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# Serial Hearing Organs in the Atympanate Grasshopper *Bullacris membracioides* (Orthoptera, Pneumoridae)

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ABSTRACT

In different insect taxa, ears can be found on virtually any part of the body. Comparative anatomy and similarities in the embryological development of ears in divergent taxa suggest that they have evolved multiple times from ubiquitous stretch or vibration receptors, but the homology of these structures has not yet been rigorously tested. Here we provide detailed analysis of a novel set of hearing organs in a relatively "primitive" atympanate bladder grasshopper (*Bullacris membracioides*) that is capable of signaling acoustically over 2 km. We use morphological, physiological, and behavioral experiments to demonstrate that this species has six pairs of serially repeated abdominal ears derived from proprioceptive pleural chordotonal organs (plCOs). We demonstrate continuity in auditory function from the five posterior pairs, which are simple forms comprising 11 sensilla and resembling plCOs in other grasshoppers, to the more complex anterior pair, which contains 2000 sensilla and is homologous to the single pair of tympanate ears found in "modern" grasshoppers. All 12 ears are morphologically differentiated, responsive to airborne sound at frequencies and intensities that are biologically significant (tuned to 1.5 and 4 kHz; 60–98 dB SPL), and capable of mediating behavioral responses of prospective mates. These data provide evidence for the transition in function and selective advantage that must occur during evolutionary development of relatively complex organs from simpler precursors. Our results suggest that ancestral insects with simple atympanate pleural receptors may have had hearing ranges that equal or exceed those of contemporary insects with complex tympanal ears. Moreover, auditory capability may be more prevalent among modern insect taxa than the presence of overt tympana indicates. J. Comp. Neurol. 465:579–592, 2003. © 2003 Wiley-Liss, Inc.

**Indexing terms: caelifera; chordotonal organ; ears; auditory evolution; acoustic insects**

Modern insect ears occur on virtually every part of the body in some taxa, from antennae to mouthparts to the sternum (Fullard and Yack, 1993). The cyclopean ear of the praying mantid aside (Yager and Hoy, 1986), this diversity of location is balanced by extreme uniformity in the number of ears; there are always two. Although hearing is restricted to only a small number of insect species, at least 19 independent origins of ears are currently recognized (Yager, 1999). Such exuberant innovation indicates that insect ears must be relatively easily derived from structures of widespread morphological distribution, and it has frequently been suggested that they arose from ubiquitous stretch or vibration receptors (Boyan, 1993; Shaw, 1994; Hoy and Robert, 1996).

Substantial support for this view comes from studies of comparative anatomy and similarities in the embryologi-

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cal development of ears in divergent taxa (Meier and Reichert, 1990; Yack and Fullard, 1990; Yack and Roots, 1992). Although elegant in conception and execution, extrapolation from these studies is limited in that results support modularity of structure but not function, and comparisons are made across species so homology of the structures is not ensured. The hypothesis that auditory organs arose from proprioceptors does, however, lead to certain expectations for changes in the sensory and morphological characteristics of peripheral structures to create functional ears (Yack and Fullard, 1993; Hoy and Robert, 1996). Anticipated sensory changes might include a rise in the number of sensory neurons in the chordotonal organ and a decrease in the threshold to airborne sound relative to that of proprioceptors. Morphological changes might include reduction in the thickness of the cuticle, enlargement of tracheal sacs, development of rigid supporting structures to isolate the chordotonal organ, and a general reorientation to bring the three ear components into the proper spatial relationship to one another. The existence of atympanate organisms that detect and respond to airborne sound clearly indicates that the lack of a frank tympanum does not preclude audition in the far field (Yack and Fullard, 1990; Cokl and Virant-Doberlet, 1997; Pflüger and Field, 1999). Hence the evolution of the chordotonal organ (CO) and peripheral structures might be independent evolutionary events and we focus here primarily on the CO proper.

We investigated the detailed structure and function of hearing organs in the bladder grasshopper, *Bullacris membracioides* (Orthoptera, Pneumoridae; Dirsh, 1965). Little is currently known about this endemic African group (Alexander and van Staaden, 1989; Alexander, 1992), but molecular data indicate that the family is relatively ancient, possibly dating from the Jurassic (Flook and Rowell, 1997). Its members are most notable for their use of exaggerated pair formation calls, although bladder grasshoppers patently lack the specialized tympanal ears common to other acridids. Their low-frequency, nocturnal signals have an acoustic reach of over 2 km (van Staaden and Römer, 1997), creating one of the largest active spaces known for invertebrate acoustic signals.

The primary aim of this study was to establish the functional morphological basis for hearing in *Bullacris membracioides.* Specifically, we used anatomical, neurophysiological, and behavioral analyses to define the extent to which this species possesses functional ears. The discovery of essentially two different ear forms means that the comparison here is both within species and with a "typical" orthopteran auditory organ. We then use these data, along with comparison with atympanate hearing in other invertebrate taxa, to draw conclusions concerning the transition in function and selective advantage during the evolutionary development of complex structures from simpler precursors.

## MATERIALS AND METHODS

### Experimental animals

Bladder grasshoppers (*Bullacris membracioides*) were collected at Inchanga (KwaZulu/Natal, South Africa) in February 1994–96 and March 1998. A total of 106 adults (females and inflated males) and 7 alternate males were used for the neurophysiological and behavioral experiments.

### Electrophysiology and receptor thresholds

The receptor axons of the segmentally repeated abdominal pleural chordotonal organs (plCOs) enter the ventral nerve cord via the segmental nerves 2 (N2s) of the respective abdominal neuromere. (In the first abdominal segment, this nerve is fused with, and commonly referred to as, nerve 6 of the metathoracic ganglion.) To determine the auditory thresholds of the plCOs in the abdominal segments 1–6 (A1–A6), extracellular multiunit recordings were made from the corresponding N2s. Animals were anesthetized with $CO_2$. The wings (in inflated males) and the pronotum were removed, and the animal was waxed ventral side up onto a small metal holder. To record from N2 of A1, A2, or A3, the cuticle above the metathoracic ganglion was removed to expose the proximal sections of the N2s; for recordings from N2 of A4, A5, or A6, a small opening was cut into the ventral cuticle above the respective abdominal ganglion. N2 was then hooked onto silver-wire hook-electrodes and covered with petroleum jelly to prevent desiccation. The recorded signal was amplified in a custom-made extracellular amplifier and monitored with headphones. Threshold curves were determined using pure tone pulses as stimuli (1-Hz pulse rate, 50-msec pulse duration, 1 msec rise and fall time); threshold was defined as the minimum sound pressure level (SPL; measured at the preparation) that elicits a response aurally discriminated from spontaneous activity in at least three of five presentations. The threshold curves of the plCO in A1 (plCO1) were measured in a sound-damped chamber (described in van Staaden and Römer, 1998).

### Behavioral thresholds and receptor ablations in females

Receptive females, including virgins, respond to the loud calling song of the inflated males with a low-intensity call of about 60 dB SPL (van Staaden and Römer, 1998). The behavioral thresholds of females were determined by playing back a digitized male calling song. For sound acquisition, adult males were put into wire-mesh cages, and their calling songs were recorded with a sound level meter placed 1 m dorsal to the animal (Bruel & Kjaer, Odense, Denmark; model 2009, 1/2" condenser microphone, type 2540, Larson & Davis [LB-Electronics, Vienna,Austria]; A weighting, RMS fast). Songs were sampled at 44 kHz on an Apple Macintosh Powerbook 520 (Apple Computer, Cupertino, CA) via the built-in 16-bit A/D sound board, edited in SoundEdit 16, version 1.0, software (Macromedia, San Francisco, CA), and stored digitally. Playback experiments were performed at night in a sound-damped room; females were placed in wire-mesh cages, and a male call was played back in SoundEdit 16 at a repetition rate of 0.3 Hz and broadcast at various intensities via a custom-made amplifier and a speaker (Dynaudio, Odense, Denmark). Alternatively, 1.7- or 4-kHz pure-tone pulses at 100- or 880-msec duration were randomly presented at 40-second intervals and 75 dB SPL. Behavioral threshold was defined as the SPL that elicits at least one female chirp in four of five stimulus presentations.

Bilateral ablations of plCO1 were performed on anesthetized females by opening the body wall at the attachment site, removing the organ, and sealing the opening with Histoacryl (Aesculap, Vancouver, British Columbia, Canada). Control animals were sham-operated. On the following day, the females were tested in playback exper-

iments as above. In all ablation experiments, the absence of neural activity from the afferent nerves was subsequently confirmed in extracellular recordings.

