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# Genital Autocleaning in the Male Cricket *Gryllus bimaculatus* (1): Structure and Function of the Genital Membrane

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We found that the genitalia of the male cricket *Gryllus bimaculatus* are equipped with an autocleaning system. The cricket keeps its genitalia clean by removing foreign matter and endogenous waste. Morphological study showed that the membrane complex consists of a median pouch and a genital chamber floor covered by small scales, each of which has a base of approximately 10  $\mu\text{m}$  in width and a fringe with 5–10 spines 3–20  $\mu\text{m}$  in length. The scales are arranged symmetrically about the midline, curving gradually in the lateral direction and continuing to the lateral pouch serving as a trash container. Observation of cleaning revealed that a small piece of artificial dirt placed on the membrane complex was conveyed over a distance of 1.3 mm to the lateral pouch in 12 minutes. Inspection of the dorsal pouch just after spermatophore extrusion in the mating stage revealed that there were patchy remnants of spermatophore material on the inner surface of the pouch, but that these were evacuated in a few minutes. Surgical elimination of the median pouch caused the formation of abnormal spermatophores with the sperm tube and attachment plate being deformed. These results suggest that genital autocleaning is indispensable for the production of a normal spermatophore in the male cricket.

**Key words:** male cricket, genitalia, scales, cleaning, spermatophore

## INTRODUCTION

Cleaning is an important behavior for animal survival (Spruijt et al., 1992). If animals do not maintain a clean body surface, they may lose normal physical functions and suffer from health problems. For example, foreign matter may physically interfere with the normal functions of various sensory organs, such as the skin and eyes. Bacteria and fungi proliferation may cause serious diseases. Furthermore, parasitic animals on the skin, such as ticks, may affect the host by delivering germs and viruses.

Thus, nearly all animals clean their body surfaces, and insects are no exception. Cleaning behavior of insects has been studied in bees (Hodges, 1952; Thelen and Farish, 1977), flies (Dawkins and Dawkins, 1976), crickets (Huber, 1955; Otte and Cade, 1976; Honegger et al., 1979; Lefebvre, 1981; Hustert, 1985), cockroaches (Eaton and Farley, 1969; Böröczky et al., 2013), locusts (Rowell, 1961; O'Shea, 1970; Pflüger and Burrows, 1978; Berkowitz and Laurent, 1996; Matheson, 1997; Page and Matheson, 2004) and praying mantises (Zack, 1978). Every part of the body surface, including the antennae, head, thorax, flank, and wings is kept clean in these insects by the use of the mouth and legs. As far as we know, however, genital cleaning in an insect has not been reported to date.

The male cricket genitalia, which are housed in the genital chamber of the last abdominal segment, have a complicated structure and are used for not only copulation but also spermatophore production (Snodgrass, 1937; Mann, 1984;

Hall et al., 2000; Sakai and Kumashiro, 2004). When the genital chamber is closed, the genitalia are protected from contamination with foreign matter. However, during copulation and spermatophore formation, foreign dirt enters the genital chamber because it is open wide (Sakai and Kumashiro, 2004). In addition to foreign matter, some domestic waste such as the remnant of spermatophore material may remain inside the dorsal pouch every time the male extrudes the spermatophore in copulation. The remnant may also cause problems in the formation of spermatophores (Kumashiro and Sakai, 2001b) as the dorsal pouch serves as a template for the spermatophore.

Recently, we found a unique cleaning system in the genitalia of the male cricket *Gryllus bimaculatus* (Kumashiro et al., 2006). Foreign dirt that fell into the genital chamber was quickly moved on the scaled membrane and collected into the lateral pouches on both sides of the genital chamber. To gain a better understanding of its mechanistic and functional significance, we investigated the morphology and physiology of the genital membrane complex.

The results showed that genital cleaning is indispensable for normal spermatophore formation in the male cricket. The results of analysis of movements in the membrane complex and its neural control are described in our companion paper (Kumashiro and Sakai, 2016).

## MATERIALS AND METHODS

### Animals

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

### Morphology of the genital membrane

The surface of the genital membrane was examined under a

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light microscope and a scanning electron microscope (SEM, JSM-5310 LV). For examination under a light microscope, tissue of the genital chamber was removed and floated on saline overnight (this material floats on saline due to its extreme hydrophobicity). This treatment facilitated to remove tissue adhering to the genital chamber. The saline contained NaCl (87.66 g), KCl (6.70 g),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (7.40 g),  $\text{NaHCO}_3$  (1.68 g) and dextrose (72.00 g) in distilled water (10 l) at pH = 7.2. After removing the muscle fibers and trachea with a pair of microscissors, the tissue was mounted on a glass well slide. For examination under a SEM, the sternite of the last abdominal segment was cut out, fixed in Bouin fixative overnight, dehydrated in an ethanol series, desiccated, and processed conventionally for SEM examination. To reproduce the expanded state of the median pouch typically seen in the stage between copulation and spermatophore preparation (Ureshi and Sakai, 2001), the median pouch was stuffed with petroleum jelly, left in Bouin fixative overnight, and then dehydrated.

#### Observations of artificial dirt movement on the membrane complex

A male cricket was restrained on the substrate and dissected to access the genital chamber. A piece of colored rubber (artificial dirt; about 80  $\mu\text{m}$  in diameter) was used as foreign object. This size was chosen by reference to various dirt attached to the margin of the genital opening. After the dorsal pouch had been removed, the artificial dirt was placed on different locations near the midline of the genital membrane complex (median pouch and genital chamber floor) with fine tweezers. The dirt moved as the membrane complex undulated. It was monitored with a video camera (SONY, Handycam) attached to a stereoscopic microscope. Since the shape of the genital membrane differs depending on the stage of the mating cycle, the following procedures were taken. In the mating stage, the median pouch was inflected under the floor (see Fig. 1B, D) so that the movement of artificial dirt was observed only on the flat genital chamber floor. In the period between spermatophore extrusion and spermatophore preparation, the median pouch expanded with hemolymph in an intact state (see Fig. 1C, E), but shrank in operated males due to loss of hemolymph. Thus, the movement of artificial dirt was observed on the median pouch artificially expanded with petroleum jelly. Under these conditions, the time it took the material to reach the lateral pouch was recorded.

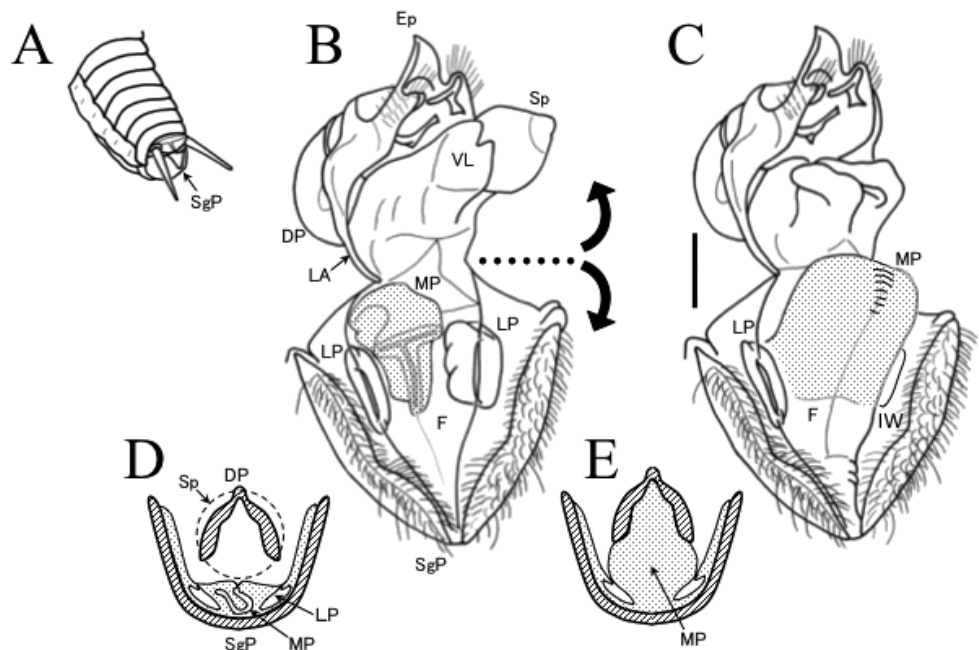
#### Measurement of time for dirt evacuation from the dorsal pouch

To measure the time for the artificial dirt (a piece of rubber as described above) placed in the dorsal pouch to be evacuated, the median pouch expanded

with petroleum jelly was first pulled out with tweezers from the dorsal pouch. The artificial dirt was placed near the midline region of the median pouch and it was restored to the original position in the dorsal pouch (see the inset figures in Fig. 9). Then, every minute after median pouch restoration, the males were operated to expose the inside of the genital chamber. When the dirt was detected around the entrance of the lateral pouch or inside the lateral pouch, the case was used for analysis. The percentage of males of each category in terms of the location where the dirt was found was determined every minute and shown in a graph (see Fig. 9).

