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Research

Sensing of Substrate Vibrations in the Adult Cicada *Okanagana rimosa* (Hemiptera: Cicadidae)

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Abstract

Detection of substrate vibrations is an evolutionarily old sensory modality and is important for predator detection as well as for intraspecific communication. In insects, substrate vibrations are detected mainly by scolopidial (chordotonal) sense organs found at different sites in the legs. Among these sense organs, the tibial subgenual organ (SGO) is one of the most sensitive sensors. The neuroanatomy and physiology of vibratory sense organs of cicadas is not well known. Here, we investigated the leg nerve by neuronal tracing and summed nerve recordings. Tracing with Neurobiotin revealed that the cicada *Okanagana rimosa* (Say) (Hemiptera: Cicadidae) has a femoral chordotonal organ with about 20 sensory cells and a tibial SGO with two sensory cells. Recordings from the leg nerve show that the vibrational response is broadly tuned with a threshold of about 1 m/s^2 and a minimum latency of about 6 ms. The vibratory sense of cicadas might be used in predator avoidance and intraspecific communication, although no tuning to the peak frequency of the calling song (9 kHz) could be found.

Key words: vibration detection, neuroanatomy, scolopidial organ, neurobiology, Hemiptera

Cicadas are in a group of insects that are well known for their acoustic communication. These intraspecific acoustic communication signals are important for sexual behavior and reproduction (Alexander and Moore 1958, Weber et al. 1988, Sanborn and Phillips 1999, Cooley and Marshall 2001, Boulard 2006, Fonseca 2014). Correspondingly, cicadas have a sophisticated hearing system, with one of the largest numbers of auditory sensory cells among the insects (about 1,000 cells) (Huber et al. 1980, Fonseca 1993, Daws and Hennig 1995/96, Fonseca et al. 2000, Strauß and Lakes-Harlan 2009). Clearly, the acoustic signal is the long-range cue for mate finding in most cicada species.

Detection of vibrations is an evolutionarily old sensory modality and serves different functions (Drosopoulos and Claridge 2006, Lakes-Harlan and Strauß 2014). In general, vibrational communication is important in many hemipteran species (Cokl and Virant-Doberlet 2003). Leafhoppers have an elaborated vibratory signaling, especially in response to approaching predators (Cocroft 1996, Cocroft et al. 2000). Primitive cicada species produce substrate-borne signals instead of airborne signals (Claridge et al. 1999). Vibratory signalling might be important also in other cicadas. It is known that during production of airborne calling song, a part of the signal energy is emitted as substrate vibrations (Stölting et al. 2002). The peak frequency of these substrate vibrations is with about 9 kHz the same as that of the airborne sound. In the short range, these signals might assist phonotaxis, especially within bushes or trees as has been shown for Orthoptera (Latimer and Schatral 1983, Weidemann and Keuper 1987, Hill 2008), and improve localisation of the caller in a group of individuals. Vibrational signals could be produced also during courtship, when different movements, including wing flips, have been noted by signaling (Dunning et al. 1979). Besides of the function in communication, any perception of vibrations may be presumed to facilitate predator detection.

For the detection of substrate vibrations insects use mainly scolopidial (chordotonal) sense organs (reviews: (Field and Matheson 1998, Lakes-Harlan and Strauß 2014)). These organs occur in different numbers in the body and appendages of probably all insects. Hemiptera possess a femoral chordotonal organ (feCO), a tibial subgenual organ (SGO), a tibio-tarsal chordotonal organ, and tarsal chordotonal organs in their legs (Debaisieux 1938, Wiese 1972, Michel et al. 1982). However, the neuroanatomy of sense organs of cicadas is only poorly known (Nishino et al. 2016). Vibrational sensitivity has only been shown in Heteroptera. In bugs, threshold curves and response properties of subgenual receptor cells have been characterized (Cokl 1983, Cokl et al. 2014).