### Light and electron microscopy

For transmission electron microscopy, plCOs were dissected out together with a small piece of attached cuticle and fixed for 2 hours at room temperature (RT) and then overnight at 4°C, in 2.5% formaldehyde and 2.5% glutaraldehyde in cacodylate buffer (0.1 M, 4.1% sucrose, pH 7.2–7.4). After washing in plain buffer (pH7.2–7.4, 2 hours, RT), specimens were postfixed in 2% osmium tetroxide in cacodylate buffer (0.1 M, 4.1% sucrose, pH 7.2–7.4, 2 hours at RT), washed in buffer again, dehydrated in a graded ethanol series, and embedded in TAAB resin (TAAB, Aldermaston, UK). Thin sections (40–80 nm) were cut on an Ultracut E Ultramicrotom (Leica Microsystems, Wetzlar/Germany), stained with 4% uranyl acetate and 4% lead citrate in 1 N NaOH, and examined and photographed on an EM 902 transmission electron microscope (Zeiss, Oberkochen, Germany).

To study their histology in light microscopy, plCOs were dissected out as above, fixed for 3–5 days at room temperature in 10% formaldehyde, and embedded in paraffin. Serial sections (5 or 10 μm) were cut on a sliding microtome (Jung SM 2000R), mounted onto slides, dewaxed, and stained with haemalaun/eosin. Alternatively, some specimens were fixed in a mixture of 2.45% formaldehyde and 2.45% glutaraldehyde, embedded in Historesin, serial-sectioned at 2.5 μm on a rotary microtome (Reichert Jung 2050), and stained with methylene-blue azur II and basic fuchsin. Microphotographs were taken on an Axiophot compound microscope (Zeiss). Images presented in this study were generated from scanned photographs (HP Scan Jet 5200C), merged, and equalized using Adobe PhotoShop 4.0 (Adobe Systems, Mountainview, CA).

### Cobalt labeling

The peripheral sensory cells in the plCOs1–6, and the central projections of the plCOs 1–3, were stained by introducing cobalt into their cut axons in N2 (axonal fills). For in situ fills, the animal was opened up and the cut end of N2 was placed in a vaseline pool containing 3% cobalt lysine solution (Gallyas et al., 1978). Alternatively, pieces of cuticle with the attached plCOs, or isolated ganglia, were dissected out together with the respective N2 and filled with cobalt lysine in vitro. Axonal diffusion was for about 24 hours at RT or 4°C. The cobalt ions were then precipitated with ammonium sulphide in insect saline. After fixation in Carnoy's solution for 1 hour at RT, the cobalt sulphide staining was silver-intensified after Bacon and Altman (1977).

### Laser interferometry

The slow, large-amplitude deflections of the lateral body wall that are part of the breathing movements were measured with a laser interferometer (Polytec OFV 3000 and OFV 501, LB-Electronics) while the activity of the plCO receptors was simultaneously recorded with electrodes as described above. The laser beam was focused on the surface of the lateral body wall, close to the lateral insertion point of the respective plCO, from a distance of 40 cm. The signal-to-noise ratio was improved by placing three to five reflecting glass spheres (Scotchlite no. 7610; weight about 0.2 μg each) on the cuticle.

## RESULTS

### General anatomy of the plCO in A1

A typical grasshopper such as the locust *Locusta migratoria* has a pair of hearing organs located laterally in the first abdominal segment (A1; Fig. 1b). The lateral body wall of this segment shows a thin (ca. 1 μm) cuticular specialization, the tympanic membrane. Associated with this membrane is the auditory receptor organ (Müller's organ), which is thought to have evolved from a plCO (Fig. 1d; Meier and Reichert, 1990). In striking contrast, the bladder grasshopper *Bullacris membracioides* lacks a thinned tympanic membrane in the lateral body wall of A1 (Fig. 1a) but nonetheless has a pear-shaped plCO much *larger* than Müller's organ present in the corresponding location (Fig. 1c). The size differential is reflected in the respective number of scolopidial sensilla: about 2,000 sensilla in the plCO1 of *Bullacris* (see Fig. 2a,b and below), versus 80 in the locust (Gray, 1960; Jacobs et al., 1999). It is attached to the pleural cuticle of A1 via two bundles of very long attachment cells. Cuticular insertions of the dorsal, thick bundle and a ventral, thin bundle are separated by more than 1 mm (Fig. 1c). Retrograde cobalt-lysine fills of nerve 2, which carries the axons of all 2,000 sensilla, show that the thin bundle (Fig. 1e) contains attachment cells for just ca. 30 of these. Within the plCO, the nerve branches into a number of separate bundles (Fig. 1e), but no clear anatomical grouping is evident among the sensilla (except for the small group forming the thin attachment branch).

Although the cuticle is nowhere as thin as a tympanal membrane in "modern" grasshoppers, some cuticular specializations are evident at the site of the plCO (Fig. 1f): a cuticular knob immediately next to the insertion point of the thick attachment bundle is surrounded by a thinner (20 μm) area of cuticle, about 1.9 mm in diameter, which is in turn separated from the surrounding cuticle (thickness about 25 μm) by a cuticular ring (thickness 80–120 μm). Figure 2 shows the arrangement and general organization of the scolopidial structures in plCO1 at the light-microscopical level. The plCO1 contains approximately 2,000 individual sensilla, as determined by counting the profiles in cross sections at the level of the attachment cells (Fig. 2a,b). Each sensillum comprises a bipolar sensory cell, a scolopale cell, an attachment cell, and a glial (Schwann) cell. Figure 2c shows the parallel arrangement of sensilla in longitudinal sections at the level of the scolopale.

A schematic overview of the ultrastructure of the distal dendritic region of the sensory cell with the scolopidium is shown in Figure 3. Distally the sensory cell gives rise to a dendrite, which is surrounded by a Schwann cell. The basal body is situated at the top of the dendrite (Figs. 3, 4a, insert). This is the beginning of the ciliary root, which surrounds the basal body in finger-like processes and then runs along the dendrite in the direction of the soma, where it splits off into several rootlets. Both ciliary root and rootlets are cross-striated with a periodicity of 60 nm. The basal body forms the basis of the cilium, which is arranged in the 9+0 structure typical of an insect sensory cilium. It is located in an extracellular canal formed by the scolopale cell (Figs. 3, 4a). In the middle of this extracellular space is an area of granular material. The canal is maintained by the scolopale rods situated in the scolopale cell. Five to seven rods are separated from each other in the middle of

