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Proprioceptors involved in stinging response of the honeybee, *Apis mellifera*.

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Abstract

Two types of mechanosensitive proprioceptor organ are present on the stinging apparatus of the honeybee: campaniform sensilla and mechanosensory hairplates. The campaniform sensilla are located on the surface of the tapering sting-shaft, which comprises an unpaired stylet and paired lancets. Each sensillum on the lancet differs from that on the stylet in terms of their topography and external morphology. The sensory afferents of the campaniform sensilla display slow-adapted firing responses to deformation of the cuticle that would be caused by the action of inserting the sting into a substrate, and their afferent signals induce and/or prolong the stinging response. By contrast, the mechanosensory hairplates are located at basal cuticular plates and on the posterior surface of the lancet valves. Two fields of hairplates on the second ramus at the ventral edge of the groove and on the antero-lateral edge of the oblong plate respond synchronously to protraction of the lancet. During the stinging response, these hairplates are likely to detect any sliding movement of the lancet and its position relative to the stylet. Afferent signals produced by them are likely to provide important information to the neuronal circuit for the generation and modulation of the stinging motor pattern.

1. Introduction

The exocuticle of insects and crustaceans contains a large number of mechanosensitive proprioceptors of various morphological types. These provide sensory information about the state and performance of exopodites and have a part in controlling the movement and posture of effectors during locomotion (reviewed by Bässler and Büschges, 1998; Pearson, 1993; Zill et al., 2004). In the honeybee, *Apis mellifera*, two kinds of external proprioceptor have been described on the stinging apparatus: campaniform sensilla and hairplates with trichoid sensilla (Hermann and Douglas, 1976a, b; Shing and Erickson, 1982). The campaniform sensilla located on the sting probably detect the depth of sting insertion by assaying the increasing cuticular deformation that occurs with successively deeper penetrations, whereas the hairplates located on the cuticular plates of the stinging apparatus are likely to be proprioceptors detecting the relative position of movable parts of the sting during the stinging response (Shing and Erickson, 1982). However, there are no published physiological studies available on the response characteristics and functional roles of these proprioceptive organs in the stinging response.

The stinging response is the culminating stage of the defensive behavior of the honeybee, involving penetration of a substrate by the shaft of the sting and the release of venom from the venom sac (Breed et al., 2004; Collins et al., 1980). Recently, the stinging response has been used for a new conditioning protocol in the honeybee, and has received much attention in the framework of learning and memory (Carcaud et al., 2009; Giurfa et al., 2009; Roussel et al., 2010; Vergoz et al., 2007). During the stinging response, in addition to the protraction of the entire shaft out of the abdomen tip, the paired ventral parts of the sting (i.e. the ‘lancet’ versus the unpaired dorsal component, the ‘stylet’) exhibit alternating rhythmic sliding (Snodgrass, 1956; Dade, 1962). This

movement results from the coordinated action of four pairs of stinging muscle (M196s, M197s, M198s, and M199s) and enables the sting to be inserted deeper into the target. A previous study described that afferent inputs to the terminal abdominal ganglion (TAG), in which the central pattern generator for the stinging movements is located, modulate the frequency of the rhythmic sliding and maintain the relationship between cycle period and burst duration of the stinging muscle activity at various frequencies (Ogawa et al., 1995). The sensory signals from the mechanosensitive proprioceptors in the stinging apparatus also appear to have an effect on the stinging motor patterns, but it is currently unknown how and which proprioceptors are involved in the modulation of the stinging response.

In the present study, we first describe the topography, detailed morphology and central projection of the proprioceptors, from which the effective stimulus to each receptor is inferred. We also analyze the electrophysiological responses of these receptors to the inferred effective stimuli, and examine the effects of the afferents on the stinging motor activity.

2. Materials and methods

2.1. Animals

Specimens of honeybees (*Apis mellifera* L.) were obtained from outdoor colonies at Okayama University. All the experiments presented were performed with foraging bees.

2.2. Preparation

Bees were anesthetized at 4°C for 20 min. The abdomen was severed from the thorax and pinned on a paraffin platform. Following longitudinal lateral-line incision, the terga of the 3–6th segments and the gut were removed to expose the stinging apparatus. To avoid any venom leaking from the preparation, the acid gland was coated with Vaseline.

2.3. Morphology

The topography and location of proprioceptors mediated by the stinging response were examined in whole sting shafts and basal cuticular plates that were removed from abdomens, under a transmission light microscope (BH-RFL, Olympus, Tokyo, Japan). The exact distribution and the external structure of proprioceptors were examined with a scanning electron microscope (SEM; T-300, JOEL, Tokyo, Japan) in isolated stinging apparatus that had been fixed with acetone (Wako Pure Chemical, Osaka, Japan), dehydrated, CO₂-critical-point dried and then coated with gold.

Sectional planes of the campaniform sensilla were examined using a transmission electron microscopy (TEM). The lancets and stylet dissected from the stinging apparatus were prefixed for 2 h at 4°C with 4% glutaraldehyde (in 0.1-M sodium phosphate buffer, pH 7.4, Sigma-Aldrich, St Louis, MO, USA). They were then post-fixed for 2 h at 4°C in a 2% OsO₄ solution in the same buffer, and finally dehydrated and imbedded in Epon 812 araldite (CY-230, Ciba Geigy, Tokyo, Japan).

Ultra-thin serial sections were double-stained with uranyl acetate and lead citrate, and observed under the TEM (H-300, Hitachi, Tokyo, Japan).

To stain the central projection of the sensory neurons innervating the proprioceptors, 10% agar gel consisting of 10% NiCl_2 was located either at the cut end of the sting (stylet or lancet) or at the end of hair sensilla on the valve for 8–24 h at 4°C. After filling, the 6th and terminal abdominal ganglia were isolated into honeybee saline (NaCl 270 mM, KCl 3.2 mM, MgCl_2 10 mM, CaCl_2 1.8 mM, NaHCO_3 7.1 mM, Dextrose 50 mM, Tris-Buffer 10 mM, pH 7.4, Wako). The nickel ions were then precipitated within the neurons by addition of rubeanic acid (Wako) to the honeybee saline. After fixation with 70% ethanol, the ganglia were dehydrated and cleared with methyl salicylate for whole-mount viewing. The ganglion stained with the nickel ion was intensified, according to the method of Bacon and Altman (1977). The stains were drawn as a whole mount using a camera lucida attached to the microscope (Olympus).

2.4. Electrophysiology

Electrophysiological experiments were performed with the exposed stinging apparatus in the above-mentioned manner. For extracellular recordings of neuronal responses of the proprioceptors, a glass suction electrode filled with honeybee saline was placed on a cut proximal stump of the lateral nerve, A8 or A9, innervating the proprioceptors. A reference electrode was placed in the abdomen. Campaniform sensilla were stimulated by a stainless steel probe attached to a micromanipulator (Narishige, Tokyo, Japan). The tip of the probe was placed on the surface of the sting, and pressed against the cuticle. The bend of the sting shaft or the barbs of the lancet led to excitation of the campaniform sensilla. To stimulate the hair sensilla on the second ramus or on

the oblong plate, a lancet was moved forward and backward alternately via a small stainless wire connected to the arm of a vibrator.

For recordings of electromyograms (EMGs), electropolished tungsten wires ($\phi = 70 \mu\text{m}$) were inserted into the stinging muscles (a protractor M198 and a retractor M199; see Ogawa et al., 1995). A reference electrode was placed in the abdomen. Recordings of the neuronal activity of sensory afferents and the EMGs were viewed on an oscilloscope and stored on magnetic tape.

3. Results

Two types of proprioceptive sense organ were observed on the honeybee stinging apparatus (Fig. 1). One is the campaniform sensilla, which are strain-sensitive mechanoreceptors distributed on the long shaft of the sting. The second type is mechanosensory hairplates located on the basal cuticular plates.

3.1. *Campaniform sensilla*

3.1.1. *Topography and external morphologies*

The campaniform sensilla on the sting were classified into two groups according to their distributions. The first group was observed on distal barbs of the lancet (Fig. 2A,C). One sensillum was associated with each of the 2nd to 7th barbs from the tip of the lancet, which has ten barbs in the honeybee (Fig. 3). The total number of the first group of sensilla was 8.11 ± 1.17 (mean \pm SD, number of samples = 45) for both sides of the lancet. The second group of campaniform sensilla was observed on wide area of the dorsolateral surface of the stylet (Fig. 2B, C). The total number of sensilla on one stylet was 63.66 ± 10.46 (mean \pm SD, number of samples =12), more than that of the sensilla on the lancet. Most of sensilla in the second group were not distributed near the mid-line of the shaft but near the lateral side instead. The topography suggests that the campaniform sensilla on the lancet detect distortions of the barb when the sting is inserted into an object, and that those on the stylet detect deformation of the entire sting shaft during the insertion. In addition to the difference in their distributions, the sensilla on the lancet and those on the stylet differed from each other in their external morphology. Electron microscopic observation revealed that surface of the campaniform sensillum on the lancet rises up quaquaversally, whereas that on the stylet hollows in the cuticle (Fig. 4A, B). The diameter of these sensilla was $2.29 \pm 0.26 \mu\text{m}$

(mean \pm SD, number of measured sensilla = 9, number of animals = 3) across the major axis. The major axis of each sensillum on the lancet corresponded to the orientation of the sting shaft (Fig. 4C).

3.1.2 Electrophysiological responses

Extracellular recording of the proximal cut-end of the lateral nerve 8 (A8; Fig. 10) including sensory afferents from the campaniform sensilla on the lancet, showed that deformation in the barb of lancet evoked a discharge of action potentials in A8 (Fig. 5A₁). The sensory afferents fired rapidly in response to the deformation stimulus. Although the spike discharge was sustained in the presence of an ongoing stimulus, spike frequency of the discharge gradually declined over the stimulus time (Fig. 5B₁). In these recordings, the discharge contained some sensory units with different spike amplitude. Bending of the sting shaft elicited bursting responses in the lateral nerve 9 (A9; Fig. 10) including the afferents from the sensilla on the stylet (Fig. 5A₂, A₃). The discharge recorded from A9 also contained several spike units. Both dorsal- and ventral-bending stimuli elicited tonic discharge of the sensory afferents in the temporal profile (Fig. 5B₂). The response of the sensilla on the stylet to the bending stimulus was not adapted as obvious in the spike frequency compared with the response to the distortion of the lancet barb. This result suggests that the afferent signals from the sensilla on the lancet respond to dynamic deformation of the barb derived from the movement of the sting into a substrate, whereas the sensilla distributed on the stylet detect the sustained distortion of the whole shaft of the sting during the insertion.

3.1.3. Stinging motor pattern evoked by stimulus to campaniform sensilla

In a preparation of the isolated abdomen as described in Materials and Methods, pinching of a tip of the lancet where the campaniform sensilla are located induced the stinging response, during which the right and left protractor muscles, M198s, rhythmically contracted in antiphase (Fig. 6A). This indicates that afferents of the campaniform sensilla on the lancet provide sensory signals triggering the stinging response. The pinching of the stylet bulb also elicited the stinging response (Fig. 6B). However, the induction of the stinging response required such a strong pinching stimulus that the stylet shaft was distorted. It thus appears that the threshold of the campaniform sensilla on the stylet for triggering the stinging response is higher than that on the lancet.

