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REPRODUCTIVE BEHAVIOUR IN THE MALE CRICKET *GRYLLUS BIMACULATUS* DeGEER

I. STRUCTURE AND FUNCTION OF THE GENITALIA

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Summary

We have investigated the morphology and physiology of the genitalia of the male cricket to establish a basis for neuroethological study of its reproductive behaviour. First, the structure of the phallic complex, including the dorsal pouch, guiding rod, epiphallus, ventral lobes and median pouch, are described, as are the muscles, cuticle, membranes and biomechanics of copulation. The innervation and sensory receptors have also been examined. Second, the functional role of the muscle in each genital organ has been determined by direct observation of muscle contraction during spontaneous or evoked movements and by analysis of the changes in movements after the ablation of the muscle. Third, for the flexible membranous organs, the ventral lobes and median pouch, the passages for haemolymph and their dynamic properties have been examined using petroleum jelly. Fourth, the sequence of coordinated motor actions performed by the internal and external genital organs, which were induced in both restrained and dissected males using newly developed techniques, has been analyzed during tethered copulation and spermatophore formation. As a result, the mechanisms of copulation and spermatophore formation are now more fully understood.

Key words: male, cricket, *Gryllus bimaculatus*, reproduction, behaviour, copulation, spermatophore extrusion, spermatophore protrusion, genital organ, innervation.

Introduction

Reproductive behaviour in crickets has been extensively studied in several species (Khalifa, 1950; Huber, 1955; Hormann-Heck, 1957; Alexander, 1961; Alexander and Otte, 1967; Engelmann, 1970; Beck, 1974; Loher and Rence, 1978; Evans, 1988; Adamo and Hoy, 1994). The behavioural repertoires and their sequence in the male reproductive cycle have been described in detail, but less attention has been paid to the mechanisms of copulation itself. Only the gross anatomy of the orthopteran genitalia has been described (Walker, 1922; Snodgrass, 1937; Quadri, 1940; Choperd, 1951; Randell, 1964; Guthrie and Tindall, 1968), and few studies have examined the movements of the genital organs during reproductive behaviour (see Alexander and Otte, 1967). Moreover, until recently, the mechanism of the copulatory motor sequence has not been studied. We have been engaged in this area of research for nearly a decade using the cricket *Gryllus bimaculatus*. The results indicated that the series of copulatory actions is carried out by way of a stimulus–response chain (Sakai and Ootsubo, 1988) and that spermatophore extrusion, performed during the final stage of copulation, is triggered by the stimulation of specialized mechanosensilla located in the genital cavity (Sakai et al., 1991). Furthermore, it was suggested that the mating stage is switched at spermatophore extrusion to the post-copulatory sexually refractory stage by a reproductive centre within the terminal abdominal ganglion *via* the brain (Sakai et al., 1995; Matsumoto and Sakai, 2000a; Matsumoto and Sakai, 2000b).

Recently, we have succeeded in establishing techniques to induce two main reproductive events, spermatophore formation and spermatophore extrusion, in dissected males. This allowed us not only to observe the movements of the genital organs and their muscle contractions, but also to record their neural activity during reproduction. In this report, we first describe the structures of the phallic complex, including the sclerites, muscles, membranes, nerves and receptors, and then their mechanistic functions and the muscle contractions responsible for the movement of each genital organ during tethered copulation and spermatophore formation. Preliminary results have appeared elsewhere (Kumashiro and Sakai, 1997).

Materials and methods

Animals

Crickets, *Gryllus bimaculatus* DeGeer, were used. They were reared in a plastic box (70 cm×40 cm×20 cm), fed a diet of insect pellets and water *ad libitum* and maintained on a

12 h:12 h light:dark photoperiod at 27 °C. For anatomical study, males were used 1–4 days after the final ecdysis. To observe the genital organs, males were used soon after ecdysis before their colouring became dark. For the analysis of reproductive actions and genital movements, males were used 1–2 weeks after final ecdysis. To increase the likelihood of immediate copulation, males were separated from females for at least 1 week before use.

Morphology

For gross anatomy, the nerve bundles and genital organs were stained with 5 % Methylene Blue prepared in insect saline (in mmol l^{-1}: NaCl, 150; KCl, 9.0; $CaCl_2{\cdot}2H_2O$, 5.0; $NaHCO_3$, 2.0; glucose, 40; Tes, 2.5, adjusted to pH 7.2 with NaOH). For scanning electron microscopy, specimens were processed using conventional procedures. The nomenclature for each organ is that of Snodgrass (Snodgrass, 1937).

Conditions for observing the movements of the genital organs

Free-moving condition

A male, with its hindwings cut at the tips to facilitate observation of the movements of the genitalia, was allowed to copulate with a female in a 100 ml beaker with the bottom lined with a sheet of paper. Under these conditions, the sequences of natural copulation and spermatophore formation were observed.

Restrained condition

A male was mounted on a cork plate with its mandibles, thorax and legs fixed with staples. In this condition, copulatory movement of the internal genital organs, such as the dorsal pouch and guiding rod, was observed through the genital cavity when the spermatophore was removed.

Abdomen-opened condition

A male was fixed with insect pins onto a cork plate in a dorsal-up position after the abdomen had been opened. The cerci, epiproct and intestines were removed, and the abdominal cavity was filled with insect saline. The movement and muscle contractions of the internal and external genital organs were closely observed during tethered copulation. The secretion and transport of spermatophore materials through the ejaculatory duct were also observed. In this condition, however, the ventral lobes and median pouch were unable to expand in the absence of hydrostatic pressure from the body fluids.

Most of the observations were conducted under a stereo microscope, and data were recorded with a video recorder (SONY, Hi8 Handycam CCD-TR3300).

Stimulation and responses

Genital movement

Movement of the genital organ was elicited by stimulation of the epiphallic sensilla with various tactile stimuli, e.g. a sharpened wooden probe or a piece of paper string. Continuous stimulation of the epiproct (the last abdominal tergite) with a cotton swab was used to elicit eversion of the phallic complex (Sakai et al., 1991).

Tethered copulation

A male was allowed to court a female for approximately 2 min before he was attached to the cork plate to facilitate the induction of copulation. A model test stimulus, mimicking the copulatory papilla of the female genitalia, was constructed from a piece of rubber (1 mm in diameter, 2 mm long) attached to the tip of a wooden bar. This was inserted into the genital cavity surrounded by the ventral region of the epiphallus and was tonically pressed against the cavity wall. This procedure allowed the internal genital cavity sensilla (Sakai et al., 1991) and those on the median and lateral processes of the epiphallus to be stimulated and resulted in a series of copulation actions like those seen in the final stage of natural copulation. The spermatophore was usually ejected after a minimum of 10 s of stimulation. During test stimulation, the abdomen of the male showed a gradual increase in vibratory movement, indicating an increase in bodily tension. Spermatophore transfer, resulting from the expansion of the ventral lobes and the median pouch, was not observed because of the absence of hydrostatic pressure. However, the end of spermatophore transfer was signalled by the occurrence of a return movement of the dorsal pouch, which occurs at the end of spermatophore transfer in the free-moving condition.

Spermatophore formation

When a male exhibited symptoms indicating ejection of the spermatophore material onto the ventral lobes, which usually occurred approximately 4 min after the end of copulation, he was fixed onto a cork plate. Then, a female, which was held inside a plastic tube, was placed in front of the male to allow him to contact her body with his antennae. Spermatophore materials were eventually ejected onto the ventral lobes 10–20 min after the onset of stimulation.

Identification of the muscles responsible for the movement of the genitalia

The muscle or muscles responsible for the movement of the genital organ were determined using a video recorder by observing their contraction at high magnification during spontaneous movement and/or responses to stimulation of the epiphallic sensilla. The tapes were reviewed at low speed for analysis. The role of a particular muscle in producing the movement of each genital organ was verified by observing the change in this movement after the destruction of that muscle with scissors. The timing of contraction of more than one muscle was analyzed, if necessary, by recording electromyograms.

Detection of haemolymph passages

The passages for haemolymph movement into and out of the ventral lobes and the median pouch were determined. Petroleum jelly was injected by touch into the two organs with a syringe in the abdomen-opened male, which enabled

dynamic changes in volume and shape to be observed. By applying external pressure with a probe, petroleum jelly was pressed out, allowing the exact exit sites to be determined.

Results

The male genital organ complex comprises two functional groups. One includes the testis, vas deferens, accessory gland and ejaculatory duct for the production and transport of spermatophore materials. The other, here termed the phallic complex (Fig. 1), includes the dorsal pouch, guiding rod, epiphallus, ventral lobes and median pouch for the formation, extrusion and transfer of the spermatophore. The main purpose of our research was to examine the skeletal structures, muscles, innervation and movements of the phallic complex.