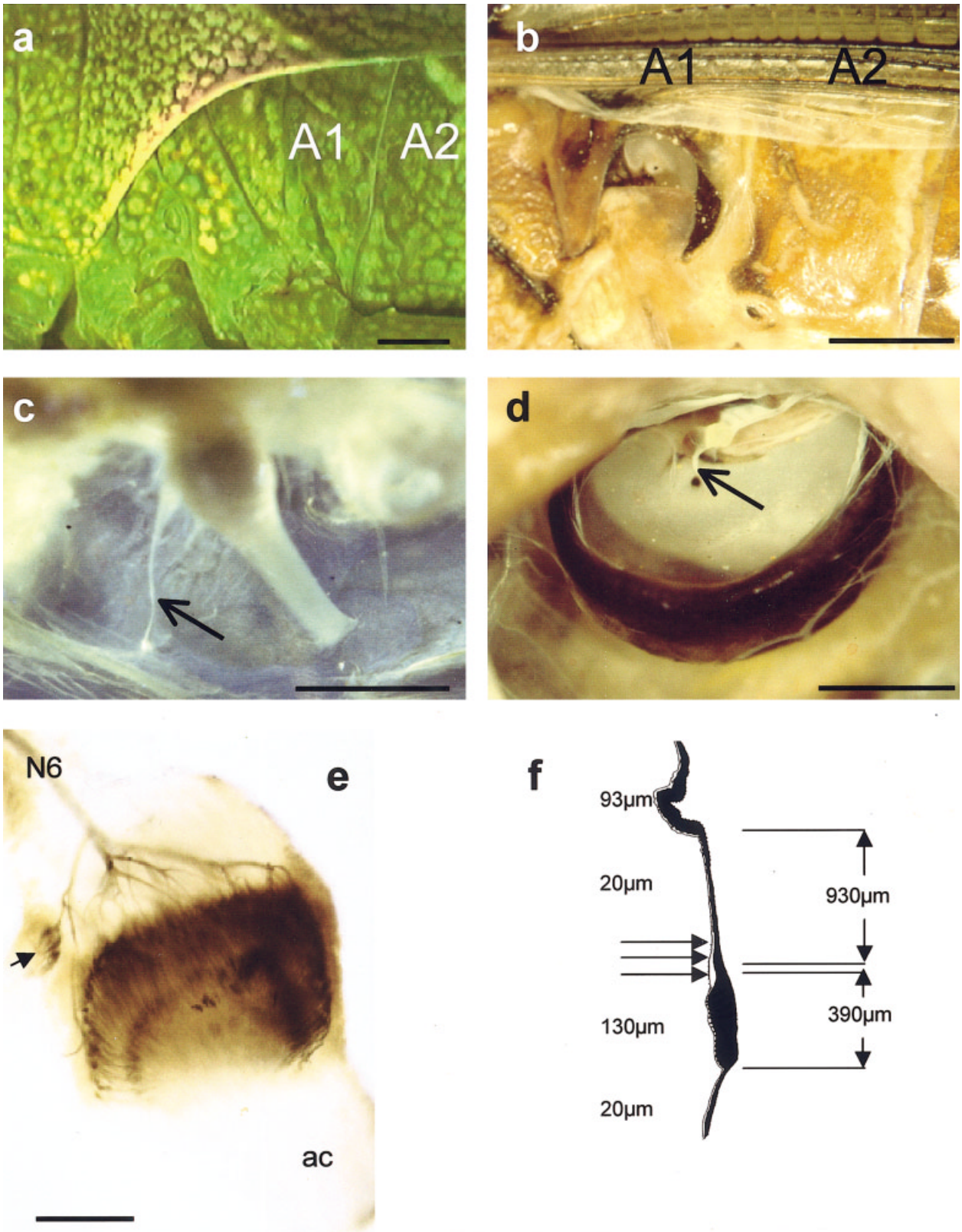



Fig. 1. Homologous chordotonal hearing organs in A1 of *B. membracioides* and the locust. **a**: External view of the location in *B. membracioides* showing the absence of an overt tympanum in the body wall of A1. **b**: External view of locust after removal of the forewing. **c**: Internal view of *B. membracioides* showing the CO with two attachment sites of the sensilla (arrow at the small bundle of attachment cells). Note the large size difference of the COs in the locust and *Bullacris*. **d**: Internal view of the so-called Müller's organ in the locust, attached to a thin, mostly translucent tympanum, with one group of sensory cells connected to the tympanum via attachment cells at the pyriform vesicle (arrow). **e**: Retrograde cobalt backfill of the CO in A1 in *B. membracioides* through nerve six (N6), showing staining of the axons, cell bodies and dendrites of the sensory cells. One group of 32 cells (arrowhead) separates from the majority and gives rise to the thin connection via attachment cells (ac; compare with Fig. 1d). **f**: Semithin section of the cuticle at the site of attachment of the thick bundle of attachment cells in A1 of *B. membracioides*. Although there is no frank tympanum at this site, the cuticle shows a degree of specialization with thickness reduced to about 20 µm, and a cuticular knob immediately next to the insertion point of attachment cells. Scale bars = 2 mm in a,b; 1.2 mm in c; 600 µm in d; 200 µm in e.

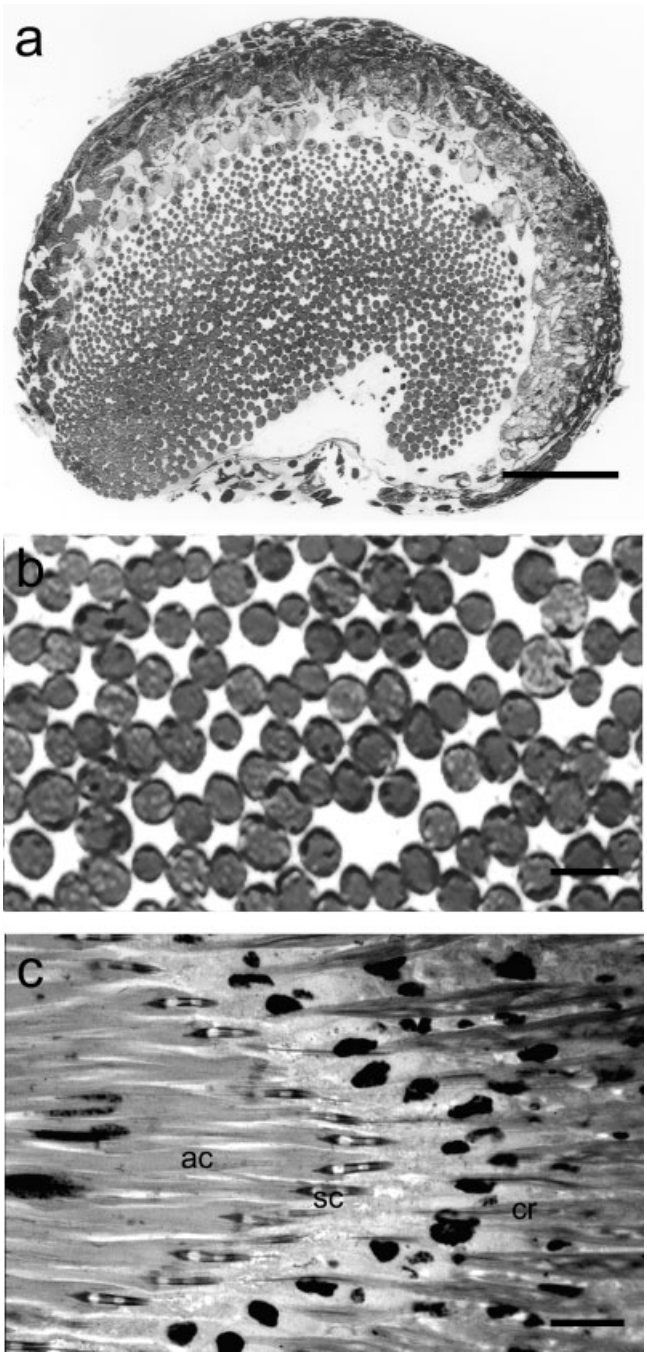



Fig. 2. Histology of the CO of *B. membracioides* in A1. **a**: Cross section of the thick bundle of sensilla at the site of the proximal end of the attachment cells. **b**: Cross section as in a, shown at higher magnification. **c**: Longitudinal section of the sensilla at the site of the scolopale. Compare with electron microscopy in Fig. 4a). ac. attachment cell; sc, scolopale; cr, ciliary root. Scale bars = 100 μm in a; 10 μm in b; 15 μm in c.

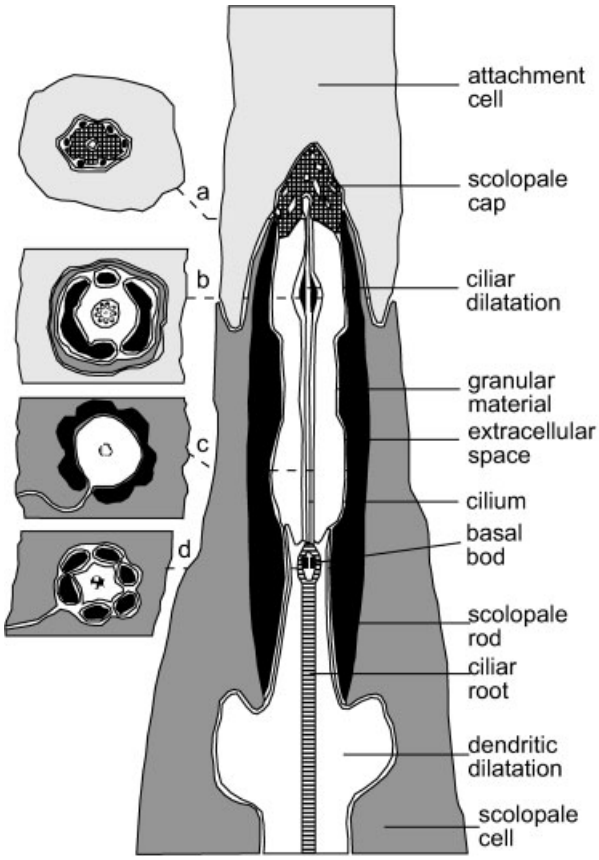



Fig. 3. Schematic overview of a scolopidium in the CO of A1, based on reconstruction of longitudinal and transverse serial ultrathin sections. Dotted lines and small letters indicate the level at which the following transverse sections were made. **a**: Cross section at the level of the scolopale cap. **b**: Cross section at the level of the ciliary dilatation. **c**: Cross section at the level where the sensory cilium runs through extracellular space. **d**: Cross section at the level of the basal body.

the scolopale, but at the scolopale edges the rods are connected to each other, forming a cylinder (Fig. 4d). The scolopale rods have an electron-dense appearance. The cilium protrudes to the scolopale cap at the tip of the scolopale cell.

