

## RESEARCH ARTICLE

# Functional relevance of acoustic tracheal design in directional hearing in crickets

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**ABSTRACT**

Internally coupled ears (ICEs) allow small animals to reliably determine the direction of a sound source. ICEs are found in a variety of taxa, but crickets have evolved the most complex arrangement of coupled ears: an acoustic tracheal system composed of a large cross-body trachea that connects two entry points for sound in the thorax with the leg trachea of both ears. The key structure that allows for the tuned directionality of the ear is a tracheal inflation (acoustic vesicle) in the midline of the cross-body trachea holding a thin membrane (septum). Crickets are known to display a wide variety of acoustic tracheal morphologies, most importantly with respect to the presence of a single or double acoustic vesicle. However, the functional relevance of this variation is still not known. In this study, we investigated the peripheral directionality of three co-occurring, closely related cricket species of the subfamily Gryllinae. No support could be found for the hypothesis that a double vesicle should be regarded as an evolutionary innovation to (1) increase interaural directional cues, (2) increase the selectivity of the directional filter or (3) provide a better match between directional and sensitivity tuning. Nonetheless, by manipulating the double acoustic vesicle in the rainforest cricket *Paroecanthus podagrosus*, selectively eliminating the sound-transmitting pathways, we revealed that these pathways contribute almost equally to the total amount of interaural intensity differences, emphasizing their functional relevance in the system.

**KEY WORDS:** Internally coupled ear, Matched filters, Acoustic communication, Insect hearing, Sound localization, Laser Doppler vibrometry

**INTRODUCTION**

Sound source localization can be of significant importance for animals, allowing them to potentially avoid predators by moving away from a threatening source (Moiseff et al., 1978; Hoy, 1992), or to communicate intraspecifically to approach singing mates or rivals (Gerhardt and Huber, 2002). Even for some parasitoids, localization is essential for finding suitable hosts (Conner, 2014; Hedwig and Robert, 2014; Lakes-Harlan and Lehmann, 2015). Small animals such as frogs, lizards, birds or mammals have ears that are separated by only a few centimeters. When the wavelength of sound is larger than the inter-ear distance, such animals may face difficulties when attempting to reliably determine the direction of a sound source (Heffner and Heffner, 1992; Köppl, 2009; Christensen-Dalsgaard, 2011). An air-filled

passage through the skull couples the ears and represents the anatomical basis for a pressure-gradient receiver in these animals (Autrum, 1940; Christensen-Dalsgaard, 2005, 2011; Christensen-Dalsgaard and Manley, 2005, 2008). In such internally coupled ears (ICEs), sound can reach both sides of the eardrum, either directly reaching the outer side or reaching the inner side by transiting from the other ear through the ear canal. This creates both time and amplitude differences between the ears that are translated into directional cues and allow sound sources to be reliably targeted (Christensen-Dalsgaard, 2011; Vedurmudi et al., 2016).

Even more extreme cases of short distances between ears, resulting in even smaller interaural time and intensity differences (ITDs and IIDs), can be found in insects (Michelsen, 1992, 1998; Robert, 2005; Römer, 2015). The distance between the ears in the forelegs of crickets, for example, may be less than 1 cm. At the same time, the wavelengths of the calling songs of these animals are many times greater than the interaural distances, and acoustic theory predicts that significant diffraction for the establishment of reasonable IIDs occurs only when the ratio of body size to the wavelength of the sound ( $l:\lambda$ ) exceeds a value of 0.1 (Morse and Ingard, 1968; Michelsen et al., 1994; Robert, 2005). ICEs have evolved in crickets, which presents a solution to this biophysical problem, but this solution is far more complex than the rather simple connection found in vertebrates (Ander, 1939; Schmidt and Römer, 2013; Römer and Schmidt, 2015). The acoustic tracheal system is a four-input system for sound that consists of two entry points through the tympana located on each foreleg and two entry points through the spiracles located on each side of the body wall behind the forelegs. The tracheal tubes of all four sound inputs are interconnected. One connection is of prime importance to the system: the cross-body trachea (transverse trachea), which connects the two ears. Sound transmitted from one side to the opposite ear has to pass through a thin membrane (septum) located in the midline of the acoustic vesicle. As the sound crosses the septum, phase delays are induced across a narrow range of species-specific calling frequencies, which results in the tuned directionality of the ear (Hill and Boyan, 1976, 1977; Michelsen et al., 1994; Michelsen and Löhe, 1995; Schmidt et al., 2011). Fine-tuning of such a system is very demanding because it requires a proper phase shift for the two ipsilateral sound components (at the outer tympanum and via the ipsilateral spiracle to the inner tympanum) for summation, and at the same time a proper phase shift for sound from the contralateral side via the connecting trachea and vesicle for cancellation.

