

# Comparative Neuroanatomy of the Mechanosensory Subgenual Organ Complex in the Peruvian Stick Insect, *Oreophoetes peruana*

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## Keywords

Neuroanatomy · Chordotonal organ · Peripheral nervous system · Mechanoreception

## Abstract

The subgenual organ complex in the leg of Polyneoptera (Insecta) consists of several chordotonal organs specialized to detect mechanical stimuli from substrate vibrations and airborne sound. In stick insects (Phasmatodea), the subgenual organ complex contains the subgenual organ and the distal organ located distally to the subgenual organ. The subgenual organ is a highly sensitive detector for substrate vibrations. The distal organ has a characteristic linear organization of sensilla and likely also responds to substrate vibrations. Despite its unique combination of sensory organs, the neuroanatomy of the subgenual organ complex of stick insects has been investigated for only very few species so far. Phylogenomic analysis has established for Phasmatodea the early branching of the sister groups Oriophasmata, the Old World phasmids, and Occidophasmata, the New World phasmids. The species studied for the sensory neuroanatomy, including the Indian stick insect *Carausius morosus*, belong to the Old World stick insects. Here, the neuroanatomy of the subgenual organ complex is presented for a first species of

the New World stick insects, the Peruvian stick insect *Oreophoetes peruana*. To document the sensory organs in the subgenual organ complex and their innervation pattern, and to compare these between females and males of this species and also to the Old World stick insects, axonal tracing is used. This study documents the same sensory organs for *O. peruana*, subgenual organ and distal organ, as in other stick insects. Between the sexes of this species, there are no notable differences in the neuroanatomy of their sensory organs. The innervation pattern of tibial nerve branches in *O. peruana* is identical to other stick insect species, although the innervation pattern of the subgenual organ by a single tibial nerve branch is simpler. The shared organization of the organs in the subgenual organ complex in both groups of Neophasmatodea (Old World and New World stick insects) indicates the sensory importance of the subgenual organ but also of the distal organ. Some variation exists in the innervation of the chordotonal organs in *O. peruana* though a common innervation pattern can be identified. The findings raise the question for the ancestral neuroanatomical organization and innervation in stick insects.

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## Introduction

Insect mechanosensory organs can show elaborate structures, by a high number of sensilla, several receptor organs in appendages or body segments, or a finely ordered projection of afferents into the central nervous system [Field and Matheson, 1998; Yager, 1999; Albert and Göpfert, 2015; Liang et al., 2017; Dickerson et al., 2021]. Comparison of such neuroanatomical data from closely related species or more diverged clades can show the evolutionary diversification of sensory organs and adaptive differences, which may also underlie species-specific behaviours [Dumont and Robertson, 1986; Katz, 2011; Barker, 2021].

Mechanoreceptors of arthropods, in particular chordotonal organs consisting of scolopidial sensilla [Keil, 1997; Field and Matheson, 1998; Liang et al., 2017], have developed complex functional morphologies. The subgenual organ (SGO) complex is an elaborate chordotonal complex of several sensory organs in the proximal tibia of orthopteroid insects [Strauß et al., 2021b]. These sensory organs respond to substrate vibrations, airborne sound, or both [Kalmring et al., 1994; Strauß et al., 2021b]. Stick insects (Phasmatodea) are among those insects with complex chordotonal organs in the tibia. While stick insects, in particular the Indian stick insect *Carausius morosus*, serve as models for neurophysiology and locomotion control [Bässler, 1983, 1993; Büschges and Gruhn, 2008; Bradler and Buckley, 2018], only very few species of stick insects have been studied in depth for the SGO complex in the tibia. The organ complex commonly includes the SGO [Čokl et al., 2006; Strauß et al., 2021b], which is highly sensitive to substrate vibrations [Dambach, 1972; Eberhard et al., 2010; Strauß and Lakes-Harlan, 2017] and also to airborne sound [Shaw, 1994]. In addition, the distal organ (DO) is placed next to the SGO and contains ca. 20 sensilla and they are arranged in a linear row. As the SGO spans the tibia transversely and the DO orients in distal direction of the tibia, the two organs are placed almost perpendicularly to each other [Strauß and Lakes-Harlan, 2013; Strauß et al., 2021a]. This combination of sensory organs and their elaborate anatomy of the DO is unique to stick insects [Strauß and Lakes-Harlan, 2013]. By the position in the haemolymph channel, the DO possibly also detects vibrations transferred along the leg [Strauß and Lakes-Harlan, 2017].

The elaborate sensory complex seen in stick insects requires further investigations regarding its evolution and sensory adaptations in mechanosensation. So far, the tibial organs have been studied in a few stick insect

