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Vibrational sensitivity of the subgenual organ complex in female *Sipyloidea sipylus* stick insects in different experimental paradigms of stimulus direction, leg attachment, and ablation of a connective tibial sense organ

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ABSTRACT

We document the sensitivity to sinusoidal vibrations for chordotonal organs in the stick insect tibia (*Sipyloidea sipylus*). In the tibia, the scolopidial subgenual organ (~40 scolopidial sensilla), distal organ (~20 scolopidial sensilla), and distal tibial chordotonal organ (~7 scolopidial sensilla) are present. We study the sensitivity of tibial sensory organs in all leg pairs to vibration stimuli as sensory thresholds by recording summed action potentials from *Nervus cruris* in the femur. The tibia was stimulated with a minishaker delivering vibrational stimuli. Because different experimental procedures may affect the vibration sensitivity, we here analysed possible effects of different experimental conditions: (1) the stimulus direction delivered in either horizontal or vertical direction to the leg; (2) recording responses only from the subgenual organ complex after ablation of the distal tibial chordotonal organ, and (3) the attachment of the leg to the minishaker by plastilin, beeswax-colophony, or freely standing legs. The tibial scolopidial organs give summed responses to vibration stimuli with highest sensitivity between 500 and 1000 Hz for all leg pairs. In the different experimental series, we find that (1) thresholds were influenced by stimulation direction with lower thresholds in response to vertical vibrations, (2) ablating the distal tibial chordotonal organ by cutting the distal-most tibia did not change the summed sensory thresholds significantly, and (3) the attachment material between legs and the minishaker (plastilin or beeswax-colophony mixture) did not significant influence the sensory thresholds against free-standing tarsi. The distal tibial chordotonal organ is a connective chordotonal organ attached to a tendon and is likely a proprioceptive organ. These results emphasise that vibrational thresholds are mainly direction-sensitive. Thus, the direction of stimulus delivery during electrophysiological recordings is relevant for comparisons of vibratory sensory thresholds.

1. Introduction

Probably all insects can detect vibrations transmitted via solid substrates or plants by mechanosensitive organs, and many insect species use specific substrate vibration signals for communication (reviewed by Markl, 1983; Gogala, 1985; Greenfield, 2002; Virant-Doberlet and Čokl, 2004; Cocroft and Rodríguez, 2005; Hill and Wessel, 2016; Yack, 2016). Substrate vibrations are detected by highly sensitive mechanoreceptors, mainly chordotonal organs containing scolopidial sensilla (Shaw, 1994; Field and Matheson, 1998). Chordotonal organs are internal mechanosensory organs located in almost all body segments (Howse, 1968; Hutchings and Lewis, 1983; Field and Matheson, 1998; Čokl et al., 2006).

The main route of transfer from the substrate is usually via the legs, and these substrate vibrations can be transmitted from the substrate through the body of an insect (Cocroft et al., 2000). In adult insects, several vibration-sensitive organs are located in different positions of the legs, like the femoral chordotonal organ and subgenual organ (Kalmring, 1985; Field and Matheson, 1998; Čokl et al., 2006; Lakes-Harlan and Strauß, 2014; Yack, 2016). These receptor organs and their physiological responses have been studied in different insect species, in particular cockroaches (Schnorbus, 1971; Shaw, 1994), the southern green stink bug (Čokl, 1983; Čokl et al., 2006), lacewings (Devetak and Amon, 1997), heelwalkers (Eberhard et al., 2010), locusts (Kühne, 1982; Kalmring, 1985), tettigoniids (Kühne, 1982; Kalmring et al., 1994, 1996), and crickets (Dambach, 1972). The most sensitive

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Abbreviations: Adb, antero-dorsal branch of *Nervus cruris*; col, colophony; CS, campaniform sensilla; DO, distal organ; DTCO, distal tibial chordotonal organ; FCO, femoral chordotonal organs; Pl, plastilin; Pvb, postero-ventral branch of *Nervus cruris*; SGO, subgenual organ; T1, foreleg; T2, midleg; T3, hindleg; TTO, tibio-tarsal chordotonal organ; X, horizontal direction; Y, vertical direction.

