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Auto-spermatophore extrusion in male crickets

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Summary

The reproductive cycle of the male cricket consists of the mating stage and the sexually refractory stage. The latter is further divided into the first refractory stage (RS1) from spermatophore extrusion in copulation to spermatophore preparation after copulation, and the second refractory stage (RS2) from spermatophore preparation to recommencement of a calling song. RS2 is time-fixed and unaffected by the female or by stress, hence RS2 is assumed to be controlled by the reproductive timer. Previously, we suggested that the timer is located in the terminal abdominal ganglion (TAG), because functional inactivation of the TAG by local cooling lengthened RS2 in proportion to cooling time. To obtain further evidence of timer localization and to examine the operation of the timer in dissected animals, we investigated the characteristics of auto-spermatophore extrusion, a phenomenon in which males eject the mature spermatophore themselves without any prior courtship. The occurrence of auto-spermatophore extrusion was 100% in dissected males with the TAG separated, compared to 1.7% in intact males. The time interval (SPaSE) between spermatophore preparation and auto-spermatophore extrusion was comparable to RS2 measured by the calling song. Spike recording from a genital motor neurone in the separated TAG indicated that burst discharge associated with auto-spermatophore extrusion occurred with a SPaSE comparable to RS2. Other efferent neurones, some of which were identified as dorsal unpaired median (DUM) neurones, showed a time-dependent spike frequency increase during SPaSE. These results strengthen our previous conclusion that the reproductive timer is located within the TAG, and demonstrate that the timer functions normally even when the TAG is separated from the central nervous system.

Key words: male cricket, *Gryllus bimaculatus*, timer, reproduction, sexual refractoriness, spermatophore, terminal abdominal ganglion, genital motor neurone, DUM neurone.

Introduction

Time dependence is an important aspect of the reproductive behaviour of male crickets (Loher and Rence, 1978; Nagao and Shimozawa, 1978; Sakai et al., 1991). It usually occurs as follows. The male begins to sing a calling song to attract females when he becomes sexually active. The calling song soon changes into a courtship song when a female approaches. When the female steps onto the male's back, the male begins to perform copulatory actions by slipping backwards underneath the female. After success in genital coupling, the male extrudes the spermatophore and transfers it to the genital chamber of the female. As soon as the male finishes copulation, his behaviour towards the female changes from courtship to aggression, that is, he enters the sexually inactive state. Subsequently, the male performs spermatophore preparation to make a new spermatophore for the next copulation. Spermatophore preparation consists of the secretion of spermatophore materials from the accessory glands and testes, their transport through the ejaculatory duct and their ejection onto the ventral lobes of the phallic complex in the genitalia. Once spermatophore preparation is initiated, the calling song occurs after a fixed time, and he again enters the sexually active state.

Thus, the male cricket has a reproductive cycle consisting of the pre-copulatory mating stage (MS) and post-copulatory sexually refractory stage (RS). The mating stage is here defined as the interval from the onset of the calling song to spermatophore extrusion in copulation. The sexually refractory stage is the interval from spermatophore extrusion to the onset of the calling song. This refractory stage is further divided into the two substages: the first refractory stage (RS1) between spermatophore extrusion and spermatophore preparation, and the second refractory stage (RS2) between spermatophore preparation and the onset of the calling song. In *Gryllus bimaculatus*, RS1 is normally several minutes if a female is readily available. However, it is considerably prolonged, occasionally more than 1 h, if no female is present or the male is subject to stressful conditions (Nagao and Shimozawa, 1987; Ootsubo and Sakai, 1992). In contrast, RS2 is time-fixed (about 1 h) and is unaffected by the female or by stress. Thus, it is called the time-fixed sexually refractory stage, and is presumably under the control of a reproductive timer (Nagao and Shimozawa, 1987; Sakai et al., 1995; Ureshi and Sakai, 2001).

In a previous report, we suggested that the timer is located in the terminal abdominal ganglion (TAG), because functional inactivation of the TAG by local cooling lengthened RS2 in proportion to cooling time (Ureshi and Sakai, 2001). In order to obtain further insight into the function and localization of the timer, an electrophysiological approach would be useful (Truman, 1978; Tomioka and Chiba, 1986). However, the male does not exhibit the calling song under dissection and restraint. As long as the male remains silent and unresponsive, there is no guarantee that the reproductive timer is functioning normally even though one can obtain some information about time-keeping from the spike activity of neurones in the TAG.

The male cricket exhibits several types of spermatophore extrusion without genital coupling, such as pseudo-copulation related spermatophore extrusion, abortion and self-cycle renewal (Beck, 1974; Sakai et al., 1991). Recently, we described a new type of spermatophore extrusion called auto-spermatophore extrusion, in which a male paired with a female extrudes the mature spermatophore without any prior courtship or copulatory actions. Auto-spermatophore extrusion was frequently observed when males were given stress by cooling of the thorax and/or abdomen after spermatophore preparation (Ureshi and Sakai, 2001). Interestingly, the time interval (SPaSE) from spermatophore preparation to auto-spermatophore extrusion was comparable to or slightly longer than RS2 measured by the mating response, which suggested that auto-spermatophore extrusion occurred shortly after the male finished the sexually refractory stage. Thus, auto-spermatophore extrusion can be used as an index of the reproductive switchover, as a substitute for the calling song or the mating response (Ureshi and Sakai, 2001).

In the present study, we investigate (i) to what extent, and when, auto-spermatophore extrusion occurs in dissected and restrained males, (ii) whether efferent neurones in the TAG show reproductive stage-specific activity for a period long enough to cover several reproductive cycles, and (iii) whether they have any time-dependent activity during the RS2. The results strengthen our previous conclusion that the reproductive timer is located within the TAG, and demonstrate that the timer functions normally in dissected males even when the TAG is separated from the rest of the central nervous system.

Preliminary results have appeared elsewhere (Kumashiro and Sakai, 1999).

Materials and methods

Animals

Crickets *Gryllus bimaculatus* DeGeer were used 1–2 weeks after the final moult. To induce the prompt occurrence of spermatophore preparation after copulation, males were isolated from females at least 1 day before use.

Definition of SPaSE

Spermatophore extrusion, which is performed by a strong contraction of the dorsal pouch, is normally elicited by stimulation of special mechano-sensilla in the epiphallus of the genitalia (Sakai et al., 1991). The moment of spermatophore extrusion is indicated by jerky ejection of the attachment plate of the spermatophore from the dorsal pouch. In contrast, the onset of auto-spermatophore extrusion is somewhat difficult to determine. The ejection of the attachment plate of the spermatophore is performed rather gradually by weak dorsal pouch contractions, as if it were a discharge of waste. However, the final ejection of the spermatophore is often accompanied by a relatively strong contraction of the dorsal pouch and abdomen, with the subgenital plate fully opened. In addition, it is always followed by the rhythmic movement (1/6 s) of the phallic complex and abdomen, which is not different from that seen after normal spermatophore extrusion (Kumashiro and Sakai, 2001a). Thus, the moment of auto-spermatophore extrusion was defined as the occurrence of a relatively strong contraction of the dorsal pouch and abdomen followed by their rhythmic contractions. The time interval between spermatophore preparation (SP) and auto-spermatophore extrusion (aSE) is abbreviated as SPaSE (the term ‘spermatophore preparation’ is equivalent to ‘spermatophore protrusion’, which has been used previously).

Induction of auto-spermatophore extrusion

To measure the rate of occurrence of auto-spermatophore extrusion and variation in SPaSE times, experiments were conducted under four different conditions. If males did not show auto-spermatophore extrusion within 2 h after spermatophore preparation, observations were terminated.

(1) Free moving: the male was placed with a female in a 100 ml beaker containing a sheet of paper.

(2) Restrained: the male was harnessed dorsal side up to a cork plate with staples and the legs restrained with plasticine. This treatment was adopted to produce persistent stress.

(3) Semi-dissected (abdomen partially dissected): the male was treated as in (2) after cutting off the wings and legs. Then, the abdomen was opened along the body axis and the digestive system only removed. The inside of the abdomen was filled with insect saline (in mmol l^{-1}: NaCl, 150; KCl, 9.0; $CaCl_2/2H_2O$, 5.0; $NaHCO_3$, 2.0; glucose, 40; adjusted to pH 7.2 with NaOH).

(4) Dissected (abdomen fully dissected). After fixing the male on the plate as in (3), the accessory glands and testes in addition to the digestive system were removed, and the paraproct and cerci were cut off. The abdomen was filled with saline. Nerve roots emanating from the terminal abdominal ganglion (TAG) were left intact except for 10d (cercal sensory nerve) and 10v (cercal motor nerve) (Edwards and Palka, 1974).