A recent comparative anatomical examination of the acoustic tracheal design in a number of crickets from different families revealed a surprising degree of variation. Tracheal elements varied in their arrangements and respective sizes (Schmidt and Römer, 2013). Because the acoustic tracheal structure is the basis of the pressure difference receiver, changes in its morphology potentially alter the sound transmission properties and directionality of the

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system. An anatomical comparison of the various acoustic tracheae of crickets revealed a novel element: a double vesicle with two separate septa (Schmidt and Römer, 2013). However, it is unclear how the proper phase delays between ipsilateral and contralateral sound pressure waves are maintained in such a system.

For crickets, it was demonstrated that receivers exhibit two frequency-dependent filters, one in the form of tuned directionality and one of tuned sensitivity, and that the two are not necessarily tuned to the same calling song frequency (Kostarakos et al., 2008, 2009). If these filters are mismatched, the crickets display reduced sensitivity or directionality, a conflict that may represent a strong selection pressure for long-range acoustic communication (Kostarakos et al., 2009; Schmidt et al., 2011). Indeed, in one rainforest cricket species (*Paroecanthus podagrosus*), the constraint of strong background noise has apparently driven the selectivity of the sensitivity filter for the conspecific carrier song frequency.

For *P. podagrosus* we also found an almost perfect match between the sensitivity and directional filters and an acoustic tracheal system with a double vesicle and dual septa (Schmidt et al., 2011). Whereas the directional filter is based on the acoustic tracheal system mainly as a result of the contralateral pathway including the septum, the physical basis of the frequency sensitivity filter is not clear in detail. It could be tuned either as a consequence of the pressure difference mechanism via the ipsilateral pathway of the tracheal system, or through intrinsic neuronal response properties of the auditory afferents.

However, the functional relevance of morphological variation in the acoustic tracheal system is still not known. Understanding the selection pressures that have driven the evolution of such variation requires a deeper understanding of the function of these elements for directionality and its potential role in matching the two filters. Therefore, in the current study, we investigated peripheral directionality in three species of closely related crickets in the subfamily Gryllinae, which differ in their acoustic tracheal morphology. These species were chosen because they produce similar carrier frequencies and magnitudes ( $I:\lambda$  ratio) in their calling songs. By examining peripheral directionality in these species, we tested the hypothesis that the elaboration of the acoustic trachea will (1) create higher values of IIDs, (2) increase the selectivity of the directional filter and/or (3) provide a better match between the sensitivity and directionality filters.

## MATERIALS AND METHODS

### Animals and study site

This study was conducted on Barro Colorado Island (9°9'N, 79°51'W) in Panama. Three species of crickets of the subfamily Gryllinae were selected: *Anurogryllus muticus* (De Geer 1773), *Gryllobates sigillatus* (Walker 1869) and *Miogryllus* sp. (Saussure 1877). In addition, selective manipulation experiments of the double vesicles were performed on *Paroecanthus podagrosus* (Saussure et al., 1897) (subfamily Podoscirtinae). Animals were collected in the wild from grassland habitats on the island, kept in plastic containers and fed with water, fish food, pieces of apple and lettuce *ad libitum* until the experiments were conducted.

### Sound recording

Songs were recorded using electret microphones (frequency range: 50–16,000 Hz; LM-09, Hama, Monheim, Germany) and digitized at a sampling rate of 20 kHz (PowerLab 4/26, ADInstruments, Sydney, Australia). Analyses of carrier frequencies were performed using audio software [CoolEdit Pro 2.0, Syntrillium, Phoenix (Adobe Audition)].

### Tracheal morphology

The acoustic tracheal systems were dissected from crickets that were freshly killed by freezing at  $-20^{\circ}\text{C}$ , and digital images were taken of the tracheal systems with a microscope-mounted camera (DCM300, Oplenic Optronics Co., Ltd, Hangzhou, China) connected to a stereo microscope (Wild M10, Leica, Wetzlar, Germany). Images were subsequently redrawn using CorelDraw X7.

### Laser Doppler vibrometer (LDV) measurements and acoustic stimulation

Animals were immobilized (Cooling Spray, Dr Henning GmbH, Walldorf, Germany) and mounted ventral side up on a small platform ( $2\times 0.4\times 0.1$  cm) using sticky wax (Deiberit 502, Siladent Dr Böhme & Schöps GmbH, Goslar, Germany). The front legs were attached to small metal rods (0.5 mm  $\varnothing$ ) and adjusted to a natural walking position. To minimize spontaneous motor activity, we removed thoracic ganglia carefully through a small opening of the ventral cuticle without damaging the connecting transverse trachea or any surrounding tissue. Leaked hemolymph was replaced with saline and the opening sealed with Vaseline again. The inner parts of the valve-like spiracles were carefully cut to allow for continuous sound input. Tympanic oscillations were acquired with a LDV (PDV 100, Polytec, Waldbronn, Germany). Glass nanobeads (0.3  $\mu\text{m}$   $\varnothing$ ) were gently applied to the posterior tympanic membrane using a fine brush, adhering on the membrane without using glue or any other additional aids to enhance the laser beam reflectance. The laser beam was adjusted to focus upon the lateral proximal edge of the posterior tympanic membrane using a XY stage (MP4/L, Brinkmann, Mannheim, Germany) and a stereo microscope (Wild M10, Leica). This area typically yielded the highest signal-to-noise ratio and most stable recordings.