#### Inspection of the dorsal pouch for the remnants of spermatophore material

To search for remnants of spermatophore material in the dorsal pouch, the median grooved fold which constitutes the inner surface of the dorsal pouch was cut out and flattened under a stereoscopic microscope. This inspection was performed both in males just after the spermatophore was artificially extruded to terminate the mating stage (Sakai et al., 1991) and in those exhibiting opening of the subgenital plate 45 s prior to spermatophore preparation (Ootsubo and Sakai, 1992). Spermatophore preparation occurred 4–10 min after spermatophore extrusion when a male was paired with a female in a 200 ml beaker (Ootsubo and Sakai, 1992).



**Fig. 1.** Genitalia of the male cricket in the two reproductive stages. **(A)** Abdomen, posterior-dorsal view. **(B)** Genital chamber and phallic complex in the mating stage, posterior-ventral view. The bottom region of the genital chamber is opened and bent ventrally to show its interior as indicated by a horizontal dotted line and curved arrows. The membranous organs (LP and MP), which are actually inflected under the floor of the genital chamber (F), are drawn in perspective. Note a number of large bristle hairs on the rim of the subgenital plate (SgP). **(C)** Genital chamber and phallic complex in the sexually refractory stage between spermatophore extrusion and spermatophore preparation. No spermatophore is present. The ventral lobes that had held the spermatophore are now shrunken and folded under the dorsal pouch. Instead, the median pouch (MP) inflated with hemolymph is actually located inside the vacant dorsal pouch (see **(E)**) before the genital chamber is bent ventrally. **(D)** Crossed sectional view of **(B)**. The circle drawn by a broken line indicates the space contour occupied by the ampulla of the spermatophore. **(E)** Crossed sectional view of **(C)**. DP, dorsal pouch; Ep, epiphallus; F, floor of the genital chamber; IW, inner wall of the subgenital plate; LP, lateral pouch; MP, median pouch; SgP, subgenital plate; Sp, spermatophore; VL, ventral lobe. These abbreviations are used in the following figures. Scale bar, 1 mm.

### Examination of the spermatophore in males with the median pouch ablated

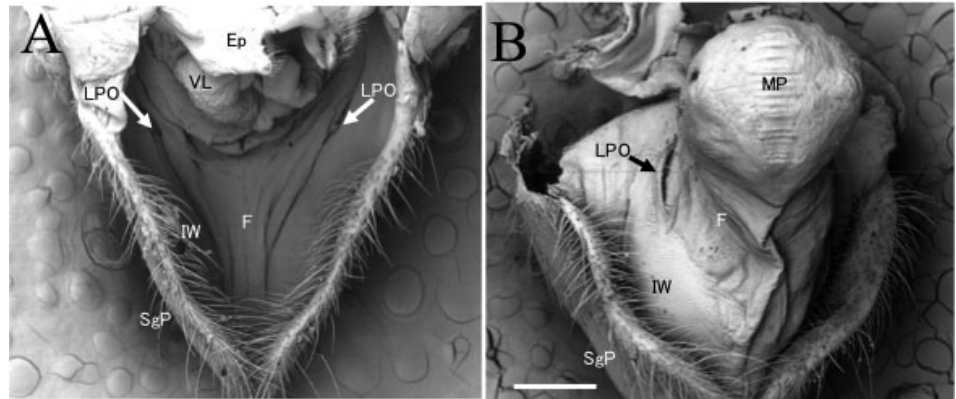
The median pouch in males three days after the final molt was cut (see Fig. 11A) with the pouch expanded by manually applying pressure onto the abdomen. The males were then reared in a group without females. Two weeks later, each male was paired with a female to let it copulate. When copulation was successful, the transferred spermatophore hanging from the female subgenital plate was quickly removed. When the spermatophore was unsuccessfully transferred and fell onto the substrate just after copulation, it was quickly picked up with tweezers. The shapes of spermatophores recovered were examined under a light microscope.

## RESULTS

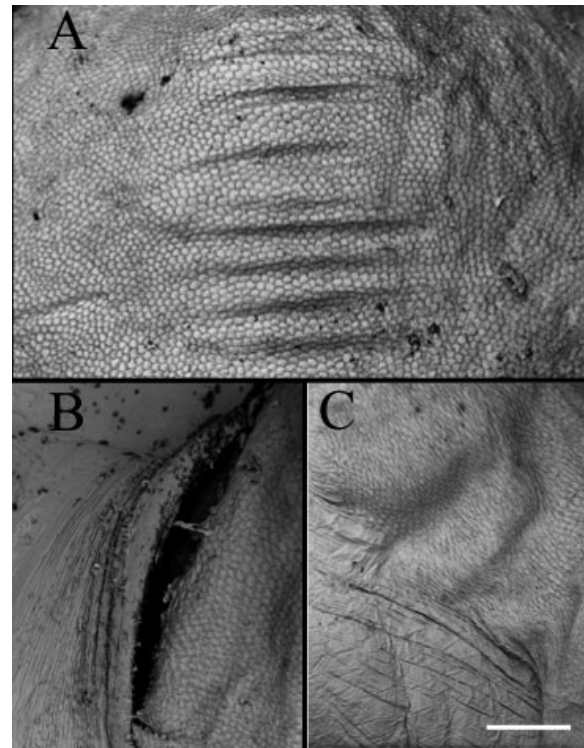
### Gross morphology of the phallic complex

The morphology of the phallic complex in the genital chamber is shown in Fig. 1. The genital chamber is housed in the last abdominal sternite called the subgenital plate (Fig. 1A). The figures on the left (Fig. 1B, D) show the state before copulation, while those on the right (Fig. 1C, E) show the state just after copulation in which the spermatophore had been extruded. Here, only the membranous organs related to genital cleaning are of concern. First, the ventral lobes (VL, Fig. 1B) resembling two fused gloves side by side are connected anteriorly to the lateral arm of the sclerotic cuticle (LA) and ventrally to the floor of the genital chamber inside the subgenital plate (SgP, Fig. 1B, D). Posteriorly, these constitute the flaps with indented fringes that hold the ampulla of the spermatophore (Sp). Second, the genital chamber floor (or simply floor: F) is formed by a semi-transparent membrane and is joined dorsally to the ventral lobes and laterally to the membrane of the inner wall of the subgenital plate (IW) (Fig. 1C). Third, the median pouch (MP) is a sac-like structure occupying the central region of the genital chamber floor. It is shrunken and hidden under the genital chamber floor (Fig. 1B, D), except for in the period between spermatophore extrusion and spermatophore preparation. The continuum of the genital chamber floor and median pouch is called here the genital membrane complex or simply membrane complex.