In (2–4), males were dissected as soon as they had finished spermatophore preparation. The male was allowed to contact a restrained female with his antennae all the time.

Irritating stimulation of the genitalia

Following spermatophore preparation, males were restrained as in the second experimental condition (Restrained). Two stimulation methods were used to irritate the

genitalia with foreign objects or by injury (Sakai et al., 1991). For stimulation with foreign objects, a piece of plasticine was pushed into the genital cavity and petroleum jelly was smeared on the cerci, pouch, epiphallus and ventral lobes. For irritation by injury, the ventral lobes were cut horizontally with scissors. Bled hemolymph coagulated around the phallic complex and served as an irritating stimulus. After either treatment, the male was paired with a female in a beaker and any induced auto-spermatophore extrusion was noted.

Abdomen-opened and restrained males often discharged diarrheatic feces during the operation. In the regular experiments feces were removed with a string of paper as soon as possible because they may cause an early induction of auto-spermatophore extrusion through persistent irritation of the genitalia (see Sakai et al., 1991). However, such feces were intentionally left in some experiments to see if they could induce auto-spermatophore extrusion soon after their feces discharge in RS2.

Spike recording

The above procedures allowed quantitative determination of the occurrence and time of auto-spermatophore extrusion but did not allow spike recording immediately after spermatophore preparation because of the time required for making preparations. Thus, in electrophysiological experiments, the operation and recordings were performed before the induction of spermatophore preparation. This procedure was somewhat inefficient because the success rate in inducing spermatophore preparation was as low as 50%.

The treatment for recording has been reported previously (Kumashiro and Sakai, 2001b). The male was fixed on a cork plate and the abdomen opened dorsally. The intestines, accessory glands, testes, periproct and cerci were removed. Particular care was taken not to stimulate the phallic complex mechanically because it caused a rapid induction of spermatophore extrusion. Nerve roots emanating from the TAG were left intact except for 10v (cercal motor nerve) and 10d (cercal sensory nerve). In most experiments, one dorsal pouch nerve, a branch of the genital nerve separated from 10v, was cut for recording and the other was left intact. In some experiments, the dorsal pouch nerves were cut bilaterally to record spikes on both sides. To isolate the TAG from the rest of the central nervous system, the connectives were cut between the 6th abdominal ganglion and the TAG.

Extracellular spikes of the dorsal pouch motor neurone (mDP) were recorded from the cut end of the dorsal pouch nerve with a glass suction electrode, amplifier (AVM-11 Nihon Kohden, Tokyo, Japan) and data recorder (PC204A Sony, Tokyo, Japan). Data were analyzed with a thermal array recorder (RTA-1100 Nihon Kohden). Spike histograms were made every 1 min with a personal computer.

Artificial elicitation of spermatophore extrusion and induction of spermatophore preparation during spike recording

Normal spermatophore extrusion in dissected and restrained males was elicited by stimulation of cavity hairs (Sakai et al., 1991) in the genital cavity with a model of the copulatory papilla of the female. Spermatophore preparation was induced by placing the male's antennae onto the female body, which was restrained in front of the male during recording (Kumashiro and Sakai, 2001b).

Backfilling

To stain dorsal unpaired median (DUM) neurones innervating genital organs including the dorsal pouch and guiding rod, backfilling was carried out with $NiCl_2$ (1 mol l^{-1}) through the cut end of the genital nerve distal to the nerve branch innervating the accessory glands. After 24 h incubation at 4°C, the TAG was cut out, reacted with rubeanic acid, fixed with formaldehyde (Sakai and Yamaguchi, 1983) and intensified by silver impregnation methods (Bacon and Altman, 1977).

Statistical analysis

For statistical analysis, the Mann–Whitney *U* test was used, and the significance value was set at P=0.05. All estimates of central tendency are given as median values (M) with 95% confidence intervals (CI).

Results

Characteristics of auto-spermatophore extrusion

Auto-spermatophore extrusion by a free moving male paired with a female is shown schematically in Fig. 1. The reproductive cycle of the male cricket is shown in Fig. 1A. The first indication of auto-spermatophore extrusion is the occurrence of abdominal contraction with the subgenital plate slightly opened (Fig. 1Bi) which starts about 1 min before auto-spermatophore extrusion. After the repetitive contractions of the abdomen, the epiphallus and ventral lobes of the phallic complex protrude backwards with the subgenital plate fully opened. The attachment plate of the spermatophore is finally pushed out from the dorsal pouch of the genitalia (Fig. 1Bii). Compared with normal spermatophore extrusion (inset above Fig. 1Bii), the ejection of the attachment plate of the spermatophore was difficult to observe.

Following this, the abdomen tip slowly moves up and down with high tension (Fig. 1Biii,iv). The extruded spermatophore is gradually pushed backward and is finally rubbed off (Fig. 1Bv). The abdomen and genitalia then began a rhythmical movement with a periodicity of approximately 6 s (Fig. 1C). Auto-spermatophore extrusion in dissected males was essentially similar to that of free-moving males.

Auto-spermatophore extrusion rate and SPaSE in different conditions

The occurrence of auto-spermatophore extrusion and the time interval (SPaSE) between spermatophore preparation (SP) and auto-spermatohore extrusion (aSE) were analyzed in males exhibiting auto-spermatophore extrusion within the 2 h observation period under the four different experimental conditions.

(1) Auto-spermatophore extrusion in the free-moving condition was examined in males paired with a female. Almost all (98.3%) of the males (*N*=414) showed normal spermatophore extrusion during copulation with females within the 2 h observation period. Only seven (1.7%) showed auto-spermatophore extrusion without prior courtship or copulatory attempts (Fig. 2A, above). The SpaSE periods were 55.5–69.5 min (median 63.2 min, CI 62–64.7, *N*=7; see small upward deflections between 50–70 min), and were comparable to an RS2 of 68.5 min (CI 54.7–80.3, *N*=414; Fig. 2A, below) measured by the calling song. (2) When males (*N*=65) were restrained on the cork plate to cause stress, the rate of auto-spermatophore extrusion increased to 30.4%. Males that exhibited auto-spermatophore extrusion under the restrained condition had a SPaSE of 92.8 min (CI 69–104, *N*=17; Fig. 2B, above), which was significantly longer than 63.8 min (CI 56.2–70.8, *N*=56; Fig. 2B, below) for RS2 measured by the calling song. The remaining 39 males (69.6%) did not show auto-spermatophore extrusion during the 2 h observation period. When they were paired with females after the end of observations, 12 males exhibited auto-spermatophore extrusion within 10 min and 27 performed courtship. (3) In addition to restraint, when males (*N*=23) were partially dissected, auto-spermatophore extrusion increased up to 78.3%. These males had a SPaSE of 67.5 min (CI 58–74.2, *N*=18; Fig. 2C, above), which was not significantly different from 63.5 min (CI 61.2–67.5, *N*=23; Fig. 2C, below) for RS2 measured by the calling song. (4) Males (*N*=42) with the abdomen fully dissected showed the highest rate of auto-spermatophore extrusion (80.9%). The SPaSE was 69.8 min (CI 63.7–87.5, *N*=34; Fig. 2D, above), which was significantly longer than 63.6 min (58.5–66.8, *N*=42; Fig. 2D, below) for RS2 measured by the calling song.

These results indicated that auto-spermatophore extrusion is normally a rare phenomenon but that it becomes increasingly frequent as stressful conditions become more severe. As a baseline for identification of RS2, the SPaSE in males under stress by abdominal dissection is established as comparable to RS2 measured by the calling song.