To conduct acoustic stimulations and measure sound-induced tympanic oscillations, we used synthetic calls (CoolEdit Pro 2.0) with carrier frequencies that ranged from 4.5 to 9.5 kHz. Stimuli consisted of single pulses (duration 23 ms, 2 ms rise and fall time) repeated every 77 ms (signal period 100 ms). Stimuli were amplified using a stereo amplifier (S.M.S.L, SA-50, Shenzhen, China) and broadcast via loudspeakers (JBL, Car GTO 19T, Los Angeles, CA, USA) at a distance of 30 cm from the animal being tested.

For each carrier frequency, 120 pulse repetitions were presented. The induced tympanic membrane vibrations were digitized at a sampling rate of 40 kHz (PowerLab 4/26, ADInstruments) and stored for offline analysis. All experiments were carried out in a soundproof environment of an anechoic chamber ( $80\times 80\times 80$  cm) at ambient temperature ( $24^{\circ}\text{C}$ ). For these experiments, we used 17 individuals of *A. muticus* (all males), 7 individuals of *G. sigillatus* (5 males, 2 females) and 4 individuals of *Miogryllus* sp. (3 males, 1 female). Because of their overall anatomical and functional similarity, we do not expect any relevant differences at the physiological level between males and females. We know from a previous study of a neotropical crickets species that there are no sex-specific differences with regard to various features of the sensitivity and directional tuning (Schmidt et al., 2011).

### Analysis of directionality

To determine the amount of frequency-dependent IIDs, we first performed contralateral stimulation with the speaker placed contralaterally at 90 deg to the longitudinal body axis, and measured the tympanic membrane vibrations elicited at a sound intensity of 90 dB sound pressure level (SPL). In three cases (i.e. three frequencies tested), we used 95 and 100 dB SPL, because

**Table 1. Summary of species-specific parameters for three species of the subfamily Gryllinae in the context of directional hearing**

	Song carrier frequency (kHz)	$\lambda$ (mm)	$l$ (mm)	$l:\lambda$ ratio	Max. IIDs (dB)	Frequency at max. IIDs (kHz)	Bandwidth directional filter 5 dB below max. (Hz)
<i>A. muticus</i>	7.03±0.26 (N=15)	48.78	4.53±0.36 (N=11)	0.09	21.69±4.59 (N=17)	6.86±0.64 (N=17)	701.63±367.14 (N=16)
<i>G. sigillatus</i>	7.15±0.07 (N=2)	47.97	3.54±0.25 (N=5)	0.07	24.53±2.84 (N=7)	7.00±0.74 (N=7)	472.00±358.37 (N=7)
<i>Miogryllus</i> sp.	7.30±0.27 (N=5)	46.67	4.50±0.43 (N=4)	0.11	28.05±3.08 (N=4)	8.00±0.83 (N=4)	382.50±238.10 (N=4)

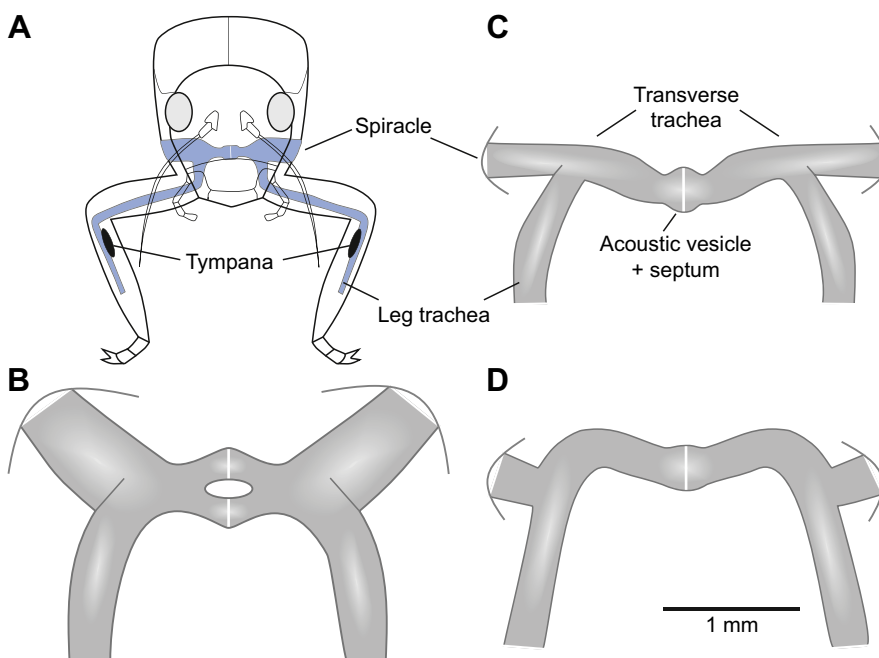
Data are means±s.d. for *Anurogryllus muticus*, *Grylloides sigillatus* and *Miogryllus* sp.  $\lambda$ , wavelength of song frequency;  $l$ , pronotum width; IID, interaural intensity difference.