In addition, two other structures (dorsal pouch and lateral pouch) are also involved in cleaning. After the spermatophore is extruded at the final stage of copulation, the shape of the membranous structure of the phallic complex changes dramatically. The ventral lobes rapidly shrink and fold under the dorsal pouch (Fig. 1C), while the median pouch in turn expands maximally with body fluid (Fig. 1C) and enters the vacant dorsal pouch (Fig. 1E). The dorsal pouch is a hollow structure made of a cuticle and membrane that serves as a template for the attachment plate and spermatophore tube of the spermatophore (see Fig. 13). There is another membranous organ (called here the lateral pouch, LP) which was previously named pocket X (Kumashiro and



**Fig. 2.** SEM photographs of the genital chamber in the two reproductive stages. **(A)** SEM photograph in the mating stage. The spermatophore was artificially removed. The median pouch (MP) is hidden under the genital chamber floor (F). The ventral lobes are artificially folded under the epiphallus (Ep) for clarity. **(B)** SEM photograph in the sexually refractory stage between spermatophore extrusion and spermatophore preparation. The epiphallus, dorsal pouch and ventral lobes are removed for clarity. IW, inner wall of the subgenital plate; LPO, lateral pouch opening. Scale bar, 500  $\mu\text{m}$ .



**Fig. 3.** Surface structure of the median pouch and genital chamber floor. **(A)** Central region of the expanded median pouch. This is part of the MP in Fig. 2B. Small scales cover the entire surface. **(B)** Lateral pouch opening. This is the enlarged LPO in Fig. 2B. Note that in contrast to the MP, the inner wall of the subgenital plate (left of the LPO) bears no scales or processes except for at the margin of the LPO. **(C)** Transition between the genital chamber floor (upper) and the inner wall of the subgenital plate (lower). This is also part of Fig. 2B. Scale bar, 100  $\mu\text{m}$ .

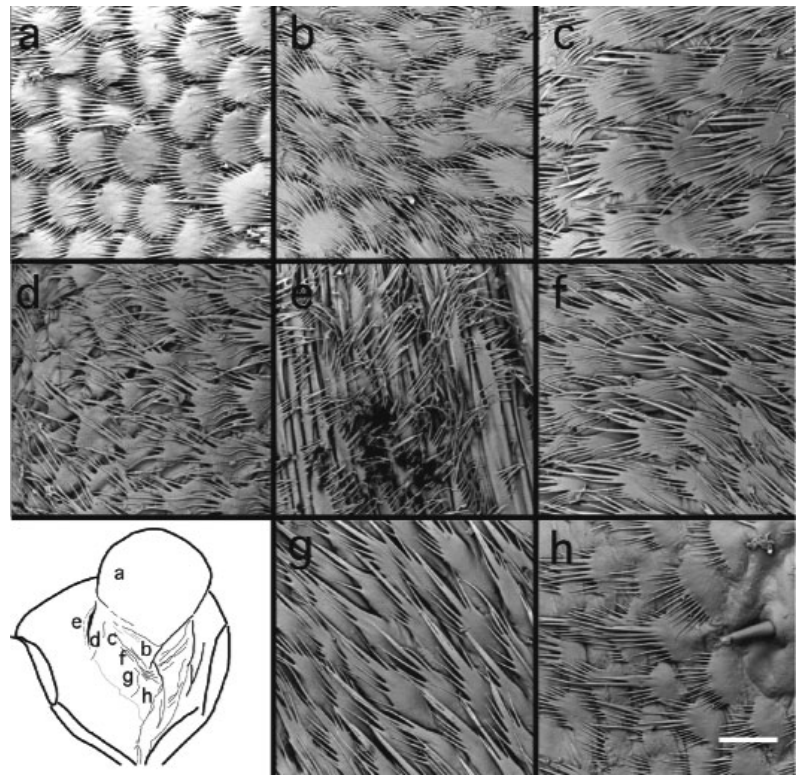
Sakai, 2001a). The lateral pouch consists of an inflected membrane situated below the genital chamber floor (Fig. 1B, D) on the right and left sides. It has a slit-like opening of

about 100  $\mu\text{m}$  in length (Fig. 1B, C) along the border between the genital chamber floor and the inner wall of the subgenital plate. In this structure, no muscle fibers or nerve innervation are present, unlike in the ventral lobes and genital membrane complex (Kumashiro and Sakai, 2001b).

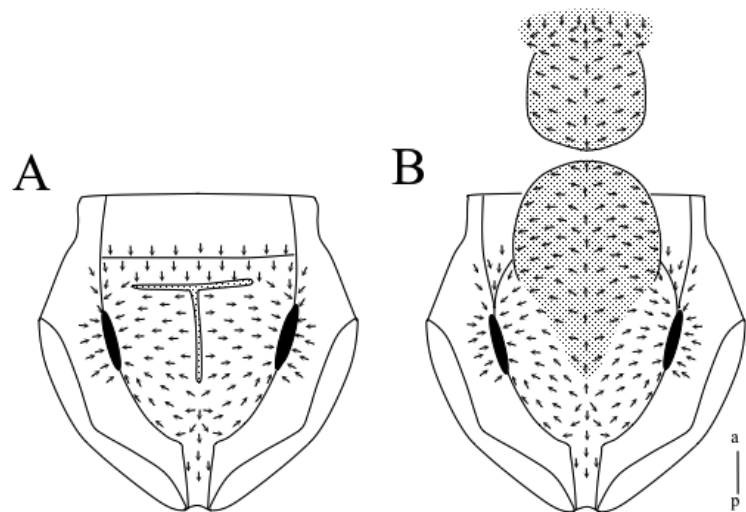
Scanning electron microscopic images of the genital chamber and phallic complex are shown in Fig. 2. Figure 2A corresponds to Fig. 1B in the state before copulation (the spermatophore having been artificially removed), while Fig. 2B corresponds to Fig. 1C in the state soon after copulation. The sample in Fig. 2B was fixed with the median pouch artificially expanded after the dorsal pouch had been removed to reproduce the state in which the expanded median pouch had been located inside the dorsal pouch. Actually the median pouch had not a spherical shape, as in Fig. 2B, but a complex shape in the dorsal pouch. This was the reverse of the appearance (not shown) of the shape of the dorsal pouch cavity, which serves as a template for the spermatophore. The dorsal midline ridge (about 40  $\mu\text{m}$  wide) of the fixed median pouch matches the width of the median groove (30–50  $\mu\text{m}$  wide and 200  $\mu\text{m}$  deep) of the median grooved fold of the dorsal pouch that serves as a template for the spermatophore tube (see Fig. 1a in Sakai and Kumashiro, 2004). The median grooved fold is a curving cuticular plate, resembling a pen point, which constitutes the inner aspect of the dorsal pouch (Fig. 10) and the median groove is a narrow ditch running in the central region of the median grooved hold (Fig. 10). The spatial relationship between the dorsal pouch and median pouch with spermatophore material is shown in Fig. 13B.

#### Surface structure of the membrane complex

The surface of the membrane complex is entirely covered by scales arranged in a slightly overlapping pattern. Each scale has an oval or hexagonal base (approximately 10  $\times$  10  $\mu\text{m}$ ) with a fringe (10–20 spines of 3–20  $\mu\text{m}$  in length). For the median pouch, the scales are generally uniform (Fig. 3A) and look like those of fish or reptiles. There are some transverse loosen creases on the midline region of the median pouch, revealing that that region forms some sags for complementary association with the median groove of the dorsal pouch. Scales are also seen on the genital chamber floor surrounding the median pouch (Fig. 3C, upper part), but are not found on the inner



**Fig. 4.** Variation of scales in the median pouch and genital chamber floor. Images a–h were taken at the locations indicated by letters a–h in the inset to the lower left. a is in the median pouch, b–d and f–h are in the genital chamber floor and e is in the inner wall of the subgenital plate. In h, a bristle hair can be seen on the right. See Fig. 2B for the reference. Scale bar, 10  $\mu\text{m}$ .