Skeleton of the phallic complex

The skeleton of the phallic complex is composed of two kinds of cuticle. The epiphallus, ectroparameres, latch-like sclerite, lateral arms, W-shaped sclerite and guiding rod are made of hard cuticle, while the median grooved fold (MGF) in the dorsal pouch and the connection between the lateral arm and the anterior edge of the proximal region of the epiphallus are made of soft cuticle (Fig. 2A–D). Hard cuticular structures serve as effectors, while soft cuticular structures serve as elastic supporters or joints. Of the latter, the MGF is a white elastic plate consisting of dorsal and ventral folds in a horizontal U shape (Fig. 2A). This structure is deformed by upward bending of the ventral fold at the curvature of the MGF (Fig. 2B). During this deformation, the W-shaped sclerite is pulled back towards the anterior region of the epiphallus (Fig. 2B) while the lateral arms are moved postero-laterally *via* the soft cuticle (Fig. 2D).

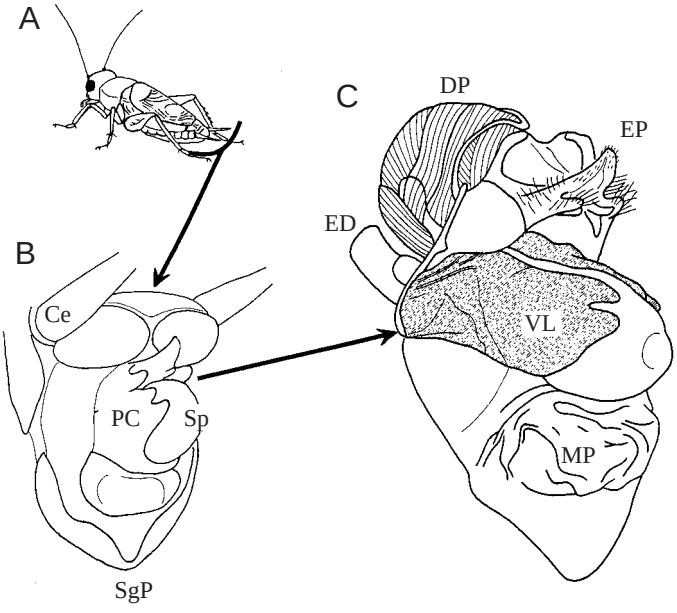

Fig. 1. The male genitalia. (A) Postero-lateral view of a male cricket. (B) A close-up of the abdomen tip. (C) Structures seen inside the abdomen tip. Ce, cercus; DP, dorsal pouch; ED, ejaculatory duct; Ep, epiphallus; MP, median pouch of the dorsal wall of the subgenital plate; PC, phallic complex; SgP, subgenital plate; Sp, spermatophore; VL, ventral lobe.

Dorsal pouch

The dorsal pouch consists of muscles, membranes, the MGF and some hard sclerites. Transparent membranes cover both sides of the MGF and the space between the MGF and the epiphallus (Fig. 2A,C). The inside of the dorsal pouch constitutes a large compressed cavity surrounded by the MGF, called the dorsal cavity, which continues dorsally to the genital cavity and opens at the posterior end of the epiphallus. The dorsal pouch serves as a mould for the attachment plate, and the guiding rod of the spermatophore serves as a threader (see insets in Fig. 2A,B).

Four thin muscles, DP1–DP4, were identified on the dorsal pouch (Fig. 3A,B). DP1–DP3 have their origins along the midline regions of the dorsal pouch. DP1 is connected with the W-shaped sclerite, DP2 with the edge of the ventral fold of the MGF and DP3 with the edge of the dorsal fold. In contrast, DP4 originates in the antero-ventral region of the MGF and ends on the posterior edge of the ventral fold.

Muscles DP1–DP4 contracted simultaneously for a few seconds in response to test stimulation with the female model, which caused a sudden deformation of the dorsal pouch *via* the bending of the MGF. This resulted in the ejection of the spermatophore attachment plate, normally resulting in spermatophore extrusion (Fig. 2B,D). The synchrony of the four-muscle contraction was confirmed by electromyography (not shown). When the spermatophore was transferred, the dorsal pouch contracted again. After this second contration, the dorsal pouch began to contract rhythmically at a rate of approximately once every 6 s (0.17 Hz). Each contraction of the dorsal pouch was similar to that seen at spermatophore extrusion, but its duration was approximately 0.5 s. When observed in the intact male, each contraction accompanied movement of the epiphallus and guiding rod, and contraction was often synchronized with strong movements of the entire abdomen. This post-copulatory dorsal pouch movement continued until subgenital plate opening, which occurred just before spermatophore protrusion. After spermatophore protrusion, the dorsal pouch showed small passive movements driven by the rhythmic contraction of the guiding rod muscles.

Guiding rod

The sclerotized guiding rod is a narrow grooved structure (0.03 mm wide and 0.2 mm deep) in the midline on the wall of the MGF, and its posterior tip (Fig. 4, GR) protrudes through the neck region of the genital cavity. The guiding rod serves as a mould to guide the whip-like spermatophore duct into the spermathecal duct of the female during genital coupling.

Two muscles, GR1 and GR2, connect the W-shaped sclerite and the proximal region of the epiphallus. GR1 originates in the medial part of the W-shaped sclerite and terminates on the proximal edge of the epiphallus, while GR2 originates on the

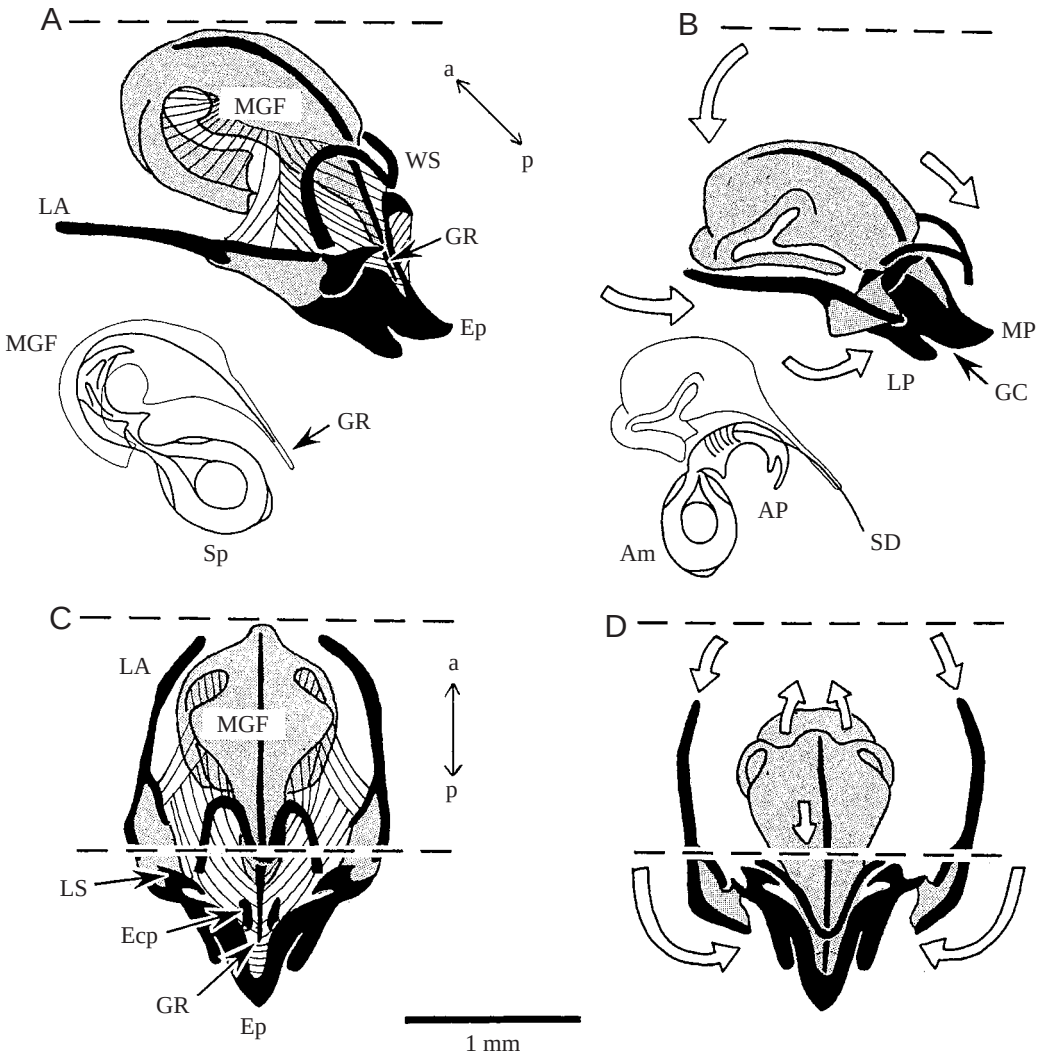

Fig. 2. Skeleton of the phallic complex. (A,B) Dorso-lateral views from the posterior. (C,D) Dorsal views. Hard cuticular elements are shown in black and soft cuticle in grey. Transparent membranes are shown by hatching (omitted in B and D). (A,C) Quiescent pre-copulatory state of the phallic complex. (B,D) Deformed state during spermatophore extrusion. Insets in A and B show the positional relationships between the skeleton and spermatophore. Am, ampulla of the spermatophore; AP, attachment plate; Ecp, ectoparamere; Ep, epiphallus; GC, genital cavity; GR, guiding rod; LA, lateral arm; LP, lateral process; LS, latch-like sclerite; MGF, median grooved fold; MP, median process; SD, spermatophore duct; Sp, spermatophore; WS, W-shaped sclerite. a, anterior; p, posterior. White arrows in B indicate the directions of movements. These conventions are applied in all the following figures. The broken horizontal lines act as a reference to indicate the degree of movement of the complex.

lateral part of the W-shaped sclerite and terminates on the inner part of the proximal edge of the epiphallus.