Here, we investigated the neuroanatomy of the vibratory sense organs of legs of the cicada *Okanagana rimosa* (Say). Furthermore, we investigated, for the first time, the neurophysiology of the vibratory sense organs of a cicada. The physiological data might also reveal whether the leg vibration sense organs are tuned to 9 kHz (peak frequency of the calling song) and thereby indicate a role in species-specific communication.

Materials and Methods

Adult females of the protoperiodical cicada *O. rimosa* were caught in the open aspen forest around the Biological Station of the University of Michigan, Pellston, MI, in July 2013 and June 2017. Animals were kept in cages with fresh aspen branches or with fresh acer branches and used within 2 or 3 d for the experiments.

Neuroanatomy of the Legs

The neuroanatomy of the legs and its sense organs was visualized by retrograde backfills of the leg nerves. The leg was isolated and preparation took place in a silicon-covered Petri dish in locust saline, pH = 7.2 (Clements and May 1974). Nerves were dissected in the coxa or femur, and free nerve endings were placed in glass capillaries filled with Neurobiotin (Vector Laboratories, Burlingame, CA). Preparations were placed in a moist chamber for 2–3 d at 4–7°C. Thereafter, the glass capillary was removed, femur and tibia dissected, and the preparations were fixed in chilled 4% paraformaldehyde (Sigma Chemicals, St. Louis, MO) solution in phosphate buffer (0.04 mol/liter Na_2HPO_4, 0.00574 mol/liter $NaH_2PO_4 \cdot 2H_2O$; pH = 7.4) for 2 h. The femur was opened dorsally and the tibia was opened ventrally with a razor blade. The legs were incubated in Cy3-conjugated Streptavidin (Jackson Immunoresearch Laboratories, West Grove, PA, 1:200 in phosphate-buffered saline with Triton (0.1369 mol/liter NaCl, 0.0027 mol/liter KCl, 0.01 mol/liter Na_2HPO_4, 0.00176 mol/liter KH_2PO_4 [all from Merck, Darmstadt, Germany], and 0.1% Triton X-100 [Roth, Karlsruhe, Germany]; pH = 7.2) for 3 d and washed three times for at least 2 h. Preparations were dehydrated in a graded ethanol (Roth) series and cleared in methyl salicylate (Merck) overnight. Backfills stainings were documented with an Olympus BH-2 microscope with a Leica DFC7000T digital camera using the Leica Application Suite (LAS, v4.9, Leica Microsystems, Wetzlar, Germany). The preparations were photographed in series of focal planes and composited with the software CombineZP (http://www.hadleyweb.pwp.blueyonder.co.uk) or ImageJ (v1.51, http://rsb.info.nih.gov/ij/) with the plugin 'Extended depth of field' (http://bigwww.epfl.ch/demo/edf/) to gain in-focus images. Schematic drawings were made during live picture grabbing (with LAS software) by using Inkscape (v0.91; https://inkscape.org/). Therefore, the Inkscape window was made transparent (Glass2k software; Chime Software v0.9.2, http://chime.tv/products/glass2k.shtml) and placed over the LAS picture. The outlines could be drawn from the live picture. Size, brightness, contrast, and background were adjusted with ImageJ and the figure panels were assembled in Inkscape.

