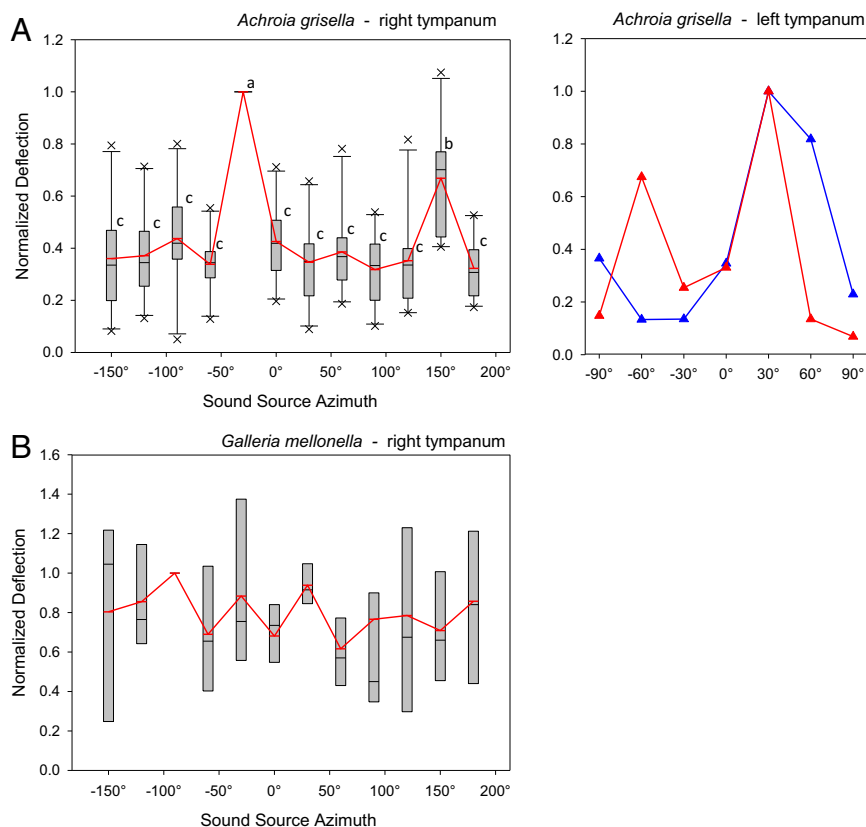


Fig. 4. (A, Left) Normalized deflection magnitude at the attachment point in the right tympanum of 10 *A. grisella* females for sound arriving from different azimuth angles. Angles are measured clockwise around the vector normal to the dorsal surface of the moth, with the body midline defined as 0°. Absolute deflection magnitudes were highest at an azimuth = -30° in 9 of 10 females; the remaining female's maximum deflection occurred at $+150^\circ$. 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 posthoc test for pairwise differences ($P < 0.001$). (A, Right) Normalized deflection magnitude in the left tympanum of two *A. grisella* females (red and blue plots). Absolute deflection magnitudes were highest at $+30^\circ$ 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 eight *G. mellonella* females. The highest deflection magnitude averaged across all eight 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 ($P = 0.625$).

The trajectories of females on the sphere, which are centered around $+30^\circ$ and -30° with respect to the synthetic song stimulus, are congruent with our laser vibrometry measurements and description of the tympana as two 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 zigzag to the left and right of a 0° heading, and while following a heading of $+30^\circ$ (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 $+30^\circ$ 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 $+30^\circ$ 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 (Fig. 2A) or losing the stimulus entirely and then regaining it via wide deviations or circling, a form of auditory scanning (Fig. 2B). 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 compared with excitation at other headings, as would be experienced by the opposite ear. Moreover, the moths have two different opportunities to lock onto the stimulus, one with the left ear and one with the right ear, and each would yield phonotaxis toward the sound source (Fig. 2A). Our model of monaural tracking predicts that, off of the sphere, a female would follow a curved trajectory in approaching a male, because a $+30^\circ$ (-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 two 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 (Fig. 5). Moreover, laser vibrometry measurements showed that tympanum deflections were equivalent for sound arriving at -90° and $+90^\circ$ (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 $+90^\circ$ also clearly refutes the possibility that the moth's body scatters high-frequency sound arriving laterally and thereby, affords a meaningful IID for localization (compare with Fig. 1; *SI Text* and Fig. S1).

