Genital Autocleaning in the Male Cricket Gryllus bimaculatus (2): Rhythmic Movements of the Genitalia and Their Motor Control

Authors: Kumashiro, Mikihiko, and Sakai, Masaki

Source: Zoological Science, 33(6) : 634-642

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zs160101

ZOOLOGICAL SCIENCE **33**: 634–642 (2016)

Genital Autocleaning in the Male Cricket *Gryllus bimaculatus* (2): Rhythmic Movements of the Genitalia and Their Motor Control

Mikihiko Kumashiro and Masaki Sakai*

Graduate School of Natural Science and Technology, Okayama University, Okayama 700-8530, Japan

Three types of genital movement, their neural controls, and functional roles were investigated to gain a better understanding of the mechanism underlying autocleaning in the male cricket. The membrane complex consisting of the median pouch and genital chamber floor shows peculiar undulation that is composed of two types of movements: a right-left large shift and small crease-like movements. The large shift was caused by contraction of a pair of muscles (MPA) located anterior to the median pouch, while the crease-like movements were caused by numerous muscle fibers extending over the membrane complex. The MPA and muscle fibers were each innervated by efferent neurons in the terminal abdominal ganglion. Experiments with artificial dirt mimicking a foreign object revealed that the crease-like movements were responsible for dirt transport, while the large shift participated in sweeping the dirt into the lateral pouch as a trash container. On the other hand, the dorsal pouch serving as a template for the spermatophore showed a jerky bending movement. Simultaneous monitoring of the membrane complex and dorsal pouch activities suggested that their movements cooperate to enable the efficient evacuation of waste in the dorsal pouch. Based on the results, we conclude that genital autocleaning supports the production of the spermatophore.

Key words: male cricket, genitalia, cleaning, rhythmic movement, neural control

INTRODUCTION

The male cricket is equipped with an autocleaning system to keep the genitalia clean (Kumashiro et al., 2006). This system is indispensable for the production of a normal spermatophore (Kumashiro and Sakai, 2016). The genital membrane complex consisting of the median pouch and genital chamber floor is entirely covered by small scales and undulates rhythmically at a frequency of 0.16 Hz (Sakai and Kumashiro, 2004). It has been shown that the scaled membrane has a crucial role in the transport of dirt and waste to the lateral pouch serving as a trash container. However, the membrane complex movement has not yet been analyzed in details. In addition to the membrane complex, the dorsal pouch serving as a template for the spermatophore exhibits a characteristic movement, i.e., jerky bending at a frequency of 0.13 Hz (Kumashiro and Sakai, 2001b). Although this dorsal pouch movement may be involved in the evacuation of waste from the dorsal pouch (Kumashiro and Sakai, 2001a), no solid evidence to support this has been reported to date.

To understand the mechanisms underlying the transport of and evacuation of objects in the genitalia, the movements, muscles, efferent neurons and function of the membrane complex were first examined. We next analyzed the function of the dorsal pouch movement and its timing in relation to the membrane complex. Our results indicate that two types of membrane complex movement and dorsal pouch movement have unique functional roles that contribute to the cleaning of the genitalia.

* Corresponding author. Tel. : +81-86-251-5789;
E-mail: masack@cc.okayama-u.ac.jp
doi:10.2108/zs160101

MATERIALS AND METHODS

Animals

Male crickets, *Gryllus bimaculatus* DeGeer, were used 1–2 weeks after the final molt.

Preparations from males in different reproductive stages

The shape of the genitalia of the male cricket changes during the reproductive cycle, which consists of the mating stage and sexually refractory stage (Ureshi and Sakai, 2001). In the state in which the male has a mature or immature spermatophore, the median pouch is withdrawn under the genital chamber floor (Fig. 1A). The median pouch and surrounding genital chamber floor undulate, but the dorsal pouch of the phallic complex is immobile in this period. In contrast, in the state in which the male has no sperpmatophore (pre-spermatophore preparation period between spermatophore extrusion and spermatophore preparation), the medain pouch expands with hemolymph to occupy the inside of the doral pouch (Fig. 1B). The median pouch and surrounding genital chamber floor undulate, and the dorsal pouch of the phallic complex also repeats a jerky bending movement in this period. Due to such state-dependent shapes of the genital organs, the membrane complex movement was examained using males in the post-spermatophore preparation period between spermatophore prepation and spermatophore extrusion, and the dorsal pouch movement was examained using males in the pre-spermatophore preparation period.

Recording of genital movement and efferent spike activity

For recording movement, the genital chamber was opened after removing the dorsal pouch, and the membrane complex was filled with petroleum jelly to keep the median pouch expanded (Fig. 2). To prevent noisy movement, all of the nerve roots emanating from the terminal abdominal ganglion (TAG) were cut except for nerve 9v and 10v (Kumashiro and Sakai, 2001b). To examine the

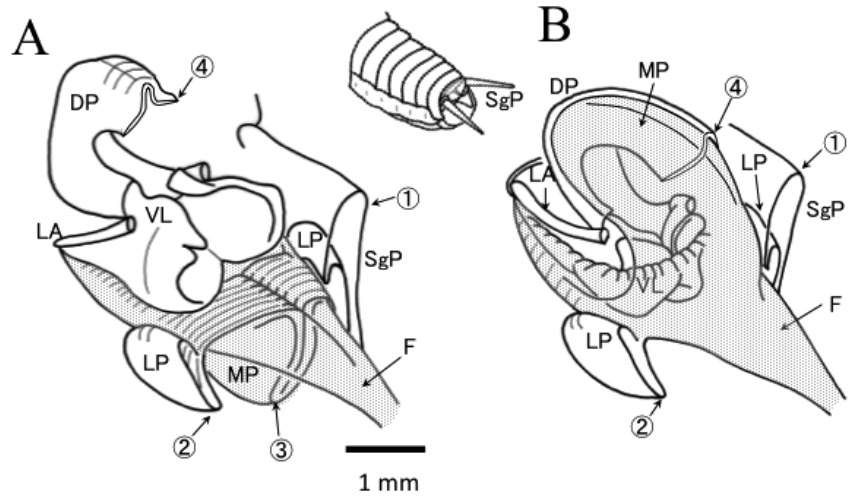

Fig. 1. Different shapes of the male cricket genitalia in the reproductive cycle illustrated as a perspective drawing. The posterior-dorsal part of the dorsal pouch (DP) including the epiphallus, guiding rod and subgenital plate (SgP) are omitted for clarification. **(A)** Genitalia in the post-spermatophore preparation. The median pouch (MP in gray) is inflected under the genital chamber floor (F in gray). The ventral lobes hold the spermatophore (not illustrated). The lateral pouch (LP) is located on the lateral verge of the genital chamber floor. The inset illustrated in the same orientation as **(A)** is a posterior-dorsal view of the abdomen to show the location of the subgenital plate housing the phallic complex. **(B)** Genitalia in the pre-spermatophore preparation period between spermatophore extrusion and spermatophore preparation. The MP expanded with hemolymph fits inside the dorsal pouch. The ventral lobes (VL) are shrunken and folded under the dorsal pouch. Circled numbers (1–4) indicate the sectional view of each structure, SgP, LP, MP and DP respectively.