In the stinging motor pattern induced by pinching of the stylet, the duration of each discharge (burst duration) and the interval between successive discharges (cycle period) of M198s, which are standard parameters for describing the stinging motor activity (Ogawa et al., 1995), were longer than those measured in the response to the lancet pinching (Fig. 6B₁). Furthermore, under a sustained pinching stimulus to the stylet, the burst duration and the cycle period became increasingly long (Fig. 6B₂). Statistic analysis showed that both of these parameters for the motor pattern induced by stimulus to the stylet were significantly longer than for those of the pattern induced by stimulus to the lancet ($p < 0.05$, independent two-sample t-test for two-tailed hypothesis, Fig. 7A_{1,2}). This elongation of the burst duration and the cycle period has also been observed when the sting was inserted into a soft object, such as a rubber block (Ogawa et al., 1995). It was hypothesized that the pinching of the stylet augmented the friction of lancet movement and prolonged the cycle period and burst duration.

Next, to clarify the function of campaniform sensilla in the modulation of the stinging motor rhythm, we analyzed the motor patterns evoked by tactile stimulus to the

abdominal sternite of the honeybee, in which the whole sting was coated and fixed with wax (Fig. 6C). In this condition, the rhythmic movement of the lancets was disturbed without exciting the campaniform sensilla on the stylet. As was seen when the sting was inserted into a rubber block, both the burst duration and the cycle period measured in the wax-coated condition were significantly longer than those in intact preparations with a non-coated, uninserted sting ($p < 0.05$, independent two-sample t-test for two-tailed hypothesis, Fig. 7 A_{1,2}). By contrast, the tactile stimulus to the abdomen induced fewer (only three) cycles of rhythmic contraction in the wax-coated condition (Fig. 6C). There were also fewer burst cycles in the whole response in the wax-coated condition compared with that in the rubber-inserted condition ($p < 0.05$, Mann-Whitney's U test, Fig. 7 A₃). This result suggests that the afferent signals of campaniform sensilla on the sting contribute to the triggering and continuance of the stinging movement, but take no part in the elongation of the stinging motor rhythm.

Furthermore, we analyzed the relationship between the burst duration and the cycle period measured from EMGs of M198, which is a useful index characterizing the stinging motor program mediated by sensory feedback (Ogawa et al., 1995). In Fig. 7B, the burst duration is plotted against the cycle period under various conditions. The motor patterns induced by stimulus of the stylet showed a linear correlation between the burst duration and the cycle period as well as the motor pattern evoked by tactile stimulus to the sternite, which is one of the characteristic stinging motor patterns (Ogawa et al., 1995, Fig. 7B₁). The coefficients of correlation are 0.9383 for the data of the stylet-stimulated pattern and 0.9607 for the data of the sternite-stimulated pattern in the rubber-inserted condition. The first-order regression lines of the correlation are $y = 0.5409x - 11.812$ for the plots the stylet-stimulated pattern and $y = 0.4394x + 3.493$ for the plots of the sternite-stimulated pattern in the rubber-inserted condition. Statistic

analysis also showed that there was no difference in the proportion of burst in the cycle period between the stylet-stimulated and sternite-stimulated patterns in the rubber-inserted condition ($p > 0.05$, independent two-sample t-test for two-tailed hypothesis, Fig. 7A₄). These results demonstrated that the stinging responses triggered by the campaniform sensilla on the stylet are the same in terms of their motor program as the response to stimulus of the abdomen (Ogawa et al., 1995). The analysis of the data of the motor patterns evoked by lancet-pinching revealed little correlation between the burst duration and the cycle period (the coefficients of correlation is 0.3338), because the plots for the lancet-stimulated patterns were more narrowly distributed than were those for the patterns evoked by stylet or sternite stimulus. However, the burst proportion of the lancet-stimulated pattern was close to that of the sternite-stimulated pattern under the uninserted condition. The input from campaniform sensilla on the lancets is also likely to evoke the stinging motor pattern. The sensory feedback for stabilizing and modulating the motor patterns probably functions successfully in the stinging response evoked by mechanical stimulus of the campaniform sensilla.

Using this analysis, we compared the stinging motor patterns recorded under the wax-coated condition with those under the rubber-inserted condition. The motor patterns under the wax-coated condition showed a correlation between the burst duration and cycle period (Fig. 7B₂). The coefficient of correlation for the motor patterns in the wax-coated condition was 0.9170. However, the first-order regression line of the correlation for the data of the wax-coated condition ($y = 0.686x - 18.928$) was steeper in its slope compared with the line of the motor pattern in the rubber-inserted condition; in addition, the proportion of burst in the cycle period recorded in the wax-coated condition was significantly larger than that recorded in the rubber-inserted condition ($p < 0.05$, independent two-sample t-test for two-tailed hypothesis, Fig. 7A₄).

It was therefore suggested that sensory feedback from the campaniform sensilla on the sting is not essential for the basic structuring of the stinging motor program but that it could make a minor contribution to the modulation of the duration of activity of the stinging muscle.

3.2. *Hairplate sensilla*

3.2.1. *External morphologies*

The mechanosensory hairplates are found on three fields of movable parts of the stinging apparatus: the first is located on the second ramus at the ventral edge of the groove, the second is located on anterolateral edge of the oblong plate, directly beneath the triangular plate, and the third is located on the posterior side of the lancet valve (Fig. 8). The plate on the second ramus comprised 20–30 hairs, which were arranged in one or two lines along the first ramus (Fig. 8A). Each hair sensillum is cone shaped, approximately $2.19 \pm 0.28 \mu\text{m}$ in root diameter and $13.65 \pm 3.21 \mu\text{m}$ in length (mean \pm SD, number of measured sensilla = 12, number of animals = 2). Their arrangement suggests that the hair sensilla on the second ramus would be able to detect the rhythmic thrusting movement of the lancet during the stinging response, because the first ramus connecting to the lancet slides on the second ramus by contraction of the protractor M198. The hairplate on the oblong plate is approximately $80 \times 50 \mu\text{m}$ in size and contains approximately 20 hairs (Fig. 8B). Each hair sensillum is also cone shaped, $3.05 \pm 0.32 \mu\text{m}$ in root diameter and $24.01 \pm 5.20 \mu\text{m}$ in length (mean \pm SD, number of measured sensilla = 18, number of animals = 2), which is slightly longer than the hairs on the second ramus. Given that this hairplate is engaged with the triangular plate, it is likely to be able to detect the relative position of the triangular plate connecting to the lancet via the first ramus, in addition to thrusting of the lancet. Mechanosensory hairs on the

valve of the lancet, which were observed using a light microscope but not with SEM, were thinner and longer than the cone-shaped hair sensilla on either the second ramus or the oblong plate (Fig. 8C). It is suggested that the hairplate on the valve is likely to detect the fluid pressure or flow velocity of the venom within the bulb of the stylet during the stinging response.

3.2.2. Electrophysiological responses

Artificial thrusting of the lancet on the stylet with a wire probe evoked spike discharges in the 9th lateral nerve (A9; Fig. 10), which includes afferent axons of the hairplates on the second ramus and the oblong plate. Some spike units with a different amplitude were contained by the discharges recorded throughout the movement. Their response to sliding of the lancet was transient and directionally sensitive: protraction of the lancet excited the sensory afferents, whereas retraction evoked few responses (Fig. 9A₁). When the lancet was moved in alternating sinusoidal or triangular waves, the spike frequency of the afferent units was altered in sync with both types of movement. There was no difference between the two types of stimulus in terms of the responses of the afferents (Fig. 9A₂, A₃): The afferents responded maximally during the protraction phase to each movement (Fig. 9B). Furthermore, it was confirmed that the firing responses of the afferents were synchronized with protraction of the lancet at different frequencies (0.5–4.5 Hz; Fig. 9C). These results showed that the afferent of the mechanosensory hairplates on the second ramus and the oblong plate could provide sensory signals synchronized with the rhythmic movement of the lancet during the stinging response. It is suggested that these afferent signals convey sensory information about the relative position of the first ramus to the second ramus or about the stroke of the lancet. The hairplates might have important roles in the modulation of the stinging

motor rhythm, such as the elongation of the burst duration and cycle period of stinging muscle activity, when the sting is inserted into a substrate.

3.3. Central projections of afferents from proprioceptors

Afferent axons from the mechanoreceptors on the lancet and the triangular and quadrate plates enter TAG via the 8th lateral nerve, A8, because these parts are developed from the 8th sternum. By contrast, axons from the mechanoreceptors on the stylet, oblong plate and sheath of the sting all of which are developed from the 9th sternum, merge into the 9th lateral nerve, A9 with motor nerve branches innervating stinging muscles, including M198, and then enter the TAG.

We stained the afferent fibers of the campaniform sensilla at the tip of the lancet and the bulb of the stylet (Fig. 10A, B). Most of projections of the campaniform sensilla of both the lancet and stylet were confined to the ipsilateral hemisphere in the TAG, although part of the axon arborizes to the contralateral side across a mid-line. Furthermore, other branches extend to the ipsilateral side of the 6th ganglion through a connective nerve cord. The branches in the projection from the stylet were fewer than those from the lancet, which might result from shortage in the uptake of the nickel into the axons. Unfortunately, we were unable to stain the afferents of the hairplates on the second ramus and the oblong plate, because it was technically too difficult to sort only the nerve roots to these sensilla. The projection patterns of afferent axons from the hairplate on the lancet valve within the TAG were similar to those of the campaniform sensilla. However, there was no process extending to the 6th ganglion (Fig. 10C).

4. Discussion

4.1. Two groups of campaniform sensilla

The campaniform sensilla are the proprioceptive organs of arthropods. They are embedded in the cuticle and are excited by compression perpendicular to their axis (Pringle, 1938). In surface view, individual receptors appear as small-diameter, domed caps. In the sting of the honeybee, campaniform sensilla are found on each barb of the lancet and the stylet (Shing and Erickson, 1982). In the current study, we showed that the two groups of campaniform sensillum on the sting differ from each other in terms of their distribution and external morphology: the first type of sensilla is located on each barb of the lancet and have a large domed cap at the surface of cuticle, whereas the second type is distributed over the whole stylet and have a small cap within a cuticular sinkhole. Their external structures appear to reflect the difference in their sensitivity and response to deformation of cuticle. The major axis of both types of campaniform sensillum, however, corresponds to the orientation of the sting shaft. This observation suggests that both types of campaniform sensilla are sensitive to bending of the sting or deformation of the barbs.

4.2. Physiological roles of campaniform sensilla

The campaniform sensilla are mechanosensitive organs that monitor forces via cuticle strain in the exoskeleton of insects (Pringle, 1938). These sensilla are arranged on various limbs of insects in groups with distinctive distributions, where cuticular strain is induced both by passive loading of the leg and during muscle-driven locomotion (Hofmann and Bässler, 1982; Hustert et al., 1981; Pringle, 1938; Spinola and Chapman, 1975). Sensory signals from the femoral and trochanteral campaniform sensilla affect the timing of the central pattern generator for insect walking, and have a

primary role in coordinating movements of the distal leg joints (Akay et al., 2001, 2004). In the cockroach, for example, sensilla responding to an increase or decrease in the force of the legs contribute to the onset of swing and reinforce leg coordination during walking (Keller et al., 2007; Ridgel et al., 1999; Noah et al., 2001, 2004; Zill et al., 2009). In our experiments, the pinching stimulus to the campaniform sensilla situated on the sting evoked the stinging response, and the stinging movement was sustained for as long as the sting was pinched. These results demonstrate that the sensory signals from campaniform sensilla on the sting have key roles in trigger and continuance of the stinging response.