species only. The leg innervation in stick insects has mainly been documented in the proximal parts of the leg [Marquardt, 1940; Godden, 1972; Bässler, 1983; Goldammer et al., 2012], while the tibia and the SGO complex have also been analysed for their innervations, with distinct nerve branches supplying the SGO and DO in *C. morosus* and *Sipyloidea sipyilus* [Friedrich, 1929; Strauß and Lakes-Harlan, 2013]. The latter belong to the major clade of Oriophasmata, the Eastern or Old World phasmids [Simon et al., 2019]. Recent phylogenomic analysis has established their sister group, the Occidophasmata, which covers the New World phasmids [Simon et al., 2019]. From this group, neuroanatomical data on chordotonal organs are still lacking. Here, the neuroanatomy of the SGO complex is investigated for the first species from the Occidophasmata, the Peruvian stick insect *Oreophoetes peruana*, by axonal tracing. This species was chosen as it is accessible for neuroanatomical investigation by its size, and its biology has been investigated for morphology [Sellick, 1998; Büscher et al., 2018], chemical defence [Eisner et al., 1997; Attygalle et al., 2021], and the phylogenetic position [Simon et al., 2019]. The neuroanatomy in *O. peruana* can be compared to homologous organs [Kutsch and Breidbach, 1994] in *C. morosus* and *S. sipyilus* [Strauß and Lakes-Harlan, 2013; Strauß, 2020]. The internal chordotonal organs are closely associated with campaniform sensilla [Strauß, 2020], which detect cuticular strain [Pringle, 1938; Zill et al., 2004]. The aim of the present study is to expand the knowledge on the SGO complex in stick insects by documenting the sensory organs, their number of sensory neurons, and their neuronal innervation pattern in *O. peruana*. The organization of the DO, for its number of sensilla and their spatial arrangement, possibly extending distally, is of particular interest. This study contributes to the comparative neuroanatomy and the evolutionary diversity of these mechanosensory structures in orthopteroid insects.

The neuroanatomy of chordotonal organs in Orthoptera like tympanal hearing organs or vibration receptor organs including the SGO is very similar between related insect species [Schumacher, 1973; Rössler et al., 1994; Lin et al., 1995; Strauß and Stritih, 2017]. However, species-specific differences in the number of sensilla in specific organs are frequently found [Schumacher, 1973; Rössler et al., 1994; Lin et al., 1995; Strauß et al., 2017]. In stick insects, this overall similarity in organ neuroanatomy and the more subtle differences in sensilla numbers were also seen in the SGO complex [Strauß et al., 2021a]. It is therefore hypothesized that the SGO complex with the SGO

and DO is present in *O. peruana* as well. Given that the elaborate DO suggests its sensory importance in stick insects, a similar neuroanatomy is also expected in *O. peruana*. This species has both females and males and allows to compare the sensory organs of the sexes, which could further indicate sensory specialization by possible differences between sexes. Overall, data from *O. peruana* add to the sensory biology of stick insects and to the study of adaptations in the SGO complex as a specialized mechanoreceptor complex.

## Materials and Methods

### Stick Insects

Individuals of *O. peruana* (Saussure, 1868) came from a crowded lab colony maintained at the Institute of Animal Physiology, Justus-Liebig-Universität Gießen. Insects were kept at ~21°C, sprayed every 1–2 days with water, and fed with fern (*Nephrolepis*) from a local garden shop. In these experiments, only adult insects were studied.

### Axonal Tracing

The sensory organs and neuronal innervation pattern in the proximal tibia were stained by axonal tracing as described previously for *S. sipylus* [Strauß, 2020]. Prior to dissection, insects were cold-anaesthetized at 4°C for 10 min. The legs were cut off in the middle femur with scissors and then placed in a glass dish covered with Sylgard (Sylgard 184, Suter Kunststoffe AG, Fraubrunnen, Switzerland) using insect pins. The legs were oriented with the ventral side up, and the cuticle was removed with a piece of a blade (Feather FA-10, 0.1 mm, Feather, Osaka, Japan) to gain access to the main leg nerve (nervus cruris). The leg was covered with *Carausius* saline [Bässler, 1977] (177.96 mmol NaCl, 17.4 mmol KCl, 25.1 mmol MgCl<sub>2</sub> × 6 H<sub>2</sub>O, Roth, Karlsruhe, Germany; 7.48 mmol CaCl<sub>2</sub> × 2 H<sub>2</sub>O, Merck, Darmstadt, Germany; 1.98 mmol Tris, from Sigma-Aldrich, St. Louis, MO, USA; dissolved in *Aqua dest.*, pH = 7.4). The nervus cruris was isolated by removing muscles and tendons with fine forceps (Dumont #5, Fine Science Tools, Heidelberg, Germany), and the nerve was cut with iridectomy scissors. For intracellular staining [Pitman et al., 1973], the free end of the nerve was transferred into a glass capillary with 5% cobalt solution (CoCl<sub>2</sub> × 6 H<sub>2</sub>O; Merck, Darmstadt, Germany, dissolved in *Aqua dest.*). Preparations were incubated for 48 h at 4°C in a moist chamber. Preparations were further processed by incubation in a 1% solution of ammonium sulphide (Alpha Aesar, Karlsruhe, Germany) in *Carausius* saline for 15 min (see Strauß and Lakes-Harlan, 2013). The legs were further rinsed in *Carausius* saline, fixed in chilled paraformaldehyde solution (4%; Sigma-Aldrich, St. Louis, MO, USA, in phosphate buffer (0.04 mol/L Na<sub>2</sub>HPO<sub>4</sub>, 0.00574 mol/L NaH<sub>2</sub>PO<sub>4</sub> × 2 H<sub>2</sub>O; pH = 7.4) for 60 min, and dehydrated in a graded ethanol series (Carl Roth, Karlsruhe, Germany, with concentrations of 30, 50, 70, 90, 96, 100% ethanol). After this dehydration process, the preparations were cleared with methyl salicylate (Merck, Darmstadt, Germany).