**Fig. 5.** Directions of the scales in the genital membrane complex. (A) Figure in the mating stage. The median pouch is inflected and thus cannot be seen. The T-shaped gray region in the center is a fissure from which the median pouch is pulled in under the floor (refer to Fig. 1B, D). Two black zones are the openings of the lateral pouches. (B) Sexually refractory stage between spermatophore extrusion and spermatophore preparation. The swollen region in gray (posterior surface) is the median pouch, which actually occupies the inside of the dorsal pouch. Upper drawing in (B) shows the backside (anterior surface) of the median pouch. Arrows surrounding the genital chamber floor indicate the directions of spines on the inner wall of the subgenital plate. For (A) and (B), the scales are arranged symmetrically about the midline, and are continuously directed toward the lateral pouch on either side except for some scales in the most posterior region of the floor. The head of each arrow indicates the side from which spines protruded in the scales. That side was defined as the direction of a scale. Direction bar: a, anterior; p, posterior.

wall of the subgenital plate across the lateral pouch opening (Fig. 3B, left side).

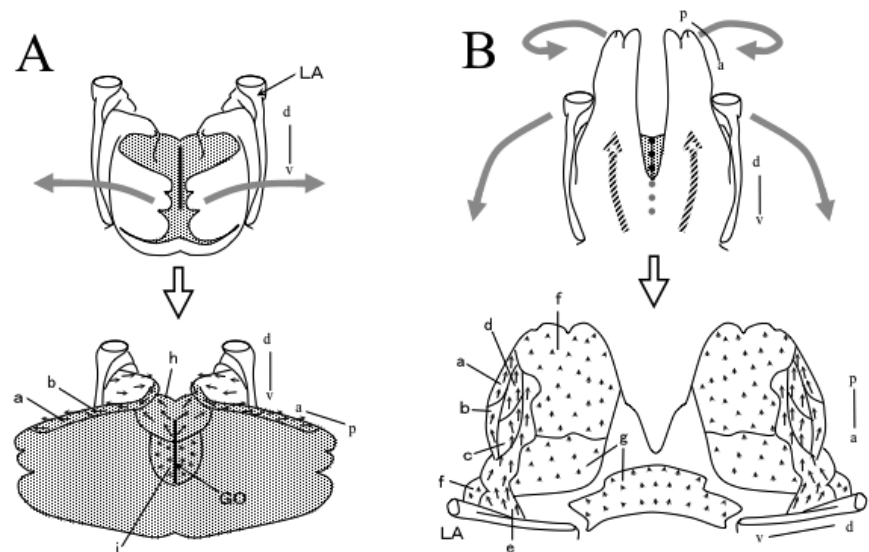
Scale morphology varies in size and shape depending upon location in the genital membrane complex (Fig. 4a–h).

Samples were obtained in the corresponding regions labeled in the inset figure (lower left). The scale in the median pouch (a) had a plate-type base of  $5 \times 10 \mu\text{m}$  in size (minor axis  $\times$  major axis) with about 20 spines (3–8  $\mu\text{m}$  in length). The longest spine protruded from the midline of the major axis of the scale and other spines were symmetric to the axis. The scales overlapped approximately one quarter to one half of the length of the neighboring scales. On the other hand, the scales in the genital chamber floor (b–d, f–h) varied in their shape and size, spine number and spine length depending on the location: a  $5 \times 15 \mu\text{m}$  base with about 15 spines (3–8  $\mu\text{m}$ ) in region (b), a  $7 \times 12 \mu\text{m}$  base with 18–25 spines (7–15  $\mu\text{m}$ ) in (c), a  $4 \times 15 \mu\text{m}$  base with 9–15 spines (4–15  $\mu\text{m}$ ) in (d), a  $5 \times 10 \mu\text{m}$  base with about 12 spines (10–20  $\mu\text{m}$ ) in (f), and a  $5 \times 9 \mu\text{m}$  base with 15 spines (4–10  $\mu\text{m}$ ) in (h). Near the transitional region to the inner wall of the subgenital plate, the scales bearing about 10 spines were much more slender and longer (6–20  $\mu\text{m}$ ) than others (g). On the inner wall of the subgenital plate across the lateral pouch opening (see Fig. 3B, left side), no scales were present, only some spines (e) protruded locally from the striped membrane forming a comb-like group. Among the scales, some bristle-type hairs of approximately 10  $\mu\text{m}$  in length were sporadically present on the posterior region of the genital chamber floor (h).

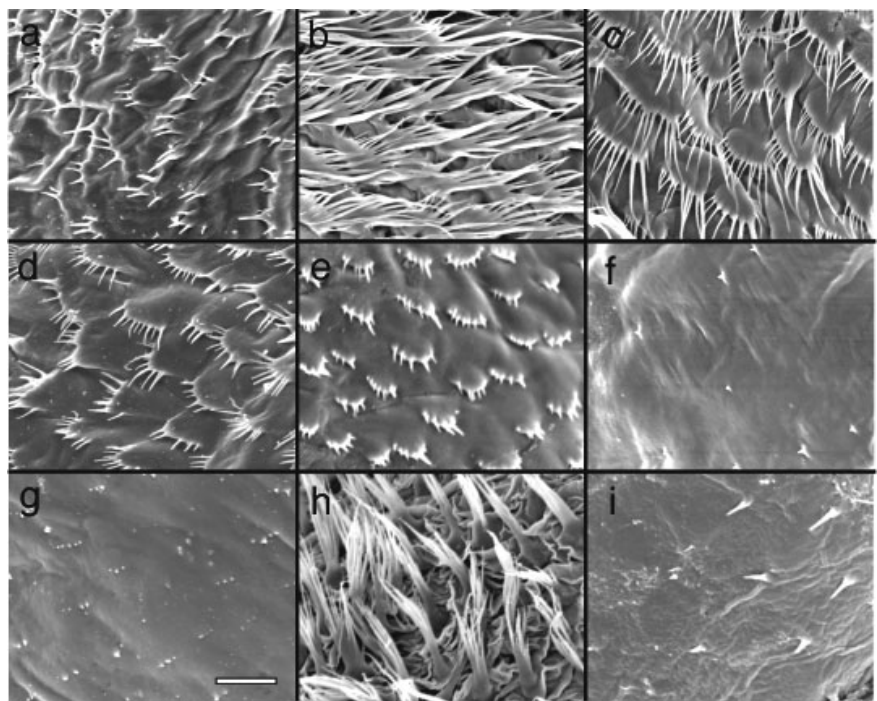
#### Direction of the scales on the membrane complex

The scale direction was mapped over the genital membrane complex (Fig. 5). Here the direction of a scale was defined as the side from which spines protruded. The scales are arranged symmetrically about the midline in a characteristic pattern. The scale direction in the genital chamber before copulation is shown in Fig. 5A, in which the median pouch is hidden under the genital chamber floor (see T-shaped region; part of the median pouch inflected under the floor, see Fig. 1B, D). In the anterior region of the floor, the scales are directed posteriorly, but after crossing the horizontal part of

the T-shaped region, the scales are directed laterally from the midline. In contrast, the scales in the posterior region of the floor are directed anterior-laterally in general, but a few of them are directed posterior-laterally or just posteriorly.



**Fig. 6.** Distribution of scales and non-scale-like processes in the ventral lobes. (A) Upper, posterior view of the ventral lobes. Lower, internal view of the ventral lobes after the flaps were opened to the right and left (refer to large gray arrows in the upper figure). (B) Upper, ventral-lateral aspect of the ventral lobes. Part of the midline (large dots) was cut to extend the lobes. Lower, whole outside view of the ventral lobes. The lateral arms were turned 90° and the flaps were extended (upward striped arrows) and then abducted to extend maximally (twisted gray arrows and downward gray arrows). Small arrows show scales or scale-like structures, and arrowheads on the surface of the lobes show non-scale-like processes. Letters (a–i) correspond to (a–i) in Fig. 7. Direction bar: d, dorsal; v, ventral.