The sting shaft of the honeybee is usually enfolded within the sting chamber located at posterior end of the abdomen. Therefore, distortion in the sting activating the campaniform sensilla is caused only if the sting is inserted into the target object. As soon as the sting is inserted, the stinging apparatus is isolated from the main body and left on the surface of the inserted object. Even after isolation from the body, the stinging apparatus continues its rhythmic stinging action so that the sting shaft can be inserted successively deeper, and the venom injected into the target. Sustained inputs from the campaniform sensilla excited by deformation in the inserted sting are likely to be effective enough to prolong the stinging action after isolation of the sting from the body of the honeybee.

We also identified campaniform sensilla on the stings of various species of *Aculeata*, including the Asiatic honeybee *Apis cerana* and four species of wasps: *Polistes chinensis* A, *Polistes rothneyi* I, *Vespa simillima* X, *Vespa mandarinia* J (Supplemental Fig. 1). The number and distribution of sensilla in *A. cerana* are identical on the lancets and the stylet with those in *A. mellifera* (Supplemental Fig. 1A₁). By contrast, differences occur between honeybees and wasps in the number and distribution of the

campaniform sensilla on the sting, although each sensillum is similar in size and morphology to those seen in honeybees (Supplemental Fig. B₁). There are more campaniform sensilla on the stylet of wasps compared with the stylet of honeybees; the total number is approximately 50, 130, 160 and over 200 in *P. chinensis*, *P. rothneyi*, *V. simillima* and *V. mandarinia*, respectively. Most of the sensilla are distributed around the bulb of the stylet in the honeybee, whereas the campaniform sensilla are located as a cluster at root of the stylet in the wasps (Supplemental Fig. 1B₂). The stylet of the wasp sting is slender without the ‘bulb’ in which the lancet valves pump and deposit the venom, because wasps eject the venom by contraction of the muscle covering a venom sac, rather than by pumping of the lancet valves (Supplemental Fig. 2). In wasps, the muscles connecting the stinging apparatus with the abdomen are well developed, and the sting is oriented to the target object. The cluster of the campaniform sensilla at the base of the stylet would, therefore, provide effective sensory signals for control of the sting toward the target. By contrast, the similar arrangement of the campaniform sensilla on the barb of the lancet between the honeybees and the wasps (Supplemental Fig. 1A₂₋₅) suggests that these sensilla provide essential signals to trigger and sustain the stinging response in various species of Aculeata.

4.3. Two types of hairplate on the stinging apparatus

The hairplate is an assembly of mechanosensory hairs in a characteristic grouping that is excited by the deflection of each hair as it comes into contact with the exopodites of the arthropod; thus, they monitor the movement of the effectors directly. In insects, the hairplates are implicated in various types of proprioceptive reflex to maintain the posture of the body and exopodites. Electrophysiological studies have shown that the hairplates at the leg joints detect the position and movement of the legs (stick insects:

Wendler, 1971; Büschges and Schmitz, 1991; cockroaches: Wong and Pearson, 1976; locusts: Bräunig and Hustert, 1985a,b). These receptors also provide sensory-feedback signals synchronized with leg movement during walking and to effect the generation of walking motor activity (Wong and Pearson, 1976; Wendler, 1966; Bässler, 1986).

In the stinging apparatus of the honeybee, the location and external morphology of the hairplates give some indication of the kind of stimulus that activates them. The hairplates with short, thick hair sensilla were identified on two fields of the basal cuticular plates, the second rami and the oblong plates, which are attached to the moving parts of the lancet as it slides along the stylet. These hairplates probably detect the skeletal movements of the first ramus or the triangular plate, and provide sensory information about the relative position and movement of the lancet on the stylet during the stinging response. By contrast, the long, thin hair sensilla located on the distal surface of the lancet valve appear to detect the flow of the venom as it fills the stylet bulb. It is suggested that the afferent signal from this hairplate has a role in monitoring the amount of venom that is drained from venom sac.

4.4. Proprioceptive feedback to the stinging pattern generator

In the stinging response of the honeybee, the rhythmically sliding movements of the lancet are produced by alternating contractions of a set of stinging muscles (a protractor, M198 and a retractor, M199). A study using EMG illustrated the relationships among three temporal components of the stinging motor pattern: the interval between successive discharges of a muscle; the duration of discharge; and the time lag between the right and left discharges of homologous units (Ogawa et al, 1995). All three components elongate linearly as the sting is inserted deeper into a soft substrate and the tension on the lancets is increased. The maintenance of these relationships and

modulation of the stinging motor pattern require sensory feedback from the proprioceptors on the stinging apparatus.

To effect the rhythmic motor pattern generation in the nervous system, the afferents from the mechanoreceptors need to provide sensory signals synchronized with the movement of the effectors. The hairplates and campaniform sensilla at the leg joint of insects provide motor neurons and interneurons in the central pattern generator (CPG) with periodic sensory signals synchronized with the stepping movement during walking, and can effect the generation of walking motor activity (reviews in Burrows, 1996; Bässler and Büschges, 1998; Zill et al., 2004). The afferents from the hairplates on the second ramus and the oblong plate discharge spikes in phase over the protraction of the lancet, and maximally respond to the peak of the lancet protraction. This result shows that these proprioceptors are capable of monitoring the correct position of the lancet during the stinging response. It is suggested that the hairplate organs on the base plates of the stinging apparatus provide the stinging CPG with proprioceptive feedback, which has an important role in the patterning of the stinging motor activity.

In the stinging response without a mechanical load to the sting shaft, the stinging muscles contract in quick rhythm and the right and left stroke of the sliding of the lancet are in synch with each other. As the sting is inserted into an object and friction of the sting increases, the burst duration of protractor, M198 becomes longer (Ogawa et al., 1995). This elongation, which was observed in the motor pattern evoked by mechanical stimulus to the campaniform sensilla on the stylet, enables a constant depth of insertion to be maintained by one contraction of M198 on each side of the stylet. This system ensures quick and certain insertion of the sting. The information about protraction of the lancet, which is provided by the hairplates, is vital to the control of lancet sliding for the motor pattern generating system.

Deafferentation, by cutting of the sensory nerves, causes the stinging motor pattern to become more variable and destabilized (Ogawa et al., 1995). The present study demonstrated that the afferents from the campaniform sensilla have little effect on modulation of the stinging motor pattern, because the burst duration is significantly longer even if sliding of the lancets is suppressed by wax coating without distortion of the sting shaft, as shown by the statistical analysis in Fig. 7. Furthermore, this deafferentation of the campaniform sensilla did not alter the temporal characteristics of the stinging motor program, including the correlation between burst duration and the cycle period of the stinging muscle activity. Therefore, it is thought that the afferent signals of the campaniform sensilla are not essential for stinging pattern generation. However, owing to technical difficulties, it was impossible to record separately the responses of the afferents from the hairplates on the second ramus and the oblong plate. Although details of the distinct response-property of each hairplate are unclear, these proprioceptors could provide important sensory feedback signals for stinging motor pattern generation. Neuronal components receiving the sensory feedback from the hairplates have been unknown in the stinging CPG. Further analysis of neuronal circuit of the CPG will provide additional details of the sensory feedback mechanism in the stinging response of the honeybee.

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Figure legend

Fig. 1. Overview of the proprioceptors on the stinging apparatus. (A) Drawing indicating location of the stinging apparatus in the honeybee (square enclosed area). (B) Schematic illustration of the distribution of proprioceptors on the stinging apparatus (lateral view). Dark-gray areas indicate a field on which the mechanosensory hairplates are located, and light-gray areas indicate a field on which the campaniform sensilla are located (see also Fig. 8). 1r, first ramus; 2r, second ramus; AGld, acid gland; Lct, lancet; Ob, oblong plate; Qd, quadrate plate; Sty, stylet; Tri, triangular plate; Vlv, valve on lancet; Vmsc, venom sac.

Fig. 2. Topography of campaniform sensilla on the sting. (A, B) Transmission light micrograph of the lancet tip viewed ventrally (A) and the stylet bulb viewed laterally (B). The preparations are whole mounted. Arrows indicate the campaniform sensilla. (C) Drawings of the lancet and stylet in a given sample, indicating distribution of campaniform sensilla on the sting. Left drawing in C shows ventral side of the lancet. Middle and right drawings show dorsal and lateral sides of the stylet, respectively. Each sensillum was marked as a black dot. The sensilla on the lancet are located at base of the each barb by ones, while most of sensilla on the stylet are distributed on the lateral side of the sting shaft from bulb to tip.

Fig. 3. Distribution of the campaniform sensilla on the lancet. Total numbers of appearance of the sensilla in 45 samples were counted along the sting: in most

of samples, the campaniform sensilla are observed at base of the 3rd, 4th and 5th barbs from the tip of sting.

Fig. 4. Electron micrographs of campaniform sensilla on the sting. (A) Scanning electron micrographs (SEM) showing external morphology of the campaniform sensillum on the lancet (A_1) and on the stylet (A_2). (B) Cross section images of the sensillum on the lancet (B_1) and on the stylet (B_2). (C) SEM images of ventral (C_1) and lateral (C_2) views of the lancet, showing alignment of the campaniform sensilla at base of the lancet barbs. Scale bars: 1 μm in A, B, 10 μm in C_1 , 5 μm in C_2 .

Fig. 5. Responses of the campaniform sensilla to sting deformation. (A) Typical recordings from afferents in the lateral nerve A8 or A9. Lower traces monitor the movement of a stainless steel probe for deformation stimulus. In A_1 , the sensilla on the lancet responded to distortion of the lancet barbs. In A_2 and A_3 , the sensilla on the stylet responded to ventralward or dorsalward bending of the stylet shaft, respectively. Schematic diagrams on right insets indicate the stimulus procedures. All responses in A were recorded from different preparations. (B) Histogram of the spike frequency in responses of large afferent units to deformation of the barbs (B_1) and ventralward bending of the shaft (B_2). Each bar shows mean firing rate in 100-ms bin, which was calculated from recordings of 4 (for B_1) or 5 (for B_2) trials in different two preparations.

Fig. 6. Electromyograms of left and right protractor muscles, M198s during the stinging responses. (A, B) Stinging motor patterns elicited by pinching stimuli to the tip of

lancets in A, or to the bulb of stylet in B₁ and B₂. Each upward arrowhead under the lower traces in A and B₁ shows a time point of transient pinching stimulus. The EMG shown in B₂ were stinging motor pattern when the pinching of the stylet was retained. A line under the lower trace in B₂ indicates the duration of the pinching stimulus. Both the duration of each discharge (the burst duration) and the interval between successive discharges (the cycle period) of M198s became longer gradually. (C) Motor pattern evoked by tactile stimulus to sternite when the whole sting was fixed with wax. In this condition, the rhythmic movement of the lancets was disturbed while the campaniform sensilla were not excited. The burst duration and the cycle period were also much longer in the stinging motor pattern in C. The traces in A, B and C were obtained from different preparations.