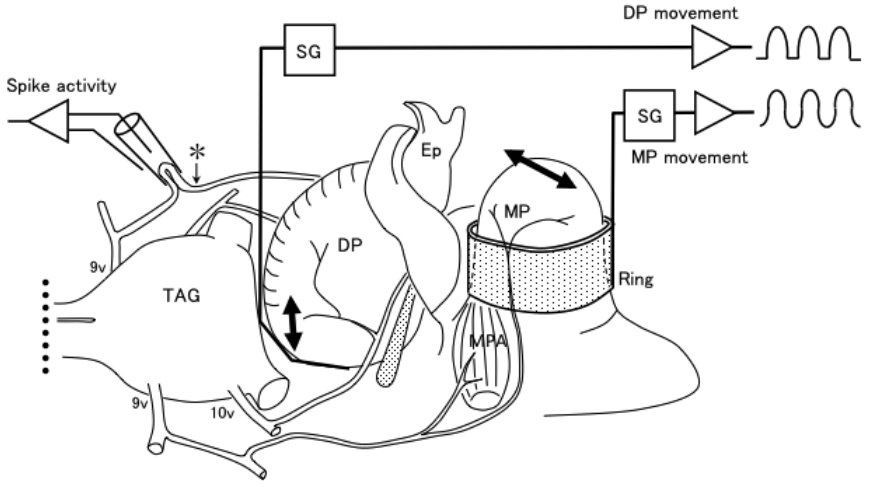

Fig. 2. Setups for recording genital movement and spike activity. Two types of movement of the phallic complex were recorded with two strain gauges (SGs). One movement is up and down bending movement (vertical two-way arrow) of the dorsal pouch (DP) and the other movement is of the median pouch (MP) in the membrane complex (oblique two-way arrow). The median pouch is fixed with a ring (Ring) to restrict the movement. Spike activity of efferent neurons innervating both the muscle (MPA) anterior to the median pouch and the muscle fibers of the median pouch was recorded *en passant* from a branch of 9v of the terminal abdominal ganglion (TAG) with a suction electrode. Recording was sometimes done from the cut end (*) of the same nerve. All of the nerves emanating from the TAG were cut at the proximal part except nerves 9v and 10v. When the influence of all of the anterior ganglia on the rhythmic movements was estimated, both the connectives were cut just anterior to the TAG (vertical dotted line). The same abbreviations are used in the following figures.

possible effect of other ganglia on the movement of the membrane complex, both of the connectives (ventral nerve cords) were cut between the terminal abdominal ganglion and the 6th abdominal ganglion (vertical dotted line in Fig. 2) during the experiments. Movements of both the genital membrane complex and the dorsal pouch were recorded with strain gauges (SGs) in dissected males as shown in Fig. 2.

Extracellular spike activity of efferent neurons associated with the membrane complex was hemi-laterally or bilaterally recorded with a suction electrode. Recording was carried out from the nerve *en route* branch of 9v (Fig. 2) or its cut end (asterisk in Fig. 2) sucked into a glass electrode. Although the nerve innervating the dorsal pouch runs in the 10v branch but not in the nerve 9v branch (see Fig. 2), some spike activity associated with the dorsal pouch movement was monitored with the electrode placed on the 9v. This phenomenon may be due to recording of efferent neurons innervating unidentified muscles (Kumashiro and Sakai, 2001a) that contract in synchrony with the dorsal pouch muscle.

Axonal filling with heavy metals

Anterograde axonal filling was carried out with 0.5 M of a cobalt and nickel mixture solution (Sakai and Yamaguchi, 1983) through the cut end of nerve 9v (see Fig. 5A). Specimens were reacted with rubeanic acid and further silver-intensified (Bacon and Altman, 1977). Axons stained by anterograde filling were traced over the membrane complex under a light microscope equipped with a camera lucida.

Surgical treatment to eliminate a particular movement

The functional roles of three kinds of movement in cleaning of the genitalia (two types of membrane complex movements and dorsal pouch movement) were examined in males with different treatments. To eliminate the large shift of the median pouch with small crease-like movements retained, the membrane complex was separated from the genitalia with the innervating nerve branch intact and placed on the surface of saline, on which it floated in a flattened manner owing to its surface tension. To inactivate muscle contraction, the 9v branch (for the membrane complex) or the 10v branch (for the dorsal pouch) was transected. In some cases, the median pouch, which was unable to make the right-directed large shift due to cutting of the ipsilateral (right) nerve, was pushed to the right by the experimenter using a small stick every time the left-directed large shift occurred (see Fig. 7, NC*).

Statistical analysis

Data for the time and speed of artificial

dirt (a piece of rubber) movement on the membrane complex or in the dorsal pouch were compared between the experimental groups. The difference in values was analyzed by the nonparametric Kruskal-Wallis test, and subsequent multiple comparisons between the groups was performed by using the Steel-Dwass method. The significance level was set as $P = 0.05$ in two-tailed tests.

RESULTS

Two types of movement in the membrane complex

The membrane complex showed a characteristic undulation at 0.16 Hz (Sakai and Kumashiro, 2004) that consists of two types of movement, a large shift and crease-like movements. In the large shift, the median pouch wriggled right to left alternately. A single shift is composed of a fast phase (between the paired fine vertical broken lines in Fig. 3A) with an averaged shift duration of 0.97 ± 0.29 s (n = 10) and a slow bumpy phase (horizontal dotted lines on the trace in Fig. 3A). The latter corresponds to a passive return to the midline and resting. In contrast, crease-like movements appeared on the shifted side of the median pouch at the fast phase (many horizontal curved lines on the median pouch hemisphere indicated by the two smaller white arrows in the two insets of Fig. 3A). Crease-like movements should have appeared on the fast phase of the record trace, but their amplitudes were so small that no record was superimposed on the steep curve of the fast phase. However, many creases about 50 μm apart were observed at the fast phase by video recording (refer to the picture in Fig. 6B). A large shift occasionally occurred in the absence of crease-like movements (shown by an asterisk in Fig. 3A, B), while crease-like movements sometimes occurred without a large shift (not shown). The visual inspection indicated that the large shift was associated with contraction of a pair of muscles located anterior to the median pouch (named MPA, Fig. 3A inset). Both types of movement continued to occur even after the two connectives were cut just anterior to the terminal abdominal ganglion (white arrow in Fig. 3B).