During the application of the test stimulus, GR2 contracted repeatedly, which allowed the guiding rod to project backwards (i.e. threading). At the moment of dorsal pouch deformation, both GR1 and GR2 contracted tonically, which is a prerequisite for the spermatophore duct to be kept in the spermathecal duct of the copulatory papilla of the female. Ablation of muscles GR1 and GR2 abolished projection of the guiding rod during dorsal pouch deformation. In contrast, during spermatophore protrusion, GR1 and GR2 showed tonic contraction that continuously pulled the dorsal pouch backwards. They then began to contract rhythmically at a rate of approximately once every 3 s (0.33 Hz).

Epiphallic complex

The epiphallic complex (Fig. 4) is composed of sclerites, including a median process and two lateral processes, a pair of ectoparamers, a latch-like sclerite, lateral arms and some soft cuticular regions. The median process serves as a hook that hangs onto the subgenital plate of the female during copulation, while the lateral processes appear to guide ectoparameres to move. The three processes and the wall of the genital cavity have bristle hairs of different lengths. A small space in the genital cavity surrounded by the epiphallus serves as a receptacle for the copulatory papilla of the female. The ectoparamers, located at the entrance of the genital cavity, are used as the claspers of the copulatory papilla of the female.

Three thick muscles (E1–E3) were associated with

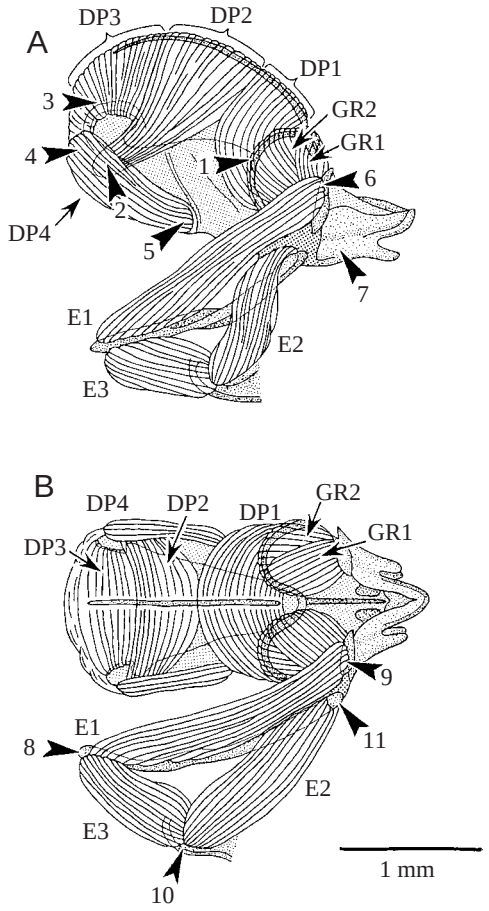

Fig. 3. Muscles of the phallic complex. (A) Lateral view. (B) Dorsal view. DP1–DP4, muscles of the dorsal pouch. GR1–GR2, muscles of the guiding rod. E1–E3, muscles of the epiphallus. Grey areas show membranes. Arrowheads indicate sclerites onto which muscles are attached: 1, W-shaped sclerite; 2, rim of the ventral fold of the median grooved fold (MGF); 3, rim of the dorsal fold of the MGF; 4, region near the curvature of the MGF; 5, posterior edge of the ventral fold of the MGF; 6, sclerite in the proximal region of the epiphallus; 7, epiphallus; 8, anterior region of the lateral arm; 9, latch-like sclerite on the anterior region of the epiphallus; 10, sclerite of the subgenital plate; 11, posterior region of the lateral arm.

movement of the epiphallic complex (Fig. 3A,B). E1 connects the anterior region of the lateral arm to the latch-like sclerite, E2 connects the anterior part of the apodeme in the last abdominal sternite (subgenital plate) to the posterior region of the lateral arm and E3 connects the same apodeme to the antero-lateral region of the lateral arm.

Although, in the dissected male, hooking was not actually induced, it was clear that at least E1 and E3 did not contribute to eversion of the phallic complex during hooking because of their muscle organization. During spermatophore extrusion, E1 contracted while E2 and E3 relaxed, causing downward movement of the lateral arms to make passages for haemolymph entry to the ventral lobes. After spermatophore extrusion, E1 and E2 contracted rhythmically, while E3 contracted tonically. These muscle contractions are the source

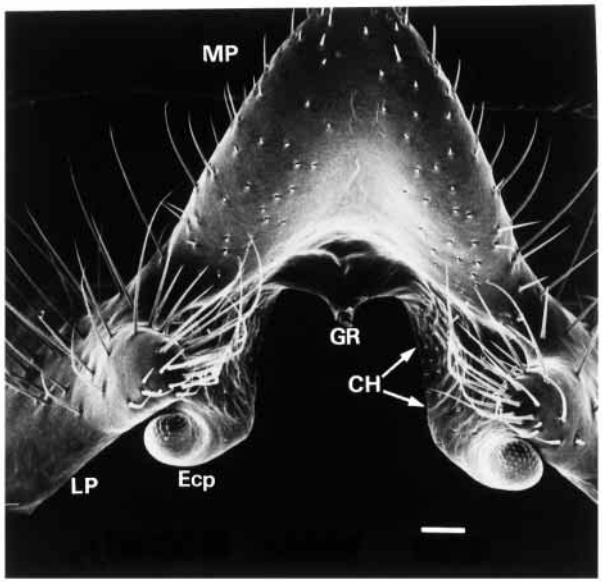

Fig. 4. Scanning electron micrograph of the epiphallus. Two arrows show the region where cavity hairs are clustered. Note the small bristle hairs. CH, cavity hairs; Ecp, ectoparamere; GR, guiding rod; LP, lateral process; MP, median process. Scale bar, 50 μm.

of the rhythmic epiphallic movements associated with dorsal pouch movement.

In the period between the opening of the subgenital plate and spermatophore protrusion, E1–E3 exhibited a contraction pattern similar to that during spermatophore extrusion, allowing the ventral lobes to extend. After spermatophore protrusion, no muscle contraction of the epiphallus was observed.

In the epiphallus, approximately 100 short bristle hairs (10–30 μm long) are present on the medial process and approximately 60 longer hairs (150–200 μm long) are present on both lateral processes (Fig. 4). On the wall of the genital cavity, slightly interior to the proximal parts of the ectoparamers, are a pair of clusters of approximately 30 small hairs (cavity hairs; arrowed region, Fig. 4). As described above, stimulation of the cavity hairs is a prerequisite for the deformation of the dorsal pouch.

Ventral lobes

The ventral lobes are flexible structures resembling a pair of gloves fused laterally (Figs 1, 5). They are composed of membranes, striated muscle fibres and soft cuticle. Their expanded state is shown in Fig. 5A. The proximal regions of the ventral lobes are continuous with the lateral arms, and the peripheral regions form a pair of flaps joined. The ejaculatory duct terminates in a fissure, termed the genital opening or endophallus, through which spermatophore materials are ejected. Striated muscle fibres in the membranes (Fig. 6C) are stretched radially and diagonally (Figs 5D, 6C) and are abundantly innervated, especially in the proximal regions (Fig. 6B).

Approximately 360 small bristle-type hairs (2–10 μm long

and 1–2 μm in diameter) are scattered over the membrane surface (Figs 5B, 7A). Larger hairs are more concentrated on the dorso-lateral surface and smaller ones on the ventral surface (Fig. 5B). Some regions of the dorso-lateral surface are covered by scale-like soft cuticle (Fig. 7B), which may prevent the lobes from being desiccated while they hold the spermatophore ampulla.