Sensory Physiology

The vibrational response of midlegs was determined by recording extracellularly from the leg nerve. Animals were fixed ventral side up on a metal holder with a wax–collophonium mixture. The cicada was dissected ventrally and the distalmost anterior part of midleg tibia was attached with superglue to a lever connected to a minishaker (Brüel and Kjær, Odense, Denmark, Type 4810; Fig. 1). The leg was adjusted to an angle of about 70° between tibia and femur and the stimulus was applied perpendicular to the body length axis due to constrains in the experimental setup (Fig. 1). The minishaker was driven by sinusoidal stimuli generated with Audacity software (v2.0.3, http://www.audacityteam.org/) and connected to an amplifier (Brüel and Kjær, Type 2706). The duration of the stimuli was 100 ms and the repetition rate was 2/s. Stimuli had carrier frequencies from 200 to 8,000 Hz. Each stimulus was given four times before the amplitude was increased by 6 dB. The amplitude was increased in six steps and the resulting acceleration was measured with an accelerometer (IDS Innomic, Salzwedel, Germany, Type KS95B) connected to a XL2 audioanalyser (NTI Audio, Schaan, Liechtenstein). The leg nerve was placed on a tungsten hook electrode (WPI, Sarasota, FL, Type TM33B05). A reference electrode was placed in the hemolymph near the recording site. The signal from the recording electrode was amplified 1,000× by an amplifier (WPI, Type ISO-80) and displayed on an oscilloscope and recorded with Labtrax Software (WPI) on a PC. The threshold was determined as the lowest stimulus intensity at which the nerve responded to at least three out of the four stimuli per frequency–amplitude presentations. Furthermore, the latency to the first spike was determined. Spike filtering with Spike2 software (v7.16, Cambridge Electronic Design, Milton, England) was used to determine different units. Responses from 13 animals were analyzed.

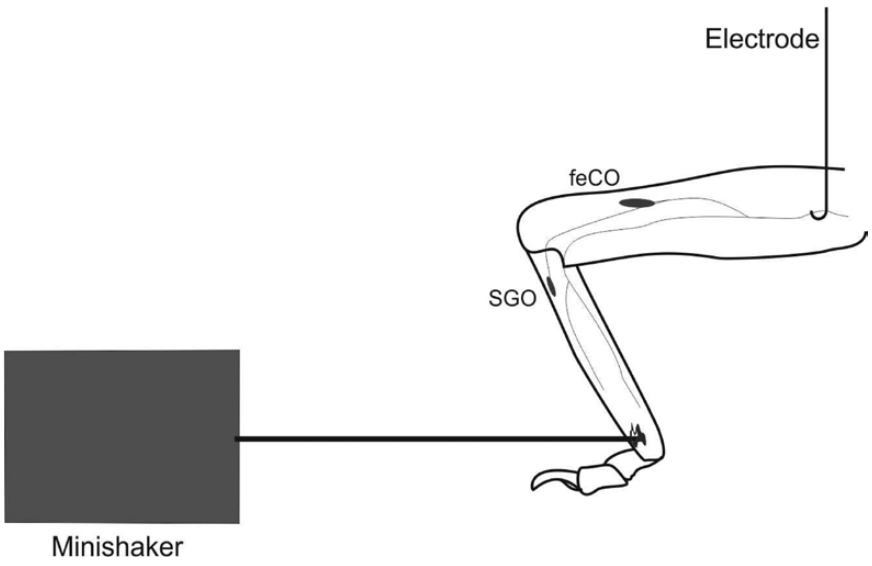

Fig. 1. Stimulation and recording setup. The midleg of a female cicada *O. rimosa* was stimulated with a lever connected to a minishaker. The lever was attached at the anterior distalmost tibia, whereby the angle between femur and tibia was about 70°. Hook electrode recordings were made from the leg nerve, which contains the axons of the two major scolopidial organs, the feCO and the SGO.

Results

Neuroanatomy

In the cicada *O. rimosa*, a main leg nerve (MLN) runs through the proximal leg segments, coxa, and trochanter (Fig. 2A). This nerve splits off several small nerves in the femur, including a thin sensory nerve (feCO nerve) and extends into the proximal tibia. Within the tibia, the nerve bifurcates and the anterior branch divides again, resulting in three nerves extending into the distal tibia (Fig. 2B). The nerve innervating the main scolopidial organs is likely a purely sensory nerve, which splits from the leg nerve in the midfemur to supply the feCO and to continue as SGO nerve (see below).

Femur and tibia are covered with a dense pattern of hair sensilla. Most of these hair sensilla are innervated by single sensory neurons, indicating a mechanoreceptive function (Fig. 2A'). The axons from the hair sensory neurons fasciculate and form small nerves, which join the MLNs at several sites. Additionally, campaniform sensilla (CS) are found at different positions, for example, CS are found in the proximal tibia near the attachment of the SGO and single CS occurs at the basis of the spurs of the ventral tibia.