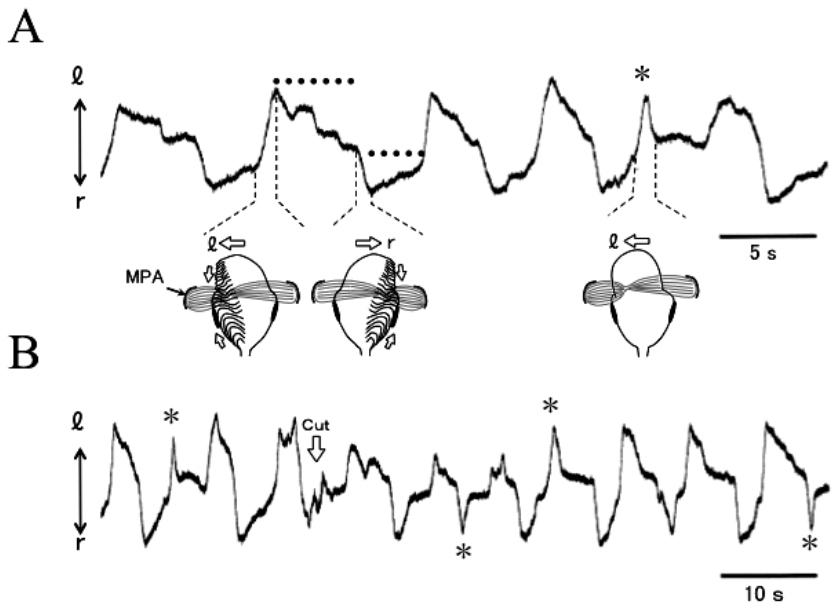

Fig. 3. Two types of movement of the genital membrane complex. **(A)** Normal rhythm of large shifts accompanied by small crease-like movements. Upward deflection (l) shows a left shift and downward deflection (r) shows a right shift. The period between the two thin vertical broken lines shows a large shift to the left or right from the midline, while the period indicated by the horizontal dotted line indicates passive recovery to the midline and some resting state following a large shift. The asterisk (*) indicates a large shift with no crease-like movements that occasionally occurred. Insets illustrate the states of the membrane complex that correspond to the periods in the analog trace indicated by broken lines. The left figure illustrates a left shifted state (indicated by the left-directed horizontal white arrow). It is accompanied by many crease-like movements (many curvy lines) in the left hemisphere of the median pouch (illustrated along the two vertical smaller white arrows). MPA indicates the pair of muscles anterior to the median pouch. The middle figure shows a shift in the right direction accompanying crease-like movements in the right hemisphere. The right figure shows a large shift (*) to the left with no crease-like movements. **(B)** Effect of connective cut on the membrane complex. The rhythmic movement continued after the connective cut (downward white arrow) between the terminal abdominal ganglion and the 6th abdominal ganglion. Asterisks indicate irregularly occurring large shifts with no crease-like movements.

Activity of efferent neurons responsible for the shift and crease-like movements

In the record at the cut end of the TAG nerve root 9v, there were always three to four different spikes (judging from their sizes) that burst at slightly different timings (n = 12). When recorded from both the left nerve (L) and the right nerve (R), their spikes were reciprocally active (Fig. 4A). That is, the burst in the left nerve was associated with the left shift of the median pouch and that in the right nerve was associated with the right shift. When only crease-like movements occurred without the shift, the frequency of the smaller spike (mMP-1) was lower [see the 1st burst indicated by dots in the record from the left nerve (L) and the 5th burst from the right nerve (R)] than that when crease-like movements occurred together with the shift normally. On the other hand, there was little change in the frequency of the larger spike (mMP-2) in either case. This suggests that mMP-1 may be responsible for the shift, and that mMP-2 may be responsible for the crease-like movements.

In another record with a better signal-to-noise ratio (Fig. 4B), two atypical cases are seen. When there was only a slight shift with normal crease-like movements, mMP-1 discharged less frequently (4th burst in Fig. 4B). Conversely, when the shift occurred normally together with weak crease-like movements, mMP-2 hardly discharged (5th burst in Fig. 4B). The burst discharges of mMP-1 and mMP-2 were then compared quantitatively in three cases: shift with the crease-like movements, only crease-like movements, and only a shift. The spike rates of mMP-1 were 61.9 ± 14.2 Hz (n = 12), 22.7 ± 9.4 Hz (n = 6) and 50.5 ± 16.4 Hz (n = 5), respectively, while those of mMP-2 were 15.4 ± 6.7 Hz (n = 12), 12.0 ± 5.3 Hz (n = 6) and 7.0 ± 5.9 Hz (n = 5), respectively. The spike rate of MP-1 was less in the case of only crease-