Just before spermatophore extrusion, the ventral lobes are projected backwards as a result of haemolymph flowing in through the openings between the lateral arm and the ejaculatory duct on both sides (Fig. 8B). Following this phase, the ventral lobes rapidly increase in volume and change shape. The flaps then instantly expand laterally, and this may help to break contact between the spermatophore ampulla and the surface of the ventral lobes. The expansion of the ventral lobes serves to lift the spermatophore and to insert its attachment plate into the genital chamber of the female. As soon as spermatophore transfer has been accomplished, the ventral lobes are pulled back inside and folded below the dorsal pouch.

After spermatophore extrusion, the ventral lobes show a series of conspicuous movements that give an overall form to the materials making up the spermatophore. Weak rhythmic movement associated with the dorsal pouch is followed by quiescence for approximately 10 s immediately prior to the ejection of a jelly-like substance from the genital opening, i.e. spermatophore protrusion. The ventral lobes then begin to move vigorously at a rate of once every 2 s (0.5 Hz) for approximately 3 min, which constricts part of the spermatophore material to form a neck between the attachment plate and the ampulla.

Artificial removal of all the spermatophore material during this stage did not interfere with spontaneous movement of the ventral lobes, indicating that the vigorous movement was not initiated by feedback from receptors stimulated by the ejected spermatophore material. However, artificial stimulation of the sensilla on the ventral lobes with a wooden probe elicited some movement. Thus, the sensilla may reinforce the production of vigorous movement of the ventral lobes. After surgical isolation of the epiphallic complex, spontaneous movement of the ventral lobes still occurred, although the movement was no longer consistent or intense. Thus, the muscle fibres of the ventral lobes may be partially myogenic. It should be noted that the spermatophore material removed immediately after spermatophore protrusion matured and formed the ampulla, attachment plate and duct over a normal time course, although their shapes were distorted.

Median pouch

The median pouch is a sack-like structure consisting of membranes and striated muscle fibres (Figs 5A,C, 6A). The ventral region is continuous with the membranous floor of the subgenital plate (Fig. 1), and the dorsal region makes contact with the ventral lobes (Figs 1, 5A). When the posterior region is expanded, a large spherical convolution forms on the dorsal side (Figs 5, 8B). Abundant nerve branches and terminals, similar to those seen in the ventral lobes, were found, particularly in association with the convolution. On the lateral margins of the pouch, a pair of pockets of unknown function are found (X in Fig. 5C). No sensilla are present on the median pouch.

During the mating stage, the median pouch is folded under the ventral lobes. During copulation, it expands as haemolymph flows in through a large opening under the ventral

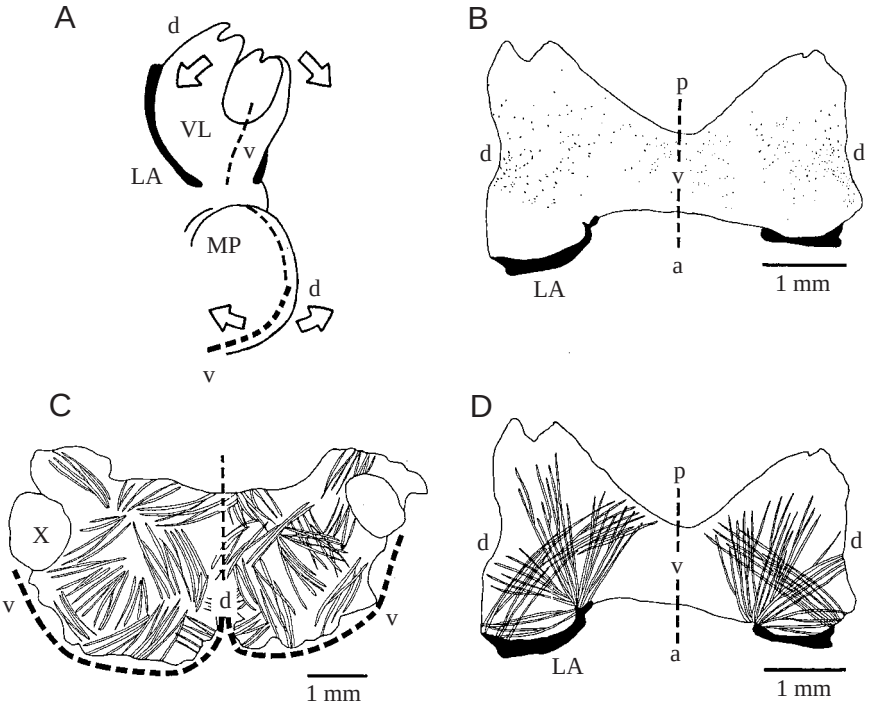

Fig. 5. The ventral lobes and the median pouch. (A) Lateral view (see Fig. 1). White arrows indicate the direction of tissue dissection required to view these structures. (B) Ventral view of flattened ventral lobes. Dots show sensilla on the surface of the ventral lobes. The broken line corresponds to that in A. (C) Inside view of a flattened median pouch first cut along the thick broken line and then opened out (see A). The broken lines correspond to those in A. String-like striated muscle fibres are sparsely distributed in sheets. (D) Ventral view of the flattened ventral lobes (see A). Note the bidirectional arrays of striated muscle sheets. X indicates the pocket of the median pouch (MP). a, anterior; p, posterior; d, dorsal; v, ventral. Scale bar, 1 mm. LA, lateral arm; VL, ventral lobe.

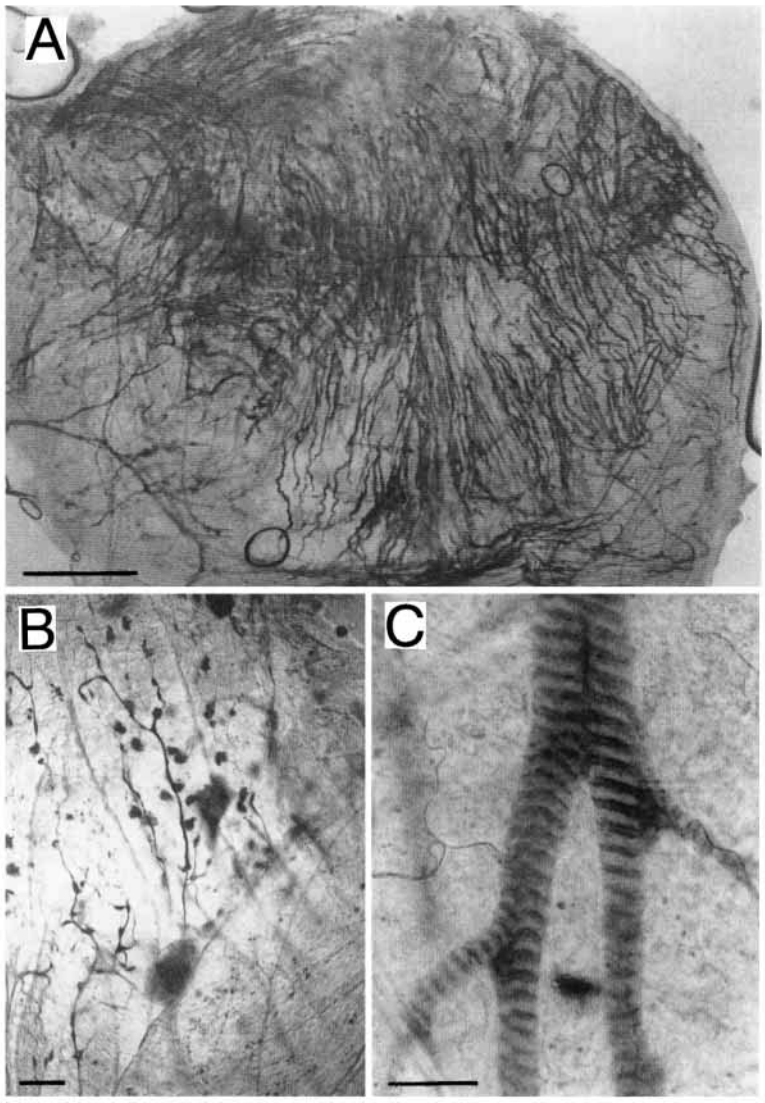

Fig. 6. Light micrographs showing the muscles and nerves in the median pouch and ventral lobes. (A) Median pouch, photographed after it had been flattened. Muscle fibres appear as dark stripes. (B) Motor nerve terminals in the proximal region of the ventral lobes where the tissue is transparent. (C) Muscle fibres in the ventral lobes showing cross-striations and anastomoses. Note the striated pattern of the fibres. Scale bars: A, 250 μm; B, 20 μm; C, 25 μm.

lobes. This enables the phallic complex to evert, i.e. hooking. After spermatophore extrusion, the median pouch expands maximally and pushes the spermatophore up from below the ventral lobes. This organ always moved spontaneously to the left and right at a rate of 0.2 Hz. During the period between spermatophore extrusion and spermatophore protrusion, the convolution of the median pouch plugged the dorsal cavity and moved inside it.