The rapidity with which *A. grisella* females found the general direction of a playback stimulus at its onset shows 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 two ears. At the end of the first part of the two-speaker trials, most females faced in the general direction of the sound stimulus at 0° (Fig. 2C). 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, because most females turned clockwise (toward the right) to reorient, and five females that turned counterclockwise 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 $+150^\circ$, 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 (SPL) in the immediate vicinity of the tympana when sound waves arrive from a source within 30° of the moth's midline. The region is ~ 1 mm in diameter and features SPLs 6–7 dB above the rest of the sound field (Fig. 6). It forms by diffraction in the ventral cleft between the thorax and abdomen, and it may increase general auditory sensitivity in *A. grisella*. Because the pressure in the region of the contralateral tympanum is 1–2 dB higher than on the ipsilateral side when sound arrives from 30° to one side of the midline, the diffraction effect may also improve directional hearing beyond the level afforded by the distinct geometry and orientations of the tympana. At the same time, finite element analysis also shows that diffraction cannot be the main factor responsible for directional hearing and generating tympanum deflection peaks for sound at azimuths of -30° and $+30^\circ$. Our analysis predicts very similar SPLs at the neural attachment point in a tympanum for sound sources at azimuths of -90° and $+90^\circ$ (Fig. 6E), and the small differences (~ 2 dB) seen between the contralateral and ipsilateral tympana for azimuths from 30° to 80° and from -30° to -80° would not account for the magnitude (~ 9 dB) and specific position (azimuth $\sim 30^\circ$) of the primary deflection peak (Fig. 4A).

A. grisella obviously localize sound sources, but the means by which they do so is less direct than in other acoustic insects (26, 27). Their putative monaural tracking is suggestive of a line-following robot (28, 29), albeit a rather inefficient one. They follow a curvilinear path toward a sound stimulus, their trajectory is often interrupted by lateral excursions to regain a signal, and any prompt arrival at the stimulus or a live male is largely because of their running speed and maneuverability. Given the importance of efficient mate finding, one must question why this protocol persists. A likely answer may be found in studying the timeline of

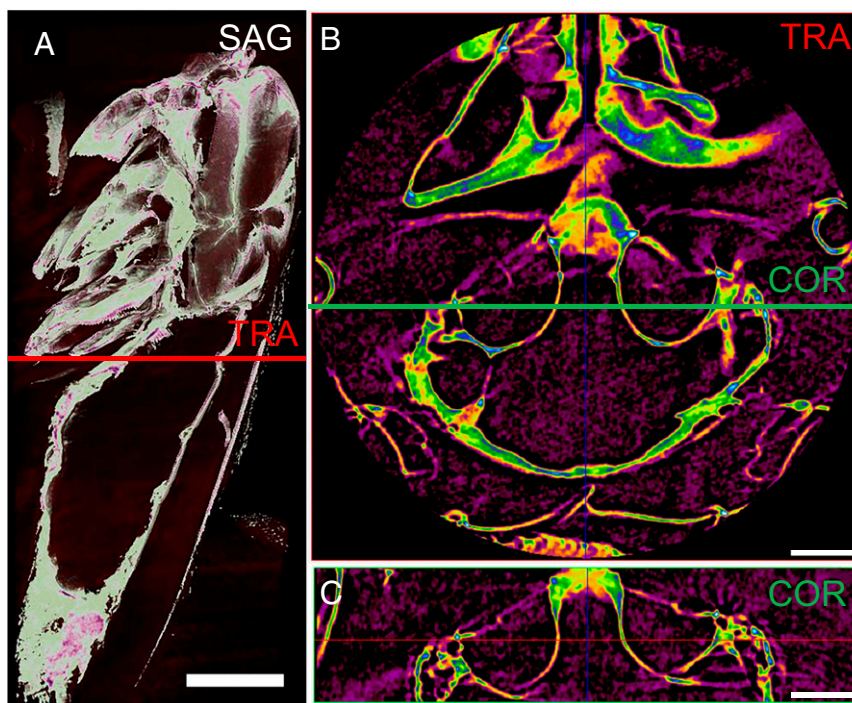


Fig. 5. X-ray CT scans of a female moth focusing on the region between the two tympana. (A) Sagittal scan (SAG) of the entire body. TRA indicates the transverse plane through the tympana on the first abdominal segment. (Scale bar: 1 mm.) (B) Transverse scan of the plane indicated by TRA in A. Yellow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