Auto-spermatophore extrusion in connective-cut males

Auto-spermatophore extrusion was examined in males with the terminal abdominal ganglion (TAG) separated from the rest of the central nervous system. All the males showed auto-spermatophore extrusion. The SPaSE was 61 min (CI 50.2–73.5, *N*=31; Fig. 3A, above), which was not significantly

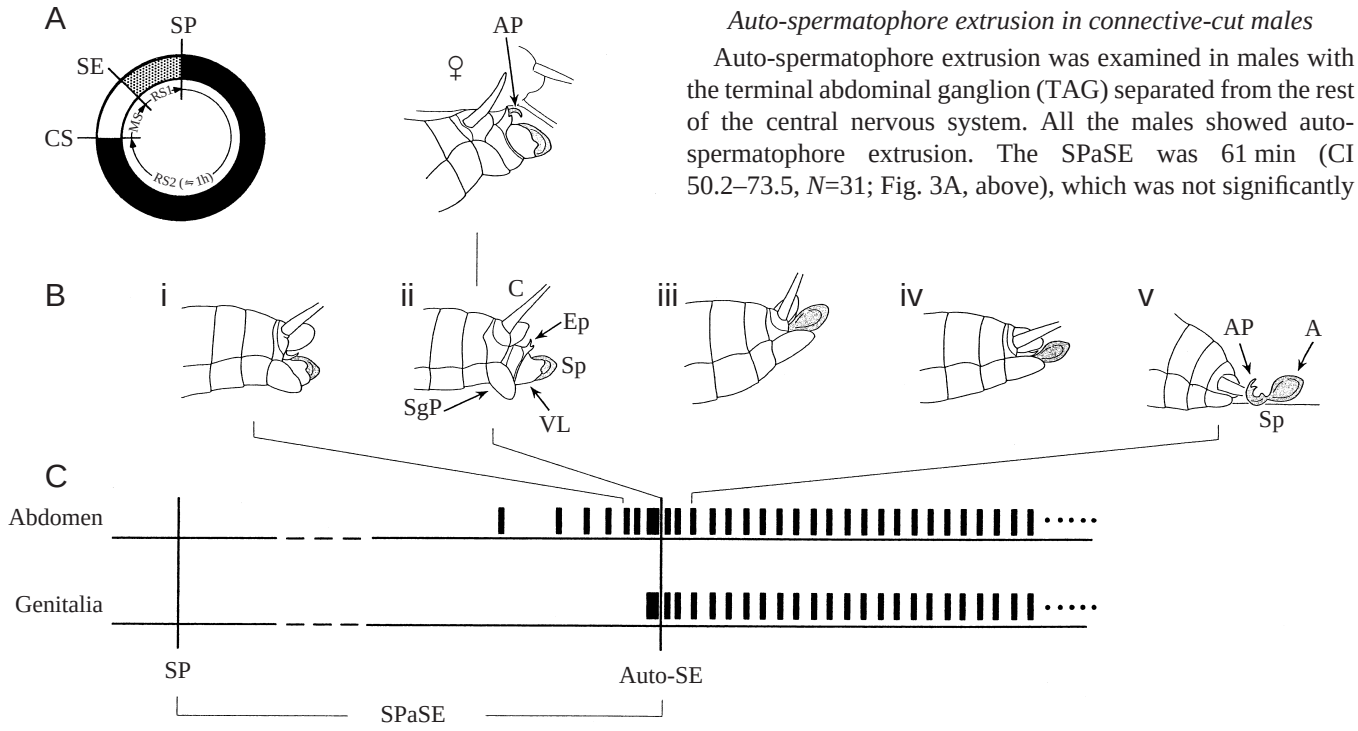

Fig. 1. (A) Diagram of the reproductive cycle of the male cricket. The sexually refractory stage is divided into the two substages: the first refractory stage (RS1, gray portion) between spermatophore extrusion (SE) in copulation and spermatophore preparation (SP) after copulation, and the second refractory stage (RS2, dark portion) between spermatophore preparation and the recommencement of a calling song (CS). The mating stage (MS, white portion) is the interval between the recommencement of the calling song and spermatophore extrusion. (B) Movements of the abdomen and genitalia before, during and after auto-spermatophore extrusion. (i–v) The sequence of motor actions in the posterior abdomen and external genitalia around auto-spermatophore extrusion. Abdominal contraction (Bi) starts some time after spermatophore preparation (SP). The spermatophore is extruded (Bii) with its attachment plate (not shown) ejected from the dorsal pouch of the phallic complex. Inset above Bii shows normal spermatophore extrusion during copulation with a female in which the epiphallus is hooking the subgenital plate of the female. The epiphallus is not seen but the attachment plate (AP) is observed from outside. The extruded spermatophore is soon removed (Biii–v). (C) Movements (shown as vertical bars) of the abdomen and genitalia. They are repeated together until spermatophore preparation is initiated. A, ampulla; AP, attachment plate; Auto-SE, auto-spermatophore extrusion; C, circus; Ep, epiphallus; SgP, subgenital plate; Sp, spermatophore; SPaSE, interval between spermatophore preparation and auto-spermatophore extrusion; VL, ventral lobe.

different from 65 min (CI 57.2–71.2, *N*=31; Fig. 3A, below) for RS2.

In the experiments reported so far, the operation always started soon after spermatophore preparation and finished within 10 min. To check the possibility that auto-spermatophore extrusion in dissected males might be caused by the delayed effect of operative shock, both the dissection and the connective cut were made at six different times (0–10, 20–30, 30–40, 40–50, 50–60, 60–70 min) after spermatophore preparation. The SPaSE values obtained from six such groups was 68.2 min (CI 51–85.5, *N*=30) compared with 65.8 min (CI 57–71.2, *N*=30) for RS2 measured by the calling song. There was no significant difference between the SPaSE and RS2 values. For comparison, the same operation was performed in males in the mating stage. They showed auto-spermatophore extrusion only 2.8 min (CI 2.0–6.8, *N*=27) after the operation.

These results indicate that the SPaSE in TAG-separated males is comparable to RS2 measured by the calling song, and that auto-spermatophore extrusion in dissected or/and connective-cut males was not due to operative shock or to the connective cut.

Effects of irritating stimulation of the genitalia on the induction of auto-spermatophore extrusion

The genitalia of intact males (*N*=17) were irritated with plasticine and petroleum jelly, and the males then tested in the free-moving condition. Three (17.6%) of them showed auto-spermatophore extrusion (SPaSEs=20.2, 49.2 and 66.5 min). The remaining 14 (82.4%) did not show auto-spermatophore extrusion but recommenced courtship later with an interval of 62.2 min (CI 56.3–72.7, *N*=14). On the other hand, in males with the ventral lobes excised (*N*=6), five showed auto-spermatophore extrusion. Their SPaSE values (14.8, 22, 30, 32.3, 59.3 min) were much shorter than their RS2 values, except for the last one.

A similar experiment was performed using TAG-separated males in which the genitalia were stimulated with diarrheatic feces discharged during the operation. All the males showed auto-spermatophore extrusion, with a SPaSE of 41.7 min (CI 35.3–50.7, *N*=21; Fig. 3B, above) which was significantly shorter than both 67.5 min (CI 59.5–75.2, *N*=21; Fig. 3B, below) for the RS2 measured by the calling song and 61 min for the SPaSE in TAG-separated males with no feces (Fig. 3A, above).

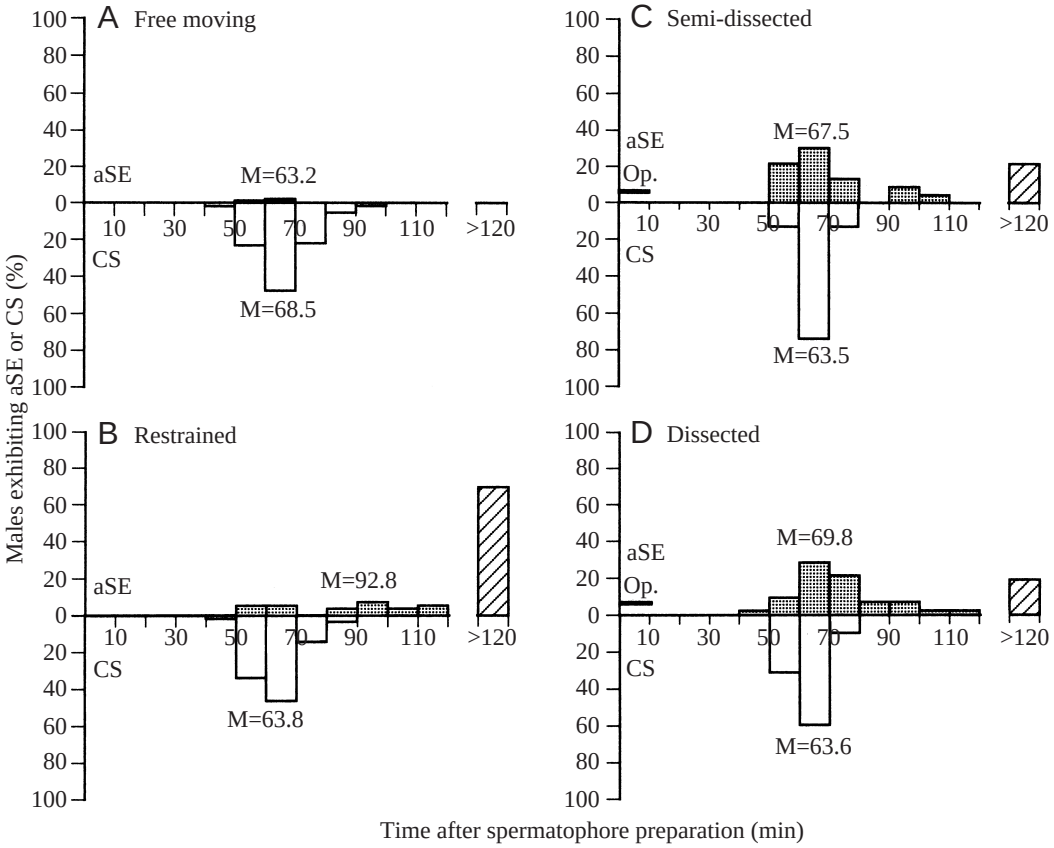

Fig. 2. SPaSE in males subjected to the four different conditions. (A–D) Percentage of males exhibiting auto-spermatophore extrusion (aSE; above) or the calling song (CS; below). (A) Free-moving condition. Note the very low percentage at 50–70 min of males exhibiting auto-spermatophore extrusion (aSE) compared to males exhibiting the calling song to a female (CS). (B) Restrained condition. (C) Semi-dissected condition. (D) Dissected condition. The columns at >120 min indicate the percentage of males that did not show auto-spermatophore extrusion within 120 min. Op. (C,D) indicates the period of the 10 min operation immediately after spermatophore preparation. M, median. These conventions are also used in Figs 3 and 10.