Ejaculatory duct

The ejaculatory duct is described here only briefly because it was not the focus of our research. It receives spermatophore material from the accessory glands (see Kimura et al., 1989; Ootsubo and Sakai, 1992) and spermatozoa from the testes, and transports them to the ventral lobes *via* peristaltic movement. We were able to observe that the spermatophore material became oval-shaped at the bottom of the ejaculatory duct approximately 30–40 s after the start of its secretion following subgenital plate opening and was transported smoothly to the ventral lobes through the ejaculatory duct approximately 10 s later. This 10 s period corresponded to the cessation of spontaneous movements of the phallic complex just before spermatophore protrusion.

Innervation

The muscles of the phallic complex are innervated by branches of nerves 10v and 9d of the terminal abdominal ganglion (after Edwards and Palka, 1974) (Fig. 9). A branch of the genital nerve (gn), separated from 10v, innervates the accessory glands in the proximal region. Several more peripheral branches innervate the ventral lobes, the ejaculatory duct, the epiphallic muscles E1–E3 and, in the most distal regions, the dorsal pouch muscles DP1–DP4 and the guiding rod muscles GR1–GR2. Only the median pouch is innervated by branches of 9v. Sensory nerves innervating the epiphallic sensilla are fused into a bundle that runs through the genital nerve and enters the terminal abdominal ganglion (see Kumashiro and Sakai, 2001).

Reproductive motor actions and their sequences

On the basis of the results presented above for each genital

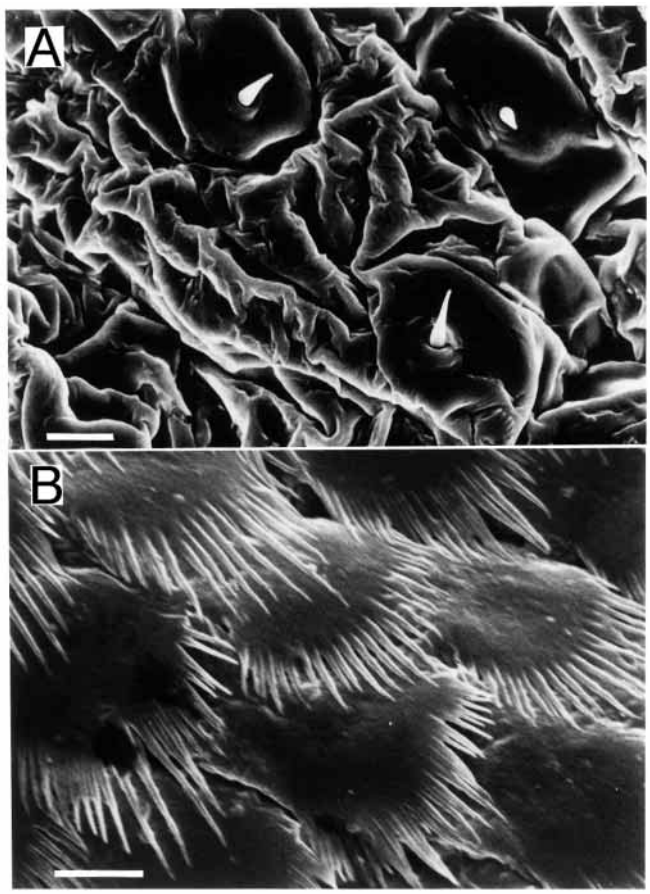

Fig. 7. Scanning electron micrographs of the sensilla and cuticular elements of the ventral lobes. (A) Sensilla located on the ventrolateral surface (see Fig. 5B). (B) Scale-like cuticle on the dorsolateral surfaces of the ventral lobes. Scale bars: A, 10 μm; B, 5 μm.

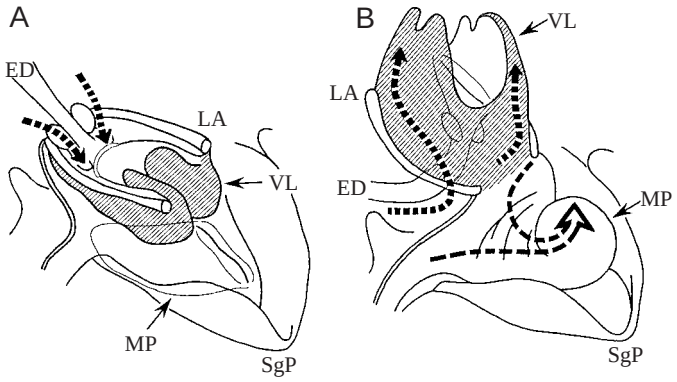

Fig. 8. Changes in the volume and shape of the ventral lobes and median pouch. (A) Quiescent state. Haemolymph cannot enter the ventral lobes (hatched area) or the median pouch. Broken arrows indicate passages for haemolymph. (B) Copulatory state. Haemolymph enters the ventral lobes through two small openings between the lateral arm and the ejaculatory duct (small broken arrows) and it enters the median pouch through a large opening below the ventral lobes (large broken arrow). MP, median pouch; LA, lateral arm; VL, ventral lobe; ED, ejaculatory duct; SgP, subgenital plate.

organ, we are able to describe the sequences of coordinated motor action during copulation and spermatophore formation.

Copulation

Copulatory motor actions involve a complex sequence of stereotyped movements. In Fig. 10, each pair of illustrations shows a lateral (upper) and a horizontal (lower) view of the phallic complex. Each circled number denotes an event such as muscle contraction, movement, etc.

Prior to the start of copulation, the phallic complex, covered by the subgenital plate, lies horizontally (Fig. 10A). The ventral lobes are extended, with some muscle tension, while the median pouch is folded below the ventral lobes. The spermatophore attachment plate lies in the dorsal pouch, and the anterior part of the ampulla is held by the ventral lobe flaps.

The stages after the start of copulation are shown in Fig. 10B–F. Expansion (1) of the median pouch causes eversion of the phallic complex (2) by which the male hooks the female. This is due to hydrostatic pressure in the abdomen and, possibly, to relaxation of the muscle fibres of the median pouch, allowing haemolymph to enter the median pouch through the large opening below the ventral lobes.

After successfully hooking the female subgenital plate with its epiphallus (3), the male begins to pull down the female subgenital plate in response to mechanical stimulation of bristle hairs on the epiphallus (not illustrated). This action then induces eversion of the copulatory papilla of the female, which naturally enters the genital cavity of the male (4).

The inserted copulatory papilla mechanically stimulates cavity hairs and some sensilla on the epiphallus which, in turn, causes contraction (5) of the guiding rod muscles GR1 and GR2. This contraction projects the rod into the aperture of the copulatory papilla (6) of the female (i.e. threading). At almost the same time, contraction of the dorsal pouch muscles DP1–DP4 (7) causes deformation of the dorsal pouch (8), which ejects the attachment plate of the spermatophore (9). Subsequent steps follow automatically without external stimuli.

The male then stops moving while maintaining its abdominal hydrostatic pressure. Contraction of epiphallic muscle E1 (10) pulls (11) the lateral arms of the phallic skeleton laterally and posteriorly, enabling the phallic complex to evert further (12). As a result, the ventral lobes are filled with haemolymph flowing in through the passages opened between the lateral arms and the ejaculatory duct, and they immediately expand fully, with flaps extending laterally (13). At the same time, the ongoing expansion of the median pouch continues (14) to lift the spermatophore high enough (15) for its attachment plate to enter the female genital opening and to fit into two swellings in the wall of the female copulatory chamber (not illustrated). During spermatophore transfer, the male raises the tip of his abdomen, pressing against the genital opening of the female and flicking with an alternating cercal movement at a rate of 3 Hz, which presumably helps to insert the long spermatophore duct deep into the spermathecal duct of the female (16).