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vibration receptor organ in the legs of many insects is the subgenual organ (Schnorbus, 1971; Shaw, 1994; Čokl and Virant-Doberlet, 2009; Eberhard et al., 2010), a scolopidial sensory organ located in the proximal tibia. The subgenual organ of orthopteroid insects usually spans the hemolymph channel and contains between 20 and 70 sensilla (Schnorbus, 1971; Houtermans and Schumacher, 1974; Rössler et al., 1994; Nishino and Field, 2003; Eberhard et al., 2010; Strauß and Lakes-Harlan, 2013). Sensory organs have been investigated by summed recordings from afferents or single sensilla recordings. They are often tuned with highest sensitivity to specific frequency ranges (Kühne, 1982; Kalmring et al., 1994; Stritih and Čokl, 2014). The subgenual organ (SGO) responds preferentially to the acceleration of vibrations (Schnorbus, 1971; Čokl, 1983; Kilpinen and Storm, 1997; review: Field and Matheson, 1998).

In addition to the subgenual organ, further vibration-sensitive organs of scolopidial sensilla occur close by, forming the subgenual organ complex (Debaisieux, 1938; Schnorbus, 1971; Kalmring et al., 1996; Field and Matheson, 1998; Eberhard et al., 2010; Strauß et al., 2014). These organs are usually located distally of the subgenual organ (distal organ or intermediate organ). In stick insects, a distal organ with ~20 linearly arranged sensilla (Strauß and Lakes-Harlan, 2013). In the distal tibia of several orthopteroid insects, a further chordotonal organ is situated, termed either the tibio-tarsal chordotonal organ (TTO) or distal tibial chordotonal organ (DTCO) (Young, 1970; Lakes and Schikorski, 1990; Mücke, 1991; Eberhard et al., 2010). In the Indian stick insect *Carausius morosus*, the distal tibial chordotonal organ is a connective chordotonal organ linked to the tendon of the retractor unguis muscles (Godden, 1972; Bässler, 1983), similar to other insects (Čokl et al., 2006; Eberhard et al., 2010). In the stick insect *Sipyloidea sipylus* investigated here, the DTCO has not been documented yet. In cockroaches, this organ is a proprioceptive organ that responds to deflexion of the tarsus (Young, 1970).

The best studied mechanosensory organ in the leg of stick insects is the femoral chordotonal organ (FCO) which has been investigated physiologically in detail (e.g. Bässler, 1983; Graham, 1985; Kittmann and Schmitz, 1992; Büschges, 1994; Bässler and Büschges, 1998; Stein and Sauer, 1999). The FCO is a bifunctional chordotonal organ detecting the leg position, while many FCO sensilla respond to low-frequency tibial vibrations (Stein and Sauer, 1999).

Less information is available about the chordotonal organs in the tibia. We have recently characterised the neuroanatomy of the subgenual organ and the distal organ, together forming the subgenual organ complex, in stick insects (Strauß and Lakes-Harlan, 2013). Both organs consist of scolopidial sensilla but their mechanosensory function was not studied in detail. Here, we investigate the vibration sensitivity of the subgenual organ complex for all three thoracic legs. In physiological experiments on vibrational physiology, the leg can be positioned or attached in different ways, and the stimulus can also be transferred in different directions. This is a relevant topic, since differences in these experimental approaches may influence the recorded vibration sensitivity (Schnorbus, 1971; Rohrseitz and Kilpinen, 1997; Michelsen, 2014). First, the direction of displacement of the leg's tibia can be in vertical direction (longitudinal axis of tibia) (Schnorbus, 1971), which strongly activates the subgenual organ (Thurm, 2001), or in horizontal direction (longitudinal axis of femur) for stimulation of the FCO (Stein and Sauer, 1999). Rarely is the effect of vibrational stimulus directions tested (Rohrseitz and Kilpinen, 1997). Second, the vibration response from all sensory organs in the tibia can be recorded, or selective organs can be ablated (Schnorbus, 1971; Devetak and Amon, 1997; Eberhard et al., 2010). In particular, the sensor organs located in the distal tibia can be ablated by heat or by cutting off the respective part of the leg to record only from the subgenual organ (Autrum, 1941; Schnorbus, 1971; Eberhard et al., 2010). Third, the attachment of the stimulated leg to the stimulator may also differ: in several studies, the leg was directly attached to a minishaker delivering stimuli (e.g. Schnorbus, 1971; Kalmring et al., 1994; Stein and Sauer, 1999) by using materials like beeswax or plastilin. Other investigations used freely standing animals (Dambach, 1972; Rohrseitz and Kilpinen, 1997). Only for cockroaches have experiments covered both stimulatory conditions, revealing an influence of attachment or free-standing legs on the sensory threshold (Schnorbus, 1971).