These results indicate that adverse stimulation of the genitalia after spermatophore preparation induces SPaSE shortening, particularly in TAG-separated males.

Motor neuronal response to genital stimulation and its spontaneous activity after spermatophore extrusion

Spike recording from the dorsal pouch nerve indicated that mDP started bursting after a relatively long latency following genital stimulation by the model of the female copulatory papilla. In the recording shown in Fig. 4A, mDP did not respond for the first 4.3 s after the onset of stimulation. Then, the discharge increased up to 120 Hz but abruptly stopped after 0.6 s. It gradually recovered over several seconds during the spermatophore transfer phase (horizontal bar in Fig. 4A). This spiking profile occurred when stimulation was removed immediately after the start of bursting (see legend of Fig. 4A), which indicates that it is centrally generated and lacks a sensory component.

After the spermatophore transfer phase, the activity of mDP changed into rhythmic bursting (1/6 s) at an intraburst frequency of 95 Hz (Fig. 4A). The burst was in synchrony with movement of the phallic complex and abdomen. If the male's antennae contacted a female, the rhythmic bursting gradually accelerated and finally stopped before spermatophore preparation. However, if female stimulation was not given, some males did not exhibit spermatophore preparation for more than 1 h. An examination of mDP spike activity (Fig. 4B) showed that the rhythmic bursting also continued for more than 1 h, though the spike rate slightly decreased around 30 min. This pattern was observed in all the preparations tested (*N*=20). A similar pattern of spike discharges was also seen in males with the connectives cut. These results indicate that the rhythmic bursting of mDP following spermatophore extrusion remains unchanged until the occurrence of spermatophore preparation.

mDP and small neurone activity after spermatophore preparation

During continuous recording of mDP for more than 2 h, the reproductive stage could be switched experimentally 2 or 3 times from the mating stage to the sexually refractory stage, using the female and artificial spermatophore extrusion technique (Fig. 5). During subgenital plate opening (the left end of the white bar in the first line in Fig. 5A), 45 s prior to spermatophore preparation, mDP became silent. At 23 min after spermatophore preparation, the spontaneous discharge of mDP appeared (Fig. 5A, asterisks, line 2), and continued sporadically with no steady increase or decrease. At 65 min, the genitalia were stimulated again to elicit spermatophore extrusion (Fig. 5B, line 1). Spike discharge vigorously increased in a manner similar to the first cycle. The temporal silence and subsequent spontaneous discharge followed as before. This time, however, the spontaneous spike rate was slightly higher than before. About 2 h after the beginning of the recording, the mating stage was switched into the sexually refractory stage for the third time (Fig. 5C). mDP showed a similar pattern as before. The changes in the spike activity of this mDP are shown quantitatively in Fig. 6A.

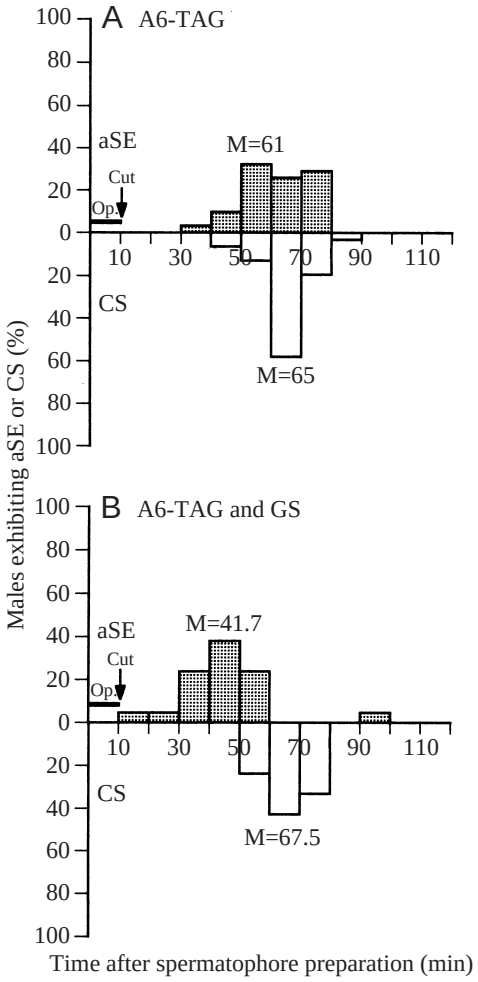

Fig. 3. SPaSE in males with the terminal abdominal ganglion (TAG) separated. (A) Connective-cut males exhibiting auto-spermatophore extrusion (A6-TAG). (B) The effect of genital stimulation (GS) with feces discharged during the operation on SPaSE in connective-cut males (A6-TAG and GS, upper). Cut, time of connective-cut. Other abbreviations and conventions as in Fig. 2.

A much smaller spike occurred in the same recording (Fig. 5). This neurone is tentatively named x neurone. The discharge pattern was different from any of the genital motor neurones so far identified (Kumashiro and Sakai, 2001b). Its discharge appeared a few minutes after spermatophore extrusion and disappeared before the occurrence of spermatophore preparation. After that, the x neurone began to discharge spontaneously at 29 min in the first cycle (Fig. 5A, line 3, small spikes) and at 31 min in the second cycle (Fig. 5B, line 3, small spikes). Then, the spike rate gradually increased and reached a plateau level (Fig. 6B). A quantitative study of x neurone spike size indicated that it comprised one (59.1%), two (31.8%), three (4.6%) or more than one neurone (4.6%) in many preparations (*N*=22). The latency of the first spike activity appearing after spermatophore preparation in each

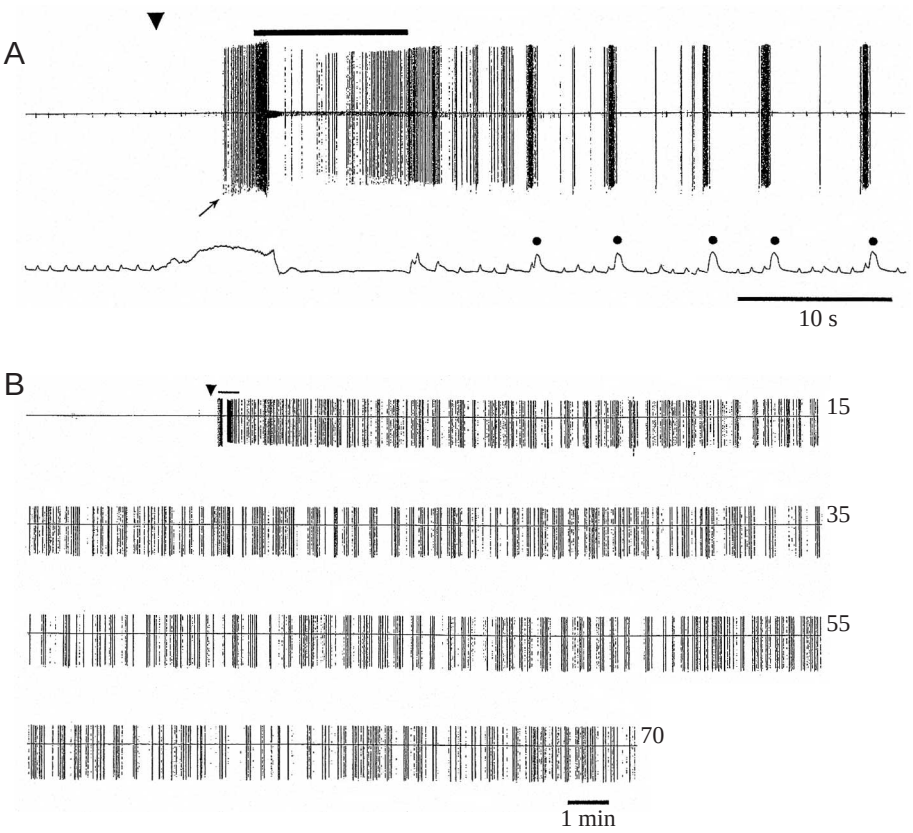

Fig. 4. Spike bursting of mDP in normal spermatophore extrusion. (A) The discharge pattern of mDP (top trace) around spermatophore extrusion induced by genital stimulation, which was started at the inverted triangle and stopped at the first bursting of mDP. mDP did not respond to the first 4.3 s, but then exhibited a strong burst (arrow), and gradually changed its discharge into a rhythmic burst after the spermatophore transfer phase (horizontal thick line). The lower trace shows the movement of the phallic complex. Each upward deflection after the spermatophore transfer phase (dots) indicates the movement of the phallic complex. (B) Discharge pattern of mDP after spermatophore extrusion. The rhythmic bursting did not stop unless the female stimulus was given. Numbers on the right indicate time (min) after spermatophore extrusion.

single x neurone was 16 min (CI 6–26, *N*=31) and the spike frequency was 0.48 Hz (CI 0.18–1.18, *N*=31) when averaged during the 5 min of the tonically discharging state 30–60 min after spermatophore preparation.