Fig. 7. Statistic analysis of the stinging motor patterns. (A) Summary of four kinds of parameters measured from EMG of M198, including the burst duration (A₁), the cycle period (A₂), the number of cycles per whole response (A₃) and the proportion of the burst duration to the cycle period (A₄). The data measured from the stinging responses to pinching stimulus to the lancet (Lct) or stylet (Sty) were showed by black bars. Gray bars indicate the data measured from the motor patterns evoked by tactile stimulus to the abdominal sternite in different conditions, in which the sting was not inserted (uninserted), inserted into the rubber block (rubber) or fixed with wax (wax-coated). (B) Relationships between the cycle period and the burst duration in the stinging motor patterns evoked by different stimuli in various conditions. Open squares indicate the data in the lancet-stimulated pattern. Open circles indicate the data in the style-stimulated pattern. Cross marks, filled lozenges and gray triangle indicate the data in the

stenite-stimulated patterns in uninserted, rubber-inserted and wax-coated conditions, respectively. Lines are first-order regression lines for each data set: solid line for the patterns in rubber-inserted condition, broken lines for the stylet-stimulated patterns in B₁ and for the patterns in wax-coated condition in B₂. Numbers of measured-cycles were 25 for the lancet-stimulation, 51 for the stylet-stimulation, 140 for the uninserted condition, 66 for the rubber-inserted condition and 25 for the wax-coated condition. Numbers of responses are 5 for the lancet stimulation, 4 for the stylet-stimulation, 45 for the uninserted condition, 8 for the rubber-inserted condition and 8 for the wax-coated condition. Numbers of animals are 3 for the lancet-stimulation, 4 for the stylet-stimulation and 6 for the sternite-stimulations in each type of conditions, respectively.

Fig. 8. External morphology of the hairplates on the stinging apparatus. (A, B) SEM images of the hairplates on the 2nd ramus (A) and on the oblong plate (B). (C) Transmission light micrographs of the hairplate on the lancet valve. Left diagram shows location of the hairplates (dark-gray areas). Abbreviations as in Fig. 1.

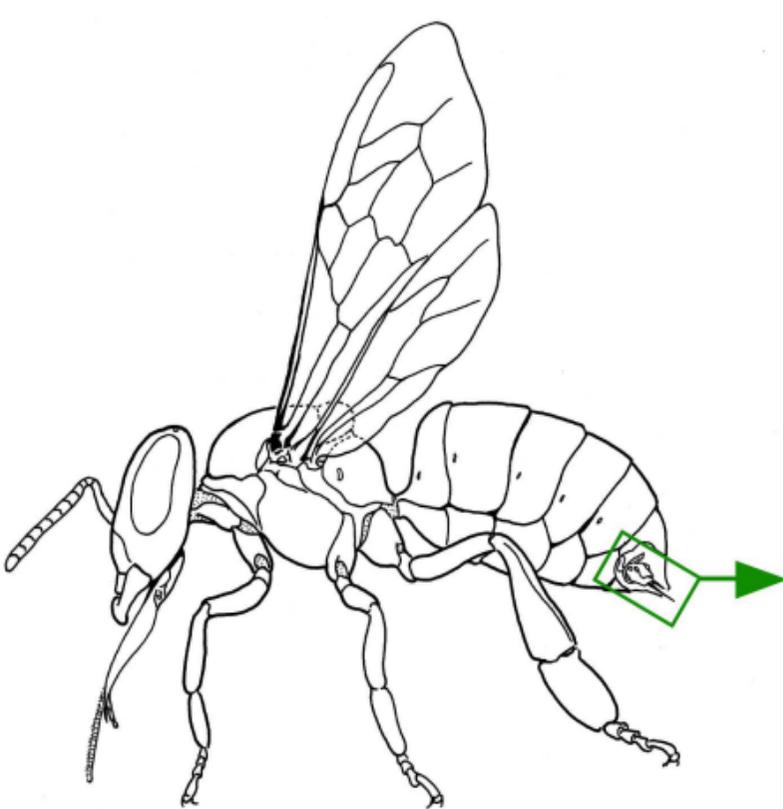
Fig. 9. Responses of the hairplates to lancet movements. (A) Typical responses to three types of the movement. A₁, A₂ and A₃ show responses to a ramp-hold-release protraction, periodic sliding in sinusoidal or triangular waves at 0.5 Hz, respectively. Upper traces show extracellular recordings of the sensory afferents within the lateral nerve A9, which innervates the hairplate on the 2nd ramus or the oblong plate. Lower traces monitor relative position of sliding lancet, in which the upward trace means protraction of the lancet. Peak-to-peak amplitude of the stimulation was adjusted to maximum stroke of the lancet sliding. All traces were

recorded from different preparation. (B) Histograms of spike frequency obtained from large afferent units. B₁ and B₂ show the responses to sinusoidal or triangular sliding, respectively. Each bar with error bar shows mean \pm SEM of the firing rate in 100-ms bin, which was calculated from 8 cycles in the recordings shown in A₂ and A₃. The spike frequency was maximum at most protracted position. (C) Responses to sinusoidal sliding of the lancet in different frequency. In C₁-C₄, the lancet was periodically moved in 0.5, 1.5, 2.5 and 3.5 Hz, respectively. All responses in C were recorded from a single preparation different from that for A and B.

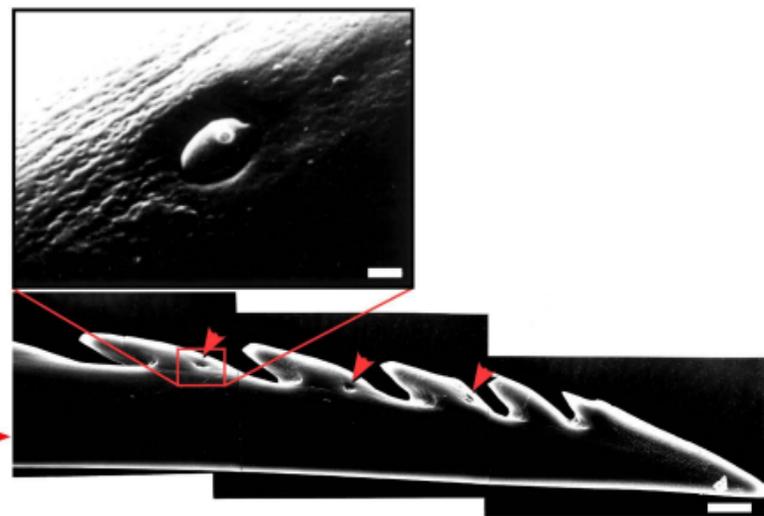
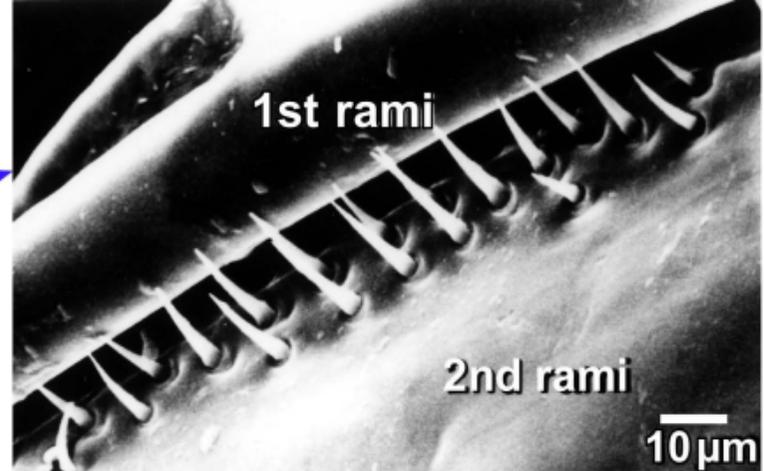
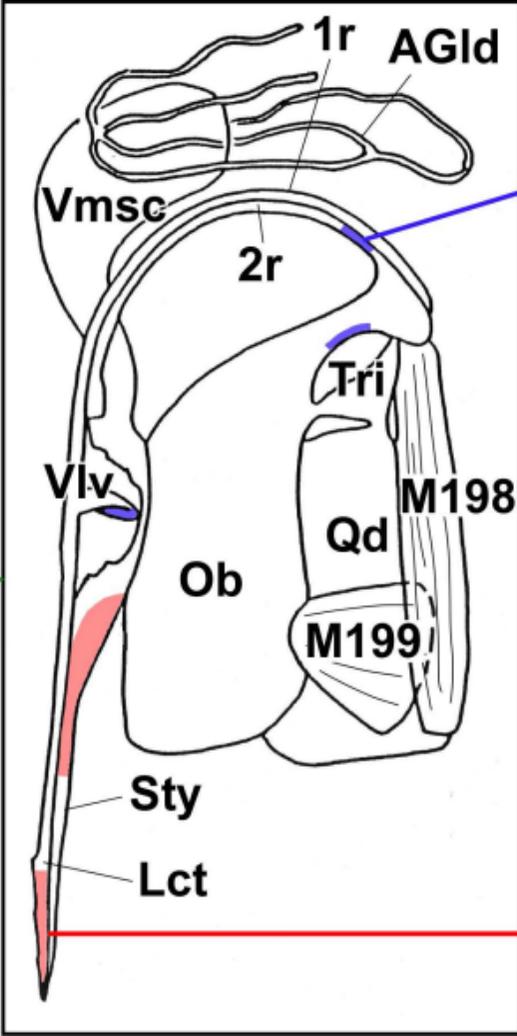
Fig. 10. Innervation and central projection of the afferents from proprioceptors on the stinging apparatus. (A, B) Drawings of the afferent fibers from the campaniform sensilla on the lancet in A and on the stylet in B within the 6th and terminal abdominal ganglia. (C) Drawing of the afferent fibers from the hairplate sensilla on the lancet valve within the TAG. 6th AG, sixth abdominal ganglion; TAG, terminal abdominal ganglion.

Supplemental Fig. 1. Arrangement of the campaniform sensilla on the sting in five species of bees and wasps. (A) Typical patterns in distribution of the campaniform sensilla on the lancet of *Apis cerana* (A₁), *Polistes chinensis* (A₂), *Polistes rothneyi* (A₃), *Vespa simillima* (A₄) and *Vespa mandarinia* (A₅). Each sensillum is marked as a black dot. On the sting of wasps, the campaniform sensilla are located at not only base of the barb but also lancet shaft. (B) SEM images showing external morphology of the campaniform sensilla on the lancet (B₁) and cluster of the campaniform sensilla located at base of the stylet (B₂) in *Polistes rothneyi*.