Two scolopidial organs have been marked by the neuronal tracer. The feCO is located dorsally in all three legs in the distal half of the femur (Fig. 2A and A'). The feCO is composed of two scoloparia: a proximal and a distal one. The proximal scoloparium contains 14–16 sensory neurons. A further distinction into two subparts, as in other hemipteran insects (Nishino et al. 2016), has not been observed by the axonal tracing. The distal scoloparium (DS) is stretched longitudinally into two parts: DS1 with four neurons and DS2 with two sensory neurons. All dendrites point distally, as the scolopidial organs are attached to ligaments connected to the tibia–femur joint.

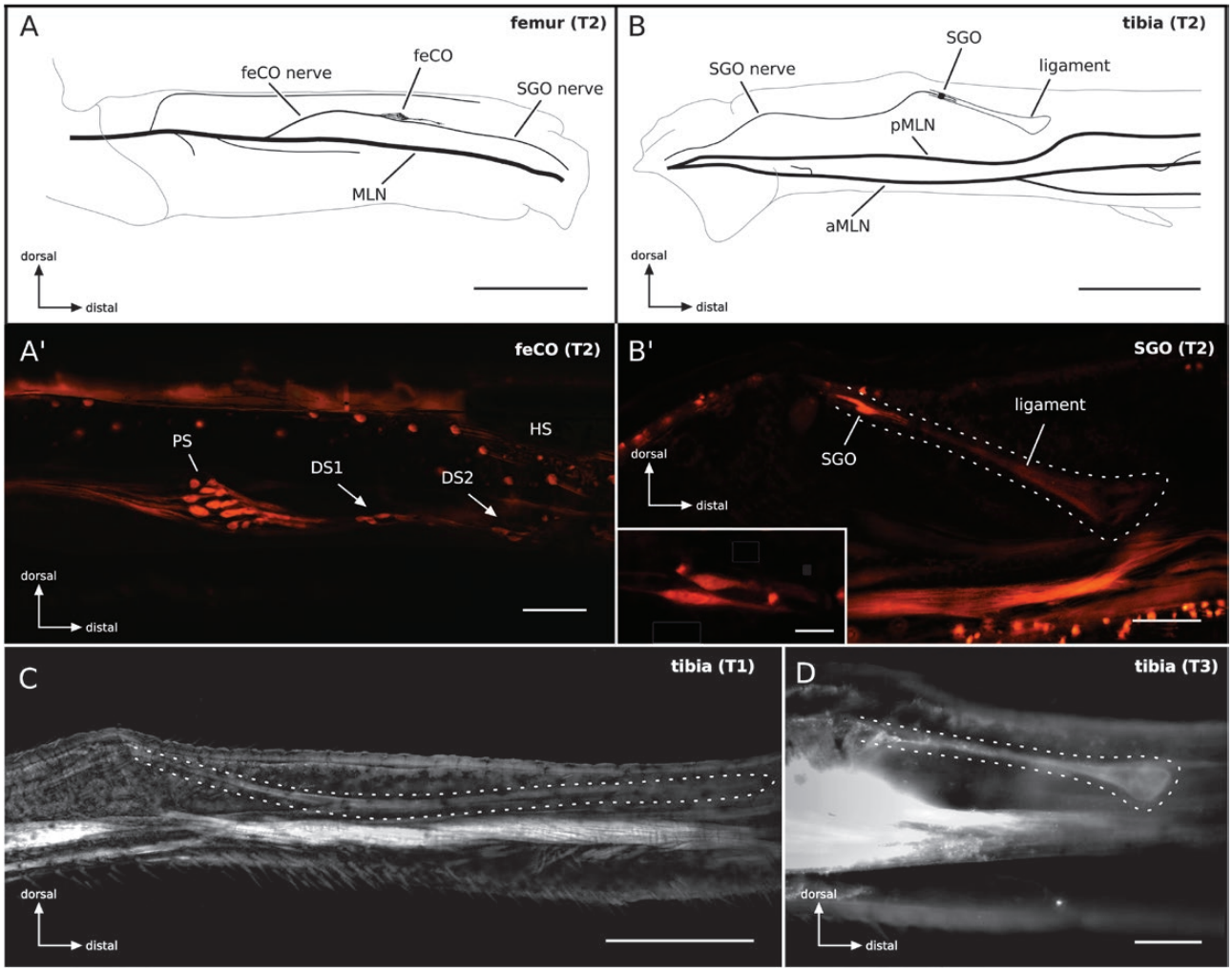

Fig. 2. Neuroanatomy of the leg innervation as revealed by neuronal tracing with Neurobiotin. (A) Schematic drawing of the nerves in the midleg femur. An MLN enters the femur (labeled after Nishino et al. 2016) and branches off several smaller nerves. The sensory axons from the feCO form the feCO nerve, which is joined distally by the SGO nerve. (A') Photo of the labeled nerve and sensory cells (neurobiotin backfill with streptavidin Cy3 visualization) in the mid femur. The feCO consists of different scoloparia, the proximal scoloparium (PS) with 14–16 sensory cells and the DS. The latter is divided into two parts (DS1 and DS2), with four and two sensory cells, respectively. Mechanosensory neurons innervate a dense array of sensory hairs (HS). (B) The main nerves in the proximal tibia. The leg nerve bifurcates in an anterior branch (aMLN) and a posterior branch (pMLN). Additionally, smaller nerves branch off and extend to the distal tibia and tarsi. The axons of the two sensory cells from the SGO run via the SGO nerve into the femur, where they join the feCO nerve. (B') The SGO scolopidial units are attached to a ligament extending into the tibia. The inset shows the two elliptically shaped sensory cells of the SGO. The hair sensilla and CS are innervated by branches from the leg nerve. (C and D) The size and shape of the ligament (outlined) of the SGO differs between the prothoracic and the meso- and metathoracic leg. In the prothoracic leg the ligament extends up to 2 mm to the distal part of the tibia (C), whereas in the meta- and mesothoracic legs the ligaments are similar in shape and about 500 µm long (B' and D). Scales: (A) 1 mm, (B) 500 µm, (A', B') 100 µm, (B') inset: 25 µm, (C) 500 µm, (D) 100 µm.