Here, we study the vibration sensitivity of the subgenual organ complex in the stick insect, *Sipyloidea sipylus*. We first give an overview of the neuroanatomy of tibial scolopidial organs in *S. sipylus*. Next, we carried out summed electrophysiological recordings in response to sinusoidal vibration stimuli, and determined the sensory threshold over a broad frequency range from 50 to 5000 Hz. To account for the possible influence of experimental parameters, we carried out recordings under different stimulatory directions. We stimulated legs (1) in both horizontal and vertical directions, (2) after ablation of the distal tibial chordotonal organ to isolate the response from the subgenual organ complex, and (3) tested for an influence of different attachments of the leg to the minishaker. For direction effects, we compare the sensitivity for directions between all three leg pairs.

2. Materials and methods

2.1. Animals

For the experiments, adult female *Sipyloidea sipylus* were taken from a crowded laboratory culture at the Institute for Animal Physiology, Justus-Liebig-Universität Gießen, maintained at 21–23 °C under a 12:12 light-dark-regime. European cultures of *S. sipylus* are parthenogenetic and consist only of females (Carlberg, 1987). Animals were fed with leaves of Rosaceae ad libitum. Water was provided by spraying the food plants and animals daily. Adult females (Carlberg, 1987) used in experiments were at least one week after final molt. Only animals with regularly formed legs including all elements were used for experiments.

The experiments described in this study comply with the principles of animal care of the Justus-Liebig-Universität Gießen and with the current law of the Federal Republic of Germany.

2.2. Sensory neuroanatomy: staining procedures and documentation

We have previously reported the neuroanatomy of the subgenual organ complex in detail for *S. siyplus* (Strauß and Lakes-Harlan, 2013). Here, we summarise these results and also show the structure of the distal tibial chordotonal organ in *S. sipylus*. To reveal sensory organs and nerves in the legs, these structures were stained by axonal tracing of the leg's main nerve, the *Nervus cruris* (Marquardt, 1940; Godden, 1972) using 5% cobalt solution (cobalt (II) chloride hexahydrate dissolved in distilled water; cobalt from Sigma-Aldrich, Steinheim, Germany). Axonal tracing was carried out on isolated legs (for details see Strauß et al., 2012; Strauß and Lakes-Harlan, 2013). Leg preparations were covered with *Carausius* saline (Weidler and Diecke, 1969; Bässler, 1977) and were incubated for 48–72 h at 4 °C. Intracellular cobalt transported through axons was precipitated with 1% ammonium sulphide solution (Alfa Aesar, Karlsruhe, Germany) in *Carausius* saline. Based on our previous study of the subgenual organ complex (Strauß and Lakes-Harlan, 2013), we have carried out further tracing experiments to stain the distal tibial chorodotonal organ (DTCO), the innervation pattern through the tibia, and the subgenual organ complex, for overview photographs. For the innervation pattern in the tibia, we focused on the midleg since this is the shortest leg in *S. sipylus* and allows complete staining by cobalt solution from proximal to distal ($n = 18$). We have made control experiments on forelegs and hindlegs which revealed identical innervation patterns ($n = 6$ each). From these preparations, the subgenual organ complex was also documented.

The innervation pattern in the tibia was revealed by extended incubation for 72 h and precipitation of cobalt in the complete tibia. To stain the DTCO in the distal tibia of the midleg, in some preparations the two