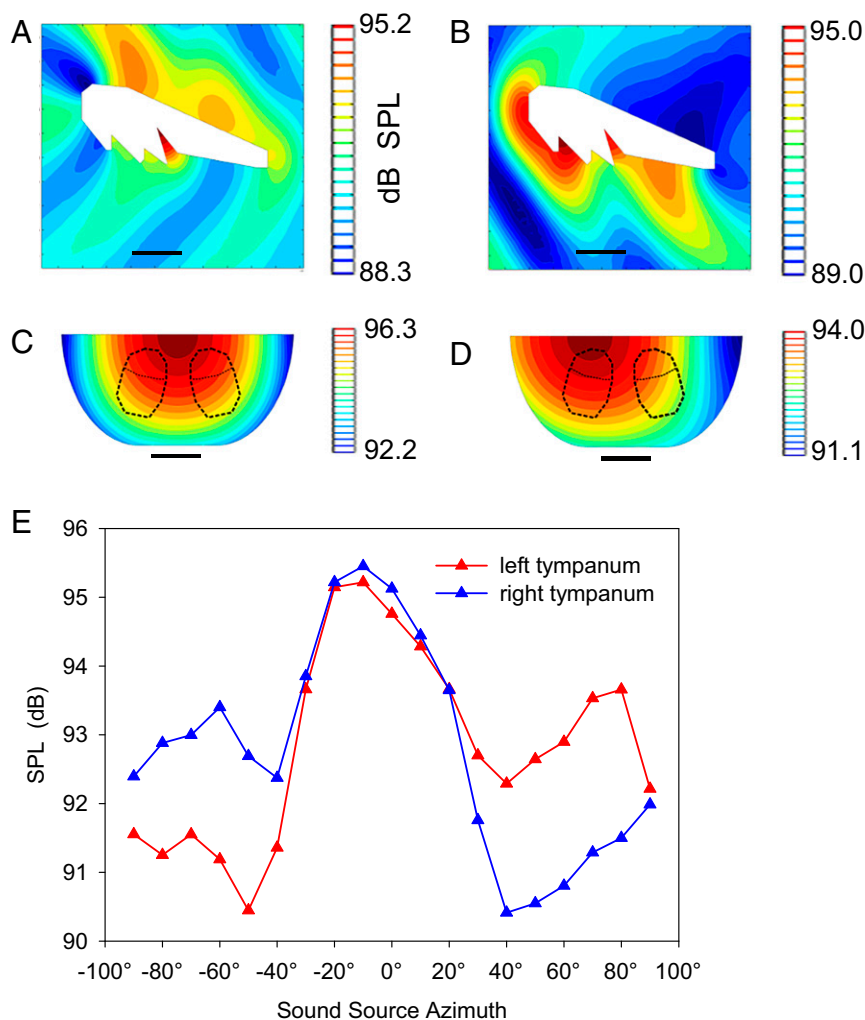


Fig. 6. SPL around a simple model of a female moth as simulated by COMSOL MultiPhysics. (A) Sagittal section, with anterior to the left, showing predicted SPLs for a 94-dB (measured at air domain boundary 10 cm from the insect) sound source situated directly in front of the moth. SPLs are 6–7 dB higher in the cleft between the abdomen and thorax, the location of the two tympana. (Scale bar: 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: 1 mm.) (C) Predicted SPLs across the ventral surface of the first abdominal segment for a sound source directly in front of the insect; approximate locations of the tympana are indicated by dashed lines, and anterior is at the top. SPL is equivalent across both tympana and reaches a maximum of 96.3 dB. (Scale bar: 500 μm .) (D) Predicted SPLs for a sound source at -30° azimuth with respect to the dorsal view of the insect ($+30^\circ$ in the ventral view shown). SPL is notably higher across the contralateral tympanum (ranging from 93.9 to 94.8 dB) than across the ipsilateral one (ranging from 93.9 to 92.1 dB). (Scale bar: 500 μm .) (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 $+90^\circ$.