Before entering the scolopale cap, the sensory cilium dilates and the 9 pairs of microtubuli surround a cylinder of electron-dense material. The scolopale cap is an extracellular structure consisting of electron-dense material penetrated by holes and cavities (Fig. 4a,b). The attachment cell constitutes the final cell of the sensillum. It surrounds the scolopale cap and is tightly attached to it by desmosomes. Attachment cells are extremely long (up to 1.4 mm), have very long nuclei, contain densely packed microtubuli, and are interconnected by desmosomes (Fig. 4c). At their distal end, attachment cells are tightly anchored to the epidermis via close interdigitation of their respective cells.

## Anatomy of the plCOs in A2–A6

In addition to the plCO in A1, five further pairs of plCOs exist in A2–A6 (plCO2–6; Fig. 5). In each of these more posterior segments, connective tissue strands span the pleural fold between the sternite and the tergite (arrows in Fig. 5b,c); the plCOs are thus suspended between the sternal apodeme on one side and their site of attachment to the lateral body wall on the other (Fig. 5c). Each pleural

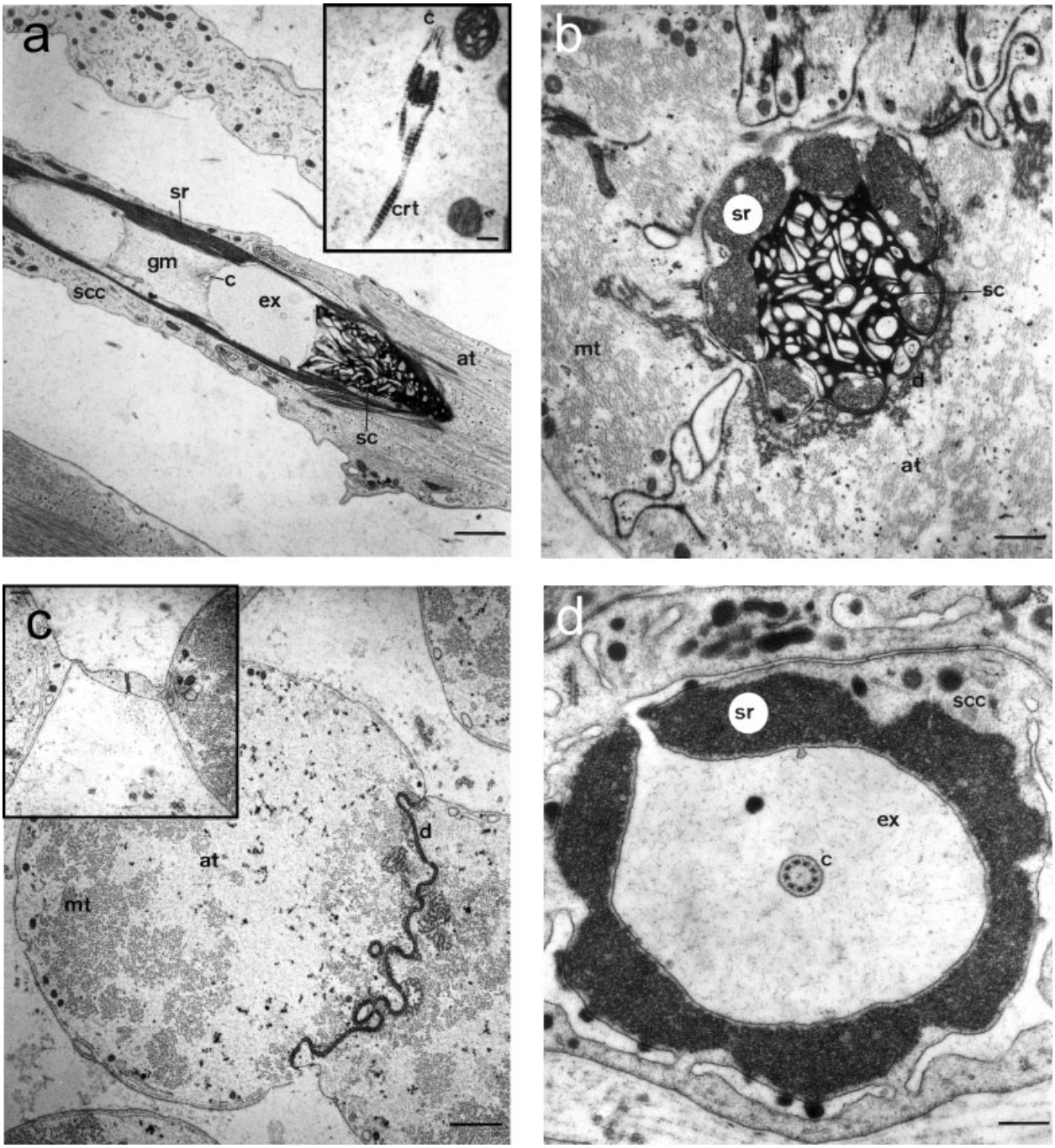
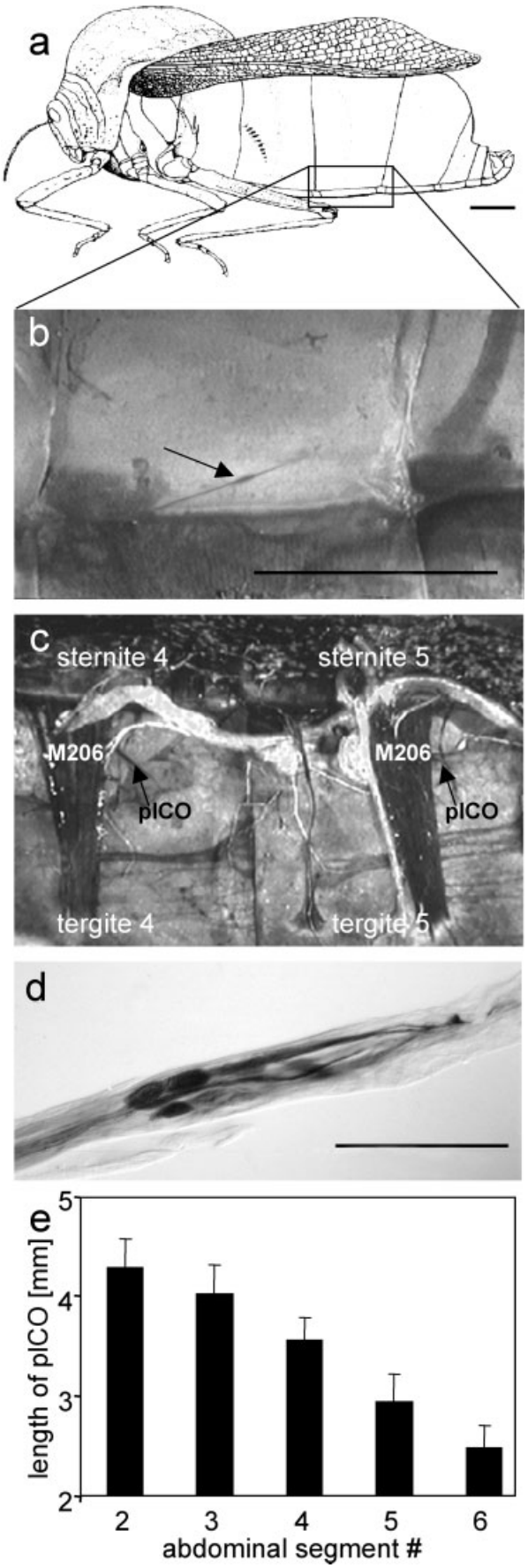



Fig. 4. Sensillum structure via electron microscopy. **a**: Longitudinal section of the scolopale (compare with Fig. 2c). The inset shows a longitudinal section of the basal body, where the ciliary root ends and the sensory cilium begins. **b**: Cross section of the scolopale cap, connected to the attachment cell by desmosomes. **c**: Cross section of attachment cells with connections by desmosomes. The inset shows another kind of connection between two attachment cells. **d**: Cross section of the scolopale in the area where the scolopale rods are connected to form a cylinder. at, attachment cell; c, cilium; crt, ciliary root; d, desmosome; ex, extracellular space; gm, granular material; mt, microtubuli; sc, scolopale cap; scc, scolopale cell; sr, scolopale rod. Scale bars = 1.7 μm in a; 0.25 μm in a (inset); 0.6 μm in b; 2.5 μm in c; 0.4 μm in d.

organ is associated with air sacs that emerge from longitudinally running tracheae. Cobalt backfills of the plCOs2–6 stained up to 11 sensilla per organ (Fig. 5d). Notably, the length of the plCOs (as measured from their point of insertion at the sternal apodeme to their attachment site at the lateral body wall) decreases from anterior to posterior (Fig. 5e). Because afferent projections from both the tympanal nerve (Rehbein, 1976; Riede et al. 1990;




Jacobs et al., 1999) and the serially homologous abdominal pleural organs (Hustert, 1978; Prier, 1999; Prier and Boyan, 2000) have been described in detail for grasshoppers, here we give only a brief summary of similar results from cobalt backfills in *B. membracioides*.