The SGO is innervated by a very thin nerve, containing the two axons of the two sensory cells of the SGO (Fig. 2B and B'). The SGO is located in the proximal tibia, close to a cuticular swelling at the dorsal side. The dendrites of the two sensory cells point distally and the scoloparium extends into a ligament. The ligament has a largely parallel orientation to the longitudinal axis of the tibia. Interestingly, the structure of the ligament differs between leg pairs of the different segments. In the foreleg, the ligament extends about 2 mm into the distal part of the tibia until it attaches to the dorsal cuticle (Fig. 2C). In the midleg and hindleg, the ligament extend for about 500 µm into the tibia and ends with a club-like shape at or close to the cuticle (Fig. 2D).

Neurophysiology

Extracellular recordings from the leg nerve revealed activity of several units, seen as background activity (Fig. 3A). Large spike amplitudes (up to 0.6 mV) occurred at spike frequencies of 4–8 AP/s, typically with a decreasing frequency during the recording until the neurons stopped firing. Such units might be motor neurons, although no movement of the leg was observed during the recording. Smaller amplitude units may have a burst like activity pattern.

In order to test the sensitivity to substrate vibrations, stimuli with different carrier frequencies and different accelerations were applied to the leg. When applying vibrational stimuli a strong neuronal response could be recorded (Fig. 3A'). The response contained large and small amplitude units. However, spike sorting did not reveal unequivocally distinct unit classes and no specific units from the different scolopidial organs (feCO, SGO) could be identified. The response to 200 Hz stimuli often contained phase-locked action potentials and in some recordings phase-lock was also observed to 300 Hz stimuli. The responses to stimuli with 500 Hz and higher frequencies were not phase locked. In many cases the responses were phasic-tonic responses.