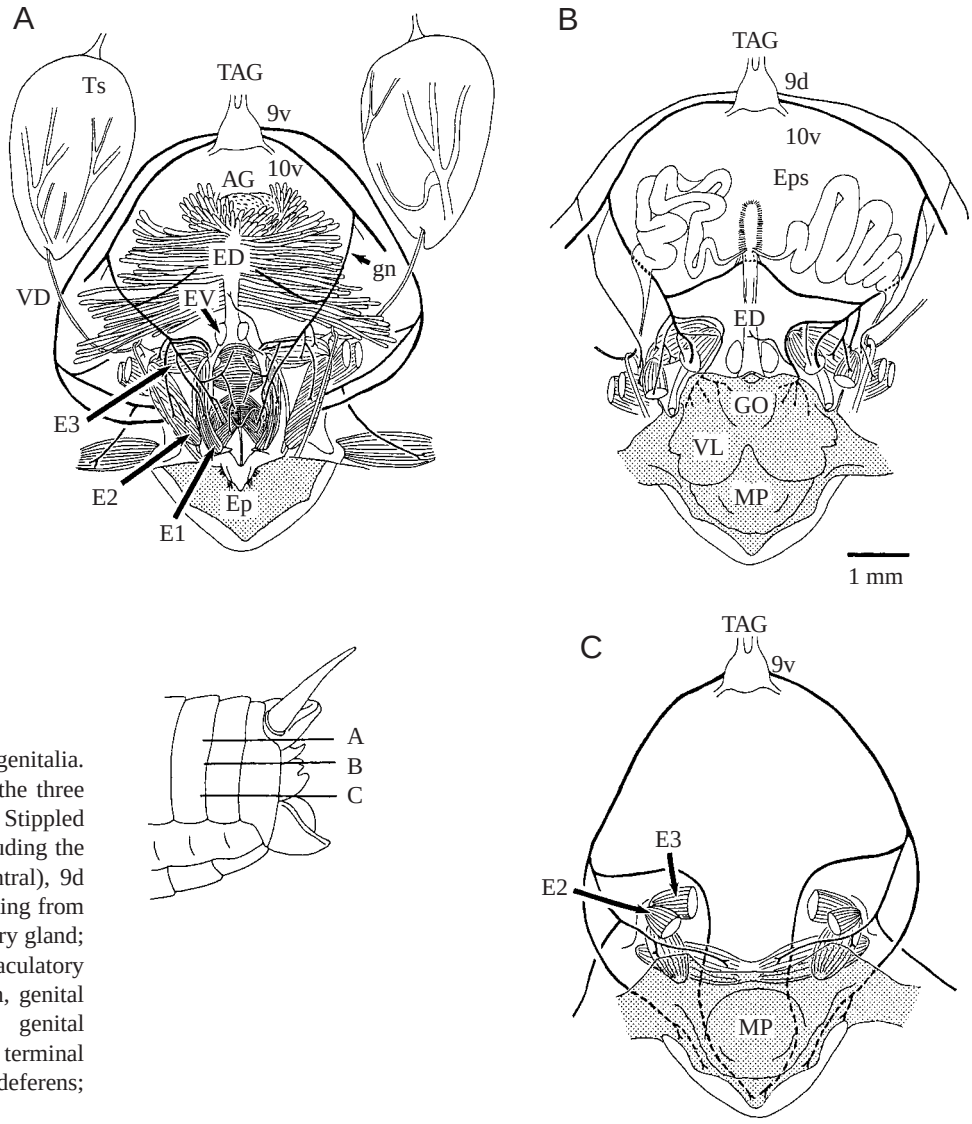

Fig. 9. The innervation of the genitalia. (A–C) Dorsal views of male genitalia at the three different levels indicated in the inset. Stippled regions show membranous structures including the ventral lobes and median pouch. 9v (ventral), 9d (dorsal) and 10v are nerve bundles emanating from terminal abdominal ganglion. AG, accessory gland; E1–E3, muscles of the epiphallus; ED, ejaculatory duct; Ep, epiphallus; Eps, epididymus; gn, genital nerve; EV, ejaculatory vesicles; GO, genital opening; MP, median pouch; TAG, terminal abdominal ganglion; Ts, testis; VD, vas deferens; VL, ventral lobe.

When the epiphallus unhooks, contraction of epiphallic muscle E3 (17) causes the phallic complex to be pulled back to its original position (18). At the same time, the ventral lobes are rapidly pulled foward and folded below the dorsal pouch (19). In contrast, the median pouch remains expanded, with its convolution moving spontaneously to the left and right within the dorsal cavity (20).

Spermatophore formation

Spermatophore formation starts with the opening of the subgenital plate, which occurs a few minutes after the end of copulation. This is followed by ventral lobe projection within 1 s and the ejection of spermatophore material (spermatophore protrusion) within 40–50 s.

Motor actions during spermatophore formation also involve a complex sequence of stereotyped movements (Fig. 11). In the quiescent state prior to the opening of the subgenital plate, the phallic complex lies in the normal position with the convolution of the median pouch inside the dorsal pouch (Fig. 11A) and the ventral lobes folded below the dorsal pouch. In this state, only the continuous contraction of the epiphallic muscle E3 (1) provides basic tension for the spontaneous movement of the phallic complex. The contraction phase of the dorsal pouch (Fig. 11B) resembles that seen during spermatophore extrusion, in which the dorsal pouch is deformed (2) and pulled backwards (3). At the same time, epiphallic muscle E1 contracts (4) to produce a small upward movement of the epiphallus (5). When the male is stimulated by the restrained female through antennal contact, the hitherto spontaneously moving phallic complex gradually accelerates its rate of contraction, and the movement changes into a tonic contraction.

At the same time, contraction of the guiding rod muscles GR1 and GR2 (6) pulls the dorsal pouch backwards (7) (Fig. 11C). Then, suddenly, the subgenital plate is opened (8) and the median pouch slips under the ventral lobes (9). Relaxation of epiphallic muscles E2 and E3 (10) everts the lateral arms of the epiphallus downwards (11) and allows