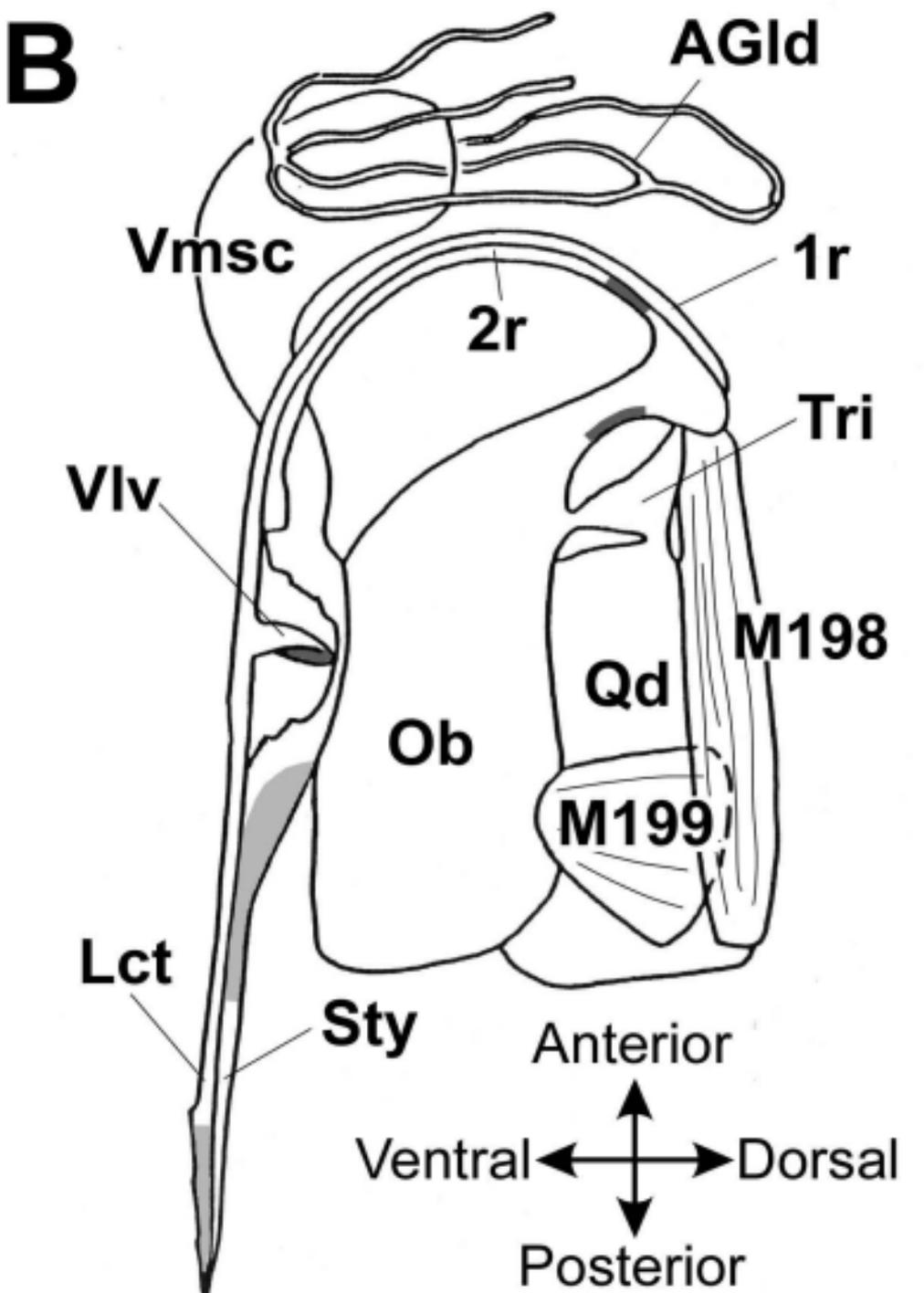
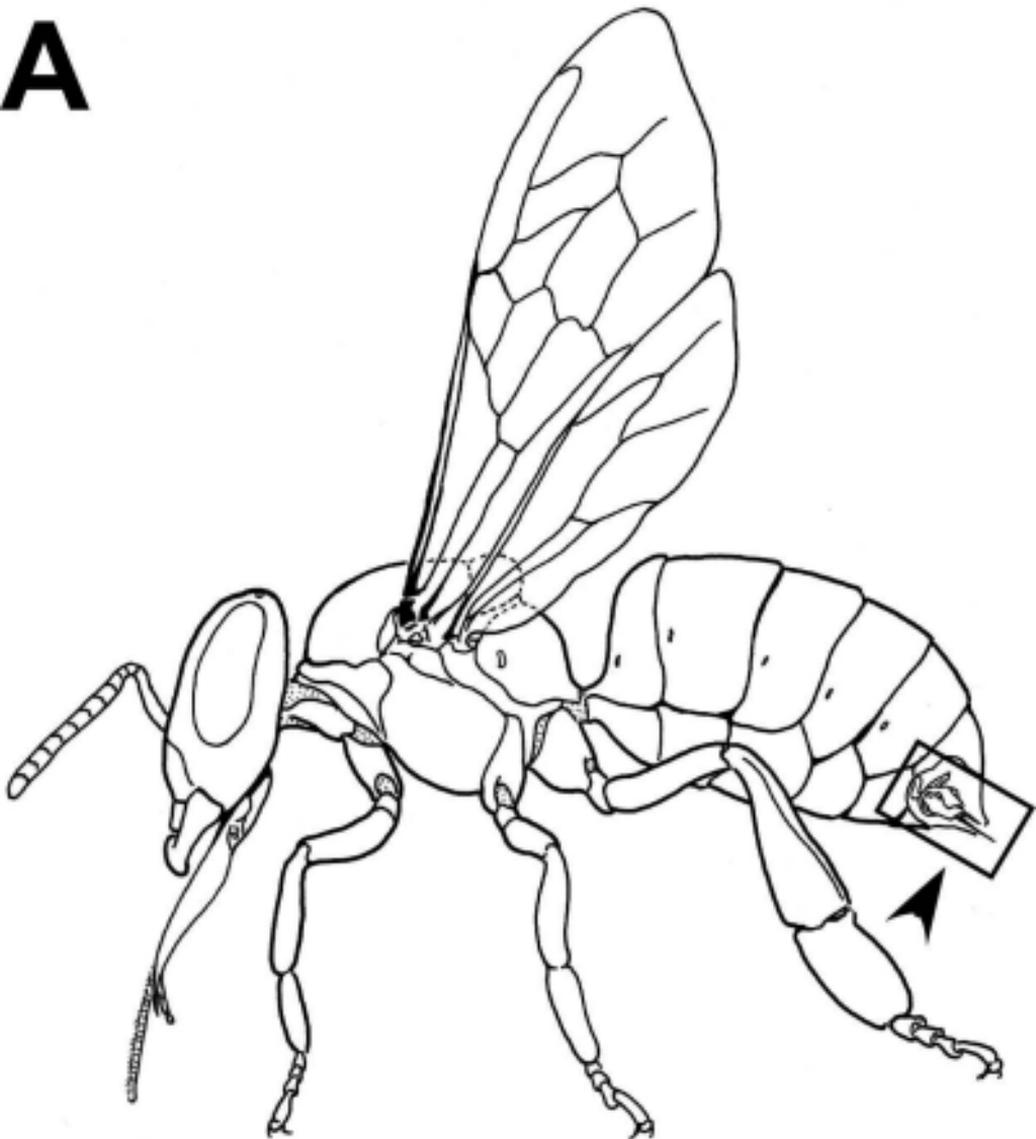
Supplemental Fig. 2. Schematic illustration of the stinging apparatus (dorsal view) in *Polistes chinensis*. Six pairs of stinging muscles (M188, M196, M197, M198 and M199) lie on three pairs of plates (the quadrate plate, oblong plate and triangular plate, which are visible after the removal of stinging muscles as shown on the right side. AGld, acid gland; BGld, alkali gland; Ob, oblong plate; Qd, quadrate plate; Sh, sheath; Tri, triangular plate; Vmsc, venom sac.



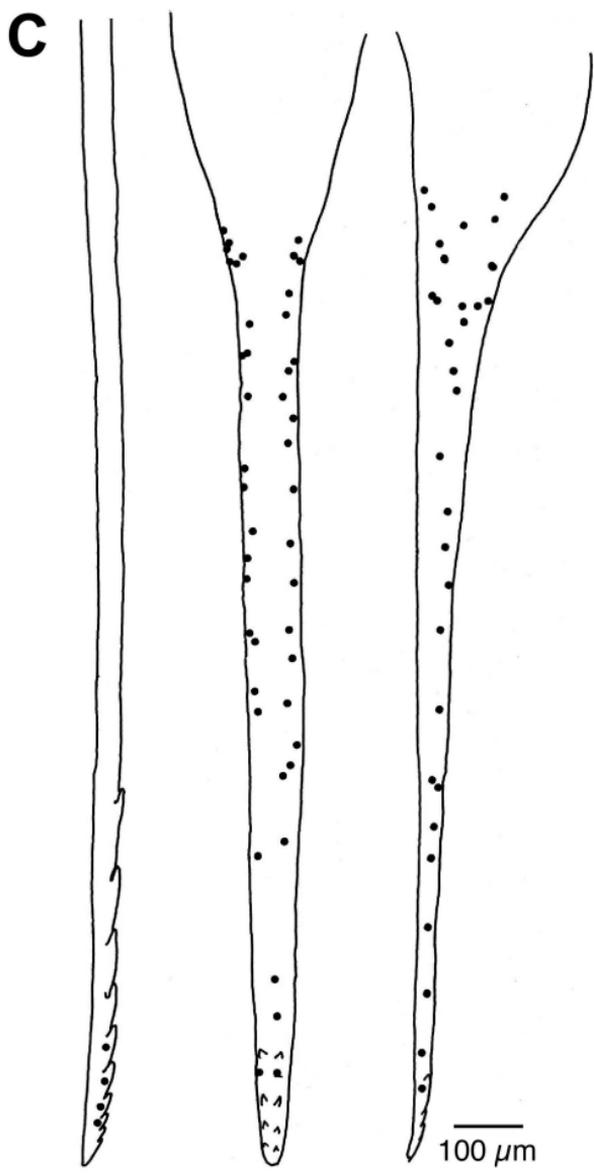
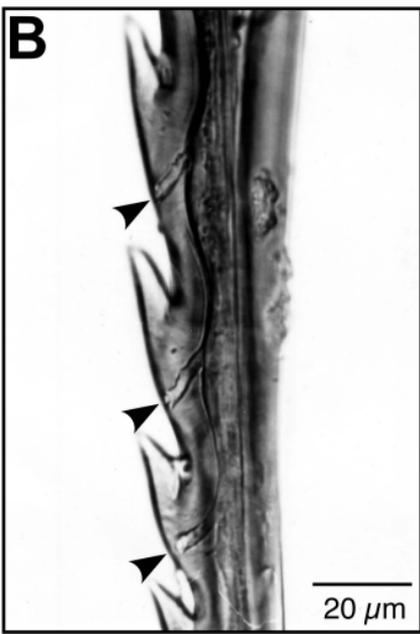
-  Campaniform sensilla
-  Hair plates