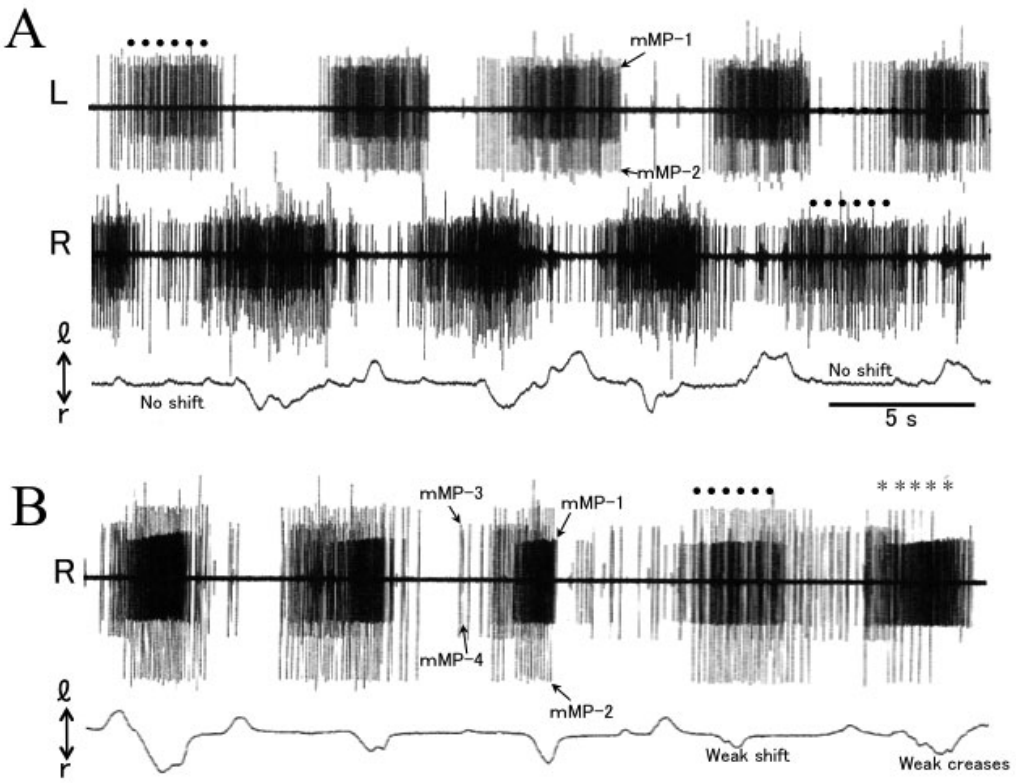

Fig. 4. Activity of efferent neurons underlying the two types of movement of the membrane complex. **(A)** Efferent neuron spikes bilaterally recorded. The bottom line shows the movement of the membrane complex (r, right direction; l, left direction). As shown in Fig. 3, the membrane complex shifted alternately to the right and left from the midline, such that the two groups of spikes were reciprocally active (see L and R). Note the portion of dotted lines: no large shifts occurred in the first burst of the left record (L) or in the 5th burst of the right record (R). In both cases, smaller spikes (mMP-1) are less active than in the other cases in which the large shift occurred. **(B)** Four kinds of spikes. In this record, four different neurons were clearly discriminated by their spike sizes. The movement shown by the dotted line indicates the case in which the shift was small, but crease-like movements were normal. In contrast, the movement shown by the asterisk line indicates the case in which the shift occurred normally, but crease-like movements were small. As seen in these two irregular cases, mMP-1 was less active in the former, while mMP-2 was less active in the latter. Other spikes, MP-3 and MP-4, were loosely associated with crease-like movements.

like movements (no shift), while that of mMP-2 was less in the case of only a shift (no crease-like movement). These results revealed that mMP-1 is responsible for the right-left shift that is caused by the muscle MPA and that of mMP-2 is responsible for the crease-like movements that are caused by the muscle fibers extending over the median pouch and genital chamber floor (see Fig. 5C). In Fig. 4B, two other intermediate-sized spikes (mMP-3 and mMP-4) can be seen. They were not synchronized well with mMP1 or mMP2, that is, they tended to discharge prior to the shift or occasionally between the shifts.

Axonal morphology of efferent neurons responsible for the crease-like movements

The genital membrane complex is lined with numerous muscle fibers that should be responsible for the crease-like movements. All of the fine branches innervating muscle fibers originated from one thick axon or axon bundle (Fig. 5B, arrow) in the preparation that was filled in an anterograde direction with heavy metals through the cut end of the nerve root 9v (arrowhead, Fig. 5A). The median pouch (MP) occupying the center of the membrane complex was innervated by a number of thin collaterals branching off several thick secondary axons. The genital chamber floor (F) occupying the periphery of the membrane complex was innervated directly by thin axons branching off the main axon (lower part of Fig. 5B). The axons on the membrane complex ran in the anterior posterior direction, roughly parallel to the muscle fibers (Fig. 5C). These left numerous synaptic buttons *en passant* on the muscle fibers (not shown here).

Movement of artificial dirt on the flattened membrane complex

To differentiate the functional roles of the two types of movement in the membrane complex, i.e., a right-left shift and crease-like movements, the shift was eliminated by cutting out the membrane complex with the innervating nerve branch left intact and placing it on saline. The flattend membrane complex (Fig. 6A, lower) showed crease-like movements at a frequency similar to that of the intact preparation with no right-left shift. A piece of colored rubber (artificial dirt, about 80 μm in diameter) placed on the midline region of the membrane complex moved toward the peripheral region (Fig. 6B). The results with five preparations showed that the times taken for the dirt to be transported to the entrance of the lateral pouch were 9.5 min, 10.8 min, 11.3 min, 13.3 min and 14.6 min with an average time of 11.9 ± 2.0 min. This value is comparable to the average time of

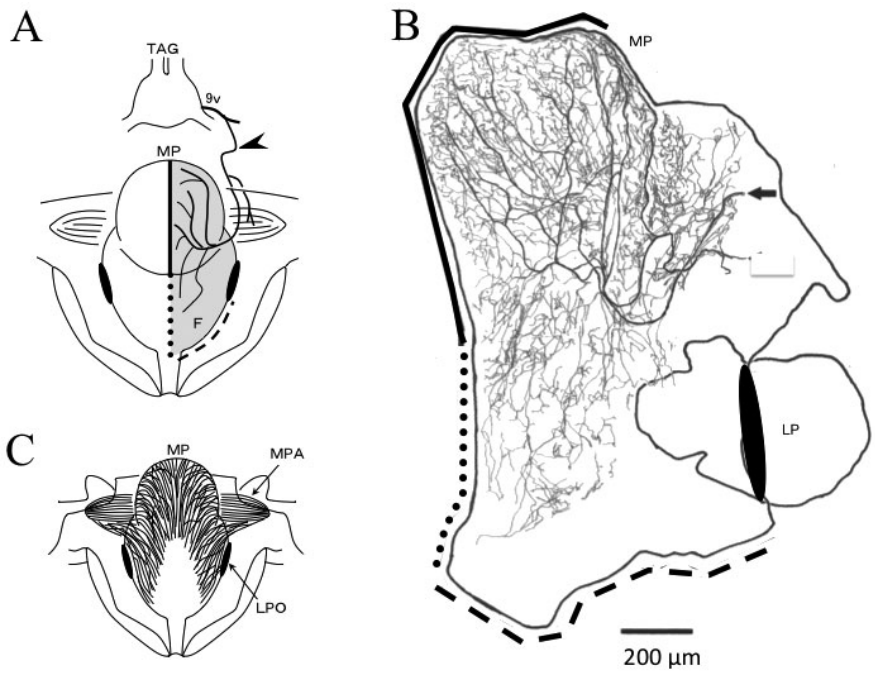

Fig. 5. Innervation of efferent neurons on the membrane complex. **(A)** Innervation by the branch of nerve 9v of the terminal abdominal ganglion (TAG) to the median pouch (MP) and genital chamber floor (F). Heavy metals were injected into the nerve branch as indicated by the arrowhead (Fig. 5A). This branch bifurcates at the periphery; one branch innervates the muscle MPA and the other innervates a number of muscle fibers in the MP and F. **(B)** Axonal arborization of the efferent neuron(s). The right half of the MP and F in **(A)** is flattened after nerve staining with heavy metals. Dense innervation is seen in MP. The arrow shows the main axon running in the nerve 9v branch. Three marks (thick line, dotted line and broken line) correspond to those in **(A)**. LP, lateral pouch. **(C)** Schematic drawing of the muscles and muscle fibers. A pair of muscles (MPA) is located anterior to the MP. The membrane complex except for the posterior region is entirely covered by muscle fibers running roughly parallel to the body axis.