These results indicated that mDP together with the x neurone can be recorded reproducibly for a period of at least several hours to reveal a stage-specific discharge pattern in the male reproductive cycle.

Neurons with such a small amplitude relative to mDP could be dorsal unpaired median (DUM) neurones, based on similar extracellular profiles recorded in the locust (Hoyle and Dagan, 1978; Bräunig, 1988; Kalogianni and Pflüger, 1992). To examine this, simultaneous recordings were made from the left and right dorsal pouch nerve. Fig. 7A shows the spike histograms of two x neurones (r/l and l), which were recorded with different spike sizes. They gradually increased their spike frequencies 20–30 min after spermatophore preparation. Their actual spikes are shown in Fig. 7B, in which one of the x neurones (r/l; inverted triangle) was recorded from both the right and left dorsal pouch nerves simultaneously while the other (l) was recorded from only the left dorsal pouch nerve. The spikes of mDPs with large amplitudes in the right and left records are shown at low gain in Fig. 7C. There is no cross-talk between these records, indicating that the simultaneous appearance of small spikes (r/l in Fig. 7B) is not due to artifacts. Simultaneous recordings of the same x neurone were obtained in 10 out of 19 preparations.

Fig. 8 shows somata of DUM neurones (30–40 μm in diameter) retrogradely stained by backfilling from the distal region of the genital nerve. Ten somata are seen in the three segments (7–9) of the TAG. Based on ten preparations, two in segment 7, four in segment 8, four in segment 9, and two in segment 10 were identified. Together, these results indicate that some of the x neurones are certainly DUM neurones.

mDP activity associated with auto-spermatophore extrusion

In these experiments, no genital stimulation was used to cause the male perform auto-spermatophore extrusion. In males with the connectives intact, mDP, which had stopped discharging prior to spermatophore preparation (Fig. 9, line 1), began to show spontaneous discharging sporadically at 33 min after spermatophore preparation (Fig. 9, line 4). However, it stopped around 40 min and remained silent. Then, spiking at a high rate suddenly appeared at 68 min, with a relatively strong burst followed at 69 min after an irregular burst that was associated with a dorsal pouch contraction (arrowhead on the bottom line in Fig. 9). In many recordings, the burst rate was 28 Hz (CI 21–74, *N*=13), and the intraburst frequency of the subsequent rhythmic burst was 87 Hz (CI 75–112, *N*=13). As in Fig. 5, x neurone spiking also appeared in the same recording (Fig. 9). Its activation pattern resembled that described previously, but did not stop discharging after auto-spermatophore extrusion.

The time of mDP bursting associated with auto-spermatophore extrusion was analyzed in 16 males (Fig. 10A). 60% of the males showed auto-spermatophore extrusion in the

2 h observation period and 40% did not. The interval between spermatophore preparation and the occurrence of a relatively strong burst associated with auto-spermatophore extrusion had a somewhat wider distribution compared with RS2 measured by the calling song. However, the median of the former was 63.7min (CI 54.7–68.3, *N*=16), which was not significantly different from 65.5 min (CI 64.2–67.3, *N*=13) for RS2.

To examine the influence of ganglia anterior to the TAG on mDP and x neurone activity, the connectives were cut 10 min after spermatophore preparation (Fig. 11, white arrow, line 2). No change occurred. In this recording, mDP had no spontaneous discharge following spermatophore preparation. At 59 min, the first spike appeared, and a relatively strong burst occurred at 61 min in association with auto-spermatophore extrusion (Fig. 11, line 7). After this, rhythmic bursting followed as in males with the connectives intact (Fig. 9). By contrast, the x neurone began to discharge 24 min after spermatophore preparation and gradually increased its spike rate (not clearly seen in Fig. 11 because of its extremely small amplitude. See inset between lines 5 and 6). Its spontaneous discharge pattern resembled that in the previous records. These results indicated that the activities of mDP and x neurones in TAG-separated males were not essentially different from those in intact males. All the TAG-separated males exhibited auto-spermatophore extrusion. The interval between spermatophore preparation and mDP bursting associated with auto-spermatophore extrusion was 59.3 min (CI 46.8–68.7, *N*=15), which is not singnificantly different from 66.2 (CI 63.5–-69.5, *N*=13) for RS2 (Fig. 10B).

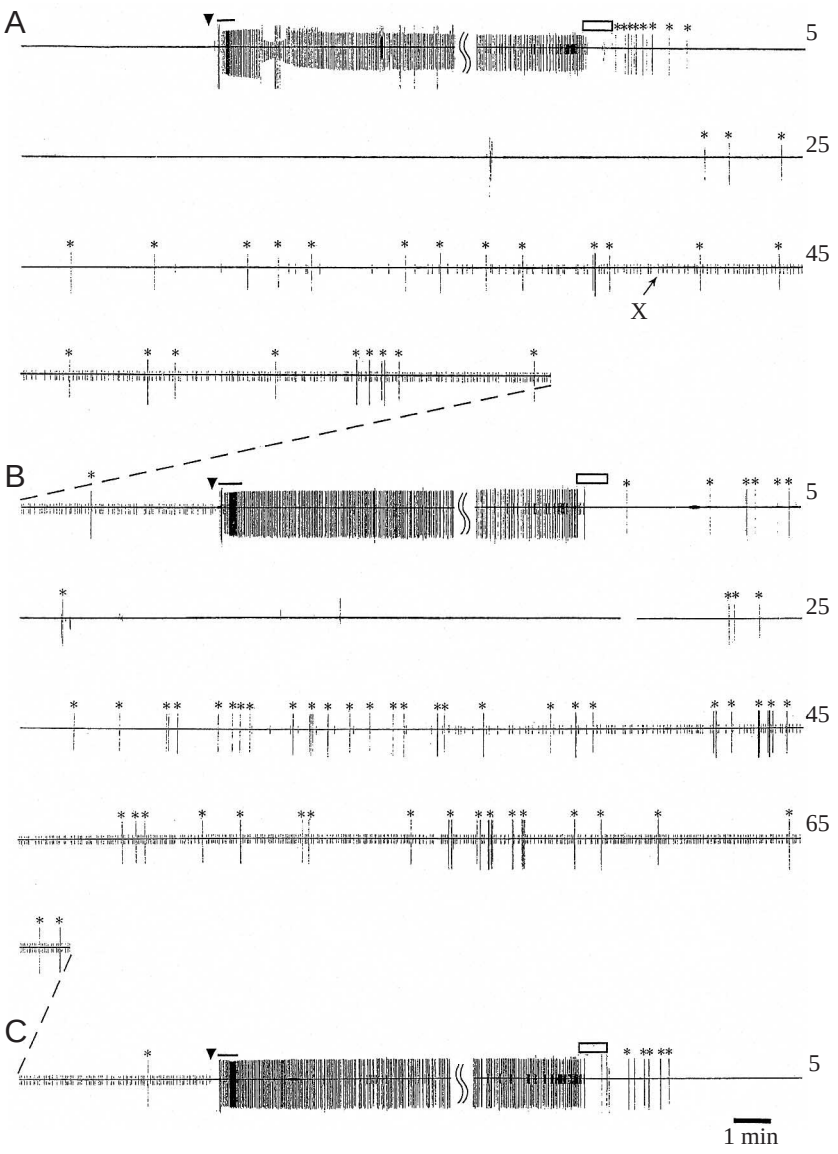

Fig. 5. Reproducibility of discharge pattern in mDP. (A–C) Long-term recordings of mDP activity (vertical double wave symbols indicate breaks). Numbers on the right indicate time (min) after spermatophore preparation. Asterisks indicate non-bursting spontaneous spikes of mDP. White horizontal bars indicate periods between subgenital plate opening (left) and spermatophore preparation (right). x, small spikes of an unidentified neurone.