vibration amplitudes were too small. Subsequently, the same stimulation was performed 90 deg ipsilateral to the longitudinal body axis, and the SPL for each frequency was reduced in 2 dB steps until the amplitude of the tympanic oscillation matched that of the contralateral stimulation. The SPLs of the stimuli were monitored and controlled with a free-field  $\frac{1}{2}$  in condenser microphone (ACO Pacific, 7052E, Belmont, CA, USA) connected to a sound level meter (Svantek, SVAN 977, Warsaw, Poland), which was positioned 2 cm in front of the animal. LDV signals were band-pass filtered (low-frequency cut-off: 1000 Hz; high-frequency cut-off: 400 Hz above the tested carrier frequency), and the root mean square (RMS) values calculated and averaged over 120 stimulus repetitions in LabChart (ADInstruments, Dunedin, New Zealand). LabChart standard digital filters are zero-phase-lag finite impulse response (FIR) filters using the ‘window method’ with a Kaiser window. The calculation of IIDs was based on the RMS differences between the contralateral and ipsilateral measurements. Within the range of tested SPLs (60–100 dB SPL), the recorded tympanal vibrations showed strong linearity. To determine the frequency sensitivity tuning of the ear, we calculated RMS values of the measured tympanic membrane vibrations to the ipsilateral stimulation at 90 dB SPL.

#### Manipulation of the acoustic tracheal system

To test the importance of the sound input of the contralateral spiracle for the generation of IIDs, we occluded the spiracle of *G. sigillatus* with Vaseline and compared the results before and after treatment with respect to the highest individual IIDs.

Because the rainforest cricket *P. podagrosus* is characterized by a large acoustic double vesicle (Schmidt and Römer, 2013), it is ideally suited for manipulation (i.e. one or both vesicles can be severed to selectively study the contribution of the vesicle to the directionality of the ear). In this species, the anterior tympanum is much larger than the posterior tympanum and, therefore, is most likely to be functionally relevant. However, the anterior tympanum is almost completely covered by a cuticular fold and could not be accessed with the laser beam. When the fold was removed, untuned directionality resulted. This finding would support the hypothesis that cuticular folds and tympanal organ slits enhance directional hearing (Autrum, 1963; Bailey and Stephen, 1978; Stephen and Bailey, 1982; Montealegre-Z and Robert, 2015). Therefore, to determine peripheral directionality in this species, we used the extracellular recordings of neuronal activity of the auditory interneuron 1 (AN1) as indicators. Sensitivity and directional tuning have already been determined in this species using this method; for details of the experimental procedure, see Schmidt et al. (2011). Pure-tone pulses with carrier frequencies ranging from 2.5 to 6 kHz were used as stimuli to determine the threshold of the AN1 in response to ipsilateral and contralateral stimulation (each 90 deg with respect to the longitudinal body axis). The threshold differences measured between ipsilateral and contralateral stimulation represented the IID for a given frequency. After IIDs of intact animals had been measured, the acoustic trachea was exposed by removing the cuticle and one of the acoustic vesicles was severed in the middle with a micro-scissor, such that only one sound path



**Fig. 1. Overview of acoustic tracheal design in three cricket species (Gryllinae).** (A) Schematic drawing of the relative position of the acoustic trachea in crickets, and illustration of individual tracheal types for (B) *Anurogryllus muticus*, (C) *Grylloides sigillatus* and (D) *Miogryllus* sp.

from the contralateral side remained. The leaked hemolymph was replaced with saline and sealed with Vaseline again. Threshold measurements were obtained, and these measurements were subsequently repeated after both vesicles were severed. For these experiments, we used 8 individuals of *P. podagrosus* (6 males, 2 females).