evolution of acoustic communication in the Lepidoptera (30). All but 1 of 10–12 independent origins of hearing in the Lepidoptera occurred since 65 Ma, the currently accepted date for the appearance of echolocation in bats (12, 31). This timing taken in conjunction with ecological patterns has led to the inference that tympanal organs and hearing ability in Lepidoptera evolved as a response to hunting by insectivorous bats. In three major moth clades Noctuoidea, Geometridae, and Pyraloidea, each including >20,000 species, hearing is ubiquitous, except in species found in geographical regions lacking bats or that are active during seasons or times of day when bats are not (32). In these latter moth species, tympanal organs and hearing ability are reduced or absent, presumably as a secondary loss (33). The hearing that had evolved in moths is simple but highly effective for avoiding and escaping predatory bats. Moths have one to four neurons per tympanum and minimum tuning over a broad frequency range, which extends well above 100 kHz in some pyraloids (34, 35). Although negative phonotaxis away from bat echolocation signals does occur in

large-bodied noctuoids (36–38) and geometrids (39), which have relatively sensitive ears that are situated laterally and afford an IID as high as 15 dB (40, 41), directional responses as such may be negligible or absent in the pyraloids (18). The members of this latter group, being generally smaller, are not superior fliers and have relatively high hearing thresholds (42, 43), features that would render the ability to localize a bat rather unimportant. Unlike noctuoids (44), a pyraloid moth may not detect a bat until it is close, and any defensive flight maneuvers might then be too slow to exploit information on the location of the bat. Consequently, just diving toward the ground if in flight and becoming immobile—and silent—if running on the substrate (45–47) appear to be their main defenses that rely on hearing.

In contrast to hearing, acoustic communication in moths is rare and only occurs among isolated species and genera in three major clades, a pattern that reflects an origin of sound signaling via a sensory bias mechanism (45, 48). In many cases, the acoustic communication is restricted to close-range courtship

(49), wherein directional hearing would not be critical, but in species, such as *A. grisella*, that transmit sound signals over longer distances, the evolution of accurate directional ability would be expected. Conceivably, 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). However, unlike *Ormia*, in which acoustic perception of host crickets 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 in which the ears still serve primarily as bat detectors, some aspects of the directional hearing mechanism in *A. grisella* may be highly derived characteristics. The differential deflection of the neural attachment point in response to sound sources at different azimuth angles around the tympanum, particularly for sources in front vs. behind, depends on specific vibration properties of two segments of the tympanal membrane. That such differential deflection is not observed in the closely related species *G. mellonella* (Fig. 4B) reinforces the notion that the directional hearing in *A. grisella* is a derived trait that 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, the simplicity of which can be a constraint. For example, the primary deflection peak of tympanal vibration is rather sharp, exhibiting changes in magnitude of 0.4 dB per degree azimuth, and one might expect the females to use this information for accurately following a $\pm 30^\circ$ heading toward the song stimulus (27). However, with only three 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. Additional 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 honey bee colonies in Florida in 2003. We reared the moths on a standard diet (16) and kept them in an environmental chamber maintained at 25° with a 12-h:12-h light:dark photoperiod. Because *A. grisella* females normally become unresponsive after a single mating, we only used virgin females in our tests. All females were between 1 and 3 d 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 compensation sphere (Syntech LC300). The stimulus was a song recorded from a single male individual in the laboratory colony that 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:analog 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 recalibrated 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 two-thirds of her wings on the day before 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 their natural milieu adjacent to honey bee 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 holding the sphere. In each trial, we obtained the female's trajectory as reconstructed with Syntech TrackSphere software from the series of her *x*, *y* coordinates in virtual space sampled at 100-ms intervals. We used these data to determine (i) a running average of the insect's azimuth heading relative to the stimulus taken over the previous 1 s, (ii) the running velocity, taken over the previous 1 s, along the trajectory, and (iii) the initial heading at the start of the trial. In a second locomotion experiment, we tested 26 females in 19-s trials that comprised 8 s of the song stimulus, 3 s of silence, and a second 8 s of song. Additionally, in a third locomotion experiment using two speakers, we tested another 26 females in 16-s trials, in which the song stimulus was broadcast from a speaker at 0° for the initial 8 s and then, a speaker at 135° for the next 8 s.