Discussion

Auto-spermatophore extrusion

Spermatophore extrusion normally occurs when cavity hairs in the epiphallus are stimulated with the copulatory papilla of the female during genital coupling (Sakai et al., 1991). It can occur, however, in different ways without genital coupling: pseudo-copulation, abortion and self-cycle renewal (Beck, 1974; Sakai et al., 1991). The spermatophore is also ejected in association with grooming when the genitalia are soiled or injured (Sakai et al., 1991). Recently, we encountered another type of spermatophore extrusion, named auto-spermatophore extrusion. This occurs following spermatophore preparation, without any prior courtship, in males that had been treated by cooling the thorax and/or abdomen (Ureshi and Sakai, 2001). Initially, auto-spermatophore extrusion was thought to be an artificially produced abnormal behaviour. However, we found that intact males paired with a female naturally exhibited auto-spermatophore extrusion with the SPaSE comparable to their own RS2 measured by the calling song. This fact reveals that auto-spermatophore extrusion is not totally an artificially produced phenomenon. It is, rather, one of the various types of spermatophore extrusion without genital coupling. Functionally, auto-spermatophore extrusion might serve to cancel the ongoing reproductive schedule when the male's physical condition is not sufficient for reproduction. Thus, it would safely prevent the male from performing energy-consuming stridulation when the spermatophore matures.

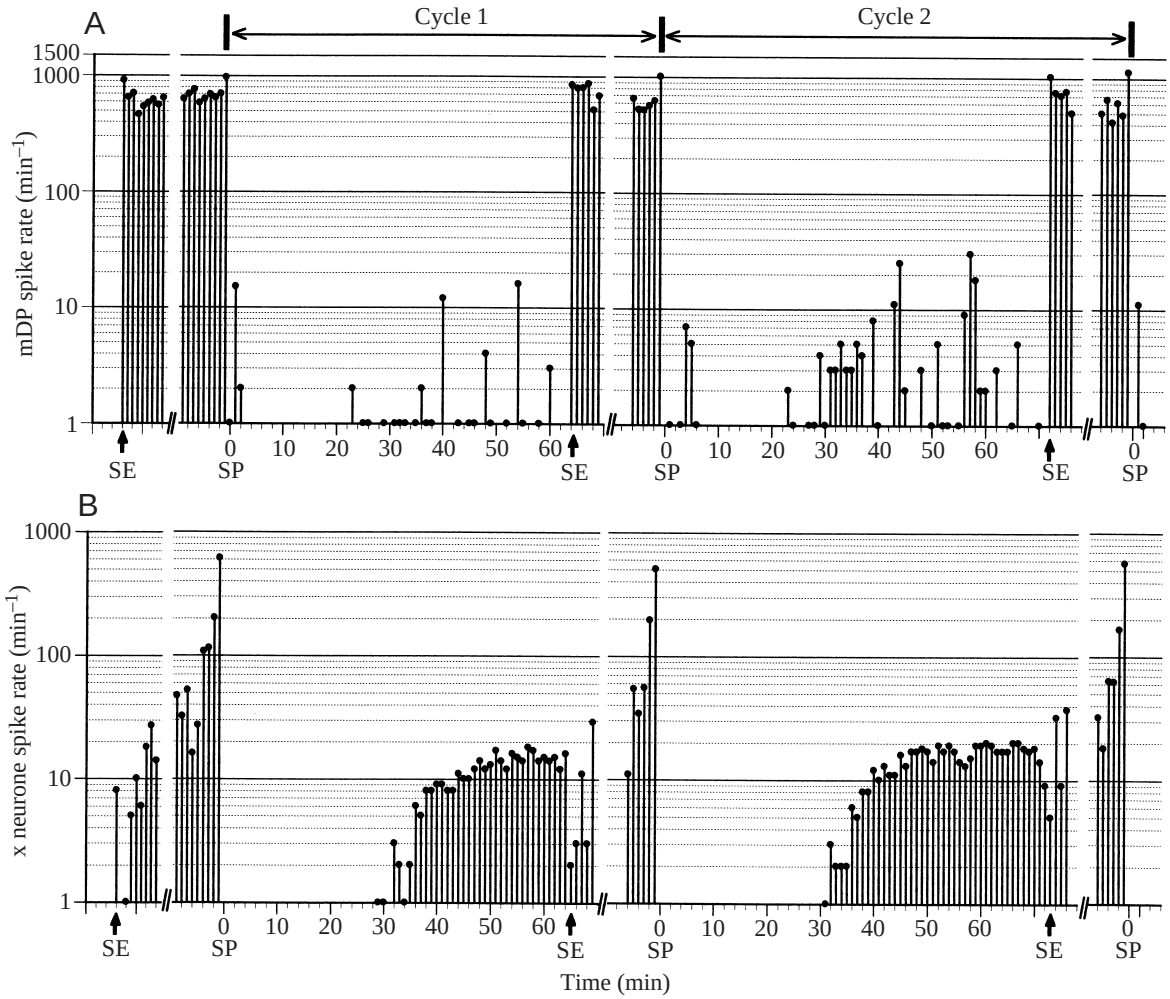

Fig. 6. Spike frequency histograms of mDP (A) and x neurone (B). Spermatophore preparation (SP) occurred at time zero. SE, spermatophore extrusion elicited by artificial stimulation. These graphs were constructed from the recording in Fig. 5.

Timer in dissected males

Our experiments indicate that auto-spermatophore extrusion is induced more frequently under stressful conditions, and that the SPaSE is comparable to RS2. The SPaSE in males with the TAG separated from the rest of the central nervous system is also comparable to RS2. Auto-spermatophore extrusion in the dissected condition was not due to the delayed effect of operative shock because the SPaSE was not lengthened when the start of the 10 min operation was delayed. These results strengthen our previous conclusion that the reproductive timer is located within the TAG (Ureshi and Sakai, 2001), and demonstrate that the timer functions normally even in the separated TAG. It is not clear why the spermatophore is abandoned rapidly after the end of the sexually refractory stage, as suggested by the fact that SPaSE is not very different from RS2. If auto-spermatophore extrusion occurs to omit performing courtship as mentioned above, the rapid switching of the reproductive stage to the sexually refractory stage by ejecting the spermatophore seems favorable for males.

Based upon the present results, previous localised cooling experiments and evidence that the biogenic amine 5-hydroxytryptophan profoundly influences the reproductive timer when administered locally in the TAG (Ureshi and Sakai, 2001; Ureshi et al., 2002), we suggest that specialized neurones in the TAG are responsible for time-keeping. A similar concept of localized dedicated neurones is emerging for another time-keeping system in insects. The circadian pacemaker appears to be localized in the lamina–medulla complex of the cricket optic lobes (Tomioka and Chiba, 1992) and in the accessory medulla of the cockroach (Petri et al., 2002).

For the role of peripheral input in auto-spermatophore extrusion, it remains unclear whether sensory afferent input flowing into the TAG is involved. In the experiments under dissection, all the nerve roots of the TAG were left intact except the cercal sensory and cercal motor nerves in TAG-separated males, although the accessory glands, testes and digestive system were removed. If the TAG was completely isolated, auto-spermatophore extrusion could have been affected because its occurrence is facilitated by artificially applied peripheral input, as discussed below. However, the

timer itself in the TAG is considered to function normally because ablation of the genital organs did not affect RS2, measured by the calling song (Sakai et al., 1991). Experiments *in vitro* preparations are currently underway to examine this point.

Auto-spermatophore extrusion stimulated by the afference from genitalia

Irritating stimulation of the genitalia applied artificially causes a grooming type of spermatophore extrusion in males in the mating stage; males with genitalia soiled by discharged feces, or with the ventral lobes incised, show spermatophore extrusion within a few minutes after stimulation (Sakai et al., 1991). This behaviour may be appropriate because the spermatophore could have been spoiled due to problems in the genitalia. The elimination of the spermatophore is not the result of passive withdrawal by scraping, since all the males subsequently do not show the recommencement of courtship but show spermatophore preparation, indicating that the reproductive stage is switched from the mating stage to the refractory stage *via* this type of spermatophore extrusion.