The afferent axons of the chordotonal organ in A1 enter the metathoracic ganglion through nerve 6 (the tympanal nerve in "modern" grasshoppers), branch within the ipsilateral median ventral association center of neuromere A1 (mVAC; Pflüger et al., 1988), and project anteriorly to form massive arborizations within the mVAC of T3. Similarly, pleural chordotonal organs in A2 and A3 enter the metathoracic ganglion via the serial homologous nerve to the tympanal nerve. The axons branch strictly ipsilaterally within the neuropile of their respective neuromere and, in addition, within the neuromere(s) of the more anterior neuropils. We observed no fibers of pleural organs A2 and A3 branching more anteriorly into the mesothoracic ganglion. However, it is possible that the stainings were simply not extensive enough to demonstrate such arborizations.

## Physiological responses of plCOs to sound

All six pairs of pCOs responded to acoustic stimulation within a biologically meaningful intensity and frequency range. However, the best frequencies and thresholds of the plCO1 differed significantly from those of the pCOs 2–6. In males, alternate males, and females alike, the best frequency of pCO1 was 4 kHz (Fig. 6a,b), which is well above the male song's carrier frequency of 1.7–2 kHz. Despite the absence of an overt tympanum, the organ is extremely sensitive at its best frequency, particularly in inflated males (absolute threshold = 12.8 ± 4.9 dB SPL; n = 5); in alternate males, this value was somewhat higher at 19.5 ± 3.8 dB SPL (n = 4; Fig. 6b). Females showed the highest variation in absolute threshold, with individual values ranging from 14 to 42 dB SPL. A comparison between old, gravid (and thus heavy) females and young, light females showed a correlation between hearing thresholds and reproductive status and/or age, with older females being less sensitive (Fig. 6a).

In contrast to plCO1, the plCOs 2–6 all had best frequencies that matched the carrier frequency of the conspecific male signal (1.5–2 kHz; van Staaden and Römer, 1998). Although it was not possible to determine the thresholds of individual sensilla in the multiunit recordings, it appears that all sensilla within one plCO are similarly tuned to frequencies between 1.8 and 2.3 kHz. The plCOs2–6 were considerably less sensitive than

Fig. 5. Characteristics of the pleural chordotonal organs (plCO) in adult male *B. membracioides*. **a**: Position of plCO in A4. **b**: External view of the plCO (arrow) after intense staining with Janus green; oriented as in a, with anterior at left. **c**: Dorsal view into the left pleural fold of abdominal segment A4 and A5, after removal of some trachea and muscle M 209. The connective tissue strands span through the pleural fold between tergites and sternites in each segment. The organ is stretched between the sternal apodeme on one side, runs under muscle M206, and attaches at the lateral body wall. **d**: Phase contrast microscopy of a plCO in A5 after retrograde backfill with cobalt stains some of the sensory cells and long dendrites. **e**: Total length of plCO organs in the abdominal segments A2–A6 of male *B. membracioides*. Length was measured from the point of insertion at the sternal apodeme to the site of cuticular attachment. Means are calculated from measurements of seven organs in each segment. Scale bars = 5 mm in a,b; 100 μm in d.

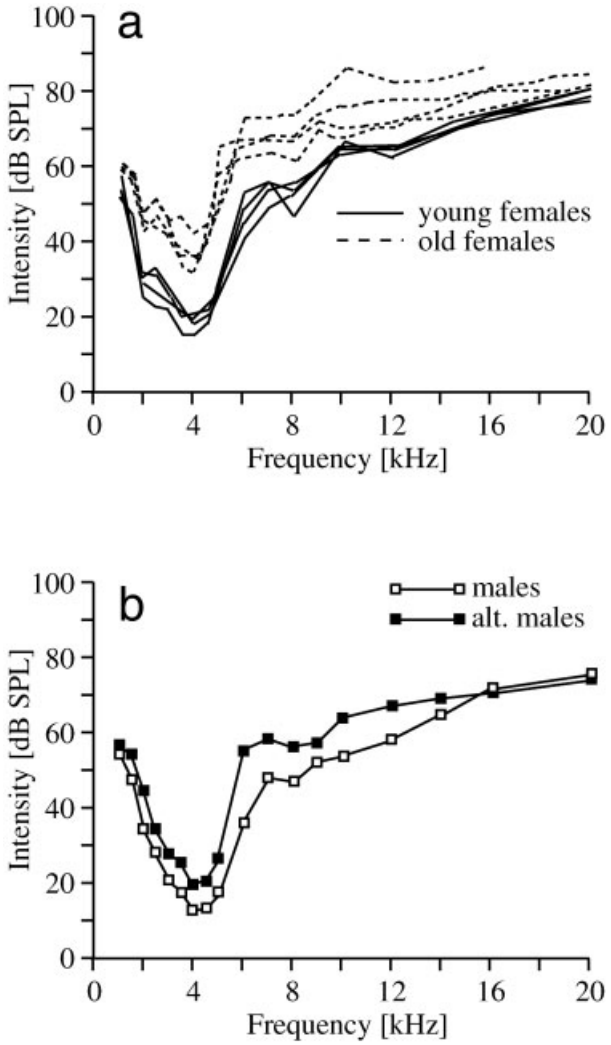



Fig. 6. Tuning curves for the CO in A1 of *B. membracioides* females (a) and males (b). **a**: Individual tuning curves for four young and four old (broken lines) females. As a result of many eggs in the abdomen of older females, they also differed significantly in weight (mean 4.87 g compared witho 3.95 g). **b**: Tuning curves of regular, inflated males (open squares; n = 13) and alternate males (n = 6; filled squares).

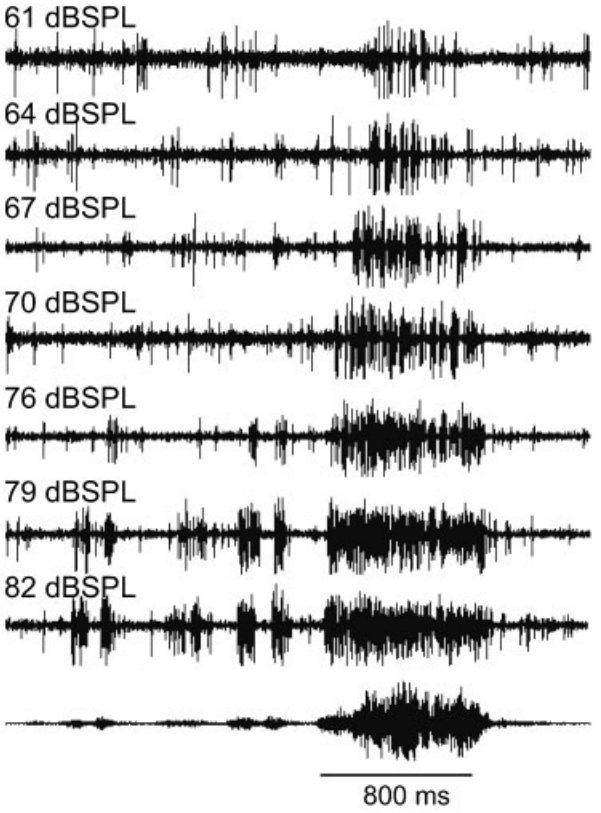



Fig. 7. Multiunit recording of the activity of a female plCO in A2 in response to increasing SPL of the male calling song. The male call consists of five low-intensity introductory syllables and a final, resonant syllable at a carrier frequency of about 1.7 kHz. Note that the temporal structure of the male call is well represented in the discharge of the fibers of the plCO at sound pressure levels about 20 dB above threshold.

plCO1, although their thresholds are still low enough for them to detect the male calling song (see below). Moreover, mean tuning curves and thresholds at 2 kHz demonstrate that sensitivity decreases from the more anterior (A2) to the more posterior (A6) segments (thresholds: mean 58–77 dB SPL; range 56–85 dB SPL). No differences in sensitivity to sound were, however, found between the plCOs2–6 of males and females.

The tuning of the plCOs2–6 to a frequency of about 2 kHz favors the detection of the conspicuously loud male calling song (98 dB SPL at a distance of 1 m). This song is highly stereotyped, consisting of five short (50–70-msec) introductory syllables of low intensity and a single long (870-msec) final note. Figure 7 shows representative responses of a female plCO2 to male calls of increasing loudness, as seen in extracellular recordings from nerve 1 in A2. The multiunit recording shows action potentials with different amplitudes (i.e., more than one unit firing), and a threshold of about 60 dB SPL to the final (loudest) syllable. With increasing SPL, the response to the last syllable intensifies, and about 15–20 dB above threshold, the temporal pattern of spikes in the nerve copies the temporal pattern of all six syllables in the calling song.