## RESULTS

### Acoustic tracheal morphology

The three species investigated had rather similar calling song frequency wavelengths ( $\lambda$ ) and also similar body sizes ( $l$ , measured as the pronotum width), which led to a  $l:\lambda$  ratio of comparable magnitude (Table 1). Their acoustic tracheal morphology, however, varied considerably (Fig. 1). The most striking morphological variation was seen in the acoustic vesicle, the central part of the transverse trachea that couples the two ears. *Anurogryllus muticus* is characterized by possession of a double

acoustic vesicle with two septa (Fig. 1B). In contrast, *G. sigillatus* and *Miogryllus* sp. each have only a single vesicle and septum of relatively similar sizes (Fig. 1C,D). The diameter of the tracheal tubes also differs between *A. muticus* and the other two species. In particular, in *A. muticus*, the branch of the tracheal tube that connects the acoustic spiracle with the transverse and leg trachea is twice the diameter of that seen in the other species. Note that these size differences cannot be attributed to the different body sizes – *A. muticus* and *Miogryllus* sp., at least, have almost identical pronotum widths (Table 1).

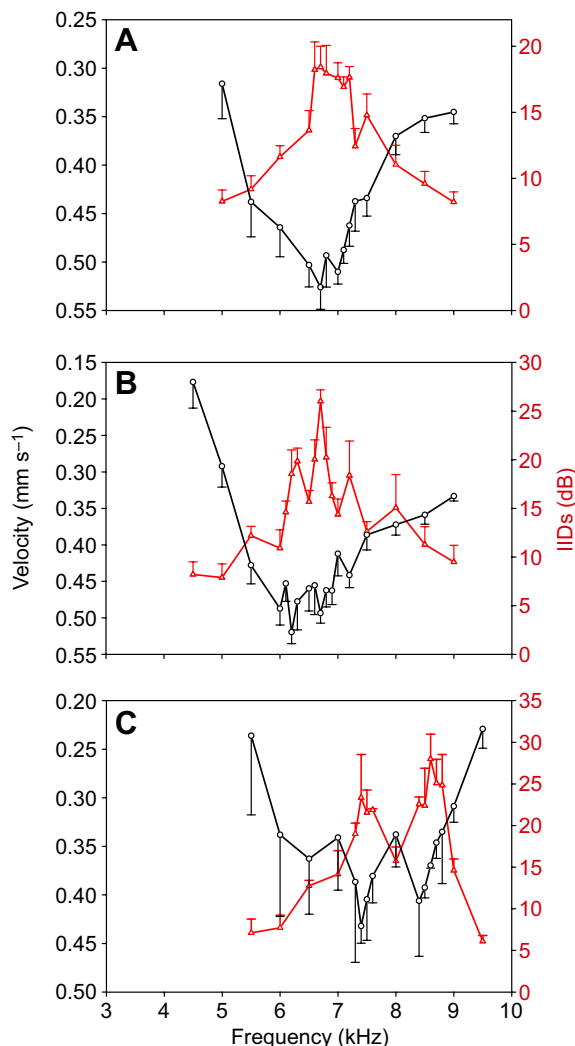
### Directionality and frequency sensitivity

The measurements of peripheral directionality revealed significant differences in the magnitudes of maximum IIDs (Table 1; ANOVA;  $F=4.362$ ,  $P=0.024$ ) between *A. muticus* and *Miogryllus* sp. (Holm–Sidak pairwise comparison;  $t=2.809$ ,  $P=0.028$ ). *Anurogryllus muticus* displayed the highest inter-individual variation of IIDs, which ranged from 13 to 29 dB at frequencies from 5.5 to 7.9 kHz. The individual maximum IIDs of *G. sigillatus* ranged from 20 to 28 dB at frequencies from 6.0 to 8.4 kHz; *Miogryllus* sp. displayed values ranging from 24 to 31 dB at frequencies from 7.1 to 8.8 kHz (Fig. 2).

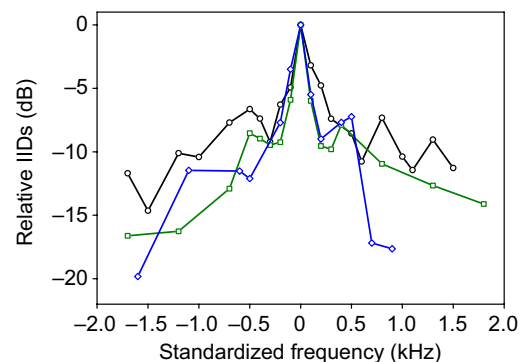
The standardization of directional tuning allowed a quantitative analysis of the sharpness of individual tuning 5 dB below peak IIDs (Table 1). *Anurogryllus muticus* had the broadest tuning of all three species (Fig. 3); however, no significant differences between the species could be found (Table 1; ANOVA;  $F=1.897$ ,  $P=0.172$ ).