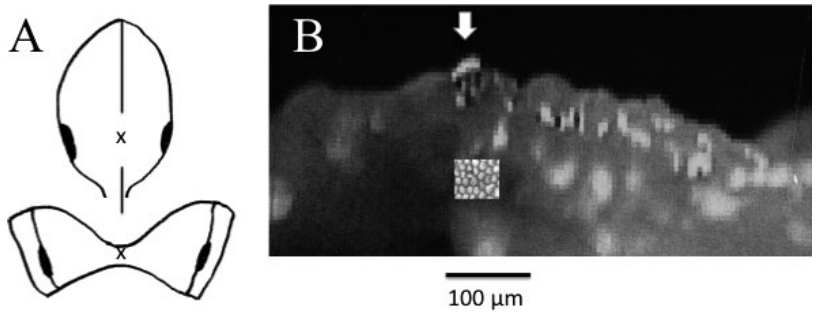

Fig. 6. Artificial dirt transport by crease-like movements on semi-intact flattened membrane complex. **(A)** Intact membrane complex viewed from above (upper) and surgically opened membrane complex viewed from the above (lower). The flattened preparation was made by transection along the midline of the upper figure (see vertical bars) and then by opening the tissue laterally. **(B)** Picture of the preparation viewed laterally. An irregularly rugged outline with each peak separated by about 50 µm is made by the crease-like movement. The white arrow indicates the piece of rubber that served as artificial dirt moving from the center to periphery (right). Inset in the center shows a picture of the scales on the median pouch for reference.

12.2 ± 2.5 min in males with the intact membrane complex (Kumashiro and Sakai, 2016).

Sweeping the artificial dirt into the lateral pouch

To understand how foreign objects are swept into the lateral pouch from its entrance, the time taken for an artificial dirt to enter that pouch after being placed near the entrance of the left lateral pouch was recorded. The experiments were carried out in three different conditions (Fig. 7): intact (I), nerve cut (NC) and nerve cut with artificial movement (NC*). The time was 138.7 ± 79.8 s (n = 7) in the I condition. In the NC condition, in which the shift of the median pouch to the right was abolished, the object near the entrance of the left lateral pouch would not enter the pouch because the entrance did not open widely. The time in this condition, 857.9 ± 105.4 s (n = 7), is the time at which the observation was terminated. It was significantly longer than that in the I condition (P < 0.005) and that in the NC* condition (P < 0.005). In the NC* condition, the immobile right median pouch was artificially moved to the right every time a spontaneous left shift occurred. As a result, the dirt entered the lateral pouch in 121.0 ± 46.5 s (n = 7). This time in the NC* condition was not significantly different (P < 0.998) from that in the intact condition.

Movement of artificial dirt in the presence or absence of the dorsal pouch

All of the experiments described above were performed using preparations with the dorsal pouch removed. In the pre-spermatophore preparation period, the expanded median pouch undulates inside the dorsal pouch while the dorsal pouch exhibits a jerky bending movement rhythmically. To understand the concreate functional role of the dorsal pouch movement, the time taken for an artificial dirt placed inside the doral pouch to come out of that pouch was measured in different conditions (Fig. 8). The results were presented in velocity (µm per min) instead of time becaue the moving distance of the dirt was different between the experiments depending on the starting point of the dirt. The velocities were 203.6 ± 155.6 µm/min (n = 24) in intact males (MD + DP), 18.1 ± 13.8 µm/min (n = 10) in males with an inactivated dorsal pouch (MP), 108.8 ± 74.9 µm/min (n = 11) in males with no dorsal pouch (MP*), and 10.8 ± 16.4 µm/min (n = 7) in males with an inactivated median pouch

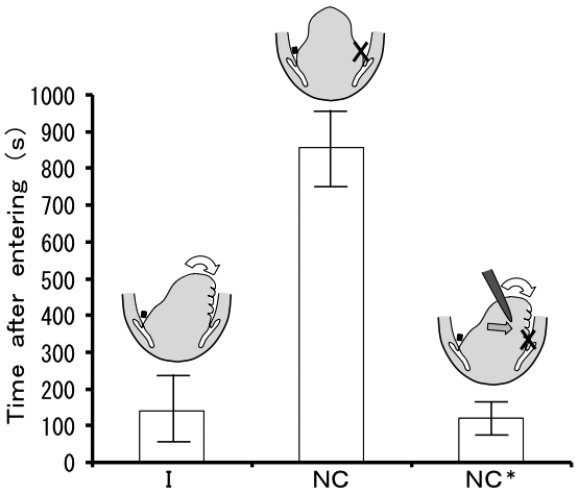

Fig. 7. Functional role of the large shift of the median pouch. A piece of rubber as artificial dirt (black dot) was placed near the entrance of the left lateral pouch (LP) and it was observed whether it went into the LP. The ordinate shows the average time (n = 7) for the object to disappear into the LP from its entrance or the time at which observation was terminated because the object would not enter the LP. I, Intact. NC, Nerve cut, that is, the right nerve branch innervating the membrane complex was cut (x). NC*, Nerve cut with assisted movement; the nerve branch was cut (x) in the same way as NC, and a large shift of the median pouch to the right (horizontal arrow) was given artificially with a small stick (oblique bar) by the experimenter.

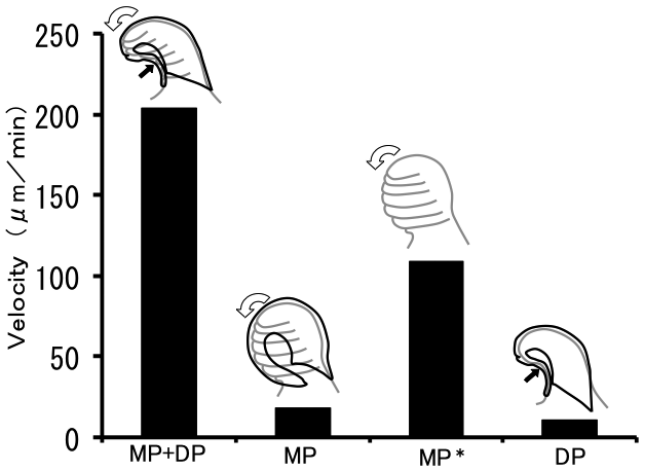

Fig. 8. Role of the dorsal pouch movement in artificial dirt removal from the inside of the dorsal pouch. The velocity of transport of the rubber piece (ordinate) was measured under four different conditions. MP + DP, Both the median pouch (MP) and the dorsal pouch (DP) were intact. MP, the MP is normal but the DP is immovable because its nerve was cut. MP*, the MP is normal but the DP was removed. DP, the DP is normal, but the MP is immovable because its nerve was cut. The inset shows a lateral view of the DP and MP. The bending movement of the dorsal pouch is indicated by a small black arrow. The large shift with crease-like movements of the MP is indicated by a curved arrow in white.