For threshold analyses, the responses were analyzed as all-or-nothing response. Thresholds to stimuli with different carrier frequencies are in the range of 1 ms^2 with a minimum at 1,000 Hz (Fig. 3B). The overall threshold is broadly tuned to vibrations from 200 to 5,000 Hz (Fig. 3B).

The latencies of the responses decrease with increasing acceleration from about 13–17 ms at threshold to 5–7 ms at larger accelerations (Fig. 3C), whereby a relatively large variation was observed. At 200 Hz, latencies at threshold were longer than at higher frequencies (1,000 Hz, Fig. 3C).

Discussion

Here, we present results on the neuroanatomy of legs and their sense organs as well as the vibrational sensitivity of the midleg of adult *O. rimosa*.

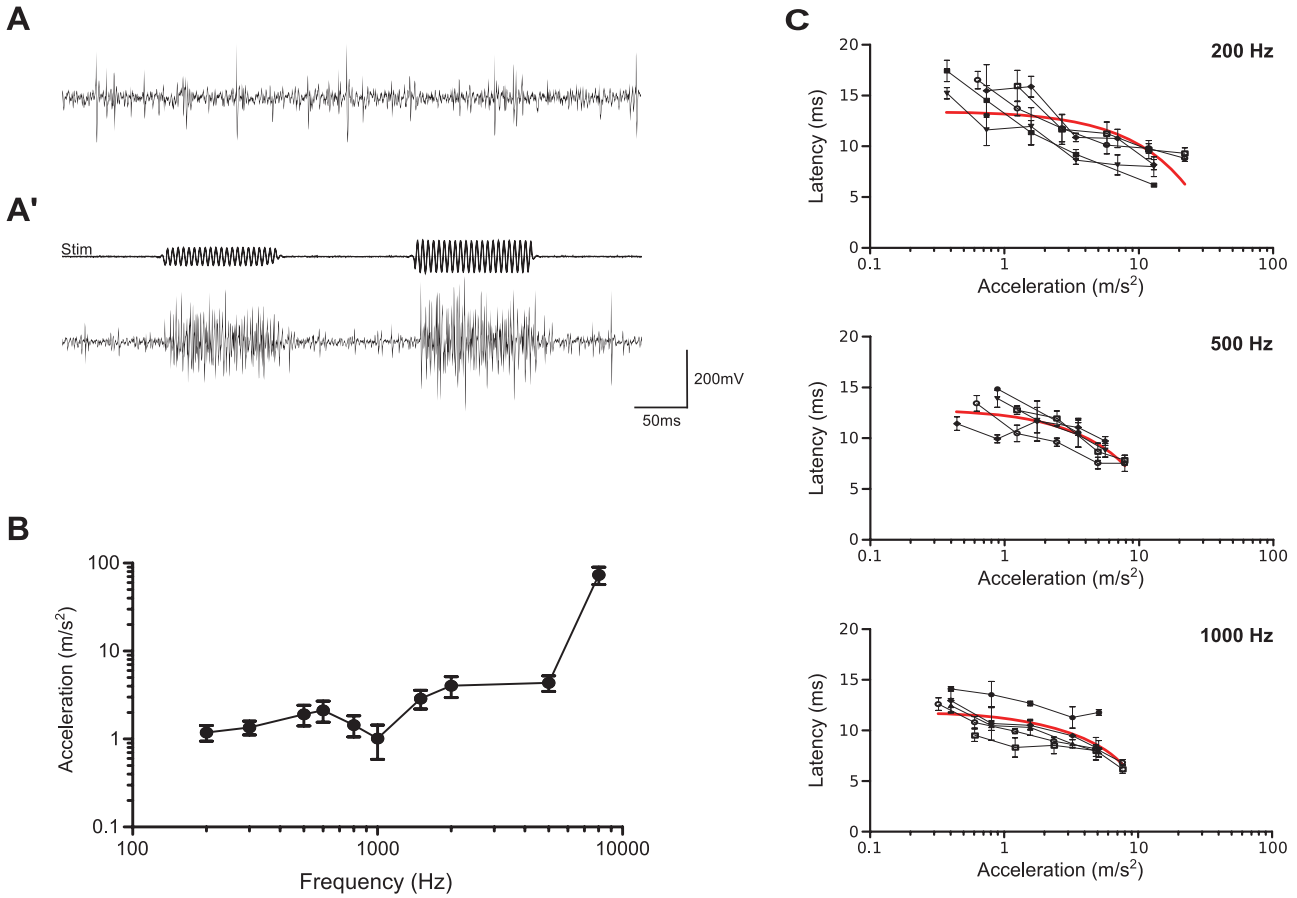

Fig. 3. Nerve recordings and threshold curve of the afferent response to vibrational stimuli of the cicada *O. rimosa*. (A) Electrophysiological recording showing stimulus independent background activity of different neuronal units. (A') Trace of recording showing different units responding to vibrational stimuli (200 Hz with 6.97 m/s^2 [left stimulus] and 12.95 m/s^2 (right stimulus) acceleration, respectively). (B) The curve shows a broad tuning in the tested frequency range, with a minimum threshold at 1,000 Hz. Data represent mean and SEM from 13 animals. (C) Latencies of the nerve responses to stimuli with three different carrier frequencies (200, 500, and 1,000 Hz). Shown are latency curves from five animals each (mean and SEM), with a linear regression line (red). Independent from the frequency, the latency decreases with increasing acceleration.

For neuronal tracing, we used Neurobiotin, which revealed fine details of the innervation. For labeling of the nervous system in insect appendages, often cobaltous or nickel salts are used, which have the advantage that the legs have not to be dissected open, for example (Strauß and Lakes-Harlan 2013). Preliminary experiments with these salts were not very successful in cicadas. Therefore, we switched to Neurobiotin for the backfill, which was visualized with Cy3-conjugated streptavidin. This method proofed to be successful, but the legs had to be dissected open along a longitudinal axis to provide access for the streptavidin. Care