The sensitivity tuning, measured as the frequency-dependent vibrational velocity of the posterior tympanum, showed a steep and rather symmetrical roll-off towards higher and lower frequencies in *A. muticus* (Fig. 2A). In *G. sigillatus*, the sensitivity also decreased strongly at lower frequencies, but was much less steep at higher frequencies (Fig. 2B). The sensitivity tuning measurements in *Miogryllus* sp. showed two peaks at 7.4 and 8.6 kHz, and steep roll-offs on either side (Fig. 2C). The absolute sensitivity measured was very similar in *A. muticus* and *G. sigillatus*, with an average of 0.53 and 0.52 mm s<sup>-1</sup>, respectively, which was about 0.10 mm s<sup>-1</sup> more sensitive than that for *Miogryllus* sp.

Fig. 2 displays a direct comparison between the sensitivity and directional tuning results. In general, a close match was observed



**Fig. 2. Sensitivity and directional tuning in three cricket species (Gryllinae).** For each species, two frequency-dependent filters were determined: the sensitivity filter (black, circles) and the directional filter (red, triangles). (A) *Anurogryllus muticus* ( $N=17$ ), (B) *G. sigillatus* ( $N=7$ ) and (C) *Miogryllus* sp. ( $N=4$ ). Data are means  $\pm$  s.e.m. Significant differences in the magnitude of interaural intensity differences (IIDs) were found (ANOVA;  $P=0.024$ ) between *A. muticus* and *Miogryllus* sp. (Holm–Sidak pairwise comparison;  $t=2.809$ ,  $P=0.028$ ).



**Fig. 3. Standardized directional tuning.** To allow a quantitative comparison of species' directional filters to be conducted, the maximum IIDs at the respective frequency of the individual tuning curves were set to zero and IIDs with lower (negative values) and higher (positive values) frequencies were averaged accordingly. *Anurogryllus muticus* ( $N=17$ ): black line, circles; *G. sigillatus* ( $N=7$ ): green line, squares; *Miogryllus* sp. ( $N=4$ ): blue line, diamonds. The bandwidth of directional filters 5 dB below the maximum (relative IIDs at 0 dB) revealed no significant difference between species (ANOVA;  $P=0.172$ ). Mean values are shown.

between the two filters, such that values of high IIDs fell within the range of highest sensitivity (Fig. 2). This observation was most striking in *Miogryllus* sp., where the two sensitivity peaks closely matched the two maxima in directionality.

### Manipulation of the acoustic tracheal system

Blocking the contralateral spiracle input had the same overall effect in all three species of Gryllinae: IIDs were strongly reduced and directionality decreased to values of about 6 dB (data not shown). The contralateral sound input was blocked in six individuals of *G. sigillatus*, which reduced maximum IIDs from 24.5 dB in the intact system to only 6.0 dB after blocking (Fig. 4; paired *t*-test,  $t=10.450$ ,  $P<0.001$ ,  $N=7/6$  control/blocked).

In *P. podagrosus*, a rainforest cricket with a double acoustic vesicle (Fig. 5), we performed a series of manipulations to evaluate the contribution of each of the two possible sound paths within the acoustic vesicle by selectively severing one or both of these paths (Fig. 5). The intact system provided on average an IID of 13.8 dB; severing one path reduced the IID to 6.6 dB, and severing the remaining path completely abolished the directionality (IID of 0.8 dB) (ANOVA repeated measurements:  $F=83.85$ ,  $P<0.001$ ; all pairwise multiple comparisons were significant  $P<0.001$ ).

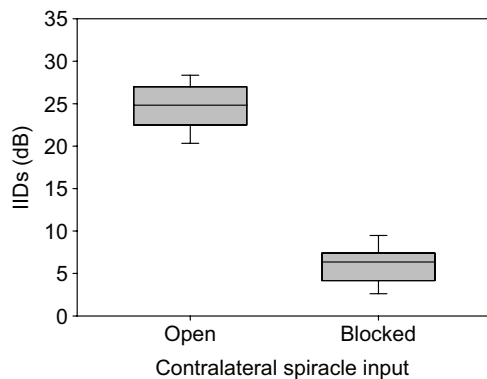
### DISCUSSION

We examined the peripheral directionality in three cricket species of the subfamily Gryllinae, selected because of differences in the structural design of their acoustic trachea. These trachea are essential for the function of the pressure difference receiver and, thus, the directionality of the ear. The individuals of the species are comparable in size, use similar calling song frequencies and had similar  $\lambda$  ratios (Table 1). Furthermore, they all inhabit patchy grassland habitats at the edges of tropical rainforests, so the ecological constraints for sound communication are also similar. Despite the morphological differences between the tracheal tubes and acoustic vesicles of the species (Fig. 1), features that certainly have effects on the properties of sound transmission, our results nonetheless provided no support for the hypothesis that a double vesicle could be regarded as an evolutionary innovation to (1) increase interaural directional cues, (2) increase the selectivity of the directional filter, or (3) provide a better match between the directional and the sensitivity filter (Schmidt et al., 2011; Schmidt and Römer, 2013). In fact, *A. muticus* had on average lower