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

Deflections were measured as the vertical distances between the highest and lowest displacements of a given point on the tympanum while the sound stimulus was broadcast. This procedure was conducted for 390 points on the tympanum, each point being measured 25 times. Data analysis was performed by Polytec v9.2 software, which sampled the vibrometer signals at 512 kHz with a sample time of 128 ms. A fast Fourier transform (FFT) was performed with a rectangular window and 25,600 FFT lines giving a frequency resolution of 7.8125 Hz. Although our sound stimulus was a pure tone, an FFT was advantageous, because it allowed us to use the vibrometer software's automatic generation of the linear frequency spectrum and corresponding coherence and cross-correlation figures. The complex average of the velocity, *V*, was calculated as the average of the real (*Re*) and imaginary (*Im*) components (representing the magnitude and the phase of oscillation, respectively) of each measurement, *V_n*:

$$\bar{V} = \frac{1}{N} \left(\sum_{n=1}^N \text{Re}(V_n) + j \sum_{n=1}^N \text{Im}(V_n) \right).$$

The velocity was then converted into a displacement in postprocessing. We focused specifically on measuring deflection at the point where the peripheral neurons are attached to the tympanal membrane.

We tested the left tympanum in two additional moths with seven broadcasts at azimuths from -90° to $+90^\circ$ and thereby, compared the stimulus orientation angle generating maximum deflection in the left and right tympana. All 12 moths remained alive throughout the series of measurements, which typically lasted 3 h. However, many moths would not survive longer testing, and this constraint precluded reliable measurements of the left and right tympana in the same individual. For each moth, measurements began at 0° azimuth and were incremented clockwise. Thus, the stimulus eliciting the primary deflection peak (Fig. 4) was the last one tested in measurements of the right ear but the second one tested in measurements of the left ear.

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

X-Ray CT Scanning. X-ray CT scans were performed using a Bruker Skyscan 1172. A moth was mounted on a block of dental wax with its dorsal side up and encased in a 5-mm-diameter plastic tube. The tube was then placed vertically, with the moth's head upward, in the imaging chamber of the scanner. A voltage of 80 kV was applied to the X-ray source, which was positioned 85.79 mm from the center of rotation of the subject. No filter was applied to the X-ray source, and images were generated with 2,664 \times 4,000 pixels at a resolution of 3.58 μm per pixel.

A total of 2,400 images were taken in 0.3° increments around one hemisphere of the moth; an average of four frames was taken at each rotation increment. The images were collected, and a volumetric reconstruction of the moth body was generated by Bruker CTvol software. These images were generated by calculating the rate of attenuation of the X-ray through the moth's body. The threshold for this attenuation signal was set manually to reveal only sclerotized

structures and ignore soft tissue of the moth. Three moths were scanned in this way, two of which were prepared by saturation in ethanol for 8 h followed by drying for 8 h. The scans were then performed the next day. The remaining moth was scanned immediately postmortem with no additional preparation.

Sound-Field Modeling. COMSOL MultiPhysics was used to simulate the 3D sound field around the moth's body. These simulations allowed us to estimate SPLs around an intact moth assuming a normal posture, particularly in the region immediately adjacent to the tympana. A floating point mesh of the thorax and abdomen was generated by the X-ray CT scanner and used as a template in SolidWorks for a simple geometrical representation of the moth body. A principal feature of this representation is the cleft between the thorax and abdomen, which is 500 μm wide at the ventral surface and extends 1,500 μm back into the moth's body. The complete model comprised the thorax and abdomen fixed in a spherical air domain 10 mm in radius. As in the laser vibrometry measurements, the sound stimulus was a pure tone of 100 kHz presented parallel to the tympanum surface, with a variable azimuth angle around the axis normal to this surface. The location of the simulated sound source was modified in 10° azimuth increments, with 0° representing a sound source directly ahead of the insect and on its midline. We set the stimulus amplitude to 94-dB SPL at the boundary of the air domain.

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