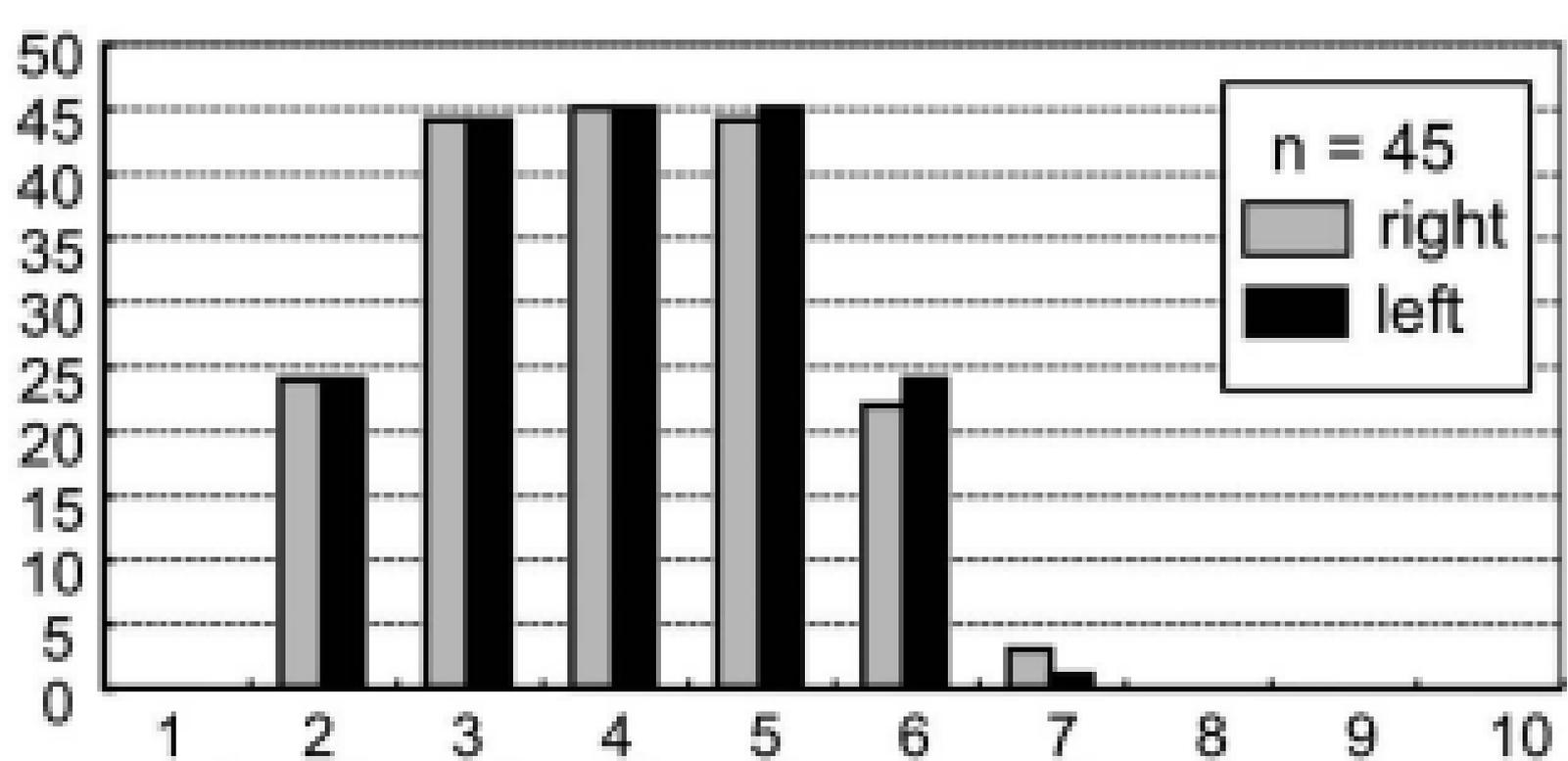
- ▲ Two types of mechano-proprioceptors were identified on honeybee stinging apparatus.
- ▲ Campaniform sensilla are located on surface of the sting barbs and tapering shaft.
- ▲ The C. sensilla detect deformation of the sting and induce the stinging response.
- ▲ Hairplates are located at skeletal plates moved during the stinging response.
- ▲ The hairplates detect stinging movements and modulate the stinging motor rhythm.



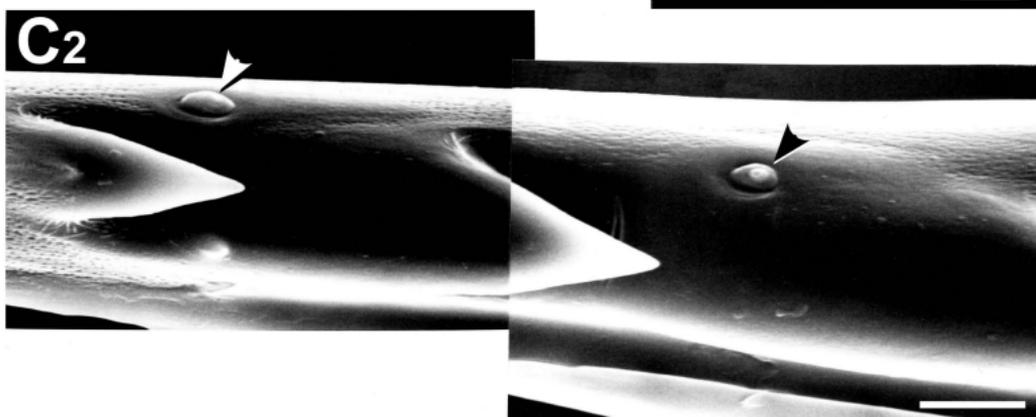
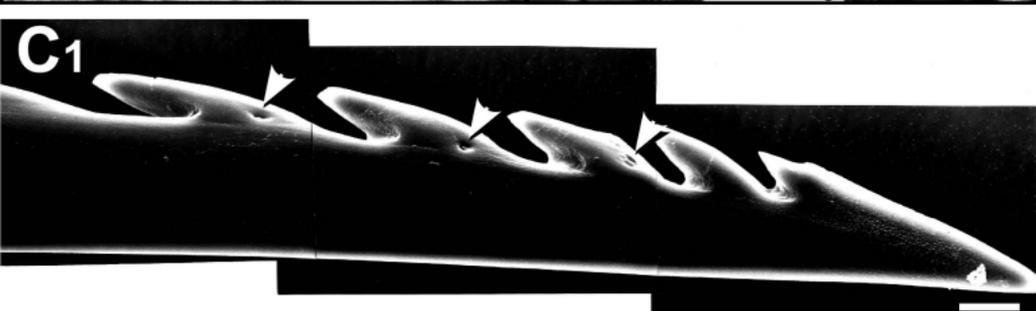
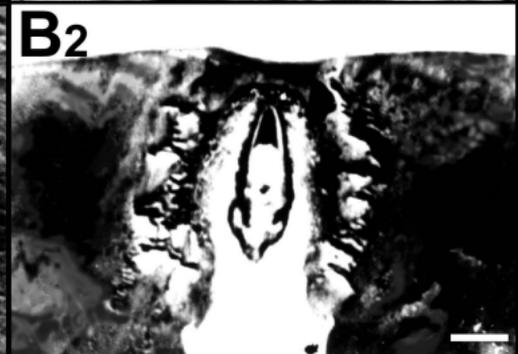
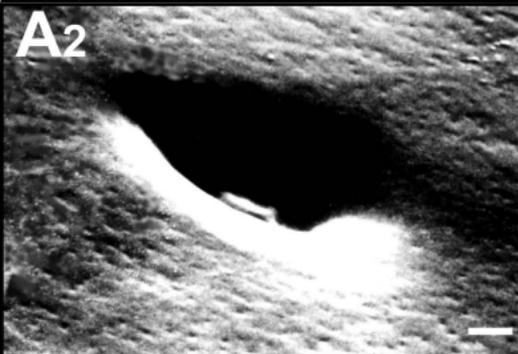
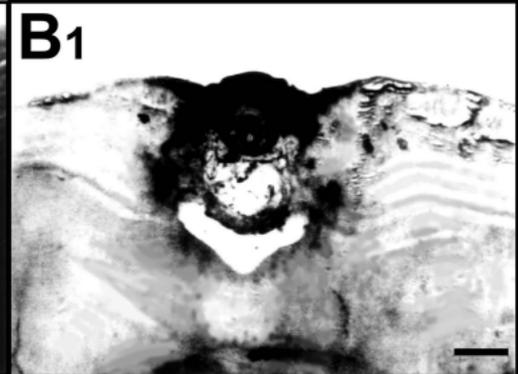
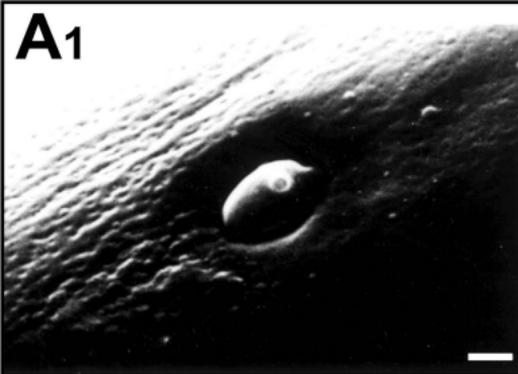
 Campaniform sensilla
 Hair plates

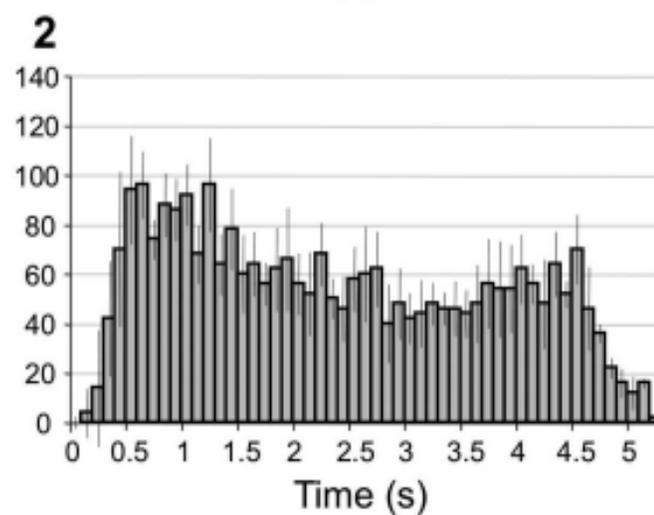
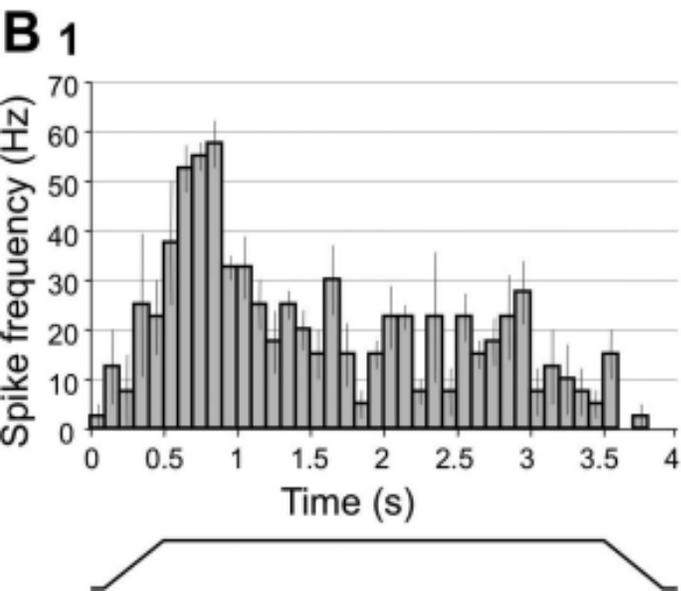
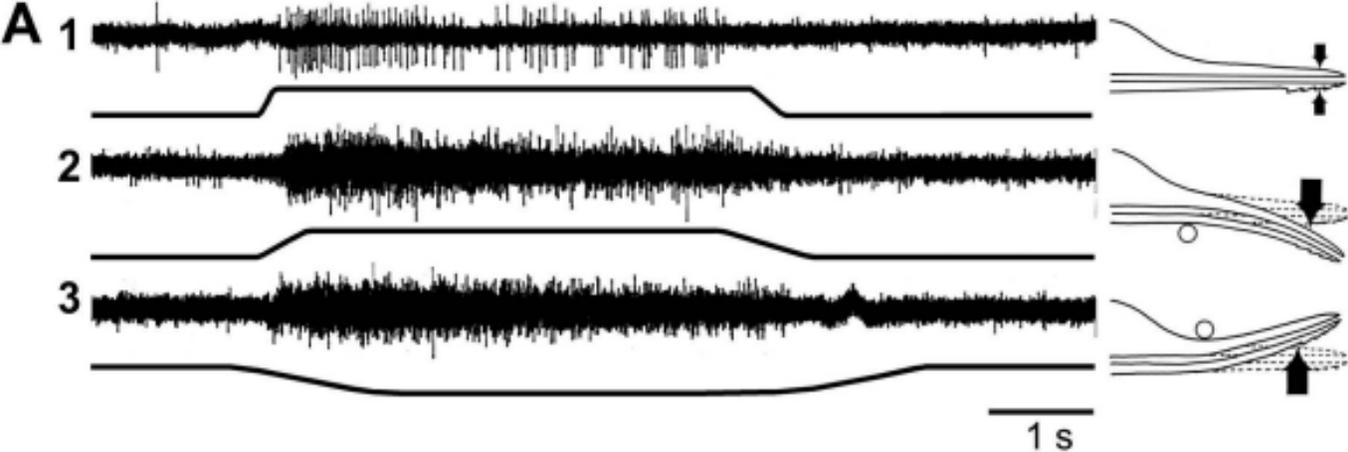