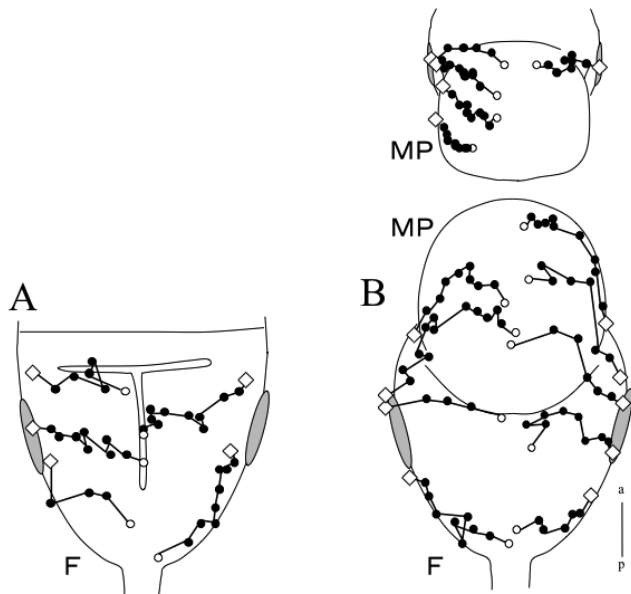
**Fig. 7.** Variation of scales in the ventral lobes. Each type (a–i) was present in the region of the ventral lobes as indicated by letters in Fig. 6. Scale bar, 10  $\mu\text{m}$ .

On the other hand, during the period between the end of copulation and the start of spermatophore preparation, the median pouch is swollen out of the T-shaped region. It in fact occupies the inside of the dorsal pouch (Fig. 5B, gray portion; refer to Fig. 1C and Fig. 2B). In this state, the scales on both sides of the median pouch are basically directed laterally, and three-dimensionally, they are directed latero-ventrally on the surface of the median pouch hemisphere. The scales on the midline region of the median pouch are all directed anteriorly (Fig. 5B). They meet with the posteriorly directed scales on the transitional zone to the genital chamber floor (upper figure in Fig. 5B). Some spine-like processes on the inner wall of the subgenital plate located along the lateral pouch opening are directed medially toward the lateral pouch opening (arrows located laterally to the zones in black in Fig. 5A, B). As a whole, when starting at any point of the membrane complex, the directions of the scales continuously lead to the entrance of the lateral pouch on either side. This scale arrangement enables every point of the membrane complex to be linked to the lateral pouch.

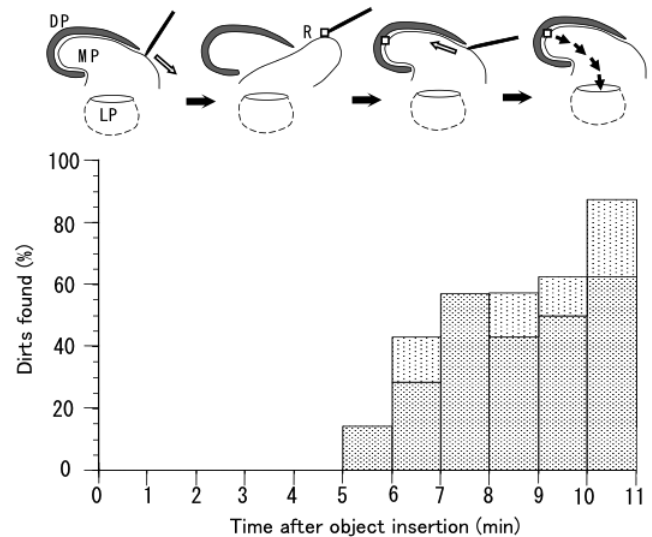
### Surface structure of the ventral lobes

The ventral lobes are also composed of the flexible membrane continuous to the genital chamber floor. The dorsal aspect of the ventral lobes in which the ampulla of the spermatophore is held, has mostly a smooth surface (Fig.

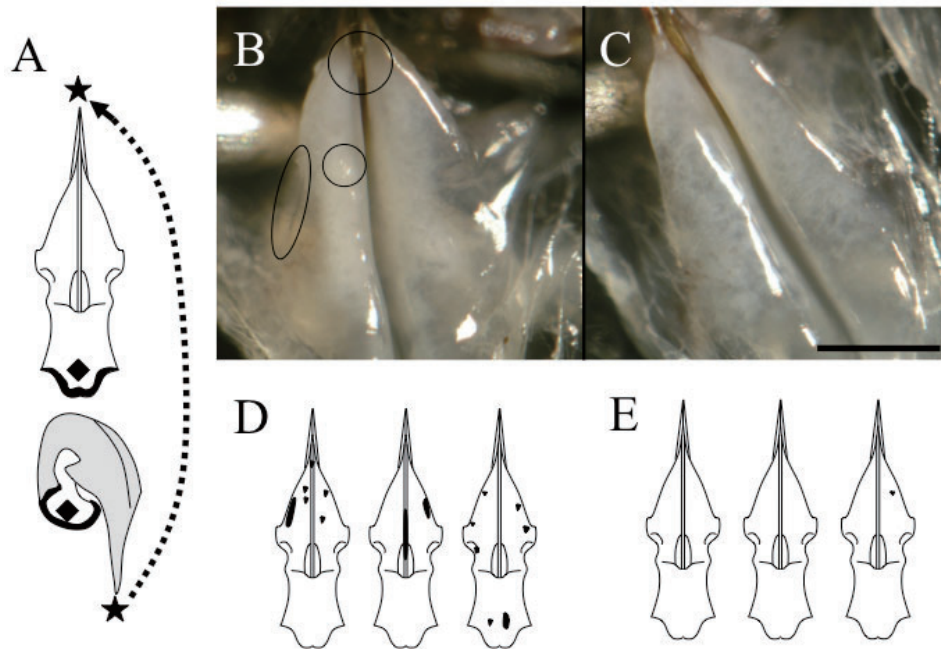
6A, gray region). The region surrounding the genital opening (GO) from which the spermatophore material is ejected at the time of spermatophore preparation (Fig. 13B) constitutes the anterior wall of the inner surface, where two different kinds of non-scale-type processes are present (Fig. 6A, Fig. 7h, i). In the ventro-lateral region of the ventral lobes, constituting the outer aspect of the lobes, various types of scales and processes were found. Scales and non-scale-type processes were grouped into three types (Fig. 6B, Fig. 7a–g): scale type (c, d and e), brush type (b and h), and thorn type (a, f, g and i). The scale types had an  $8 \times 10 \mu\text{m}$  (minor  $\times$  major axis) oblong-shaped base with about 13 spines (3–14  $\mu\text{m}$  long) in (c), a  $10 \times 10 \mu\text{m}$  lozenge-shaped base with 5–9 spines (2–5  $\mu\text{m}$ ) in (d), and a  $6 \times 7 \mu\text{m}$  oval-shaped base with 2–6 spines (1–3  $\mu\text{m}$ ) in (e). The brush type had spines that were rather wavy: 6–12 spines (2–20  $\mu\text{m}$ ) in (b) and about five spines (3–12  $\mu\text{m}$ ) in (h). The thorn type possesses 2–5 spines (2–5  $\mu\text{m}$  long) emanating in groups at irregular intervals in (a), a single spine (1  $\mu\text{m}$ ) sporadically in (f), 2–5 spines (1  $\mu\text{m}$ ) on the fringe of the scale-like protrusion about 9  $\mu\text{m}$  apart in (g), and sporadic single spines (2–6  $\mu\text{m}$ ) spaced at an interval of about 14  $\mu\text{m}$  in (i). In addition to these types, there are a number of small bristle-type hairs on the lateral-ventral regions of the outer aspect (Kumashiro and Sakai, 2001a). These findings indicated that typical scales are located in limited regions of the outer aspect, while non-scale-like processes were present in