*Bullacris* males call at night, when the transmission conditions for their calls are close to ideal in the natural habitat; attenuation rates close to that of geometric spreading alone result in hearing distances of 1.5–1.9 km for plCO1 (van Staaden and Römer, 1997). Unlike plCO1, however, plCOs2–6 are tuned to the male carrier frequency. Together with the high SPL of the male call, this suggests that plCOs2–6 also respond at considerable sender-receiver distances. Figure 8 shows theoretical distance-response functions constructed by combining field data for the mean nocturnal SPL of the male call at various distances in the habitat with the intensity-response functions of plCOs obtained in the laboratory. Because plCO2 has a mean threshold of 60 dB SPL, it will be activated in the field at distances of about 80–100 m to a calling male. With decreasing distance, it is activated more strongly according to both its intensity-response–function and the attenuation properties of the transmis-

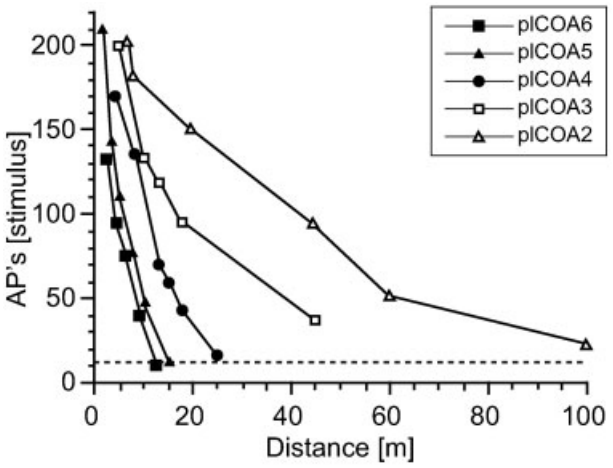



Fig. 8. Distance-response functions of the discharge of pleural chordotonal organs (plCOs) in A2–A6 in response to the male calling song, recorded in a single female. The distance-response function for each CO was obtained by using data for the attenuation of male calling song in the field from van Staaden and Römer (1998) and transferring these values into the IR-function obtained in the laboratory. Note that each plCO exhibits a different detection distance (intersection with the dashed line, representing the mean spontaneous activity in all five organs). For each given distance, starting from the maximum detection distance for plCO in A2 of about 80–100 m, the relative sensory activity within the set of plCOs varies. The distance of the male from the female is thus, at least partly, encoded in the output of these serially repeated hearing organs.

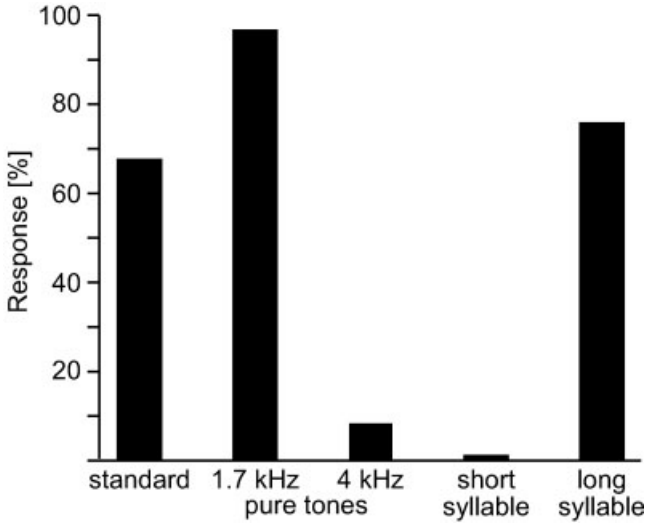



Fig. 9. Acoustic response of females to a standard male call and to various synthetic models of the call. Females never responded to models in which the last syllable was shortened from 860 to 200 msec, and they responded significantly more often to a pure tone of 1.7 kHz (optimal frequency of plCOs in A2–A6) compared with 4 kHz (optimal frequency of CO in A1).

sion channel. More posterior plCOs have higher thresholds and are therefore activated at closer range. The least sensitive plCO in A6 reaches threshold only at distances close to 10 m and exhibits a steep distance-response function with shorter distances. Consequently, both the number of activated plCOs and the level of activation within each plCO provide reliable sensory information to a female about her distance to a calling male.

## Behavioral function of pleural organ receptors

It has been previously shown that females respond to the male call with an acoustic reply after bilateral ablation of plCO1, an experiment that clearly establishes the role of the more posteriorly located plCOs as functional hearing organs (van Staaden and Römer, 1998). Playback experiments further reveal that this behavioral response is elicited much more reliably at 1.7 than at 4 kHz (Fig. 9). With a playback intensity of 75 dB SPL, a pure tone of 4 kHz elicited a female response in only 8% of presentations, whereas a pure tone of 1.7 kHz was successful in 96% of presentations (n = 76). This is true even though 75 dB SPL is about 40 dB above the threshold of the hearing organ in A1 in the average female (compare with Fig. 5), but only 15 dB above the threshold of the most sensitive pleural organ in A2. Moreover, in order to evoke acoustic responses from receptive females, a male call must exhibit a long-duration syllable, such as the final syllable in the natural male call. In playback experiments, females never responded to syllables reduced in duration to 200 msec.

## Exteroceptive function of plCOs2–6

The above experiments clearly confirm the role of plCOs as "ears," far-range exteroceptors for airborne sound. However, because of their location in the pleural fold, we cannot exclude, a priori, a function as proprioceptors for monitoring ventilatory or other movements, as shown in other grasshoppers (Hustert, 1975). We therefore recorded the ventilatory movements of a tergite by using laser interferometry, while simultaneously recording electrically from the corresponding plCO; Figure 10 shows such an experiment in A3. In the absence of acoustic stimuli, the multiunit discharges in the nerve were not correlated to the ventilatory movement of the tergite, even though the ventilatory movements were of high amplitude in this preparation. However, the response of the plCO receptors to sound was clearly modulated by the ventilatory movements. When the preparation was stimulated with a pure tone (1.8 kHz for 5 seconds), the response was strongest during inspiration, i.e., when the pleural fold, and thus the strand with the plCO, were stretched.

# DISCUSSION

In modern grasshoppers, hearing is mediated by tympanal sensory organs situated laterally in the first abdominal segment. Following the design principles common to all tympanate insects, such ears are composed of a thin membrane backed by a tracheal air space. Connected to the membrane are mechanoreceptors that transform mechanical energy into neural signals. Bladder grasshoppers of the ancient family Pneumoridae are clearly deficient in the first diagnostic feature characterizing tympanal hearing in insects, namely, a localized, often translucent thinning of the cuticle (the tympanum; compare with Fig. 1a,b). They do, however, share with other insect hearing organs both the typical chordotonal sensilla and the air-

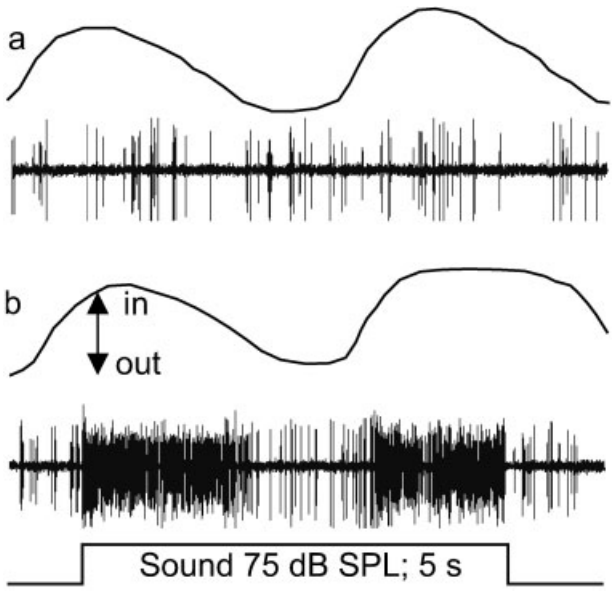



Fig. 10. Simultaneous recording of tergite movement and activity of plCO receptors in abdominal segment 3. Movement was measured close to the site of attachment of the plCO by means of laser interferometry without mechanical contact. **a**: There is no correlated activity in the receptors as a result of large-amplitude in-and-out ventilatory movements. **b**: The response to simultaneously presented low-frequency sound is strongly downmodulated with expiration, i.e., when the pleural folds are not expanded, and the pleural organ is not stretched.

filled sac of tracheal origin (Hoy, 1998). Typical tympanal membranes of acridid grasshoppers, crickets, or parasitoid flies are formed by the apposition of a thin exocuticle and a tracheal air sac and are between 1 and 5 μm thick (Michel, 1974; Michel and Petersen, 1982; Robert et al., 1994). However, the lateral body wall at the site of attachment of the CO in the abdominal segment A1 of *B. membracioides* is about 20 μm thick. This approximates the dimensions in the third larval instar of locusts, which exhibit a sensitivity about 20 dB less than that of the adult ear (Petersen et al., 1982). Decreased sensitivity in association with reduced tympanal development has also been reported for several other acridid species (Riede et al., 1990).