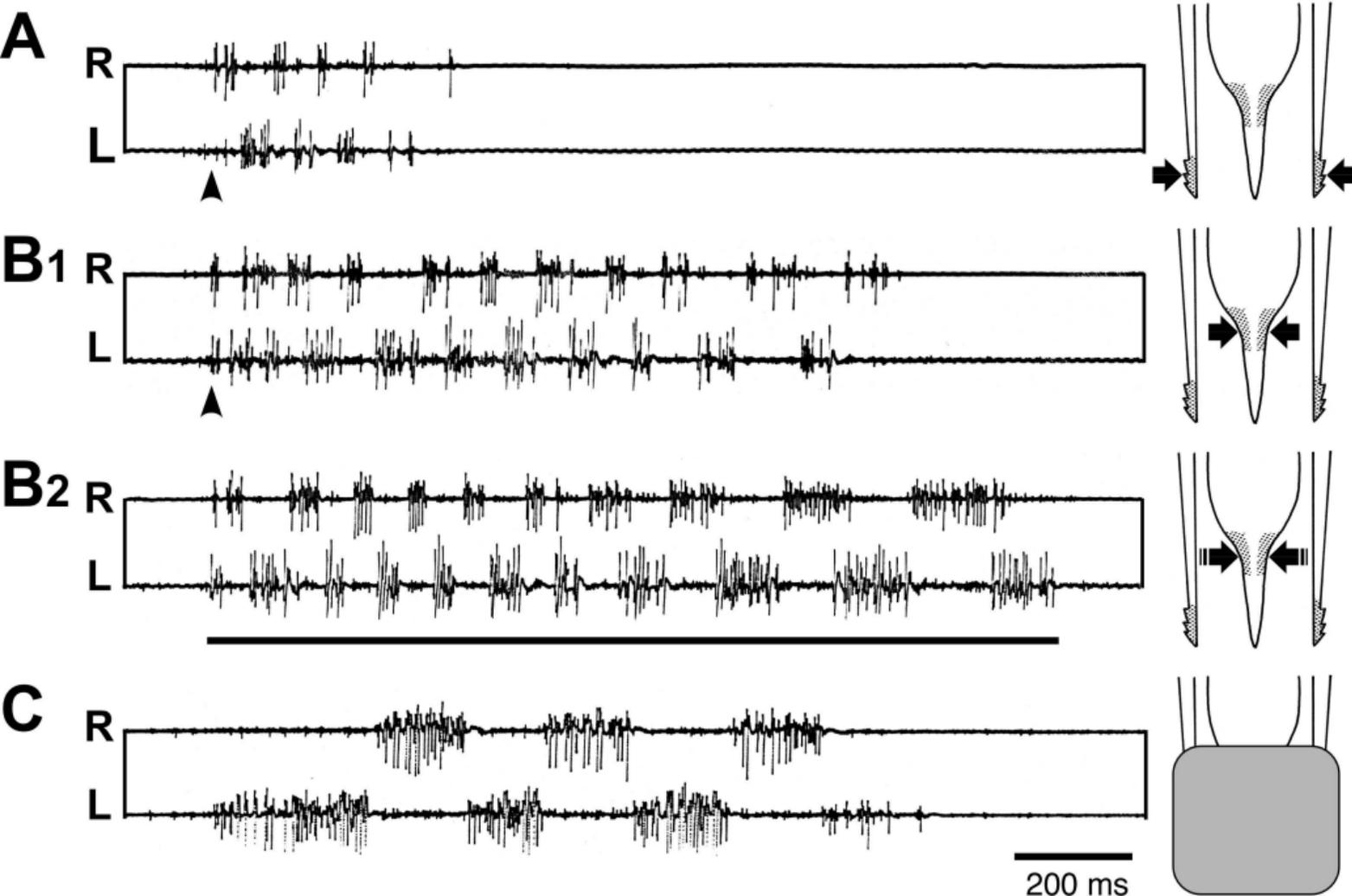
Number of C. sensilla

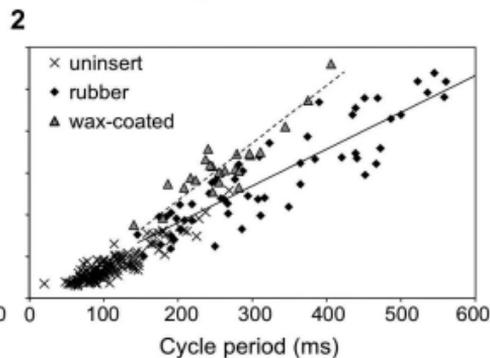
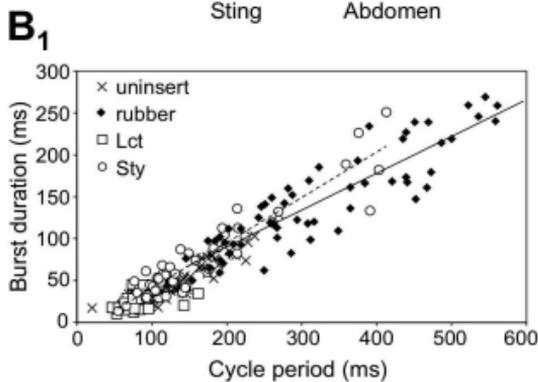
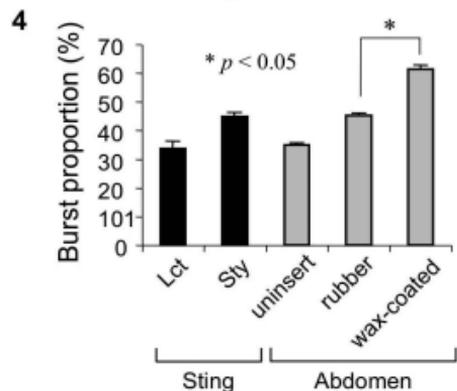
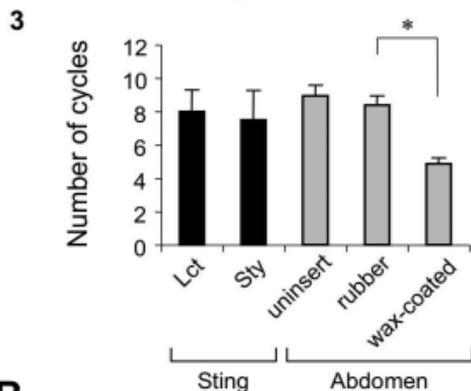
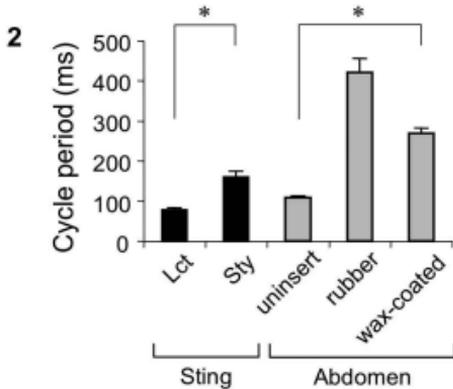
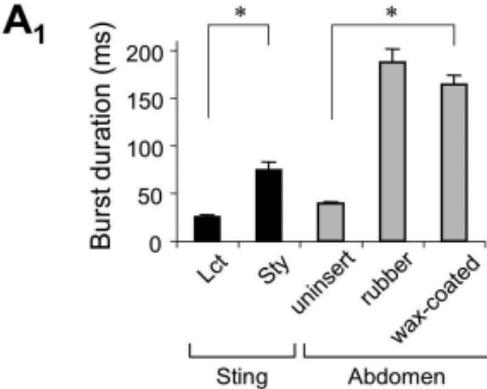