was taken to analyze only intact tissues in the preparations. In all legs of the cicada, we found a similar sensory organization with a femoral (feCO) and subgenual (SGO) scolopidial sense organs (except for the ligament). Additionally, to the scolopidial organs, numerous mechanosensory hair sensilla, hair plates, and CS form the leg sensory system.

The feCO is innervated by a small nerve (5B1) from the MLN. The feCO is divided in different scoloparia, like in most other insects (Field and Matheson 1998). We could not detect an anatomical distinction into a ventral and a dorsal scoloparium as has been described for the cicadas *Lyristes bihamatus* (Motschulsky) (Hemiptera: Auchenorrhyncha) and *Terpnosia nigricosta* (Motschulsky) (Hemiptera: Auchenorrhyncha) (Nishino et al. 2016). The cell number of the feCO in *O. rimosa* is in the range of these other Hemiptera. This number (about 20) is smaller than in many other insect species; for example, in Orthopterans several hundred units in the proximal scoloparium and about 40 in the DS have been reported (Field and Pflüger 1989, Matheson 1992). It is not known what determines the number of cells, but the number is not correlated to the overall animal size, as *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) has with about 75 sensory cells (Shanbhag et al. 1992) also a higher cell number in its feCO than *O. rimosa*.

The MLN runs through the femur and splits off into different branches in the tibia, similar to a pattern often seen in insects (Mücke 1991). From all nerves, numerous small branches innervate various parts of the epidermis and especially the hair sensilla. The SGO consists of only two sensory units, as has been described for other cicadas (Debaisieux 1938, Nishino et al. 2016), and bugs (Michel et al. 1982). The two axons form a very small nerve, passing the feCO until it fasciculates with the leg nerve. Thus, a tibial sensory complex as in orthopterans and phasmids with a subdivision of scolopidial organs, probably with different functions in detection of vibrations (Nishino and Field 2003, Strauß and Lakes-Harlan 2013, Lakes-Harlan and Strauß 2014), is not present in Hemiptera. This finding is somewhat surprising as many Hemiptera have an elaborate vibrational communication.