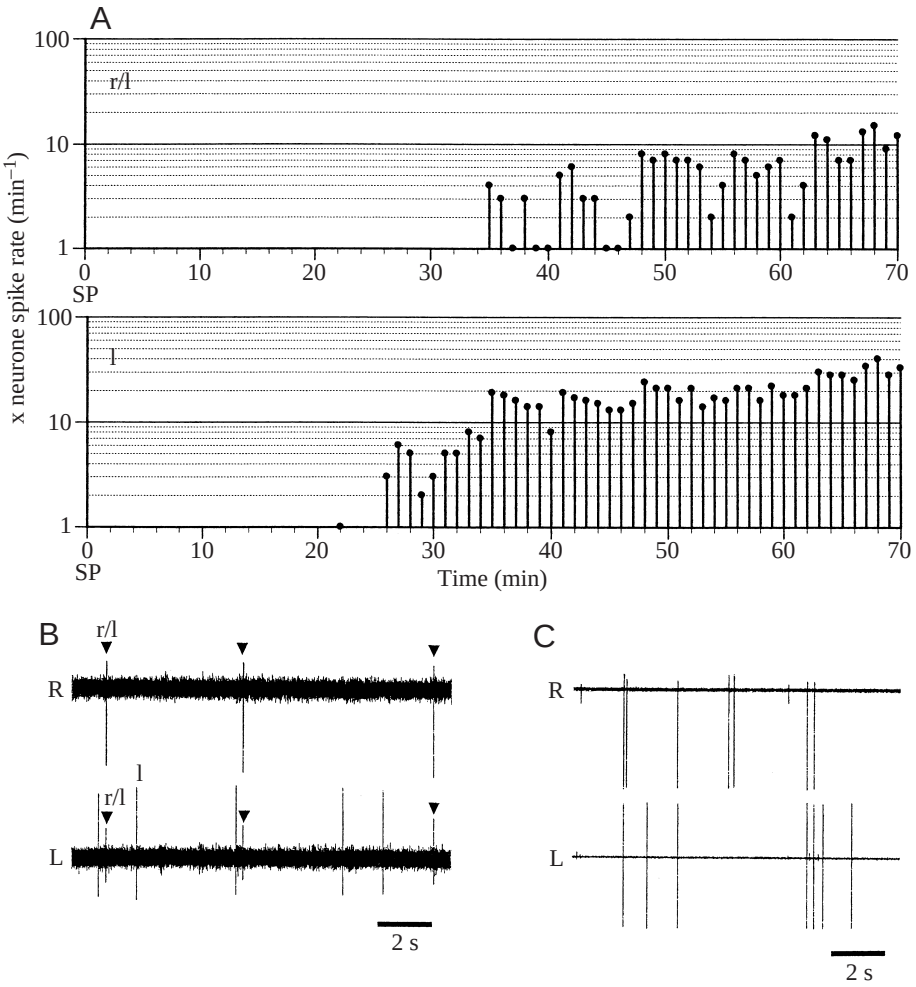

Fig. 7. An x neurone identified as a DUM neurone. (A) Spike frequency histograms of two x neurones recorded from the right and left dorsal pouch nerves. r/l, spike simultaneously recorded from both the nerves; l, spike recorded from the left nerve only. (B) Recordings from the right (R) and left (L) nerves, as shown in A, at high gain. Inverted arrows indicate a synchronized spike of r/l neurone. (C) Different portion of the same recording as in B at low gain. Large spikes are from mDP. Note the absence of crosstalk.

In the present experiments with males in the sexually refractory stage, stimulation of the phallic complex with plasticine and petroleum jelly had a minor effect since most of the males did not show auto-spermatophore extrusion during RS2. In contrast, stimulation of the ventral lobes by injury was much more effective: the immature spermatophore was ejected not long after the operation. The male may actively throw the spermatophore away in response to something abnormal happening in the genitalia. Stimulation of the genitalia with feces also caused facilitation of auto-spermatophore extrusion in the TAG-separated males. Their SPaSE was not so short as that in males with the ventral lobes injured but it was significantly shorter than RS2. This may be due to the effect of the irritating stimulation together with lack of inhibition from the ganglia anterior to the TAG, including the brain (Matsumoto and Sakai, 2000a,b; Kumashiro and Sakai, 2001b).

From these observations, it is concluded that adverse stimulation of the genitalia can facilitate the occurrence of auto-spermatophore extrusion, particularly in males with the connectives transected. This cautions one to be aware of feces discharged during the operation and to remove any as quickly as possible. Otherwise, one may inadvertently mistake the shortened SPaSE for the original preset timer as estimated by RS2.

mDPactivity in the reproductive cycle

So far, long-term extracellular spike recording has been successfully carried out using simplified preparations of insects. The activity of motor neurones and brain neurones associated with ecdysis-related motor patterns were recorded in a developing moth for several hours after dissection (Truman, 1978). Even longer recordings, of up to several days, have been made from optic lobe neurones underlying the circadian rhythm in crickets and cockroaches (Tyshchenko, 1973; Tomioka and Chiba, 1986, 1992; Colwell and Page, 1990). In the present experiments, extracellular spike recording from one of the genital motor neurones was used as a monitor for the reproductive stages and events in the male cricket. mDP

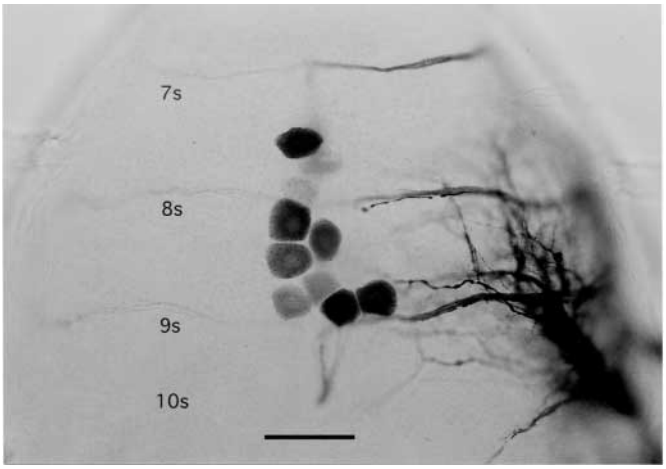

Fig. 8. DUM neurones retrogradely stained in the terminal abdominal ganglion (TAG). 7s–10s indicate the four segments of the TAG after Edwards and Palka (1974). Dendrites to the right are those of genital motor neurones, including mDP. Scale bar, 100 μm.

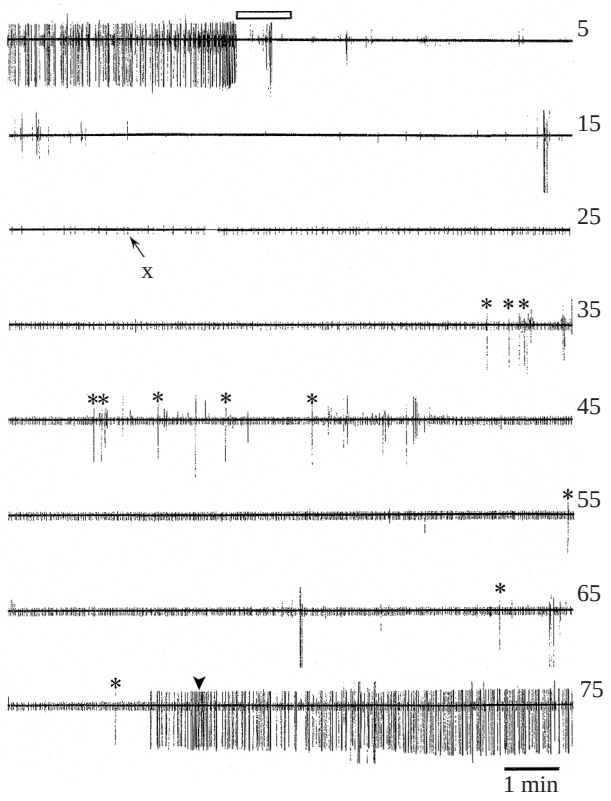

Fig. 9. Activity of mDP in males with connectives intact. Rhythmic burst discharge is seen in the first and last lines, and sporadic spiking (asterisks) elsewhere. The break in line 3 is the time of cassette tape exchange. Arrowhead on bottom line, onset of auto-spermatophore extrusion. White bar and 'x', as in Fig. 5.