Overall, the study used legs from 6 female and 4 male animals. In total, the nerve pattern was analysed in 53 legs from 10 animals.

### Microscopy and Documentation

For microscopy, the cuticle was removed around the fixed tissue by carefully cutting at the dorsal and ventral side of the tibia along the tibia's main axis with a piece of a blade, and by removing the cuticle with forceps from the internal tissues and neuronal components. The leg preparations were mounted in methyl salicylate on a glass slide, viewed with an Olympus BH-2 microscope (Olympus, Shinjuku, Japan), and photographed with a Leica DFC 7000 T camera (1,920 × 1,440 pixel) attached to the microscope using the Leica Application Software V4.9 (Leica Microsystems CMS GmbH, Wetzlar, Germany). Stacked photographs were generated from series of photographs using CombineZP. The photographs were carefully adjusted for brightness and contrast and further assembled and labelled in CorelDraw 11 (Corel, Ottawa, Canada).

The innervation pattern of the sensory structures in the tibia was documented by drawing (Leitz microscope with a drawing attachment; Leitz, Wetzlar, Germany). The schematic was redrawn digitally using CorelDraw 11.

### Terminology of Sensory Structures, Tibial Nerves, and Tibial Nerve Branches

The terminology for chordotonal organs and sensilla groups follows that used for *C. morosus* and *S. sipylus* [Strauß and Lakes-Harlan, 2013]. The terminology for tibial campaniform sensilla follows the grouping established for *C. morosus* [Zill et al., 2011]. The terminology for the axes of the tibia follows Ball and Field [1981].

The main leg nerve is the nervus cruris [Marquardt, 1940] from which different nerve branches innervate the muscles and contain axons from sensory organs [Godden, 1972; Bässler, 1983]. The terminology for the tibial sense organs used here numbers the nerve branches ordered from proximal to distal [Bässler, 1983] in the tibia, following the numbering established for *S. sipylus* [Strauß, 2020]. This is applied to first- and second-order nerve branches of chordotonal organs and campaniform sensilla.

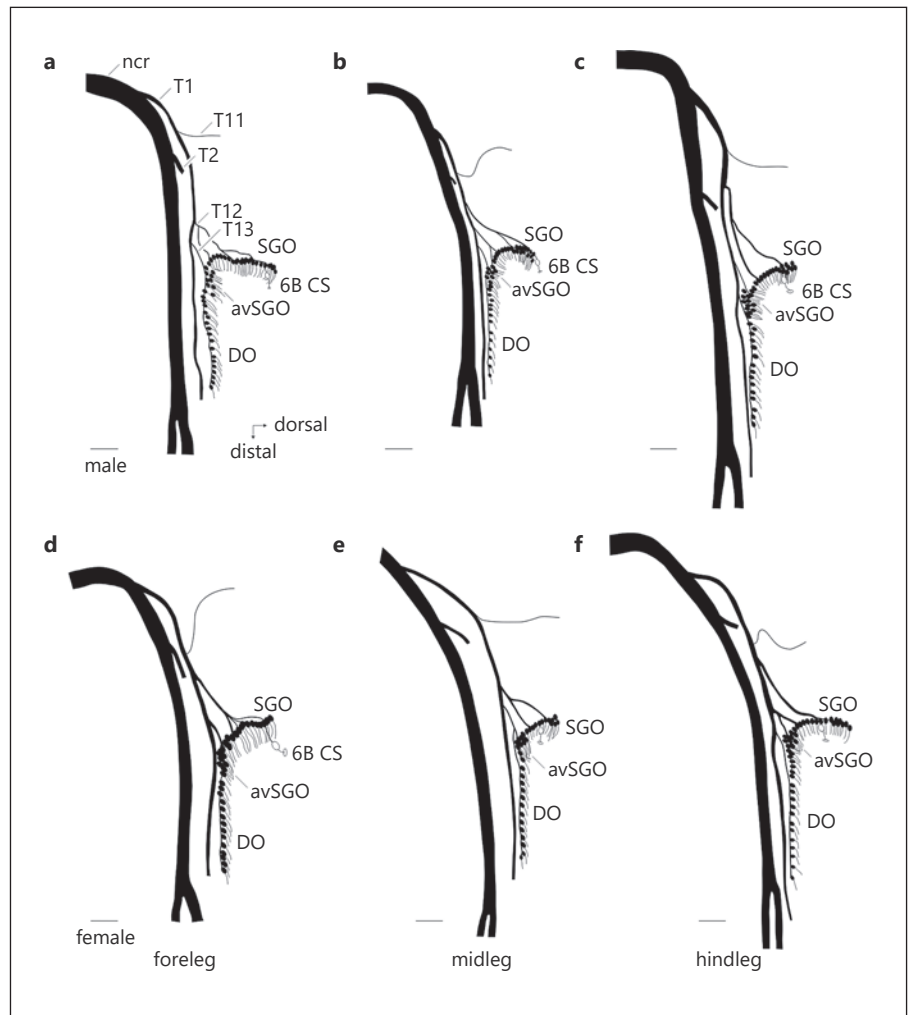
### Statistical Analysis

For statistical analysis of the variation of branching patterns between different sensory organs, GraphPad Prism 4 (GraphPad, San Diego, CA, USA) was used for a two-sided  $\chi^2$  test.