Despite the lack of an overt tympanum, the sensitivity of the pneumorid ear in A1 is extremely high. Average thresholds of 13 dB SPL (inflated males) or 20 dB SPL (young females) at 4 kHz are the best sensitivity ever reported for grasshoppers. We currently have little indication how this high sensitivity comes about, although microscanning laser-Doppler vibrometry revealed highest levels of vibrations of the body wall at 4 kHz in A1 (Robert, van Staaden, and Römer, unpublished data).

There are striking similarities and differences between the chordotonal organ in A1 of *B. membracioides* and modern grasshoppers. In contrast to the locust ear, which has about 80 individual sensilla, the organ in A1 of *B. membracioides* is composed of about 2,000 sensilla (Fig. 2a,b), close to the highest number reported for the auditory organ of cicadas (Doolan and Young, 1981). This contrasts markedly with only 11 sensilla found in the more posterior pleural chordotonal organs in A2–A6, which is similar in modern grasshoppers (10–15) and *B. membracioides*. It is unclear whether 2,000 sensilla is the ancestral or derived condition due to mutations such as those in the *rhomboid* or *abdominal*-A genes that affect the number of sensilla in serially homologous organs of *Drosophila* (Meier et al., 1991). Increasing mechanosensory sensilla might stem from the evolution of specialized adaptive behavior, such as "vibrational sounding" in some parasitoid wasps (Wäckers et al., 1998; Broad and Quicke, 2000).

To deposit their eggs, females need to find hosts concealed in the plant substrate. To do this, they produce pulses of sound by tapping the substrate with their antennae and detecting the reflected echoes with subgenual organs (SGOs) in the legs. Only females perform this task, and their SGOs are particularly enlarged compared with males. In the subfamily Orussidae (Hymenoptera) SGOs contain 300–400 sensilla per organ (Vilhelmsen et al., 2001), which is an order of magnitude higher than most insects (Field and Matheson, 1998). This small wasp taxon shares prominent plesiomorphic characters with the symphyta (Vilhelmsen, 2000); thus the high number of sensilla may well represent the plesiomorphic character in the Hymenoptera, rather than the endpoint of very specialized mechanosensory function. The second difference concerns the length of the attachment cells in A1. In *B. membracioides* these are about 1.4 mm long, significantly longer than the 0.1 mm found in modern grasshoppers (Gray, 1960). In this regard, the organ more closely resembles the pleural system, where the length of attachment cells varies between 0.8 and 2.5 mm.

An obvious similarity in the arrangement of sensilla in the A1 hearing organ of modern grasshoppers and *B. membracioides* is the separation of attachment sites for two subgroups of sensilla at the cuticle and tympanum, respectively. In the locust ear, the high-frequency d-cell receptors are one of four (Gray, 1960; Römer, 1976) or three (Jacobs et al., 1999) groups of receptors in the ear attached to the pyriform vesicle, which is separated from the attachment sites of the remaining groups (Fig. 1b,d). *B. membracioides* also exhibits a distinct separation between the attachment sites of a group of 32 sensilla from the mass of 2,000 sensilla by more than 1 mm at the cuticle of the lateral body wall (Fig. 1c,e). This morphological arrangement suggests that the group of 32 sensilla may well represent the ancestral precursors of the 12–14 high-frequency d-cells in modern grasshoppers. However, we currently have no information as to the possible differential tuning of receptors in the A1 organ of *B. membracioides*. Tuning curves for receptors in A1 (Fig. 6) were obtained from whole nerve summed action potential recordings, revealing the highest sensitivity to be at 4 kHz. It may well be that in the afferent nerve carrying more than 2,000 axons of very small diameter, the responses of the small fraction of 32 axons possibly tuned to high frequencies are overshadowed by the larger number tuned to 4 kHz.

Alternatively, the situation in *B. membracioides* may resemble that of cicadas, in which recordings of summed action potentials from the afferent nerve show a uniform tuning to low frequencies between 3 and 6 kHz, but interneurons are sharply tuned to different frequencies (Fonseca et al., 2000). The separation of axons into various fiber bundles (Fig. 1e) after retrograde backfilling into the

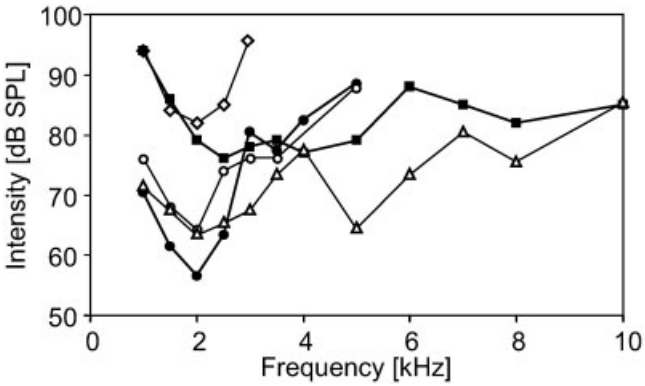



Fig. 11. Comparison of the tuning of plCO of *B. membracioides* with different atympanate CO in various insects; prothoracic CO attached to the prothoracic cervicosternite in the locust (Pflüger and Field, 1999; open triangle); CO in the posterior metathorax of an atympanate moth (Yack and Fullard, 1990; open diamond); tibial organ in the cockroach leg (Cokl and Virant-Doberlet, 1997; closed circle); sexually dimorphic, midline ear of the praying mantis (Yager, 1990; closed square); plCO3 in *Bullacris* (open circle). Note that all organs are most sensitive at frequencies around 2 kHz, despite their rather different position and morphological arrangement in the body.

organ may represent some form of morphological, and possibly functional, frequency grouping within the sensilla in A1. The nonuniform distribution and range of diameters of attachment cells certainly warrants further investigation, and single-fiber recordings of either auditory afferents or single interneurons of the auditory pathway are required to determine whether the organ in A1 is capable of frequency discrimination.

The pleural organs in the segments A2–A6 are all tuned to a frequency around 2 kHz, which is close to the carrier frequency of the male calling song at 1.8 kHz (van Staaden and Römer, 1997, 1998). Because this is true for both males and females alike, tuning cannot be attributed to the mechanical resonance properties of the inflated abdomen present only in the adult male. Furthermore, male and female pleural organs do not differ significantly in their thresholds. Thus, the abdominal resonator acting as an efficient sound radiation device does not simultaneously enhance the sensitivity of the serially homologous organs. This finding poses an interesting question regarding the reason for such "matched" tuning to the male call.

Here we argue, by comparison with COs on various body parts of other insects, that highest sensitivity close to 2 kHz is a common tuning property of such organs and not an adaptation to the specific carrier frequency of the *B. membracioides* male call. Figure 11 illustrates examples of tuning curves reported in the literature for a variety of CO forms including a prothoracic CO attached to the prothoracic cervicasternite in the locust (Pflüger and Field, 1999), a CO in the posterior metathorax of an atympanate moth (Yack and Fullard, 1990), sensilla in the atympanate tibial organs of cave crickets (Cokl et al., 1995), the tibial organ in the cockroach leg (Shaw, 1994; Cokl and Virant-Doberlet, 1997), and the sexually dimorphic, midline ear of the praying mantis (Yager, 1990). No doubt this list could be supplemented with other examples of insect hearing noted by Yack and Fullard (1993), which require further anatomical and/or physiological study. The cases illustrated are all most sensitive to a narrow frequency range between 1.4 and 2.5 kHz, but they exhibit variable thresholds from 55 to 80 dB SPL, almost identical to the range of thresholds covered by the plCOs in segments A2–A6 of the ancestral insect studied here.

Despite differences in the fine structure of the COs and location on the insect's body, the sensilla are tuned to similar frequencies. (A somewhat broader tuning to frequencies between 1 and 5 kHz at thresholds higher than 70 dB SPL has been found in the forelegs of the atympanate bushcricket *Phasmodes ranatriformes* (Tettigoniidae), which is assumed to represent the ancestral condition in the evolution of the hearing organ in the forelegs of ensiferans (Lakes-Harlan et al., 1991). This suggests that mechanical tuning is unlikely and favors an electrical explanation. If this is the ancestral condition of COs for the detection of airborne sound in acridids or pneumorids, it would represent a preadaptation (exaptation) for the evolution of a long-distance male call, at a frequency at which the female organs are most sensitive. Further steps in the evolutionary transition to tympanal organs may simply increase either the range of detectable frequencies and/or the sensitivity to ultrasound.