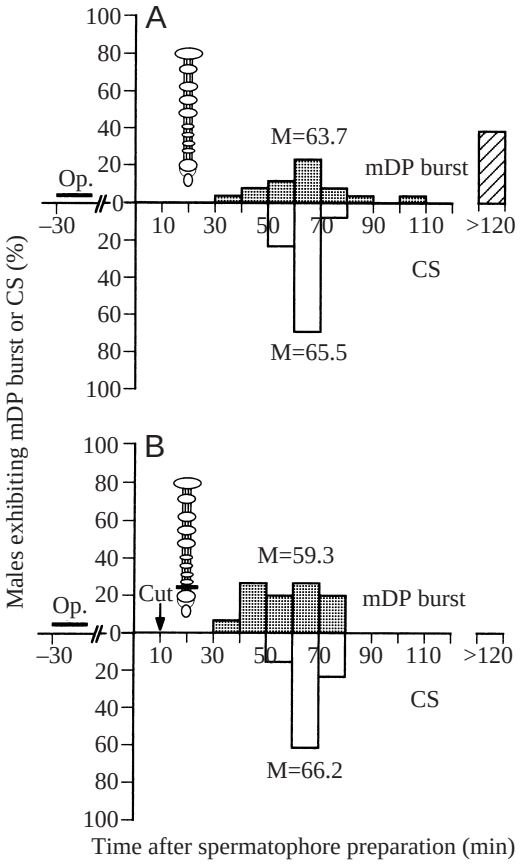

Fig. 10. (A,B, above) Occurrence of mDP bursting associated with auto-spermatophore extrusion. (A) Males with connectives (inset) intact. 40% of males (slashed bar) did not show burst discharge within 2 h. (B) Males with the connectives (inset) cut. 'Op.' indicates a 15 min operation performed before the start of recording, and 'Cut', the time of connective cut. (A,B, below) Males that recommenced their calling song (CS) after spermatophore preparation in the free-moving condition. M, median numbers.

was particularly suitable because it is singly recorded from the dorsal pouch nerve and its spike burst causes spermatophore extrusion *via* deflection of the dorsal pouch (Kumashiro and Sakai, 2001b). The results revealed that mDP shows a characteristic activity during the two sexually refractory stages: rhythmic bursting in RS1 and sporadic discharge in RS2. Furthermore, these discharge patterns were recorded reproducibly in the same preparation for more than 2 h without any change in activity due to deterioration or artifact.

In the reproductive cycle, in which the stages are switched *via* normal spermatophore extrusion and spermatophore preparation, mDP stops rhythmic bursting when the subgenital plate opens 45 s prior to spermatophore preparation. Subsequently, mDP begins to discharge spontaneously, but only sporadically. This makes sense because mDP is a motor neurone responsible for contracting the dorsal pouch muscles, which are not used during RS2. There is a weak time-dependency even in the separated TAG: mDP is completely silent in the initial state following spermatophore preparation and becomes active some time later. However, the spontaneous spike rate of mDP was too low and variable among preparations to clarify this time-dependency. More significant

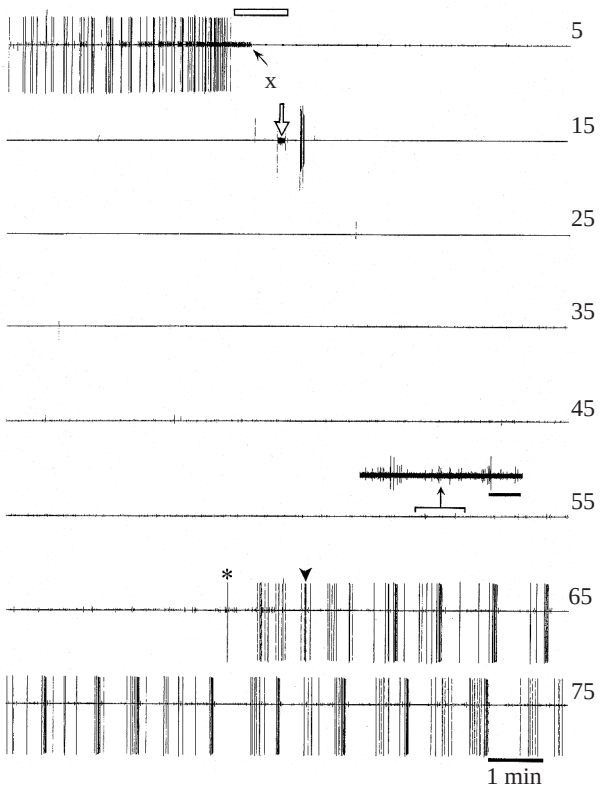

Fig. 11. Activity of mDP in males with the terminal abdominal ganglion separated. The open arrow indicates the time of connective-cut. Inset between lines 5 and 6 shows an enlarged recording of the indicated portion in which spikes with at least three different amplitudes are discerned. Scale bar for inset, 10 s. Conventions are the same as in Fig. 9.

information concerning timer output might be obtained by recording from other genital motor neurones, such as those innervating the ventral lobes and median pouch, because they have higher spontaneous frequencies and show gradual changes in activation patterns after spermatophore preparation (Kumashiro and Sakai, 2001b).

Nevertheless, the results demonstrate that neurones in the TAG can withstand long-term spike recording in the dissected condition. Conveniently, spike recording is a much more reliable method for monitoring the state of the reproductive stage and the time of events than movement of the phallic complex, because the changes in the spike rate of mDP are so distinct and stage-specific.

mDP activity associated with auto-spermatophore extrusion

In the experiments without genital stimulation, mDP showed auto-spermatophore extrusion-associated burst activity with a SPaSE comparable to RS2. However, the burst frequency was much less (28 Hz) than 137 Hz (CI 110–185, *N*=16) (Kumashiro and Sakai, 2001b) in normal spermatophore extrusion induced by genital stimulation (see Fig. 4A). Furthermore the spike burst in the former was always preceded by irregular discharges mixed with some weaker bursts, while in the latter strong bursting occurred only once, with a long latency of several to 10 s after the start of genital stimulation. This difference was not due to the presence or absence of a sensory response component in the burst discharge, because mDP neither responded simply to genital stimulation nor reduced its discharge when the stimulus was quickly removed at the moment of the bursting (Fig. 4A). As for stress in dissected males under recording conditions, no difference was seen between auto-spermatophore extrusion and normal spermatophore extrusion. There must be different neural mechanisms underlying the release of auto-spermatophore extrusion and the trigger of normal spermatophore extrusion that are presumably intrinsic to the center for spermatophore extrusion in the TAG, which may include a neural circuit for excitation, inhibition and rebound excitation to the genital input.

After the spermatophore transfer phase, mDP activity changed into rhythmic bursting with an intraburst frequency of 87 Hz, which was comparable to 95 Hz (CI 94–115, *N*=11) after normal spermatophore extrusion (Kumashiro and Sakai, 2001b). The burst cycle was also similar to that (1/6 s) seen after normal spermatophore extrusion. These changes are reliable indicators of the occurrence of auto-spermatophore extrusion, even in cases where the spermatophore is not actually extruded in males using the accessory glands and testes removed, as in the present experiments. Additionally, it should be noted that mDP is not a timer neurone and its burst does not indicate the moment of the time-up itself of the reproductive timer. However, recording mDP is useful for future studies in even more simplified preparations to monitor the reproductive state.

Time-dependent neurone activity

The spontaneous spike activity of one or more small neurones (x neurones), simultaneously recorded with mDP, had a much clearer time-dependency than mDP; firing began 10–30 min after spermatophore preparation, then gradually increased in frequency and reached a plateau level. The tiny spike amplitudes suggest that these are not motor neurons (Hoyle and Dagan, 1978; Bräunig, 1988; Kalogianni and Pflüger, 1992). Although all the x neurones were not tested, some showed simultaneous spike discharge in the right and left dorsal pouch nerves, indicating that they are dorsal unpaired median (DUM) neurones, as reported in the thoracic ganglion (Evans and O'Shea, 1977; Hoyle and Dagan, 1978; Gras et al., 1990; Thompson and Siegler, 1991; Burrows, 1996; Baudoux and Burrows, 1998; Grolleau and Lapied, 2000; Bräunig and Pflüger, 2001) and the abdominal ganglion (Lange and Orchard, 1984) of the locust. Other x neurones, recorded from only one side of the dorsal pouch nerve, may also be DUM neurones, based upon similar activation pattern and spike rates. The missing spontaneous contralateral spike may have been hidden under the background noise because of the small size. In addition, we stained several DUM neurones, together with genital motor neurones including mDP, by backfilling through the genital nerve. The pattern of soma distribution was similar

to that reported in the TAG of the male cockroach (Sinakevitch et al., 1996). At present, it is unknown which DUM neurone somata send their axons to the dorsal pouch nerve, from which we recorded x neurones with mDP, because backfilling through the thin dorsal pouch nerve hardly stained DUM neurones. The presence of time-dependent activity in the TAG suggests that the timer output may not be 'all or none', as seen in behavioural output such as the sudden emission of calling after the 1 h silence, but rather is graded to influence different neurones at different times after spermatophore preparation. It is necessary to investigate DUM neurones further and to determine the origin of their time-dependent activity.

In conclusion, the present results strengthen our previous suggestion that the reproductive timer is located within the TAG, and demonstrate that the timer functions normally in the separated TAG once it is triggered at spermatophore preparation. The gradual increase in the spike activity of efferent neurones in the TAG gives an insight into the fashion of the timer output. The next step is to seek timer neurones in simplified preparations.

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