(DP). The velocities in both the MP condition ($P < 0.001$) and DP condition ($P < 0.001$) were significantly smaller than that in the MP + DP condition. The velocities in the MP condition ($P < 0.001$) and DP condition ($P < 0.005$) were also significantly smaller than that in the MP*condition. At the same time, the velocity in the MP + DP condition was larger than that of the MP* condition, though the differece was not significant ($P = 0.220$). A comparison of the results in the DP condition and MP + DP condition indicates that the crease-like movements of the median pouch are critical, and that the bending movement of the dorsal pouch may not be involved in the evacution. On the other hand, a comparison of the MP and MP + DP conditions indicates that the bending movement of the dorsal pouch is critical, and that the crease-like movements may not contribute to the evacutation (although a critical role for the crease-like movements in object transport has already been established, as described above). However, when the DP and MP condition are compared with the MP* condition, the crease-like movements of the median pouch are obiously critical for the evacuation, and the immobilized dorsal pouch in the MP condition actually hinders the evacuation by the crease-like movements in the MP* condition. Finally a comparison of the MP* with MP + DP conditions indicates that the bending movement of the dorsal pouch facilitates evacuation.

Timing of the median pouch and dorsal pouch movements

The spike activity of efferent neurons innervating the membrane complex was recorded with a glass electrode attached to the nerve *en route* branch of 9v (see Fig. 1). At the same time, the bending movement of the dorsal pouch was recorded with a strain gauge. In Fig. 9, thickly aggregated intermediate spike bursts associated with the dorsal pouch movement and large spike bursts associated with the median pouch movement can be seen. The former are not efferent neurons innervating the dorsal pouch but possibly some neurons innervating other muscles that contract in synchrony with the bending movement of the dorsal pouch (see methods). However, it can be a better indicator of the occurrence of dorsal pouch movement than the movement record. Two kinds of the spike bursts occurred at different timings since their frequencies are slightly different: 0.16 Hz for the median pouch and 0.13 Hz for the dorsal pouch. Accordingly, at one timing, dorsal pouch-associated spikes are activated in the period between two successive bursts of the median pouch spikes, while at another timing, both groups of spikes are active nearly at the same time. In the former case in which the dorsal pouch-associated spike burst occurred between the first burst (Fig. 9A1) and second burst (Fig. 9A2) of the median pouch spikes, the second burst of the median pouch spikes became stronger than the first. When the dorsal pouch–associated spike burst occurred at almost at the same time as the median pouch spike burst (Fig. 9B2), the median pouch burst was inhibited (white horizontal bar). However, when the dorsal pouch-associated spikes ceased bursting, the median pouch spike burst quickly recovered (Fig. 9B2). Furthermore, facilitation was found in the median pouch spike burst that occurred just after dorsal pouch activity stopped (compare Fig. 9A1 with Fig. 9A2, and compare Fig. 9B1 with Fig. 9B2) and even occurred in the next burst (compare Fig. 9C1, C2 with Fig. 9C3). These results indicate an intimate relationship between median pouch activity and dorsal pouch activity.

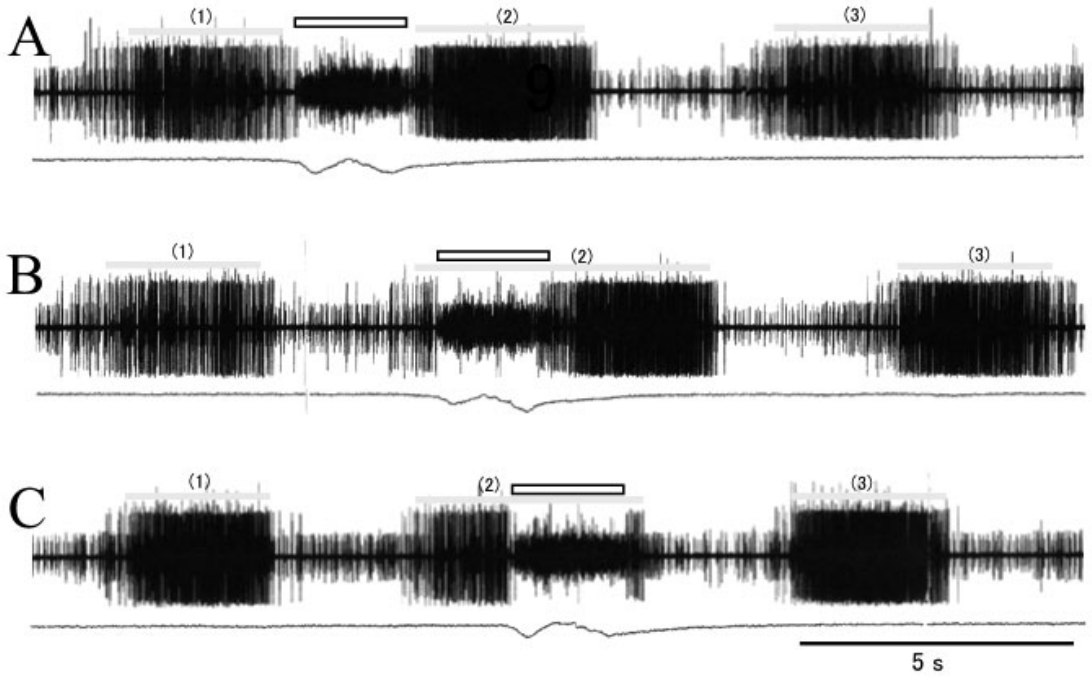

Fig. 9. Timing of spike burst of the membrane complex and movement associated with the dorsal pouch. **(A–C)** Spike activity recorded from the branch of nerve 9 and movement recorded from the dorsal pouch (see Fig. 2). **(A)** This shows the case in which dorsal pouch movement (white bar) occurred in the interval between the two membrane complex neuron bursts (large spikes). The second burst (2) of the membrane complex is more intense than the first (1). **(B)** This shows the case in which dorsal pouch movement occurred in the earlier phase of the membrane complex neuron burst. The membrane complex activity was inhibited during the dorsal pouch movement (2) but was then facilitated after the cessation of dorsal pouch movement when compared with the first burst (1). **(C)** This shows the case in which dorsal pouch movement occurred in the later phase of the median complex neuron burst (2). The burst was inhibited, but the next burst was facilitated (3) when compared with the first (1) and second bursts (2).