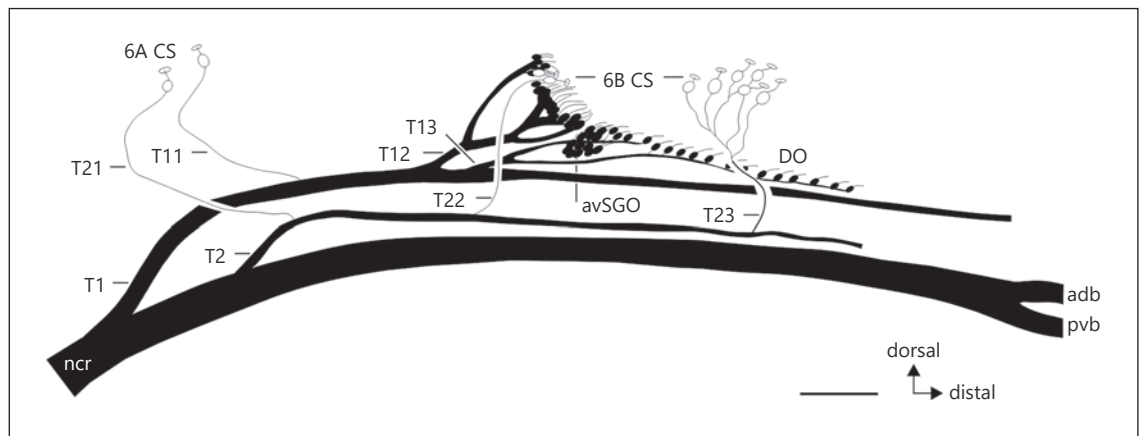
## Results

### Sensory Organs in the Subgenual Organ Complex in *Oreophoetes peruana*

The SGO complex is located in the proximal tibia of all legs (online suppl. Fig. S1; for all online suppl. material, see [www.karger.com/doi/10.1159/000525323](http://www.karger.com/doi/10.1159/000525323)). In *O. peruana*, it consisted of the SGO and DO (Fig. 1, 2), closely associated with campaniform sensilla in two groups termed 6A and 6B (Fig. 2). The SGO was placed almost perpendicularly to the tibial main axis (Fig. 1–3a). The sensilla of the DO were oriented in the distal direction of the tibia (Fig. 1–3e). This overall organization of sensory

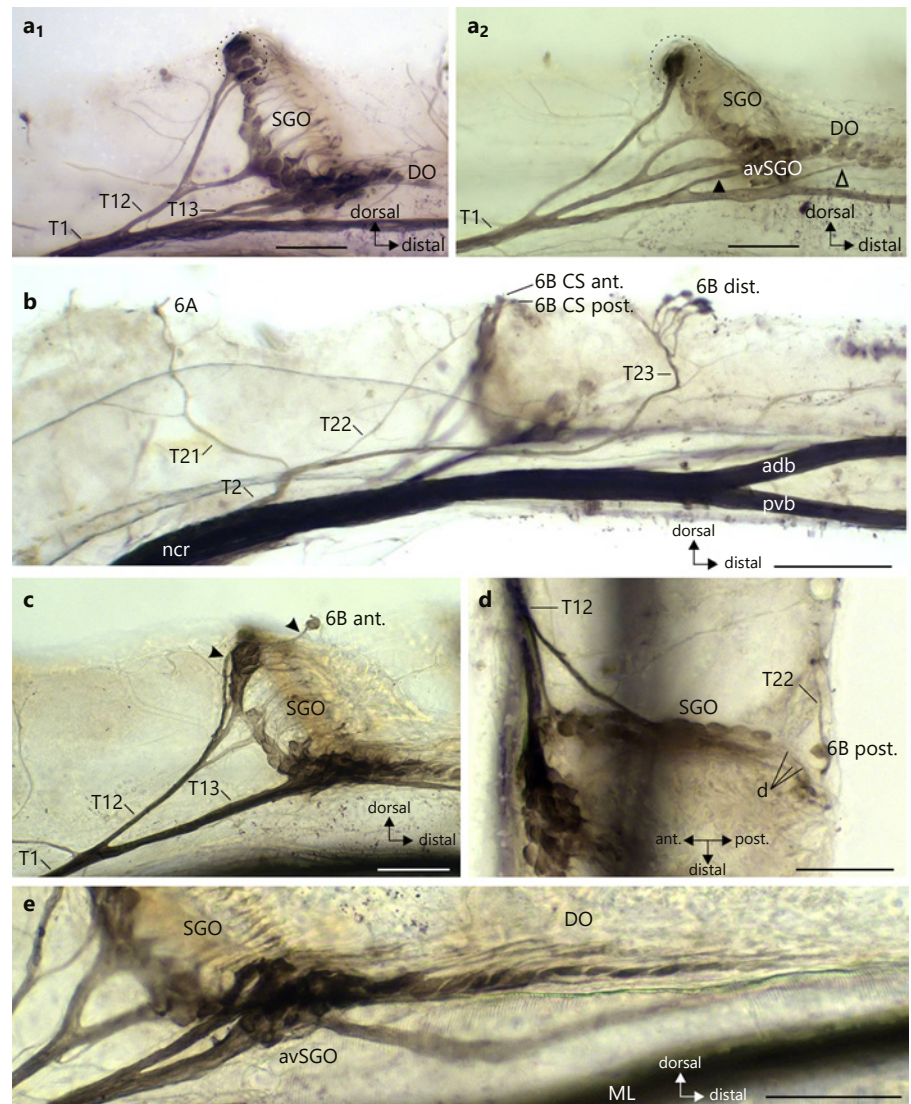


**Fig. 1.** Schematic of the neuroanatomy of the subgenual organ complex in all leg pairs of male (a–c) and female (d–f) *O. peruana*. Nerve branches are numbered in (a). Scales = 100  $\mu$ m. avSGO, anterior-ventral subgenual organ; DO, distal organ; ncr, nervus cruris; SGO, subgenual organ.