The cicada *O. rimosa* is not known for communication via substrate vibration. However, substrate vibrations can occur as a by-product during calling (Stölting et al. 2002) and might be elicited during courtship. Especially, in the first case, we might expect a species-specific tuning of the threshold to vibratory stimuli at the peak frequency of the calling song at 9 kHz.

We found a broadly tuned threshold curve from 200 to 5,000 Hz, with a decrease of sensitivity at higher frequency. This wide range and lack of high-frequency tuning indicates no tuning to species specific signals. However, tuning of single receptor cells might deviate from the tuning found in the summed nerve recording.

The minimum frequency tuning of the two subgenual receptor cells in the bug *Nezara viridula* (Linnaeus) (Hemiptera: Pentatomidae) has been determined with around 200 Hz for one receptor and with 600–1,000 Hz for the other receptor (Cokl 1983). These receptor cells are similarly attached as those in *O. rimosa* and it may also be that in *O. rimosa* both receptors react differently. The structural basis of the different tuning is not known. In the bug, the threshold values were around 0.01 m/s^2 and therefore much lower than in *O. rimosa*. In general, threshold determination based on single-cell recordings reveals lower values than those from extracellular recordings (Kalmring et al. 1996). The minimum at around 1 kHz observed in the threshold curve of *O. rimosa* may also indicate that this relates to one unit, similar to the one unit in the *N. viridula*. Vibration receptors in bush crickets also have minima around 800–1,000 Hz (Kalmring et al. 1996). In the *N. viridula*, reactions to up to 5 kHz have been recorded (Cokl 1983), mirroring the responses to high-frequency stimuli in *O. rimosa*. Further studies with single-cell recordings are needed to evaluate the tuning of the different sensory units. In respect to the SGO, it will also be interesting to compare the responses from the foreleg and the midleg of *O. rimosa*. The ligament of the SGO is different in the foreleg compared to the mid- and hindleg (see results and Nishino et al. 2016) and it is unknown how the different attachments affect the physiology of the sensory units.

Besides the SGO, which is thought to be the most sensitive receptor organ for substrate vibrations (Autrum and Schneider 1948), other sense organs of the leg can monitor vibrations as well. It has been found in other species that parts of the feCO react to vibratory stimuli (Field and Pflüger 1989, Matheson 1992, Büschges 1994, Stein and Sauer 1999, Lakes-Harlan and Strauß 2014). Such a response likely occurs also in *O. rimosa*, especially in a low-frequency range, as in Orthoptera. Additionally, CS reacts to mechanical stimulation. These sensilla show a phase-locked response up to about 200 Hz stimuli (Kühne 1982) and the phase-locked response observed in *O. rimosa* may have originated from CS.

Yet, another vibration sensitive organ might be the adult hearing organ, which has not been investigated here. This organ is situated ventrally in the second abdominal segment and it has been proposed that this organ might have a function in vibration detection in nymphs (Lakes-Harlan and Strauß 2006). The rationale is that it is a large sensory organ with approximate 1,000 units (Fonseca et al. 2000) and that it is energy-costly to produce and maintain such an organ only for adult hearing, keeping in mind that the postembryonic development may take up to approximately 17 yr in periodical cicadas. Thus, the organ might have a sensory function in nymphs, where it certainly cannot function as auditory organ (Strauß and Lakes-Harlan 2009). Behavioral experiments show that nymphs react to vibrational stimuli (Lakes-Harlan and Holzhauer 2015). The proposed nymphal function of the 'auditory' organ may still be present in the adult cicada. Whether it can respond to substrate vibrations is a subject of future experiments.

In summary, cicadas have a complement of vibration receptors in their legs and summed recordings of the leg nerve did reveal a broad tuning to vibrational stimuli. Future single-cell recording will show whether the receptor cells are differently tuned and adapted to specialized functions, like detection of substrate vibration from the calling song.

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