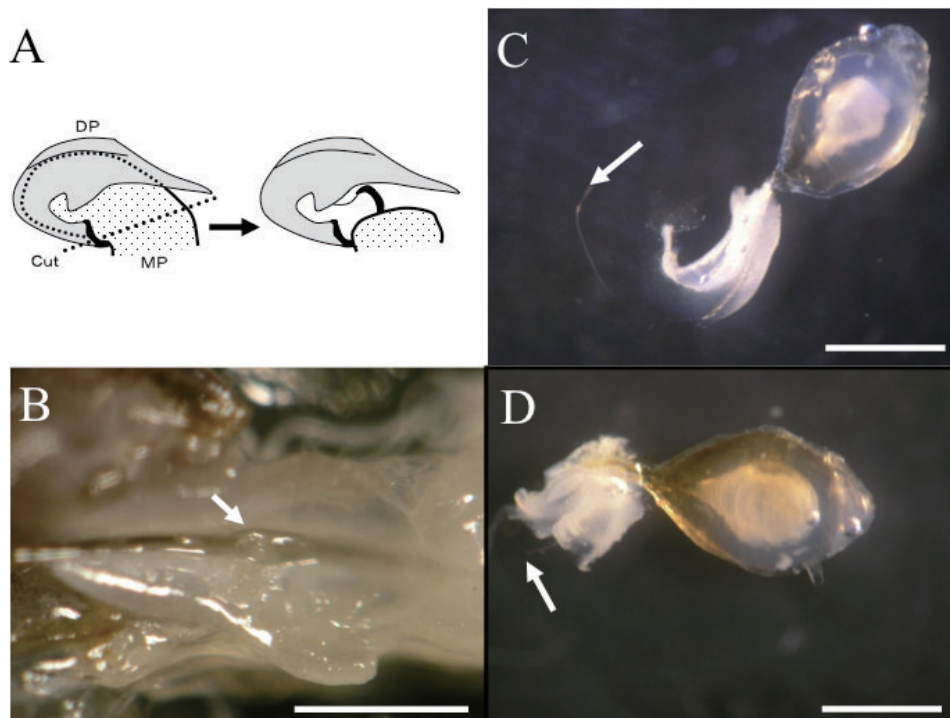
**Fig. 8.** Trajectory of artificial dirt on the genital membrane. **(A)** Tracks of artificial dirt (a piece of rubber) in the mating stage (refer to Fig. 5A). Five tracks on the genital chamber floor. **(B)** Tracks of artificial dirt in the stage between copulation and spermatophore preparation (refer to Fig. 5B). The lower figure shows nine tracks of artificial dirt in the posterior region of the median pouch and genital chamber floor. The upper figure (i.e., the back side of the lower figure) shows five tracks in the anterior region of the median pouch. In each track, the white circle indicates the starting point, the black circles indicate *en route* points, and the white square indicates the end point. The time between two successive points was 1 min. The gray zone in the floor is the opening of the lateral pouch. Direction bar: a, anterior; p, posterior.



**Fig. 9.** Time when artificial dirt was found near or in the lateral pouch after exiting the dorsal pouch. The abscissa shows the time (every minute) of inspection after insertion of the rubber piece as artificial dirt in the dorsal pouch. The ordinate shows the occurrence (%) of cases in seven or eight males every one minute. The case in which the dirt was found near the lateral pouch opening is shown by a light gray bar and the case in which the dirt was found in the lateral pouch is shown by a deep gray bar. The upper part shows the procedure for treatment. First (leftmost), the median pouch (MP) was drawn out from the dorsal pouch (DP) with a needle. Second, artificial dirt (R) was placed in the central region of the median pouch. Third, the median pouch was returned to the dorsal pouch. Fourth (rightmost), the artificial dirt moved toward the lateral pouch.



**Fig. 10.** Remnants of spermatophore material on the median grooved fold of the dorsal pouch. **(A)** The median grooved fold in the inner surface of the dorsal pouch. The upper figure shows the median grooved fold artificially extended from the normal position (below). **(B)** Median grooved fold of the dorsal pouch immediately after the spermatophore was extruded by artificial stimulation in males in the mating stage. Remnants of spermatophore material are seen in the circles. **(C)** Median grooved fold just at the time when the subgenital plate is opened prior to spermatophore preparation. No remnants are seen. **(D)** Sketches of three samples obtained from males just after spermatophore extrusion. Dark portions show remnants of the spermatophore. Note the left and middle preparations in which remnants are choking the median groove, which acts as a guide for the spermatophore tube. **(E)** Sketches of three samples obtained from males at the time when the subgenital plate was opened 45 s prior to the occurrence of spermatophore preparation. Nearly no remnants are seen. Scale, 500  $\mu\text{m}$ .



**Fig. 11.** Abnormal spermatophores produced by males with the median pouch ablated. **(A)** Method of operation. The median pouch (MP) was cut while it was expanded in the dorsal pouch (oblique dotted line). **(B)** Some remnants of spermatophore material are adhering to the median groove (white arrow) in a male whose median pouch was removed. **(C)** Normal spermatophore. White arrow indicates the tip of the spermatophore tube. **(D)** Abnormal spermatophore in a male whose median pouch was transected. The ampulla of the spermatophore is apparently normal, but the attachment plate is malformed and the spermatophore tube is lost (white arrow). Scale, 1 mm.



wide regions. The three types of processes on the ventral lobes were essentially directed posteriorly (Fig. 6B).

#### Route of movement of artificial dirt on the membrane complex without the dorsal pouch

The positional change of a small piece of colored rubber (artificial dirt; 80  $\mu\text{m}$ ) placed at various points near the midline region of the genital membrane complex was recorded. Nineteen cases are shown in Figs. 8, 9 of which started from the genital chamber floor (Fig. 8A, B) and 10 of which started from the median pouch (Fig. 8B). The starting point is shown by a white circle and the end point is shown by a white square. For the genital chamber floor, the object moved laterally when placed in the anterior region of the floor, while it moved anterior-laterally when it was placed in the posterior region. Although the object occasionally made several turns in different directions *en route*, it eventually arrived at the opening (gray zone) of the lateral pouch. For the median pouch, the dirt placed on the anterior (5 cases in the upper figure of Fig. 8B) and posterior (5 cases in the lower figure of Fig. 8B) aspects directed laterally or posterior-laterally and arrived at the opening of the lateral pouch. Each route roughly corresponded to the directions of the scales (see Fig. 5) on which the dirt moved. The arrival time for the dirt starting at the midline region of the median pouch ranged from 8.2–14.8 min with an average time of  $12.2 \pm 2.5$  min ( $n = 7$ ).

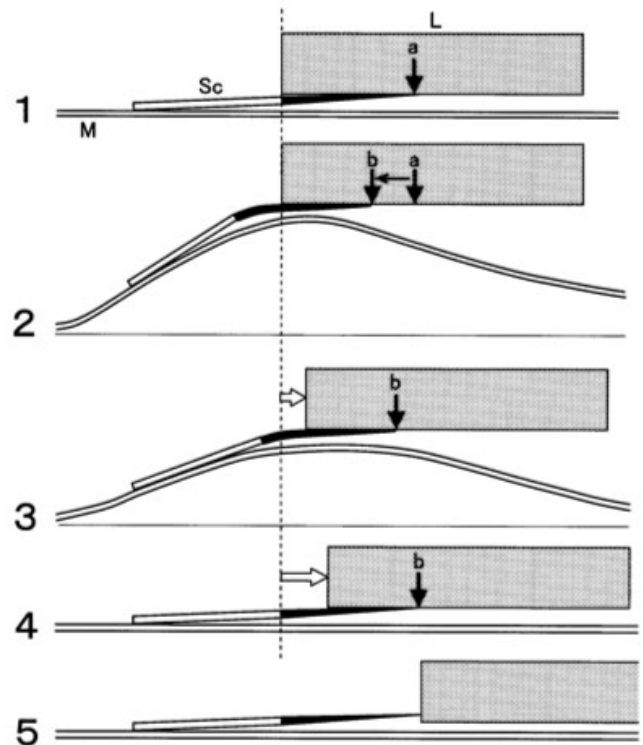
#### Evacuation of artificial dirt from the dorsal pouch

In the previous experiment in which artificial dirt was placed on the midline region of the membrane complex, the dorsal pouch had been removed before the experiment. However, in the pre-spermatophore preparation period following spermatophore extrusion, the expanded median pouch occupies the cavity of the dorsal pouch (Fig. 1C, E). Thus, experiments were performed in males with the dorsal pouch intact. The procedures are shown in 4 figures in Fig. 9 inset. The median pouch was first pulled out of the dorsal pouch and the artificial dirt was placed on the midline regions of the median pouch, and then the median pouch was restored into the dorsal pouch. The time of the earliest case in which the dirt was found outside the dorsal pouch was 5.7 min. Thus, from 5–10 min after the start, inspection was made every minute in 44 males (seven males for 5–6, 6–7, 7–8, 8–9 min, and eight males for 9–10 and 10–11 min). The percentage of the cases in which the dirt was found at the entrance of the lateral pouch (light gray) or inside the lateral pouch (dense gray) increased with time as shown in the graph (Fig. 9). It finally reached 88% in one minute from 10 to 11 min just before the observation was terminated.