**Fig. 2.** Innervation of the subgenual organ complex in *O. peruana*. Redrawn midleg innervation in *O. peruana* (female), viewed from posterior. Nerve branches are numbered from proximal to distal. Sensilla in chordotonal organs are shown in black, campaniform sensilla in white. Scale: 100  $\mu$ m. adb, anterior-dorsal branch of the nervus cruris; avSGO, anterior-ventral subgenual organ; CS, campaniform sensilla; DO, distal organ; ncr, nervus cruris; pvb, posterior-ventral branch of the nervus cruris; SGO, subgenual organ.

**Fig. 3.** Neuroanatomy of the subgenual organ complex in *O. peruana*. **a** Innervation of posterior SGO (encircled) with dense cell bodies is from the nerve branch T1. The entire SGO is innervated by one nerve branch (**a1**; T12) or two nerve branches (**a2**; nerve branches not numbered). (**a2**) The DO can also be innervated by two nerve branches, with the proximal branch from the proximal cell bodies (filled arrow head) and the distal branch from the distal cell bodies (open arrow head): anterior view. **b** Innervation pattern of the posterior nerve branch T2 to campaniform sensilla 6A (T21), the proximal 6B campaniform sensilla (posterior: T22), and the distal 6B campaniform sensilla (T23): posterior view. **c** The axon for the anterior proximal 6B campaniform sensillum (arrowhead) merges with the nerve branch innervating the SGO (T12): anterior view. **d** The posterior proximal 6B campaniform sensillum is innervated by nerve branch T22 independently of the SGO (innervated by T12: ventral view). **e** The DO in lateral view extends into the distal tibia: anterior view. Scales: (**a**, **c–e**) = 100  $\mu$ m; (**b**) = 200  $\mu$ m. adb, anterior-dorsal branch of the nervus cruris; ant., anterior; avSGO, anterior-ventral subgenual organ; CS, campaniform sensilla; d, dendrites; DO, distal organ; ncr, nervus cruris; post., posterior; pvb, posterior-ventral branch of the nervus cruris; SGO, subgenual organ.



organs and their innervation pattern was similar in all leg pairs and in both females and males (Fig. 1). In addition, the numbers of sensilla were similar between the sexes for both organs. The SGO contained 37–54 sensilla (female: 37–54,  $n = 6$ ,  $N = 31$ ; male: 38–54,  $n = 4$ ,  $N = 21$ ) and the DO 16–23 (female: 16–23,  $n = 6$ ,  $N = 31$ ; male: 17–23,  $n = 4$ ;  $N = 21$ ) (online suppl. Table 1).

#### The Main Tibial Nerve Branches

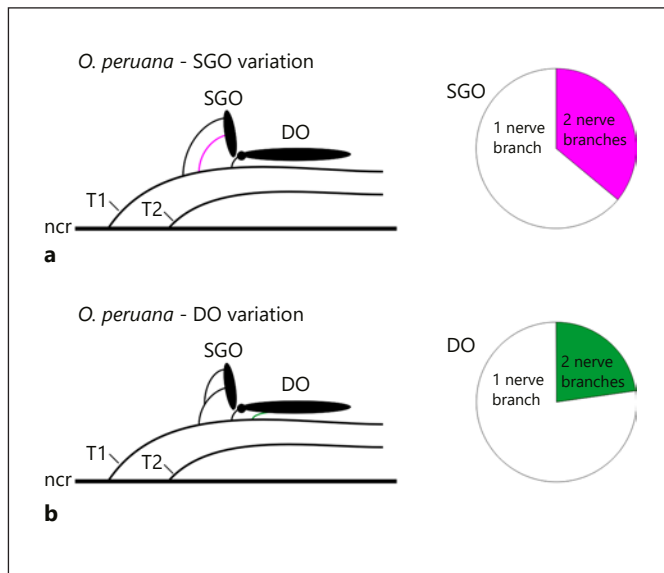
The proximal tibia was supplied by two nerve branches from nervus cruris, T1 and T2 (Fig. 2). Both branches had further nerve branches splitting off (T11, T21, etc.), which contained axons from specific sensilla in the tibia. The two most proximal nerve branches (T11, T21) supplied campaniform sensilla (6A) (Fig. 2). For 6A, at least

one sensillum occurred on the anterior and posterior sides. The following nerve branches in distal direction supplied a chordotonal organ and/or campaniform sensilla (see below).

#### Innervation of the Subgenual Organ Complex and Campaniform Sensilla in the Tibia

The innervation of sensory elements on the anterior side of the tibia was via nerve branch T1, of those on the posterior side via T2. The more medially located distal 6B campaniform sensilla are supplied by the nerve branch T23 (Fig. 2, 3b).