Surprisingly, a CO associated with a well-developed tympanum at the base of the forewing in the blue cracker butterfly, *Hamadryas feronia*, exhibits both tuning and sensitivity similar to that of the atympanal COs summarized in Figure 11 (Yack et al., 2000). The authors speculation that this Vogel′s organ represents a degenerate bat detector is corroborated by the changes in hearing found in diurnal moths: a loss of high-frequency sensitivity and tuning to lower frequencies (Fullard et al., 1997; Surlykke et al., 1998). It remains to be seen whether these ears show morphological modifications similar to those reported for some acridid species (Riede et al., 1990).

Shaw (1994) presented a hypothesis for the evolution of a hearing organ in ensiferans through modification of a pre-existing, vibration-detecting CO, the subgenual organ. He creates a scenario in which the plesiomorphic character is an SGO suspended in hemolymph between opposite walls of the tibia, as is found in some extant termites. Because the vibrations are transmitted through the hemolymph, the organ is relatively insensitive to airborne sound. Coupling such a system to a tracheal expansion, as in the cockroach, increases the sensitivity to vibrations and preadapts the system to respond to airborne sound entering the tracheal system through spiracles in the body wall. Further modifications such as the development of one or two tympana, and a sophisticated tracheal system associated with the CO, facilitate the detection of sound in crickets and bushcrickets. Similarly, physiological and anatomical evidence suggests that the prosternal ear in parasitoid flies evolved from a preadaptive, vibration-sensitive CO in non-hearing flies (Lakes-Harlan et al., 1999).

In the case of the plCOs of *B. membracioides,* we have shown that they do not function as proprioreceptors in the context of abdominal ventilation but respond at biologically meaningful intensities to the male calling song (Figs. 7, 8). This contrasts with the homologous abdominal organs in modern grasshoppers and locusts, in which receptors function as proprioreceptors for monitoring ventilatory movements, but not sound (Hustert, 1975). Hence, the organs in *B. membracioides* are well suited for responding to sounds produced by mates and rivals. Using

bilateral ablation experiments with the COs in A1, we have indeed shown that the behavior of females (acoustic responses during duetting) is mediated via the pleural organ receptors (van Staaden and Römer, 1998). However, although the discharge of the pleural organs in *B. membracioides* was not modulated with large ventilatory movements of the lateral body wall, a proprioreceptive response is still possible in other behavioral contexts; for instance, egg deposition by the female involves large telescope-like extension and retraction of the abdominal segments. This would be similar to the prothoracic cervicosternite CO in the locust, which combines features of a proprioreceptive mechanoreceptor and a hearing organ (Pflüger and Field, 1999).

Although the pleural receptors do not respond to ventilatory movements alone, their response to airborne sound is modulated by ventilation (Fig. 10). During inspiration, when the stretching of the ligament formed by the attachment cells within the pleural fold is highest, the response to sound is maximal. In the experimental situation of the restrained animal, in which lateral body wall deflections through ventilatory movements can be unusually large (Hustert, 1975), we found that acoustic responses may be downmodulated by about 15 dB during expiration (Fig. 10). One may argue that this renders the processing of signals too noisy for reliable signal detection or discrimination. However, the acoustic behavior of females responding to males at different distances (calls of different SPLs) is rather reliable and does not indicate high variance (van Staaden and Römer, 1998). This could come about either by less pronounced modulation of acoustic responses in unrestrained animals, or by acoustically interacting insects, reducing the amount and/or frequency of ventilation.

Our results confirm developmental studies by Meier and Reichert (1990), showing that in abdominal segments A2–A8 of grasshoppers, receptor groups differentiate at the same location as the auditory tympanal organ in segment A1. Abdominal receptors form the pleural COs in the adult insect, demonstrating that the auditory organ is ontogenetically a segmental specialization of a serially repeated system of COs. They also investigated the embryonic development of these receptor groups in another atympanate grasshopper (*Heide amiculi;* Eumastacoidea), confirming that the plCO in A1 develops in the same position as those in modern grasshoppers, but because the receptors do not become associated with the cuticular and tracheal apparatus of functional ears, the insect is deaf. Precisely which parameters of the cuticle and association with the tracheal apparatus turn a proprioreceptive CO into a sound-detecting device, albeit with reduced sensitivity, remains to be determined. Morphological comparison of such insects completely deaf to airborne sound and the plCOs in *B. membracioides* should prove enlightening.

The plCOs of *B. membracioides* in segments A2–A6 are all tuned to the same frequency around 2 kHz but exhibit different thresholds; those positioned anteriorly are more sensitive than the posterior ones. This results in a range fractionation of responses to the male call at different distances. Although the most sensitive plCO in A2 has a threshold of 60 dB SPL (a full 40 dB higher than the CO in A1), it responds at distances of about 80–100 m from the male. This is an extraordinarily high value if one considers that the tympanal organ in A1 of modern grasshoppers shows detection distances to conspecific mating calls in the field of only 2 m (Gilbert and Elsner, 2000; Lang, 2000). Even the least sensitive plCO in A6 of *B. membracioides* responds at such a short distance. One should point out, however, that this is due to both the extremely intense, resonant male call, and the excellent sound transmission conditions that result from temperature inversions after sunset (van Staaden and Römer, 1997). In contrast, the SPL of calling songs in other grasshopper taxa is rather low. Moreover, they contain frequencies up to 40 kHz and are thus more strongly attenuated in the grass habitat close to the ground (Gilbert and Elsner, 2000).

TABLE 1. Comparison of Pleural Chordotonal Organs (pICOs) of *B. membracioides*

| Ear | No. of sensilla | Best frequency (kHz) | Sensitivity (mean ± SD) |
|---|---|---|---|
| pICO1 | 2,000 | 4.0 | 20.1 ± 7.9 |
| pICO2 | 9–11 | 1.5–2.0 | 58.0 ± 3.4 |
| pICO3 | 9–11 | 1.5–2.0 | 67.2 ± 4.2 |
| pICO4 | 9–11 | 1.5–2.0 | 71.9 ± 5.3 |
| pICO5 | 9–11 | 1.5–2.0 | 75.2 ± 7.6 |
| pICO6 | 9–11 | 1.5–2.0 | 77.8 ± 4.7 |

The range fractionation observed in the plCOs of the abdominal segments A2–A6 is a common principle in sensory systems (Cohen, 1964) and applies to proprioreceptors as well as to exteroreceptors (for review, see Field and Matheson, 1998). Although we do not know why the anterior plCOs are more sensitive than the posterior ones, there is an obvious correlation between sensitivity and length of plCOs, as measured from the point of insertion at the sternal apodeme to the site of cuticular attachment (Fig. 5e). This points to a mechanical basis for range fractionation, as has been reported for the proprioreceptive femoral chordotonal organ (feCO) in the locust hind leg (Field, 1991; Shelton et al., 1992). In this organ, the viscoelastic ligament joining the feCO to the tibia consists of strands (attachment cells) of different lengths, which are sequentially tightened during flexion of the tibia. This leads to a range fractionation of stretch-sensitive responses during tibial flexion and of relaxation-sensitive responses during tibial extension.

We have shown in a previous paper that the plCOs of females are involved in acoustic duetting with males; females still responded to the male call after bilateral removal of the COs in A1. Moreover, females add approximately one syllable to their acoustic response for each 3-dB increase in SPL of the male call, which may well represent the behavioral outcome of the physiological range fractionation of plCOs (van Staaden and Römer, 1998). This implies some form of temporal and/or spatial integration at the neuronal level of the auditory pathway. Our own results on the afferent projection of plCO afferents from A1 to A3, and the more extensive survey by Prier and Boyan (2000) of afferent projections of pleural, tympanal, and winghinge COs in the locust, have shown that they all arborize in areas of neuropil such as the median ventral association center (mVAC). This projection pattern is a feature of CO afferents in all tympanate and several atympanate insects (Pflüger et al., 1988; Boyan, 1993). In a systematic survey of synaptic inputs of afferents onto identified interneurons in the locust, Prier and Boyan (2000) found that neuron 714 received excitatory and bilateral input from all the serially homologous

COs tested, from the second thoracic to the seventh abdominal segment. Another interneuron (531) receives input only from the (tympanal) afferents of the first abdominal segment. Such convergence of synaptic input onto auditory interneurons and spatial summation may account for the observed graded behavioral acoustic responses of females.

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