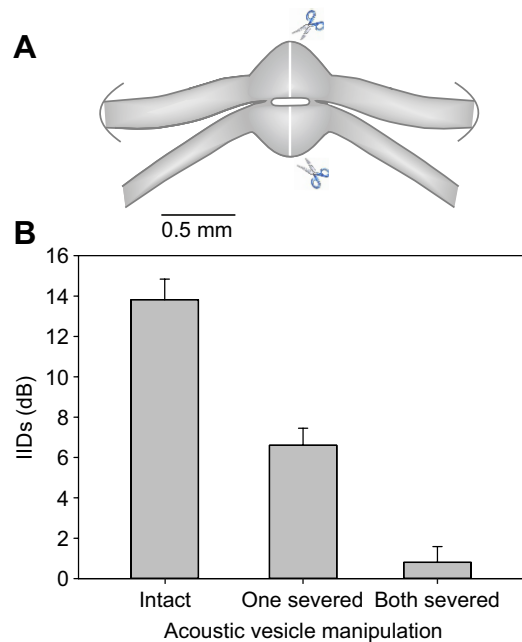
maximum IIDs than the species with a single acoustic vesicle, although it possessed an elaborate acoustic structural design that included a double acoustic vesicle and two septal membranes. Similarly, the sharpness of the directional filter in *A. muticus* was lower than that of either *G. sigillatus* or *Miogryllus* sp., indicating that this filter is less narrowly tuned to the best frequency (Fig. 3).

In a pressure difference receiver, sharply tuned directionality of the ear is only possible when the proper phase relationship exists between the pressure wave components that arrive at the outer and inner sides of the tympanum. The medial septum of the acoustic vesicle has been identified to play a crucial role in this context (Michelsen et al., 1994; Michelsen and Löhle, 1995). In a tracheal system that contains dual septa, however, this situation appears to be more complex. Pressure waves from the contralateral side divide and pass the two septa before rejoining, and these waves need to be transmitted in a coordinated fashion to induce the proper phase relationship in combination for directionality of the ear to be effective. It is not clear how phase differences within the acoustic tubes occur in a species such as *A. muticus*, and to what extent sound transmission in dual septa might explain the broader directional tuning observed in this species as compared with that seen in *G. sigillatus* and *Miogryllus* sp. (Table 1).

One hypothesis with regard to the evolution of more elaborate acoustic tracheal structures such as a double vesicle addressed the sensitivity and directionality matching between the two frequency-dependent filters (Kostarakos et al., 2009; Schmidt et al., 2011; Römer and Schmidt, 2015). High background noise in species-rich habitats may represent a selection pressure for the evolution of more-selective sensitivity filters in rainforest crickets, allowing them to overcome signal-masking effects (Schmidt et al., 2011). In this case, mismatching in the tuned directional filter would be detrimental for intraspecific communication, as either sensitivity or directionality would be reduced for a given calling song frequency.



**Fig. 4. Quantitative measure of IIDs before and after the contralateral spiracle was blocked.** For *G. sigillatus*, blocking the contralateral spiracle significantly decreased the magnitude of IIDs (paired *t*-test;  $P<0.001$ ). Boxes show the range from the 25th to 75th percentiles, error bars above and below the box indicate the 90th and 10th percentiles, and the solid horizontal line depicts the median value.



**Fig. 5. Effect of acoustic vesicle manipulation in *Paroecanthus podagrosus*.** (A) Manipulation of the acoustic tracheal system by selectively severing one or both vesicles. (B) Severing one ( $N=7$ ) or both vesicles ( $N=6$ ) revealed a significant effect on the magnitude of IIDs versus the intact control ( $N=8$ ) (means  $\pm$  s.e.m.; ANOVA repeated measurements:  $F=83.85$ ,  $P<0.001$ ; all pairwise multiple comparisons were significant  $P<0.001$ ).

Because the rainforest cricket *P. podagrosus*, native to an environment with a high level of nocturnal background noise, had a sharply tuned sensitivity filter that almost perfectly matched the peripheral tuned directionality, and a double acoustic vesicle, the matching hypothesis was attractive (Schmidt et al., 2011). This hypothesis was further supported by the finding that two species of field crickets (*Gryllus bimaculatus* and *Gryllus campestris*, both equipped with a single vesicle) displayed considerable mismatching between the filters (Kostarakos et al., 2008, 2009). However, Hirtenlehner et al. (2014) found that the filters of individuals from different populations of *G. bimaculatus* and *G. campestris* matched well, demonstrating that matching or mismatching is not necessarily a hardwired species-specific feature. Indeed, in the present study, we found that the two filters matched well irrespective of species-specific differences in acoustic vesicle morphology (Fig. 2).