The SGO was supplied exclusively from T1, including the posterior sensilla in the SGO (Fig. 2, 3a<sub>i</sub>). Two campaniform sensilla (proximal 6B) were located close to the



**Fig. 4.** Innervation pattern and nerve branch variation in the subgenual organ complex of *O. peruana*. **a** Variation of the SGO innervation by one or two nerve branches. **b** Variation of the DO innervation by one or two nerve branches.

SGO (Fig. 2, 3b). The axon of the anterior 6B cell body was associated with nerve branch T12 also supplying the SGO sensilla (Fig. 2, 3c). Importantly, the posterior 6B cell body was separated from the SGO sensilla (Fig. 3d), and its axon formed the nerve branch T22. In the preparations analysed here, the posterior SGO sensilla were never innervated from nerve branch T2. Some variation occurred for the SGO innervation (Fig. 3a, 4a). The SGO sensilla were innervated by one nerve branch (Fig. 3a<sub>i</sub>; found in most cases of 64.15% of preparations,  $n = 53$ ) or two nerve branches (Fig. 3a<sub>ii</sub>; b; found in 35.85% of preparations). The SGO sensilla at the anterior side occur in a dense group, the anterior-ventral SGO (avSGO) (Fig. 1, 3a<sub>ii</sub>, e).

The DO was supplied from T1 (Fig. 2, 3b). The DO sensilla formed a linear set (Fig. 3e). The DO innervation was independent of the distal 6B campaniform sensilla placed at the level of the DO (Fig. 2). Some variation was also noted in the innervation of the DO sensilla (Fig. 4b), either by one nerve branch (Fig. 3a<sub>i</sub>; found in most cases of 77.36% of preparations,  $n = 43$ ) or by two nerve branches (Fig. 3a<sub>ii</sub>; found in 22.64% of preparations). In the cases of two nerve branches, the more proximal one supplied the proximal DO sensilla (Fig. 1f, 2, 3b). The differences in innervations by one or two nerve branches between the SGO and DO are statistically significant ( $\chi^2$  test,  $p = 0.0438$ ,  $\chi^2 = 4.063$ ,  $df = 1$ ). The DO has a more consistent

innervation by a single nerve, jointly with the avSGO (Fig. 2, 3e). Despite the variation in nerve branches, a common innervation pattern could be shown with a single nerve branch for SGO and DO (Fig. 5a).

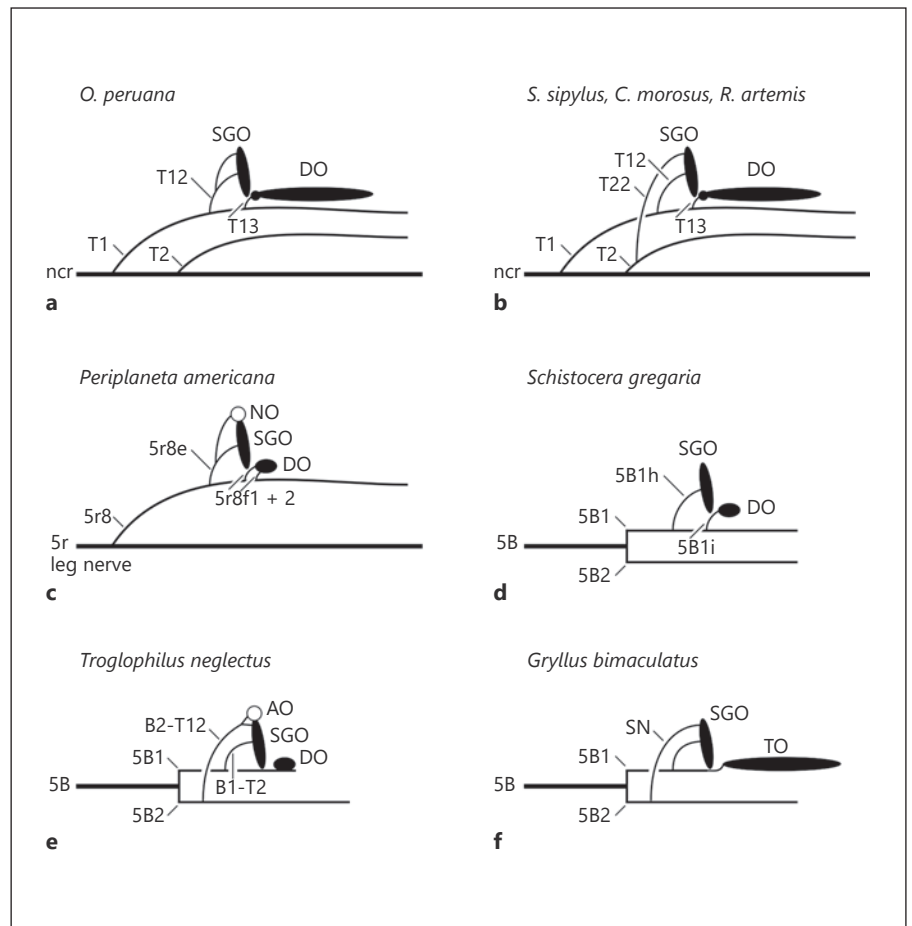
A rare variation was also seen in the innervation of the distal 6B campaniform sensilla, as the sensilla in two preparations were innervated directly from the nervus cruris (online suppl. Fig. S2).