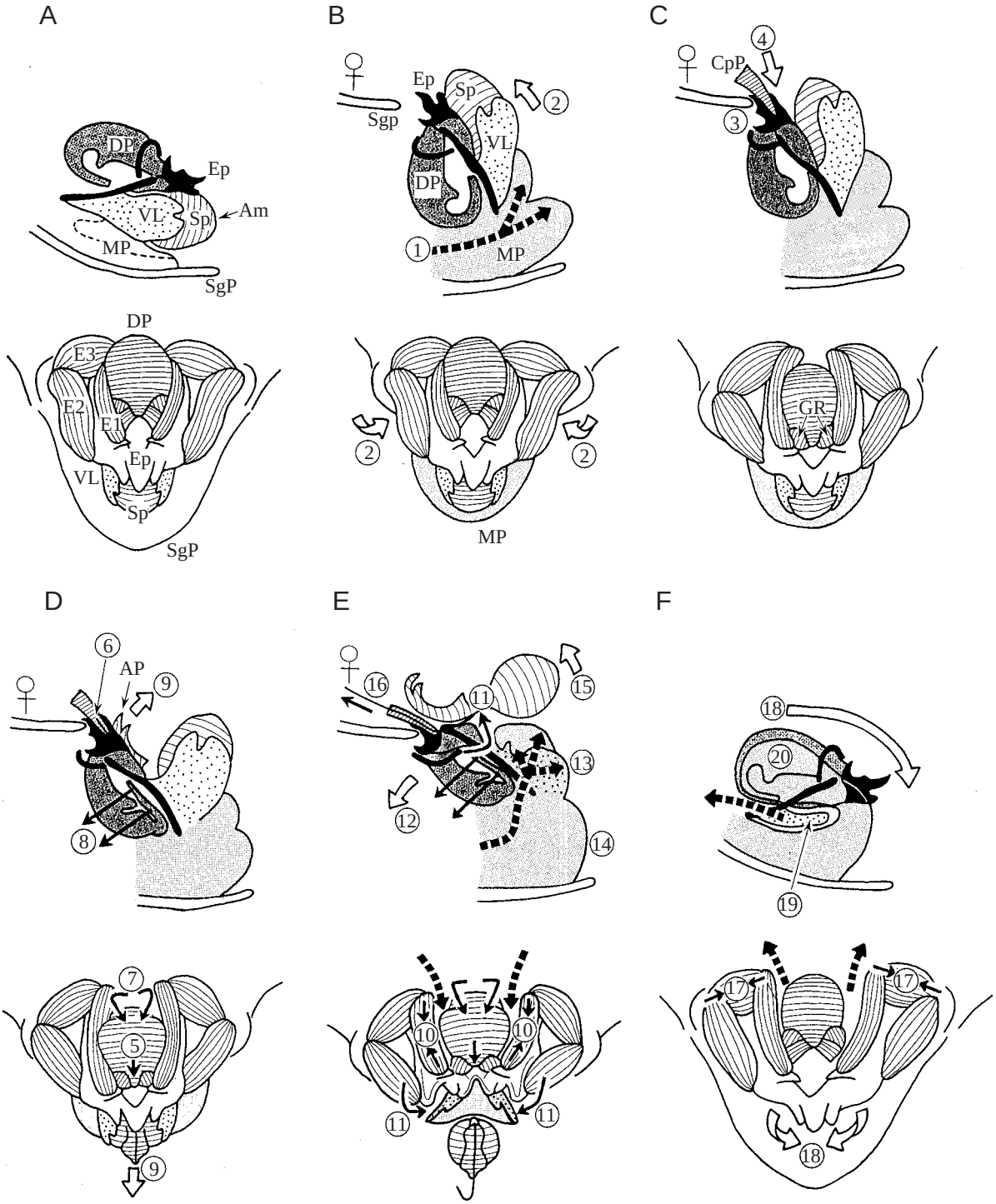

Fig. 10. Diagrams showing the sequence of copulatory motor actions. In each pair of illustrations, the upper diagram shows a lateral view (muscles omitted) and the lower diagram shows a horizontal view with some parts from the upper section omitted. Local movements are shown by small arrows, large movements by white arrows and haemolymph flow by broken arrows. Circled numbers indicate events during copulation discussed in the text. (A) Before copulation. (B) During hooking. (C) Insertion of the female copulatory papilla into the genital cavity following successful hooking. (D) Insertion of the guiding rod into the spermathecal duct through the aperture of the female copulatory papilla and subsequent ejection of the spermatophore attachment plate from the dorsal pouch (i.e. spermatophore extrusion). (E) Spermatophore transfer. (F) After copulation. See text for explanation. Am, ampulla of the spermatophore; AP, attachment plate; CpP, copulatory papilla; DP, dorsal pouch; Ep, epiphallus; E1–E3, muscles of the epiphallus; GR, guiding rod; MP, median pouch; SgP, subgenital plate; Sp, spermatophore; VL, ventral lobe.

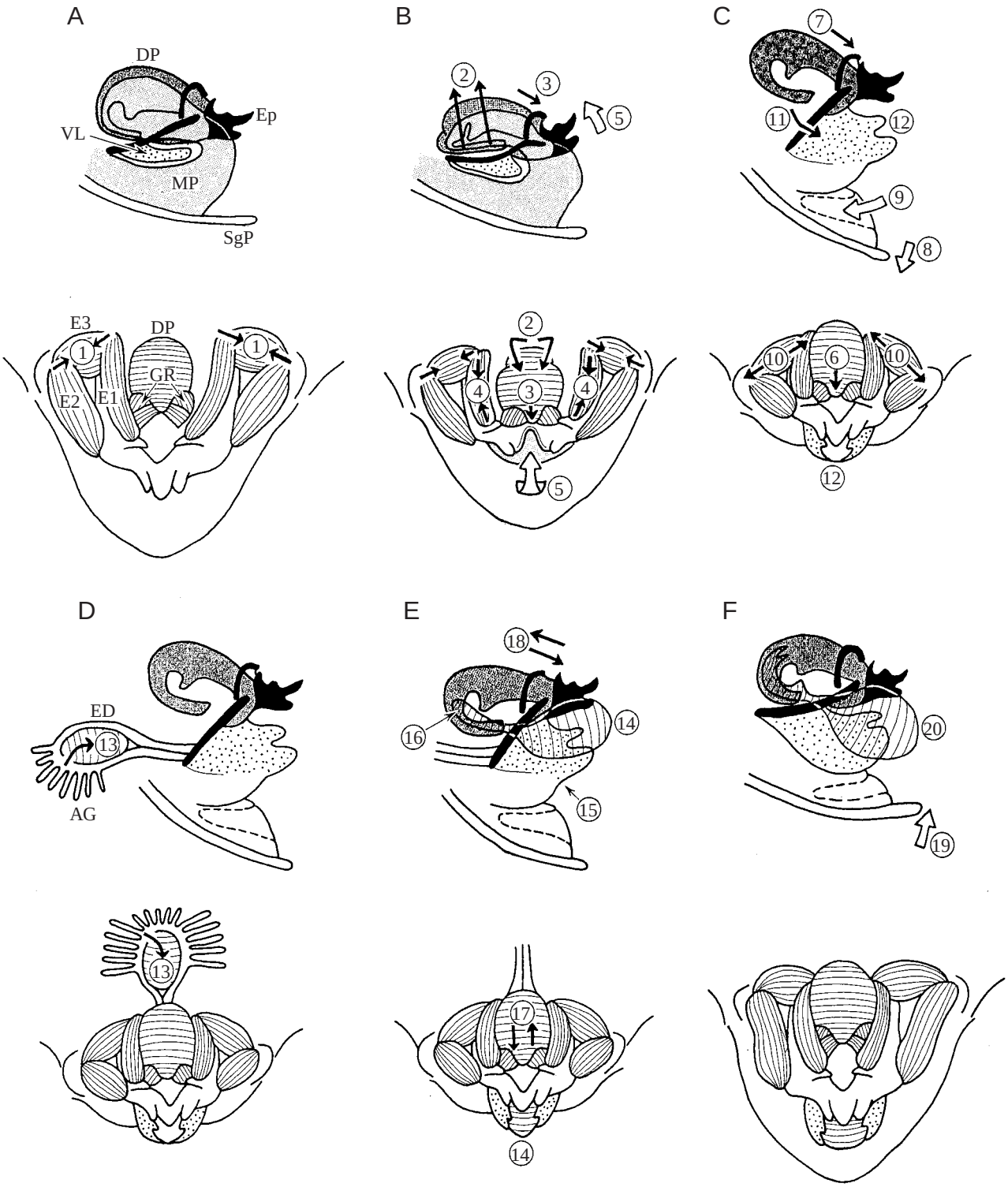

Fig. 11. Diagrams showing the sequence of spermatophore formation. Conventions are as in Fig. 10. (A) Quiescent state of the phallic complex between spermatophore extrusion and spermatophore protrusion. (B) Spontaneously contracted state. (C) Subgenital plate (SgP) opening occurs shortly before spermatophore protrusion and extension of the ventral lobes (VL). The median pouch (MP) is instantly folded below the ventral lobes. (D) Secretion of the spermatophore material occurs into the bottom of the ejaculatory duct (ED) from the accessory glands (AG) and testes. (E) Spermatophore material is ejected onto the ventral lobes through the genital opening (i.e. spermatophore protrusion). The anterior part of the spermatophore material is pushed into the dorsal pouch (DP) to form the attachment plate and the duct of the spermatophore. (F) Twenty minutes after spermatophore protrusion. See text for explanation. Ep, epiphallus; E1–E3, muscles of the epiphallus; GR, guiding rod.

haemolymph to enter and unfold the ventral lobes (12). However, the ventral lobes themselves maintain some tension to form the receptacle for spermatophore material.

A few seconds later, spermatophore materials are secreted from the accessory glands and testes into the bottom of the ejaculatory duct (13) (Fig. 11D). They become oval-shaped within approximately 30 s, are transported to the genital opening by peristaltic movements of the ejaculatory duct and are ejected onto the ventral lobes 10 s later (14). During transport, all movement of the phallic complex stops, and the proximal parts of the ventral lobes then begin to exhibit vigorous movements (15). As a result, the immature spermatophore is divided into two parts (Fig. 11E): the anterior part, which is compressed into the dorsal cavity to form the attachment plate and duct of the spermatophore (16), and the posterior part, which is held by the ventral lobes to form the ampulla. As a result of the contraction of the guiding rod muscles (17), the dorsal pouch is moved passively (18).

During the subsequent 20 min (Fig. 10F), the phallic complex gradually loses its rhythmic movement, the subgenital plate is slowly closed (19), and finally the spermatophore matures with a hardened and transparent ampulla (20).

Discussion

To date, there has been no complete account of the functioning of insect genital systems. Previous studies have described the gross morphology of the genital organs (Walker, 1922; Snodgrass, 1937; Quadri, 1940; Choperd, 1951; Randell, 1964) and genital nerves (Seabrook, 1968) and/or muscles (Guthrie and Tindall, 1968; Grossman and Parnas, 1973; Snell and Killian, 2000). Studies of the movement of the genital organs are limited (Alexander and Otte, 1967; Beck, 1974; Loher and Rence, 1978), and a single study has addressed some aspects of motor control (Snell and Killian, 2000). Thus, the neuroethology of reproductive behaviour in insects remains an almost unexplored field of research.

Most information about copulation and spermatophore formation in the male cricket has come from behavioural observations in the free-moving condition (Khalifa, 1950; Huber, 1955; Hormann-Heck, 1957; Alexander, 1961; Alexander and Otte, 1967; Engelmann, 1970; Beck, 1974; Loher and Rence, 1978; Evans, 1988; Adamo and Hoy, 1994) and from anatomical observations at particular stages in the reproductive cycle (Hohorst, 1936; Ootsubo and Sakai, 1992). The functional roles of mechanosensory receptors in mediating copulation were deduced through ablation experiments (Sakai and Ootsubo, 1988; Sakai et al., 1991; Snell and Killian, 2000). However, to understand the cause and effect of each motor action in the reproductive sequence, direct observations of movement, muscle contractions, cuticle mechanics and membrane dynamics during reproductive behaviour are required. We have made these observations and established an understanding of the structure and function of the genital system in the male cricket, including the mechanisms of copulation and spermatophore formation.