DISCUSSION

Difference in functions of the two types of the movement in the membrane complex

We found that the male cricket showed characteristic movements in the membrane complex of the genitalia (Kumashiro and Sakai, 2001b). The membrane complex, which consists of the median pouch and the genital chamber floor, has two types of movement: a right-left large shift and small crease-like movements (Kumashiro et al., 2006). The latter was previously referred to as “wavy” movement (Kumashiro et al., 2006); however, in the present report we have changed the name to “crease-like” movements. These occur on the same side as that to which the median pouch has shifted. For the muscles underling two types of movement, the shift is caused by contraction of a pair of muscles located anteriorly to the median pouch (MPA; Kumashiro and Sakai, 2001a), while the crease-like movements are caused by contraction of numerous muscle fibers extending over the membrane complex (Kumashiro and Sakai, 2001a).

Surgical separation of the membrane complex revealed that the crease-like movements are of primary importance for the transport of objects on the membrane complex. A number of small creases about 50 μm apart occur in synchrony or with a slight time delay from the center to periphery of the membrane complex. These should produce unidirectional driving force via the elastic nature of the spines of the scales on the membrane surface as we suggested (Fig. 12 in Kumashiro and Sakai, 2016). Thus, foreign susbtances on the membrane complex are smoothly conveyed to the lateral pouch on either side of the genital chamber.

In contrast, the large shift of the median pouch does not greatly help transport of the object in the experimental condition in which the membrane complex is exposed by removing the dorsal pouch. Instead, the shift plays a crucial role in sweeping the foreign substance into the lateral pouch. This is schematically summarized in Fig. 10A. The artificial dirt that has been conveyed to the entrance of the lateral pouch (Fig. 10A-1, black dot on the right side of F) can slip through the widely opened entrance (Fig. 10A-2, small vertical arrow) when the median pouch is shifted to the contralateral side (Fig. 10A-2, thick black arrow). When the median pouch is shifted to the ipsilateral side (Fig. 10A-3, thick white arrow), the dirt is pushed into the pouch (Fig. 10A-3, black arrow) by the tips of the scale spines, which function as latches (Fig. 12 in Kumashiro and Sakai, 2016).

Thus, the two types of movement of the membrane complex are functionally different: the crease-like movements play a role in that transport of objects on the membrane complex and the large shift participates in object sweeping into the lateral pouch.

Here, we should briefly describe the lateral pouch, which is made of a locally depressed sac-like structure of the membrane complex. It is not associated with muscles or muscle fibers, and thus cannot contract itself. Dirt and waste accumulate inside the pouch but cannot be evacuated. The

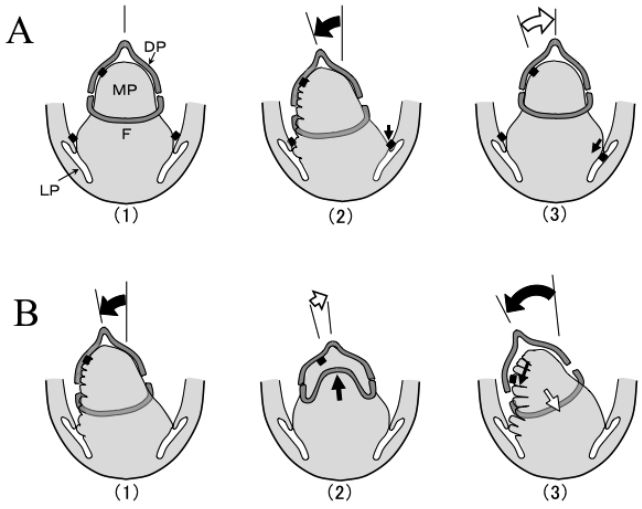

Fig. 10. A model of genitalia cleaning by three different movements. **(A)** Sweeping dirt into the lateral pouch (LP) by the shift of the median pouch (MP). The shift of the MP (2, large black arrow) from the resting state (1) toward the contralateral side to the dirt (1, right dot on F) on the genital chamber floor (F) causes the opening of the ipsilateral LP entrance to make the dirt enter the LP (2, small vertical arrow for dot). When the MP returns to the resting position (3, white arrow), the dirt is pushed into the LP by the spines of the scales in the F (3, small black arrow for dot). **(B)** Cleaning the inside of the dorsal pouch (DP) by cooperative action of the DP and MP. The dirt (1, black dot on MP) is hardly moved by the crease-like movements when the MP makes a shift to the left (1, large black arrow) because the dirt is sandwiched between the MP and DP. However, when the MP movement occurs (3, large black arrow) immediately after the DP movement (2), the dirt is smoothly evacuated. This is because the crease-like movements are strengthened (3, small black arrow), and some space is made between the DP and MP by the bending (2, upward black arrow) and returning (3, downward white arrow) of the DP.

content of the lateral pouch increases day by day and finally becomes full in a month (Kumashiro et al., 2006).

Neural control of the membrane complex

As described above, the membrane complex showed two types of rhythmic movement. The rhythm is intrinsically generated within the TAG since it continued even after the connectives were cut at the point just anterior to the TAG. Our previous spike recording from nerve 9v branch of the TAG revealed that there are two groups of discharges, m(1)MP and m(2)MP, both of which showed rhythmic bursts at slightly different timings (Kumashiro and Sakai, 2001b). The neuron group m(1)MP consisting of 2–3 different spikes was associated with the median pouch movement, while the neuron group m(2)MP consisting of 1–2 different spikes was associated with the dorsal pouch movement. In the present study, these findings were confirmed by improved spike recording at a more distal point of the 9v. At least four different neurons were identified and they should belong to the group of m(1)MP spikes. One of them (mMP-2) having largest spike, is closely related to the contraction of muscle fibers over the membrane complex, which is responsible for the crease-like movements. Another (mMP-1) was associated with the muscle (MPA) contraction underlying the large shift of the median pouch. Two other efferent neurons (mMP-3 and mMP-4) are loosely synchronized with mMP-1 or/and mMP-2. These may innervate other muscles located near the MPA that have not yet been identified (Kumashiro and Sakai, 2001a).

Anterograde staining from the nerve 9v branch disclosed that there was one main axon or axon bundle in the membrane complex. Thin axon collaterals emanated from the main axon arborized over the median pouch and genital chamber floor, leaving numerous axon terminals on their muscle fibers. It should be noted that secondary axons were thicker, and tertial axons were more dense in the median pouch than in the genital chamber floor. This difference in the axonal arborization suggests that the crease-like movements in the median pouch are more vigorous than those in the genital chamber floor surrounding the median pouch. The spatial distribution of axon terminals suggests that they might underlie some spatiotemporal pattern of the crease-like movements that enables smooth object transport from the center to periphery.