## Discussion

### Neuronal Innervation Pattern of the Proximal Tibia

For leg chordotonal organs, the neuroanatomy and innervation pattern has been documented for diverse insect taxa [Debaisieux, 1938; Lakes and Mücke, 1989; Mücke, 1991; Field and Matheson, 1998]. Further investigation of the neuroanatomy is essential for particular sensory organs alongside physiological analysis, or for the sensory organs in species relevant for evolutionary aspects. The SGO complex allows to study the diversification and adaptation of mechanosensory organs. While the SGO is a sensitive detector of substrate vibrations, additional chordotonal organs developed next to it. Their sensory adaptations and morphologies show how mechanosensors can diverge. The present study reports for the first time the neuroanatomy of the SGO complex from a species of Occidophasmata, the New World stick insects. The SGO complex consists of two chordotonal organs, the SGO and the DO. The tibial innervation in *O. peruana* is consistent with data from previously analysed stick insects from the Oriophasmata [Strauß, 2020], since identical nerve patterns occur in the tibia. However, few anatomical differences occur in *O. peruana* in the innervation of the SGO by specific nerve branches and in the variability of innervation patterns. Despite some variation in the number of innervating nerve branches for chordotonal organs, a common innervation pattern for the chordotonal organs (Fig. 4, 5a) and the campaniform sensilla can be documented. Such variation in innervation is common in leg chordotonal organs [Lakes and Mücke, 1989]. The overall pattern of tibial nerve branches and of sensory elements is identical among species of stick insects studied so far (compare Strauß and Lakes-Harlan, 2013; Strauß, 2020; Strauß et al., 2021a). A comparison of the variability in other sensory organs in the more proximal parts of the leg (trochanter, femur) like campaniform sensilla and the femoral chordotonal organ requires neuroanatomical studies among further species of stick insects.

**Fig. 5.** Comparison of innervation pattern in the subgenual organ complex of stick insects and other orthopteroid insects. Schematic of the common innervation pattern in *O. peruana* (a), *S. sipylus*, *C. morosus*, and *R. artemis* (b), *P. americana* (Blattoidea) (c), *Schistocerca gregaria* (Orthoptera: Acrididae) (d), *Troglophilus neglectus* (Orthoptera: Ensifera) (e), and *Gryllus bimaculatus* (Orthoptera: Ensifera) (f). Note the differences in innervating nerve branches for the SGO. AO, accessory organ; DO, distal organ; ncr, nervus cruris; NO, Nebenorgan; SGO, subgenual organ; SN, subgenual nerve; TO, tympanal organ. (c) Adapted by permission from Springer Nature, Journal of Comparative Physiology A, Die subgenualen Sinnesorgane von *Periplaneta americana*: Histologie und Vibrationsschwellen, Schnorbus, 1971; (d) adapted by permission from Springer Nature, Zoomorphology, Innervation pattern and sensory supply of the midleg of *Schistocerca gregaria* (Insecta, Orthopteroidea). Mücke, 1991; (e) adapted from John Wiley and Sons, The accessory organ, a scolopidial sensory organ, in the cave cricket *Troglophilus neglectus* (Orthoptera: Ensifera: Rhabdiphoridae), Strauß and Stritih, 2016; (f) adapted by permission from Springer Nature, Das Tympanalorgan von *Gryllus bimaculatus* Degeer (Saltatoria, Gryllidae), Zeitschrift für Morphologie der Tiere. Michel, 1974.



**Chordotonal Organs in the Subgenual Organ Complex**  
The chordotonal organs in *O. peruana* are organized as in other stick insects [Strauß et al., 2021a]. In *O. peruana*, few notable differences to Oriophasmata were seen in the innervation of SGO and DO (Fig. 5a, b). This conforms to the suggestion that within taxa of orthopteroid insects, the SGO complex is highly similar.

**SGO:** the SGO is exclusively innervated by T12, but never from T2 (Fig. 2, 4a). In *C. morosus* or *S. sipylus* are the posterior SGO sensilla innervated by a distinct nerve branch, T22 (Fig. 5b). There is no specialization or simplification notable in the posterior SGO sensilla in *O. peruana*, as the sensilla resemble those of other stick insect species by forming a rather dense group of somata [see Strauß, 2020]. Further, the number of SGO sensilla (~40) is similar to that of the other stick insects (Strauß and Lakes-Harlan, 2013; Strauß et al., 2021a), consistent with a fully developed SGO in *O. peruana*. In sum, there are no indications of an evolutionary regression of a part of the

SGO. The SGO innervation pattern is thus simpler in *O. peruana* than in other stick insects.

In a comparative perspective, there are patterns in the SGO innervation known from related insect taxa that resemble the SGO innervation in *O. peruana*. The innervation of the entire SGO from one nerve branch is similar in locusts (*Schistocerca gregaria*) [Mücke, 1991] and cockroaches (*Periplaneta americana*) [Schnorbus, 1971] (Fig. 5c, d). In other insects like crickets or cave crickets (both Ensifera), the SGO is innervated from two separate nerve branches (Fig. 5e, f).

The switching of innervating nerves between groups of sensory neurons of chordotonal organs can be supported from other comparative studies [Strauß et al., 2016; Strauß, 2017]: the accessory organ is a small group of scolopidial sensilla located close to the SGO in several orthopteroid insects, but this organ is lacking in stick insects [Strauß, 2017]. The accessory organ is usually innervated together with a single CS by a distinct nerve branch.