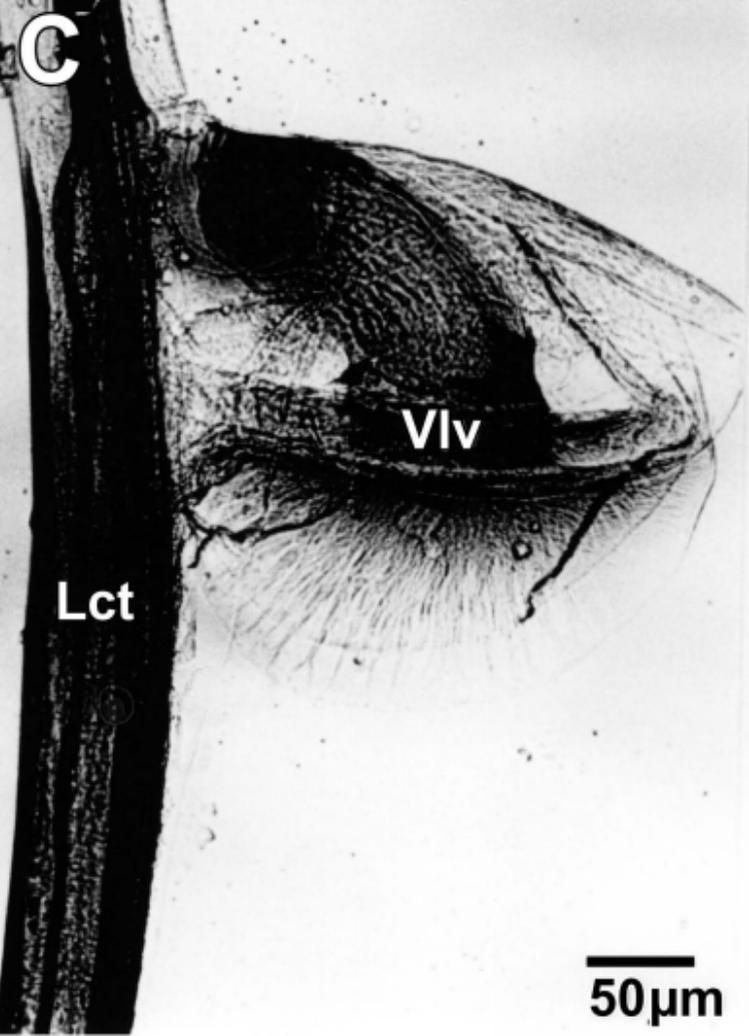
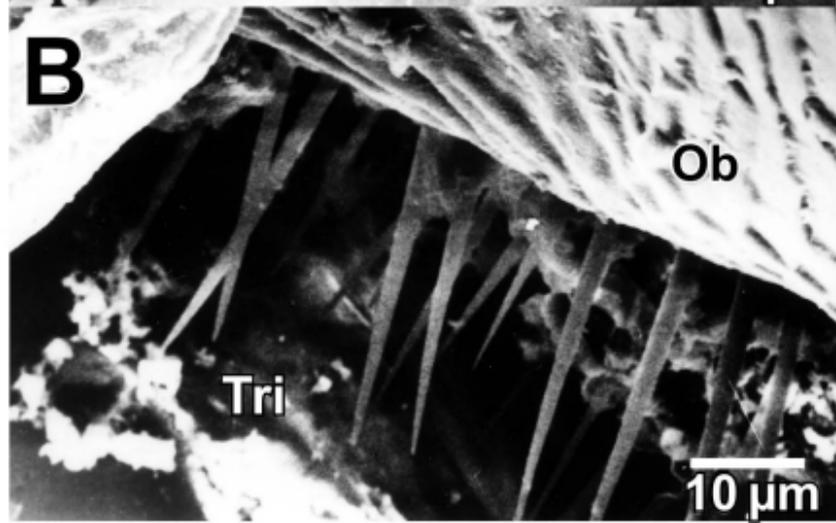
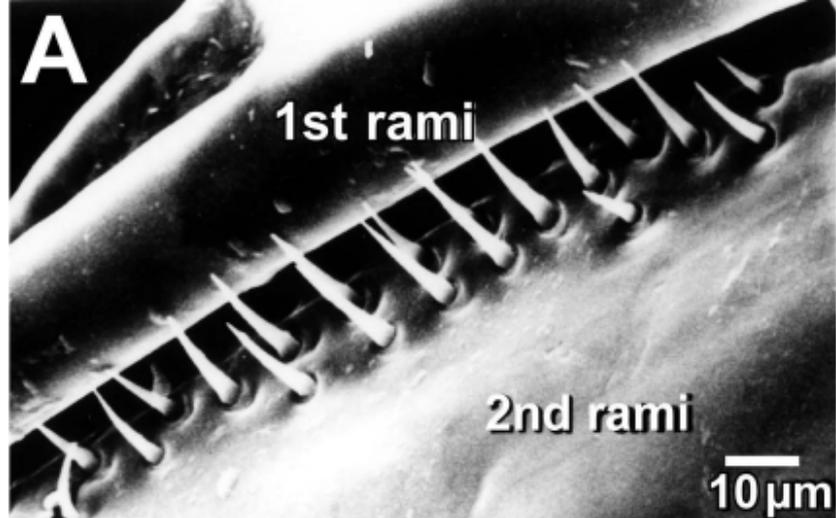
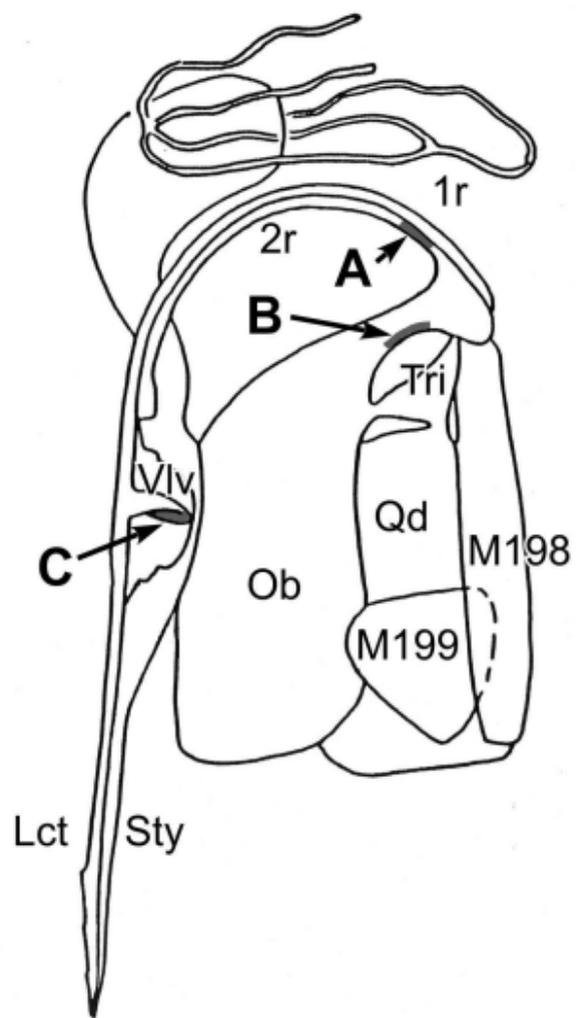
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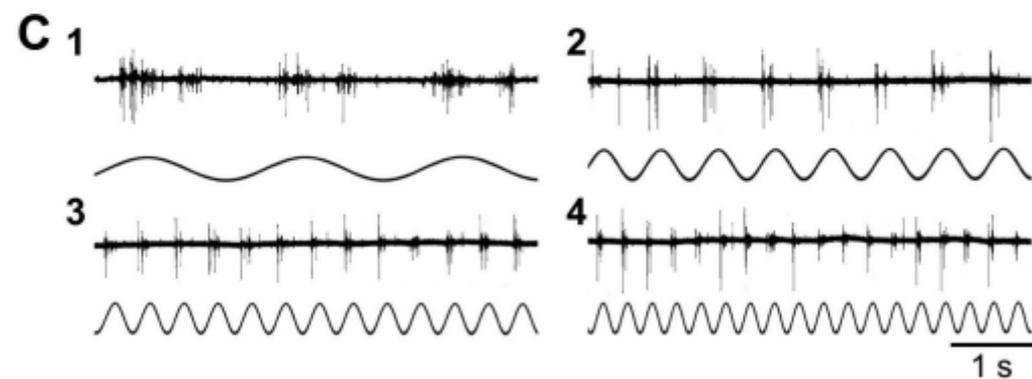
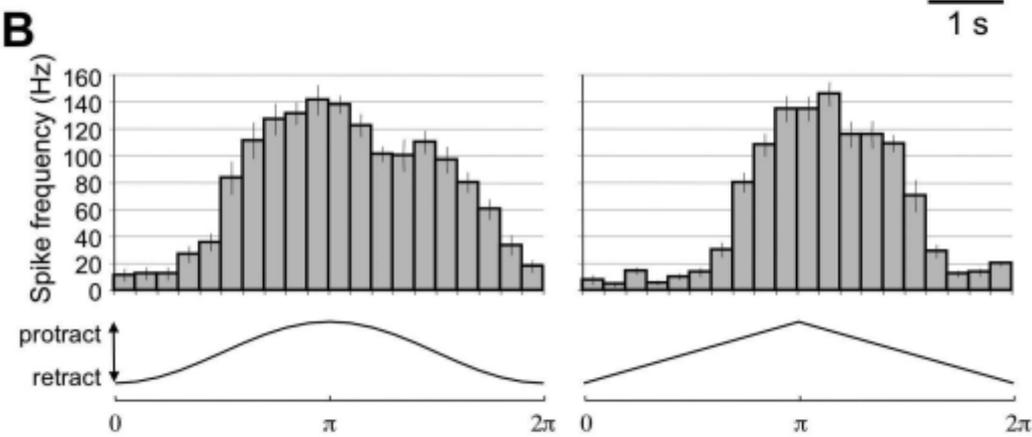
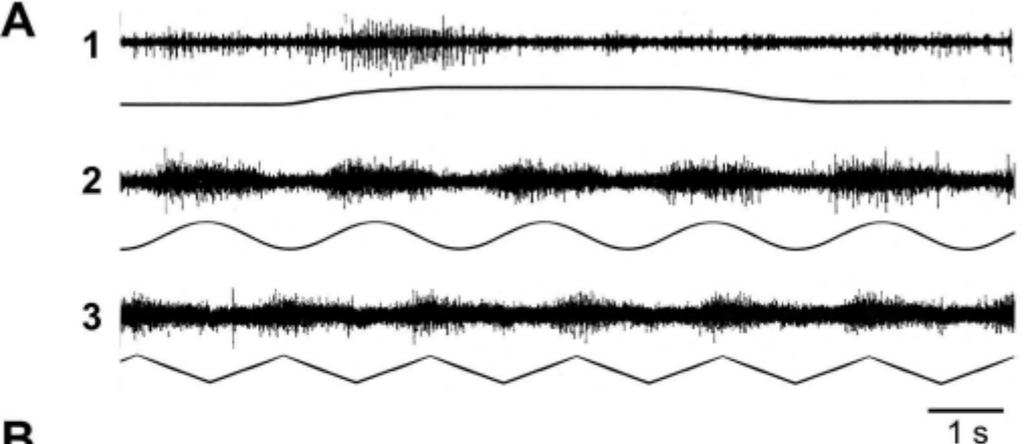


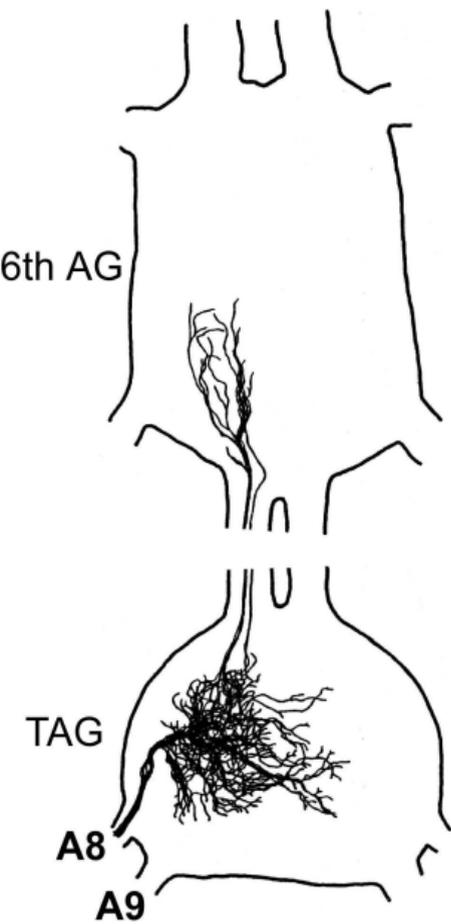










A**B****C**