Our data confirm the matched filter hypothesis (Capranica and Moffat, 1983; Wehner, 1989), as the carrier frequency of the male's calling songs matches the receiver's auditory sensitivity, facilitating acoustic communication by maximizing the signal-to-noise ratio. Because of the small size of the *G. sigillatus* population on Barro Colorado Island, we were able to record calling songs from only two males. Nonetheless, males of this species are known to use song frequencies between 6 and 7 kHz (Walker, 2014), a frequency range that corresponds with the receiver's highest sensitivity and directionality filter (Fig. 2B). For *Miogryllus* sp., however, this is only partly true because the highest frequency sensitivity and maximum IIDs for two out of four individuals were shifted well above the males' calling song frequency (Table 1, Fig. 2).

Our results did not indicate that the double vesicle lent a higher adaptive value as compared with the single acoustic vesicle, at least not in the species investigated. From data collected for about 40 cricket species (Schmidt and Römer, 2013; A.K.D.S. and H.R., unpublished data), we are currently aware of only these two acoustic vesicle variants (double, single). However, because phylogenetic relationships among these species are still unresolved, it is not yet clear which of these vesicle types constitutes the derived or ancestral condition.

In many, if not all, crickets, the prothoracic (acoustic) spiracles can be completely closed, so preventing the entrance of sound via this input. Although this has not yet been tested, we assume that animals keep their spiracles open during phonotaxis to optimize the pressure difference receiver function and, thus, the directionality of the ear. We performed two kinds of experiments to test the role of the contralateral pressure component via the transverse acoustic trachea and vesicle. Blocking the contralateral spiracle dramatically reduced the amount of IIDs, verifying that sound input is important for directionality (Fig. 4). Nonetheless, despite the prominent role it plays in directionality (Michelsen and Löhe, 1995), blocking the contralateral sound input did not completely abolish the directionality of the system; in *G. sigillatus*, the remaining IIDs were on average 6 dB and could reach 9 dB (Fig. 4). Such a magnitude cannot be explained by diffraction due to the presence of the cricket body. In *G. bimaculatus*, with a  $l:\lambda$  ratio of 0.09, Michelsen et al. (1994) calculated the intensity difference around the body by diffraction to be less than 1.5 dB.

The second manipulation experiment was performed to elucidate the role of the double vesicle in detail, as well as the roles of each of the two separate sound paths within the vesicle. We examined the directionality of the ear in an intact *P. podagrosus* system, and after severing either one or both of the sound paths to the ipsilateral ear. The results clearly showed that the two paths contribute almost equally to the total amount of IIDs in the intact system (Fig. 5).

Moreover, when both paths were severed, the remaining IID was  $0.80\pm 0.78$  dB, which is quite close to the value that has been calculated for the small effect of diffraction on directionality in the absence of the pressure difference receiver (Michelsen et al., 1994).

Other manipulation experiments have been conducted on various parts of the acoustic tracheal system (Boyd and Lewis, 1983; Schmitz et al., 1983; Weber and Thorson, 1989), particularly to study the specific role of the septum within the acoustic vesicle as a phase shifter. These experiments yielded different results for different cricket species, and even different populations of the same species (Wendler and Löhe, 1993; Löhe and Kleindiest, 1994; Hirtenlehner et al., 2014). Perforation of this soft membrane reduced IIDs in the field cricket *G. bimaculatus* from 10 to 1–2 dB (Michelsen and Löhe, 1995), whereas the same amount of perforation in two other populations of the same species reduced directionality by 5.2 and 7 dB, but a tuned directionality of about 7–8 dB still remained after the manipulation (Löhe and Kleindiest, 1994; Hirtenlehner et al., 2014). Even under field conditions where the local directional information at the position of a receiver can be strongly impaired (Rheinlaender and Römer, 1986; Gilbert and Elsner, 2000; Kostarakos and Römer, 2010), septum perforation had little influence on the localization ability of females. Surprisingly, the same kind and amount of septum perforation in the closely related field cricket *G. campestris* resulted in the almost complete elimination of directionality (Hirtenlehner et al., 2014). Septum perforation reduced the average IIDs by more than 11 dB – twice as much as was observed for *G. bimaculatus*. These results illustrate the incompleteness of our knowledge with regard to the contribution of the particular anatomical components of the pressure difference receiver to tuned directionality.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

A.K.D.S. and H.R. designed the experiments and drafted the manuscript. A.K.D.S. conducted and analyzed the experiments.

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