In some phaneropterine bushcrickets, the accessory organ is innervated jointly with the SGO, while the nerve branch remains to innervate the single CS [Strauß, 2017]. This innervation pattern of a single posterior campaniform sensillum is similar to *O. peruana*, while nerve branch T22 persists (Fig. 3d). Hence, a switch of the innervating nerve for the chordotonal organ is likely also for the posterior SGO sensilla in stick insects. It is unclear if differences in the innervation pattern could result in a different projection pattern of afferents in the central nervous system and allow, for example, for a better somatotopic resolution of afferents in central neuropils. Apart from possible functional or adaptive explanations, another important aspect for traits are conditions derived from ancestral organizations [Riedl, 1978; Dumont and Robertson, 1986; Schwenk and Wagner, 2003]. It remains to be analysed in the more basal lineages of stick insects which innervation pattern is plesiomorphic for this group.

**DO:** The organization of the DO and the number of DO sensilla in *O. peruana* is highly similar to *C. morosus*, *S. sipylus*, and *R. artemis* [Strauß et al., 2021a]. The DO shows the linear organization of sensilla seen also in those Old World stick insects (Oriophasmata), suggesting a shared pattern for the sensory organs across stick insects. This broad presence of the linear DO in stick insects supports its relevance in mechanosensation. In *O. peruana*, there is no unique specialization apparent. The sensilla in the SGO complex are sensitive to substrate vibrations [Strauß and Lakes-Harlan, 2017], but the DO is not studied further for the sensory physiology.

#### Variation in the Innervation Pattern

Among individuals from one species, some variation in the innervation pattern of chordotonal organs occurs [e.g., Lakes and Mücke, 1989; Strauß et al., 2014]. Such variation is likely due to differences in axonal pathfinding during embryonic development of the central nervous system [Keshishian and Bentley, 1983; Lakes-Harlan and Pollack, 1993; Sanchez et al., 1995]. Functional implications for such peripheral variation are unclear, since the information processing likely depends on the central projection of axons. Similar variation for the SGO was also observed in *S. sipylus* [Strauß, 2020]. However, the extent of variation is notable in *O. peruana*, especially for the DO. For the SGO, the proportions of preparations were statistically not significant between *O. peruana* and *S. sipylus* [ $\chi^2$  test,  $p = 0.0641$ ,  $\chi^2 = 3.429$ ,  $df = 1$ ; data on *S. sipylus* from Strauß, 2020]. However, the DO in *S. sipylus* was always innervated by a single nerve

branch [Strauß, 2020]. This is in contrast to *O. peruana*, where ~23% of preparations show two DO nerve branches (Fig. 3a<sub>ii</sub>). These differences in proportions were statistically highly significant ( $\chi^2$  test,  $p < 0.0001$ ,  $\chi^2 = 25.99$ ,  $df = 1$ ). The reason for these differences in the DO neuroanatomy is not apparent, since both species show otherwise great similarity in the DO anatomy and the number of DO sensilla (*S. sipylus*: averages of 20–22 between leg pairs [Strauß and Lakes-Harlan, 2013]). Similar data on innervation variability are so far not documented for other species such as *C. morosus*. In *O. peruana*, the distal 6B CS can be innervated directly from nervus cruris (online suppl. Fig. 2) in a very rare and aberrant innervation pattern (in 2 of 53 preparations, 3.8%). This was also rarely documented in *S. sipylus* [Strauß, 2020, 1 in 44 preparations, 2.3%].

#### Phylogenetic Considerations of the Sensory Innervation in Phasmatodea

While the elements of the insect nervous system are often conserved during evolution [Meier and Reichert, 1995; Yager, 1999; Katz, 2011], the sensory organs, supplying nerves, and neuronal circuits can also change [Strausfeld, 1970; Strauß, 2017; Tosches, 2017] or even become reduced [King, 1983]. The SGO complex of stick insects shows a unique neuroanatomy among the orthopteroid insects [Strauß and Lakes-Harlan, 2013]. So far, only few species of stick insects have been investigated for this mechanosensory complex. The presence of the SGO and the DO supports a common, elaborate complex in stick insects or at least for the Neophasmatodea (Oriophasmata + Occidophasmata) [Simon et al., 2019]. This is consistent with a common neuroanatomy of sensory organs within a taxon, while numbers of sensilla and to some extent the innervation patterns can be modified. However, further neuroanatomical studies, especially of Occidophasmata species, could support this. Such studies can also give insights whether the innervation patterns are generally different between the two major clades of Phasmatodea. Finally, including data from the sister group to Neophasmatodea, the Aschiphasmatinae [Simon et al., 2019] would support the reconstruction of the ancestral innervation pattern in Euphasmatodea. The established phylogeny of Phasmatodea [Simon et al., 2019] allows for a character analysis at the levels of homologous sensory organs and peripheral nerves to infer their evolutionary changes in this group and to evaluate the anatomical robustness and modifications in specific lineages.



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## Statement of Ethics

The experiments documented 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. No specific ethics approval is required for research on insects under these regulations.

## Conflict of Interest Statement

The author has no conflicts of interest to declare.

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## Author Contributions

Johannes Strauß was responsible for the conceptualization of the study, experiments and analysis, design of figures, writing of the original draft, and funding acquisition.

## Data Availability Statement

The data on sensory neurons in the subgenual organ complex of *O. peruana* are available as supplementary data (online suppl. Table 1).

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