Muscle anatomy

A recent description of the genital muscles of *Acheta domesticus* (Snell and Killian, 2000) has revealed apparent species differences from the muscles described above. It is useful to relate differences in terminology to muscle similarities and differences in the two genera. In *Gryllus bimaculatus* (present study), the epiphallic muscles E1, E2 and E3 correspond to the dorsal pouch levator (DL), epiphallic abductor (EpAb) and epiphallic adductor (EpAd) of *A. domesticus*, respectively. The guiding rod muscles of *G. bimaculatus*, GR1 and GR2, correspond to the ectoparamere levator (EcL) of *A. domesticus*. However, the dorsal pouch muscles differ. The largest of three muscle sheets of the *A. domesticus* dorsal pouch protractor (DP) appears to correspond to DP1 in *G. bimaculatus*, but the other two muscle sheets are absent from the latter. In contrast, muscles DP2–DP4 of *G. bimaculatus* are absent from *A. domesticus*.

Copulation by chain reaction

Copulation behaviour in the male cricket *Gryllus bimaculatus* is stereotyped and rapid (completion takes 23 s). The male takes approximately 3 s from the start of backward walking to successful hooking (Sakai and Ootsubo, 1988) and 20 s from hooking to the end of spermatophore transfer to the female (Sakai et al., 1991). Although the male sometimes fails in copulation, the female is to blame in most cases. What mechanisms underlie this amazing performance? A plausible mechanism is a chain reaction, which is widely proposed for courtship, in which the male and female exchange signals to enable pair formation and mating (Tinbergen, 1951). However, it is not clear whether motor actions such as positioning the body axis and coupling the genitalia during copulation are also carried out by a chain reaction. If this were the case for copulation, then one motor act should stimulate some mechanosensory receptors, which in turn should elicit another motor act, and so on.

Previously, we have shown that copulatory actions in the cricket can be explained by a stimulus/response chain (Sakai and Ootsubo, 1988; Sakai et al., 1991): the female mounting onto the back of the male elicits an intense posture in the male that causes tight contact to be made between the male cerci and the female abdomen. As a result, bristle hairs on the cercal dorso-medial regions are stimulated, which causes the male to walk backwards under the female. When the end of the abdomen of the male reaches the subgenital plate of the female, backward walking stops because cercal contact with the abdomen of the female is lost. Instead, the last abdominal tergite and the proximal regions of the cerci are pressed by the subgenital plate of the female, which causes a hooking movement in the male *via* stimulation of bristle hairs on the tergite and cerci. After successful hooking, the male begins to pull down the subgenital plate of the female. This movement leads the female to evert her copulatory papilla and, within 5.6 s, the copulatory papilla is inserted into the genital cavity of the male (genital coupling). The male then extrudes the spermatophore in the next 3.8 s and transfers it

to the female within 9.1 s of spermatophore extrusion. All movements cease approximately 1 s later, when the epiphallus unhooks. That is, all the steps of the copulation sequence proceed by a chain reaction, which is consistent with the speed and constancy of the behaviour of the male. Our present results substantiate the relationships between the cause and effect of each motor action in the later stages of copulation.

Automatic actions in copulation

The spermatophore extrusion and transfer that occur in the final stage of copulation are unusual motor actions in a number of respects. First, they cannot be instantly elicited by the key stimulus. Second, once triggered, the motor actions go to completion even if the key stimulus is removed or severe disturbances are presented (Sakai et al., 1991). Third, the movements of different genital organs are spatially and temporally coordinated. For example, after genital coupling, the guiding rod is projected first, as shown by its immediate reaction to the test stimulation; the spermatophore is then suddenly extruded, and the ventral lobe and median pouch begin to expand. Although each movement takes place in an automatic manner, its time course is temporally coordinated. In the case of spermatophore transfer, the spermatophore, which is extruded by dorsal pouch deformation, is lifted smoothly with the ampulla sitting on the expanding ventral lobes and with the attachment plate remaining straight, while the tip of the abdomen vibrates. This sequence enables the spermatophore attachment plate to be inserted correctly into the female genital opening and attached to the convolution of the genital chamber. To achieve this, the ventral lobes and median pouch must expand at an appropriate speed, which possibly depends on their size, shape and elasticity. The properties of the soft cuticle in the epiphallic skeleton and the sizes of the passages are also important in regulating haemolymph flow into the two membranous structures. A similar role for the haemolymph is seen during oviposition by female locusts (Rose et al., 2000). On the other hand, neural motor patterns coordinate the muscles of the epiphallus, ventral lobes and median pouch. From these observations, it appears that copulation by the male is dependent both on the elaborate structures of the genital organs, including the disposition of mechanoreceptors, and on the activity of central pattern generators.

Snell and Killian (Snell and Killian, 2000) recently described spontaneous genital movements in decapitated male crickets and took these to be spermatophore threading during spermatophore transfer in intact males. However, no evidence is available for this. A similar observation was first made in decapitated praying mantis and cockroaches by Roeder et al. (Roeder et al., 1960), who suggested that, although the abdomen of a decapitated cockroach makes various movements, including slight peristaltic movements of the terminal abdominal segments and movements of the phallomeres, that continue for several hours, such movements lacked adaptive behavioural significance. It should be stressed that, in contrast to the repetitive nature of such movements in decapitated males, the specific movement for spermatophore extrusion of ejecting the attachment plate of the spermatophore, which is coupled with projection of the guiding rod (i.e. threading), occurs only once in response to the key stimulation to the epiphallus. The rhythmic movements of the genitalia that occur after decapitation are, therefore, unlikely to represent the spermatophore threading observed during copulation. Instead, such movements are likely to represent those observed preceding self-spermatophore extrusion (so-called ‘abortion’; Sakai et al., 1991; see Kumashiro and Sakai, 2001) or those observed after spermatophore extrusion, as described below.

Fuction of rhythmic movement of the phallic complex

When the male has accomplished copulation, the phallic complex begins to move rhythmically, with the median pouch convolution plugging the dorsal cavity. This movement is not due to surgical artefacts because the movement can be partially observed from the outside in intact males. Although the rhythmic movements continue until the subgenital plate opens, they occasionally slow after the removal of the female or are temporarily stopped by noxious stimulation such as pinching the antenna. This suggests that the emergence of the rhythm is gated by the brain, since inhibition of copulation by noxious stimulation is imposed by the brain (Matsumoto and Sakai, 2000a). The function of the rhythmic movement is not clear. It may reflect some preparatory activity for the excretion of spermatophore material into the ejaculatory duct, or cyclical deformation of the dorsal pouch may be necessary to clean residual spermatophore material from the inner surface of the dorsal cavity. After spermatophore protrusion, the phallic complex exhibits another rhythmic movement that results mainly from contraction of the guiding rod muscles. The fuctional role of this movement is also unknown, although it may facilitate compression of the anterior part of the ejected spermatophore material into the dorsal cavity.

Spermatophore formation by a central motor programme

A few minutes after copulation, the rhythmic movement of the phallic complex accelerates and finally changes to a tonic contraction. These actions and subsequent spermatophore protrusion, however, rarely occur within an hour of copulation if the male has been isolated from the female (Ootsubo and Sakai, 1992). This suggests that spermatophore protrusion is a separate type of behaviour, although it is initiated shortly after spermatophore extrusion when the female is available nearby. Once the pouch opens, all the subsequent steps, including the transfer of spermatophore materials into the bottom of the ejaculatory duct, their transport through the duct, ejection onto the ventral lobes and the partition and compression of the anterior part of the spermatophore, proceed automatically. The execution of these steps does not require the actual performance of each previous motor action in the sequence, i.e. it does not depend upon a chain reaction. For example, the secretion of the

spermatophore material occurs even if pouch opening is hampered (the pouch cannot be opened in abdomen-opened males since the pouch is pressed onto the cork plate), and the post-copulatory rhythmic movement ceases approximately 50 s after pouch opening in males with their accessory glands removed (data not shown). The spermatophore itself follows its own programme of maturation (Gregory, 1965; Khalifa, 1949; Mann, 1984). These observations indicate that, unlike the chain reaction mechanism for copulation, spermatophore formation is carried out by a central motor programme. The substrate for the programme must be located within the terminal abdominal ganglion because all the steps proceeded normally in males in which the connectives between the sixth abdominal ganglion and the terminal abdominal ganglion had been cut immediately after pouch opening (Kumashiro and Sakai, 2001).

It can be concluded that copulation behaviour and spermatophore formation in the male cricket are subserved by elaborate genital structures and distinct motor programmes in the central nervous system. The next step is to identify the neurones innervating the genital organs and to record their activity during reproductive behaviour.

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