#### Remnants of spermatophore material in the inner surface of the dorsal pouch

When the inside of the dorsal pouch was inspected for the remnants of spermatophore material in males immediately after the spermatophore was extruded, residual white spots were found on the surface of the median grooved fold of the dorsal pouch (Fig. 10B). Some spots were found on the curved cuticle of the fold, which serves as a template for the hooks of the attachment plate (Fig. 10B, D), and others

choked the narrow median groove (Fig. 10B, D-left, middle), which serves as the cast for the spermatophore tube. Even in small quantities, they could cause malformation of the spermatophore tube to render the spermatophore useless by preventing the release of spermatozoa through that tube. In contrast, almost no white spots were found in the dorsal pouch when subgenital plate opening occurred at a median time of 4.6 min ( $n = 12$ ) after spermatophore preparation (Fig. 10C, E). The average number of spots per male was 4 ( $n = 12$ ) when inspected immediately after spermatophore extrusion. However, when inspected at subgenital plate opening, 7 males had no spots and 5 males had 1.6 spots on average. In addition, the spots were larger in males immediately after spermatophore extrusion. These results indicate that spermatophore material certainly remains in the dorsal pouch when the spermatophore is extruded and is



**Fig. 12.** Model of dirt transport on the undulating membrane with scales. **1.** Load (L) equivalent to dirt is on a scale (Sc) of the genital membrane (M). The tip of the scale is indicated by an arrow **a**. **2.** When the membrane is raised, the scale slides toward the left with respect to the left end of the load {see the horizontal thin arrow between the arrow **a** (original position of the spine tip) and arrow **b** (new position of the spine tip)}. This is based on an elastic nature of the spine of the scale (black part of Sc) and an inelastic nature of the plate of the scale (white part of Sc). **3.** When the membrane begins to fall, the load is lowered with no positional change in relation to the spine of the scale. As a result, the load is slightly displaced to the right (white arrow). **4.** The scale returns to the original position. **5.** When the load slips off the spine, the spine resists movement of the load in the opposite direction. In the figure, the load appears to be floating but is actually supported by nearby scales (not illustrated here). This model is not a summary of the results but to explain how the dirt is moved by undulation of the scaled membrane.

quickly evacuated from the dorsal pouch.

### Effect of median pouch ablation on spermatophore formation

The shape of the spermatophore was examined in males that had been reared in isolation for two weeks after surgical removal of the median pouch (Fig. 11A). Males with the median pouch ablated showed various abnormal spermatophores. An example is shown in Fig. 11D in which the spermatophore had a deformed hook of the attachment plate with no spermatophore tube (compared with the normal spermatophore indicated by a white arrow, Fig. 11C). The shapes of abnormal spermatophores were divided into two groups. One group is mildly abnormal types that had a locally deformed medial hook of the attachment plate and a shorter sperm tube, and the other group is severely abnormal types that showed entire deformation of the attachment plate and spermatophore tube. The spermatophores in all of the operated males ( $n = 20$ ) were normal in 15% of the males, mildly abnormal in 65% and severely abnormal in 20%. In intact males ( $n = 13$ ), the spermatophores were normal, mildly abnormal and severely abnormal in 92.3%, 7.7% and 0% of the males, respectively. Inspection of the inner surface of the dorsal pouch in males with the median pouch ablated revealed that many spermatophore remnants clogged the median grooved fold (indicated by a white arrow, Fig. 11B) presumably due to the repetitive production and spontaneous extrusion of the spermatophore (Sakai et al., 1991) during 2 weeks in isolation.

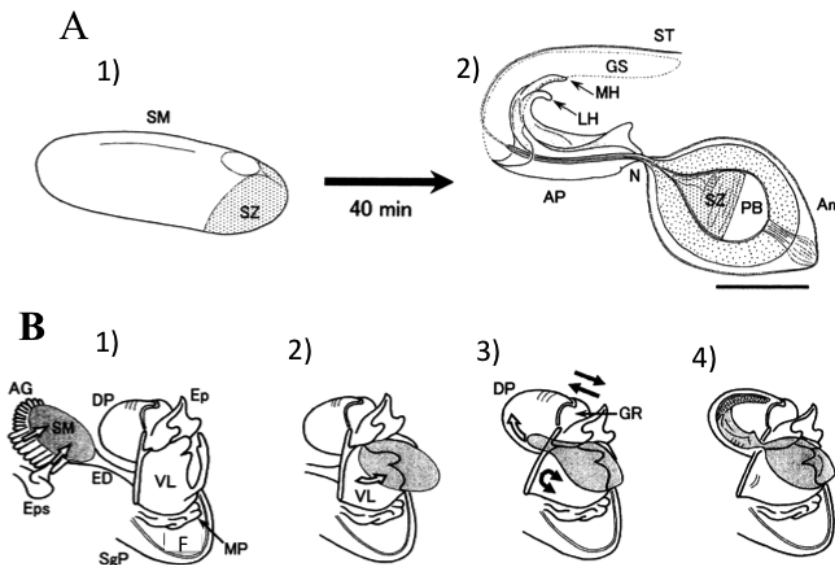
## DISCUSSION

### Function of the scaled membrane in the genitalia

Our results showed that the genital membrane in the male cricket is entirely covered by small scales with spine directionality. The scales are symmetrically arranged about the midline to the margin of the genital chamber. Similar scales are known to be present in other organs in insects. Butterflies have scales on their wings (Yoshida and Aoki, 1989), which are about 10 times larger than those of the cricket genitalia. One of the functions of the butterfly scales is to repel water due to surface tension. The same effect was found in the scales of the cricket genital membrane as seen in its highly hydrophobic nature (see methods section). This property of the scales is suited for preventing dirt or waste from adhering to the surface of the genital membrane.

Our experiments with artificial dirt used as a foreign object showed that material on the membrane complex was smoothly conveyed to the lateral pouch following the direction of the scales. A similar kind of transport has been observed in the inner surface of the ovipositor in orthopteran female insects (Austin and Browning, 1981). Although the shape and arrangement of the processes are considerably different among species, those of the grasshopper *Caedicia* sp. (Orthoptera: Tettigoniidae) (Austin and Browning, 1981) are close to those of the median pouch of the male cricket. Their processes are directed distally along the shaft of the ovipositor and function as latches to assist in pushing the egg unidirectionally so as to pass it to the exit. The ovipositor is not a simple cuticle tube but consists of four quadrifid mobile valves. The two left and two right valves move back and forth alternately. Such movement produces a driving force for transporting the egg. On the other hand, the genital membrane in the male cricket does not oscillate back and forth. Instead, it undulates up and down at a rate of 0.16 Hz (Kumashiro and Sakai, 2001a).