Our previous study with retrograde staining from the branch of nerve 9v marked 6–9 somata of neurons in the lateral region of the TAG (Kumashiro and Sakai, 2001b). The recent study in which axonal filling was performed at a more distal site of the same nerve branch revealed that there were four somata in the lateral part of the TAG (Kumashiro et al., 2008). Immunocytochemical study combined with retrograde and anterograde staining using Lucifer Yellow showed that the largest one of the four backfilled neuron somata was serotonergic, and that only one serotonergic axon extended collaterals over the membrane complex (Kumashiro et al., 2008). These results suggest that the neuron (mMP-2) with the largest spike and innervating muscle fibers is serotonergic. A similar serotonergic neuron was identified in the TAG of the male cricket *Acheta domesticus* whose peripheral target of that neuron was the posterior basal muscles of the spermatophore sac (Hustert and Topel, 1986; Elekes et al., 1987).

Here it should be mentioned something about efferent neurons so far described. First, somata of the efferent neurons, which were stained retrogradely from the cut end of the nerve 9v branch, were all found in the motoneuron cluster region (soma ventrally and dendrites dorsally) in the lateral part of the TAG (Kumashiro and Sakai, 2001b; Kumashiro et al., 2008), and the largest serotonergic neuron soma was also located in the same region (Kumashiro et al., 2008). Second, octopaminergic efferent neurons like dorsal unpaired median (DUM) neurons, which are not classified as motoneurons, but as modulatory neurons, innervate both visceral and skeletal muscles (Bräunig and Pflüger, 2001). However, efferent spikes (mMP-1–mMP-4) recorded from the nerve 9v branch cannot be those of DUM neurons because they did not occur simultaneously when recorded bilaterally. Third, a piece of membrane fragment cut out from the median pouch showed a weak twitch sporadically (partially myogenic). Application of serotonin to that fragment increased the occurrence of the spontaneous twitch but that of octopamine did not (Kumashiro et al., 2008). This result revealed that the crease-like movement is facilitated by the serotonergic efferent neuron possibly mMP-2.

Cooperative evacuation by both the membrane complex and the dorsal pouch

As has been discussed, the membrane complex of the genitalia is involved in transporting and sweeping objects to keep the genitalia clean. The process is carried out by the two types of movement in the membrane complex. There is another rhythmic movement, i e., a jerky bending movement of the dorsal pouch. This may also be involved in the cleaning of genitalia (Kumashira and Sakai, 2001b). To determine its functional role, evacuation speed was measured in different conditions. The results showed that the speed of object transport in males with the dorsal pouch removed was slower than that in males with the dorsal pouch intact. This finding supports the notion that dorsal pouch movement contributes to evacuation. However, the speed of evacuation is much slower when the dorsal pouch is inactivated, which suggests that evacuation is hindered by an immobilized dorsal pouch. In such a condition, the remnants of spermatophore material inside the dorsal pouch may hardly move, being pressed by the inner surface of the dorsal pouch onto the surface of the median pouch. That is, the jerky bending movement of the dorsal pouch is necessary for evacuation.

Based on the results of this study (Fig. 9), the mechanism of object evacuation is summarized in Fig. 10B. When the median pouch shows a large shift without preceding dorsal pouch bending, the artificial dirt in the dorsal pouch is moved only slightly by the crease-like movements of the median pouch, possibly because the object is sandwiched between the median pouch and the dorsal pouch (Fig. 10B-1). When dorsal pouch bending occurs at nearly the same time as or immediately after the median pouch movement (Fig. 10B-2), the inner surface of the dorsal pouch may be separated temporarily from the median pouch so that the dirt is moved easily by the crease-like movements. Furthermore, evacuation can be facilitated by strengthening crease-like movements when they occur following dorsal pouch movement (Fig. 10B-3).

Together with the results reported in a companion article (Kumashiro and Sakai, 2016), the results of the present study show that genital autocleaning is indispensable for the production of the spermatophore in the male cricket.

ACKNOWLEDGMENTS

The authors thank Y. Tsuji for his histological work, and H. Nishino for his critical reading of the manuscript. This project was supported by a Grant-in-Aid for Scientific Research (18570069) from the Ministry of Education, Science, Sports and Culture to M. S.

REFERENCES

Bacon JP, Alatman JS (1977) A silver intensification method for cobalt-filled neurons in wholemount preparation. Brain Res 138: 359–363

Bräunig P, Pflüger H-J (2001) The unpaired median neurons of insects. Advances in Insect Physiology 28: 185–266

Elekes K, Hustert R, Geffard M (1987) Serotonin-immunoreactive and dopamine-immunoreactive neurons in the terminal ganglion of the cricket, *Acheta domesticus*: Light- and electron-microscopic immunocytohemistry. Cell Tissue Res 250: 167–180

Hustert R, Topel U (1986) Location and major postermbryonic changes of identified 5-HT immunoreactive neurons in the terminal ganglion of a cricket (*Acheta domesticus*). Cell Tissue Res 245: 615–621

Kumashiro M, Sakai M (2001a) Reproductive behaviour in the male cricket *Gryllus bimaculatus* DeGeer. I Structure and function of the genitalia. J Exp Biol 204: 1123–1137

Kumashiro M, Sakai M (2001b) Reproductive behaviour in the male cricket *Gryllus bimaculatus* DeGeer. II Neural control of the genitalia. J Exp Biol 204: 1139–1152

Kumashiro M, Sakai M (2016) Genitalic autogrooming in the male cricket, *Gryllus bimaculatus* (1): Structure and function of the genital membrane. Zool Sci 33: 623–633

Kumashiro M, Tsuji Y, Sakai M (2006) Genitalic autogrooming: a self-filling trash collection system in crickets. Naturwissenshaften 93 (2): 92–96

Kumashiro M, Iwano M, Sakai M (2008) Genitalic autogrooming in the male cricket, *Gryllus bimaculatus* DeGeer. Acta Biol Hung 59 (Suppl): 137–148

Sakai M, Kumashiro M (2004) Copulation in the cricket is performed by chain reaction. Zool Sci 21: 705–718

Sakai M, Yamaguchi T (1983) Differential staining of insect neurons with nickel and cobalt. J Insect Physiol 29: 393–397

Sakai M, Taoda Y, Mori K, Fujino M, Ohta C (1991) Copulation sequence and mating termination in the male cricket *Gryllus bimaculatus* DeGeer. J Insect Physiol 37: 599–615

Ureshi M, Sakai M (2001) Location of the reproductive timer in the male cricket *Gryllus bimaculatus* DeGeer as revealed by local cooling of the central nervous system. J Comp Physiol A 186: 1159–1170

(Received June 2, 2016 / Accepted August 21, 2016)