We propose a mechanism for the transport of an object on the scaled membrane. As shown in Fig. 12, a small foreign object (load) lying on the scale can be moved as follows. When the scale is pushed up by a spontaneous upward movement of the genital membrane, the spine of the scale slightly slips off the load due to its elastic nature (Fig. 12-1, 2). During returning downward, the object is lowered with no positional change in relation to the spine of the scale (Fig. 12-3). As a result, the load is slightly dislocated to the right (Fig. 12-4) and is prevented by the spine from moving in the opposite direction when it comes off the scale (Fig. 12-5). When the upward movement is large, the load is dislocated larger and can be slipped off the scale at a stroke. When the load extends over many scales moving at slightly different timing (Kumashiro and Sakai, 2016), it can be dislocated further



**Fig. 13.** Spermatophore and its formation process. **(A)** Spermatophore material and mature spermatophore. 1) Spermatophore material (SM) just excreted from the accessory glands (AG) and spermatozoa (SZ) from the testis into the ejaculatory duct (ED). 2) Matured spermatophore 40 min after spermatophore preparation. Am, ampulla; AP, attachment plate; GS, gelatinous substance; LH, lateral hook; MH, median hook; N, neck; PB, pressure body. ST, sperm tube; Scale, 100  $\mu$ m. **(B)** Sequence of spermatophore formation. 1) Excretion of SM and SZ into the ED at SgP opening. 2) Spermatophore preparation. 3) Casting of the attachment plate and sperm tube of the spermatophore in the dorsal pouch and formation of the ampulla of the spermatophore in the ventral lobes. 4) Maturation of the spermatophore. DP, dorsal pouch; Ep, epiphallus; F, genital chamber floor; GR, guiding rod; LP, lateral pouch; MP, median pouch; SgP, subgenital plate.

in a single undulation. The movement of spines during undulation was qualitatively discerned with a higher magnification stereoscopic microscope (Kumashiro et al., 2008).

#### Regional difference in scale shape and spine direction

The membrane complex bears different types of scales depending on the location in the genital chamber. The scales may exhibit unique functions owing to their different morphologies. For example, those in the median pouch are uniform with a plate-type base and relatively short spines. This shape may be suitable for scraping waste adhering to the narrow median groove of the dorsal pouch (30  $\mu\text{m}$  wide and 200  $\mu\text{m}$  deep, Sakai and Kumashiro, 2004), which serves as a template for the spermatophore tube (7  $\mu\text{m}$  in diameter and 2 mm in length, see Fig. 13A-2). The scales on the genital chamber floor are more variable in shape. There was a tendency for those in the medial region to be ovoid and those in the lateral region to be slender. Such a difference may simply reflect a gradational variation during development and may have no relation to function. However, there is the possibility that they are arranged in the most suitable places so as to transport a foreign object as efficiently as possible according to the geometry of the genital chamber floor.

Furthermore, the membrane complex has a characteristic scale arrangement about the midline. For the genital chamber floor, scales in the anterior region are directed posteriorly and then laterally, while those in the posterior region are directed anterior-laterally in general. Scales in the median pouch in contrast are mostly directed lateral-ventrally (Fig. 5B), suggesting that objects are conveyed from the dorsal pouch to the lateral pouch. When the scales are followed according to their spine direction, either side of the lateral pouch opening is reached regardless of the starting point in the membrane complex. This suggests that the object in the genital membrane is moved in the directions of the spines and transported to the lateral pouch, which serves as a waste container (Fig. 5A). In fact, our experiments using artificial dirt showed that the dirt was transported along that route as expected. It should be stressed that the scales in the midline region of the median pouch are all directed anteriorly. This arrangement suggests that no dirt is transported to the posterior end of the median pouch, where the guiding rod is located. The guiding rod is used as a sheath for ejecting the spermatophore tube into the copulatory papilla of the female during spermatophore extrusion (Kumashiro and Sakai, 2001a). Thus, the guiding rod is prevented from being obstructed by waste produced in the dorsal pouch as described below.

#### Functional significance of scale-like processes on the ventral lobes

In the ventral lobes, scale-type and non-scale-type processes lie mainly on the outer surface. Typical scales are present only in the dorsal regions of the flaps and are arranged in the posterior direction. The ventral lobes do not undulate except for in the state in which the spermatophore material is pushed into the dorsal pouch soon after it exits the genital opening (Fig. 13B-2, 3). It is clear that the scales serve as a repellent for the dirt due to their hydrophobicity. However, it is not clear why the scales are localized only in

the dorsal regions of the flaps. The dorsolateral regions of the ventral lobes cover the dorsal aspect of the spermatophore ampulla, which may be frequently soiled with feces that have fallen from the perianal region located just above. The scales may help brush off dirt on the ventral lobe flaps when they are drawn under the dorsal pouch immediately after copulation (see Fig. 1B, C).

Thorn-type processes are present around the genital opening in the anterior wall of the ventral lobes, and are all directed laterally (see Fig. 6A). These processes may facilitate the movement of the slimy spermatophore material out of the ejaculatory duct and onto the inner surface of the ventral lobes at the time of spermatophore preparation (Fig. 13B-1, 2). Similar processes were also observed on the inner surface of the ejaculatory duct (not shown), which is certainly favorable for transporting spermatophore material toward the genital opening. In contrast, brush-type processes were located on the upper part of the genital opening, where they were arranged first in the upward direction and then in the anterior direction (Fig. 6A). This directionality may be helpful to push the anterior part of the spermatophore material into the dorsal pouch (Fig. 13B-3).

#### Speed of artificial dirt transport with respect to spermatophore preparation

The results showed that artificial dirt lying on the exposed genital membrane is conveyed from the central region to the lateral pouch in about 12 min. Since the distance between the inside of the dorsal pouch and the entrance of the lateral pouch is about 1.3 mm, the speed of dirt transport is approximately 1.8  $\mu\text{m}/\text{s}$ . In the intact condition, however, the dirt is sandwiched between the inflated median pouch membrane and the inner surface of the dorsal pouch. Nonetheless, the dirt was evacuated from the dorsal pouch (in 88% males) in 11 min, which is roughly comparable to that in the experiment performed without the dorsal pouch. This suggests that there must be some mechanism for evacuation, which is discussed in a companion article (see Kumashiro and Sakai, 2016).

On the other hand, it is known that the male cricket exhibits spermatophore preparation in about 6 min after spermatophore extrusion (Ootsubo and Sakai, 1992). Spermatophore preparation is the event in which spermatophore material being ejected from the ejaculatory duct is cast into the dorsal pouch via the ventral lobes to form a new spermatophore (see Fig. 13B-2, 3). The 5–11 minutes needed for evacuation of the dorsal pouch (Fig. 9) is a little longer than that needed for spermatophore preparation. However, spermatophore preparation in 6 min after spermatophore extrusion occurred when the male continuously received chemo-tactile stimulation from a nearby female. In a natural condition the male is not expected to make spermatophore preparation within one hour because the male is normally separated from the female soon after copulation (Ootsubo and Sakai, 1992). As described below, genitalia inspection after spermatophore extrusion indicated that the dorsal pouch almost finished evacuation 5 min after spermatophore extrusion. Thus, the cleaning speed is sufficient to provide for spermatophore preparation.

### Significance of dorsal pouch cleaning

Previously, we postulated that the inside of the dorsal pouch may be stained with the remnants of spermatophore material (Kumashiro et al., 2006). However, until now, no solid evidence has been provided. Our inspection of the dorsal pouch showed that patchy gelatinous substances were adhering to the median grooved fold in males immediately after spermatophore extrusion. Although such waste matter was small in quantity, it may cause a serious problem for spermatophore formation. In fact, some of the remnants were choking the median groove, which serves as a template for the spermatophore tube. However, almost no waste was found when inspection was carried out several minutes after spermatophore extrusion. Thus, dorsal pouch cleaning is completed very quickly. Furthermore, it was demonstrated that the inside of the dorsal pouch in males with the median pouch ablated was full of coagulated waste when inspected two weeks after the operation. Nearly all of the males with the median pouch ablated failed to produce a normal spermatophore, which suggests that genital autocleaning is indispensable for the production of a normal spermatophore.

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