Chapter 16
Reproductive Behavior and Physiology in the Cricket *Gryllus bimaculatus*

Masaki Sakai, Mikihiko Kumashiro, Yukihisa Matsumoto, Masakatsu Ureshi, and Takahiro Otsubo

Abstract *Gryllus bimaculatus* males have a reproductive cycle consisting of a mating stage and a sexually refractory stage. During the mating stage, the male exhibits distinct behavior that encompasses three main stages: calling, courtship, and copulation. The last stage, copulation, is carried out in a fixed manner by the stimulus-response chain. The final copulatory act, spermatophore extrusion, is caused by stimulation of mechano-sensilla in the epiphallus during genitalia coupling, which terminates the mating stage. The sexually refractory stage starts with spermatophore extrusion, during which the male is rather aggressive and does not exhibit any mating behavior. A male first shows spermatophore preparation when stimulated by the female, then forms the new spermatophore, and finally recommences the calling song, i.e., the start of the mating stage. Physiological investigations reveal that the male mating behavior is mainly controlled by the brain and the terminal abdominal ganglion (TAG), which exerts three types of inhibition on the pattern generators for mating behavior. The brain also facilitates sexual excitation via octopamine. One of the conspicuous features of the reproductive behavior in *Gryllus bimaculatus* is that the sexually refractory stage between spermatophore preparation and the start of calling song is time fixed at around 1 h. Experiments utilizing the targeted cooling of the central nervous system indicate a presence of a time-measuring mechanism ("timer") that is located within the TAG. Long-term spike recordings of neurons also support the presence of such a timer within the TAG. Finally, the occurrence of mating-like actions in larval nymphs is discussed. All of these findings have now generated a large body of work that will

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help establish *Gryllus* as a new experimental system for studying reproductive behavior and physiology in crickets and other insect species.

Keywords Male cricket • *Gryllus bimaculatus* • Reproductive behavior • Nervous system • Postembryonic development

16.1 Introduction

There are about 2500 species of crickets known in the world. Some of them live near human habitats and have aroused human interest since the ancient days (Hsu 1929). The male crickets have complex behavioral repertoires such as calling, courtship, copulation, and fighting, and they have been extensively studied since the 1950s (Choperd 1951; Huber 1955; Hörmann-Heck 1957; Alexander 1961; Alexander and Otte 1967; Beck 1974; Loher and Rence 1978; Evans 1988). More recently, crickets have been used in neuroethological research (Huber et al. 1989) because of their comparatively large-sized nervous system and their suitability for surgical experimentation. The range of these studies is very broad, encompassing song production (Bentley 1969), song recognition and sound localization (Weber et al. 1981; Loher et al. 1993), startle response to wind (Palka et al. 1977; Pollack and Hoy 1989; Shimozawa and Kanou 1984), reproduction (Sakai and Kumashiro 2004a), death feigning (Nishino and Sakai 1996), and fighting and status decision (Simmons 1986; Adamo and Hoy 1995; Stevenson et al. 2000; Iwasaki et al. 2006). Here, we focus on cricket mating behavior and its physiological mechanisms, and we describe our work using *Gryllus bimaculatus* as a model system.

16.2 Reproductive Behavior

16.2.1 Male Cycle

In order to manifest reproductive behavior, crickets should possess sexually mature ovaries and the testes. The adult male performs mating behavior consisting of calling, courtship, and copulation when it is paired with the female. However, these actions depend upon the internal state of the male, which changes cyclically via copulation. That is, the male has a reproductive cycle consisting of two stages (Fig. 16.1). The period between the start of the calling and the end of copulation is defined as the mating stage. Immediately after copulation, the male becomes sexually inactive and aggressive even toward the female. This period between the end of copulation and the restart of the calling song is named the sexually refractory stage. Only after this refractory period is over can the calling behavior start again. As in many animals, the male has the dominant, active role in mating behavior, so
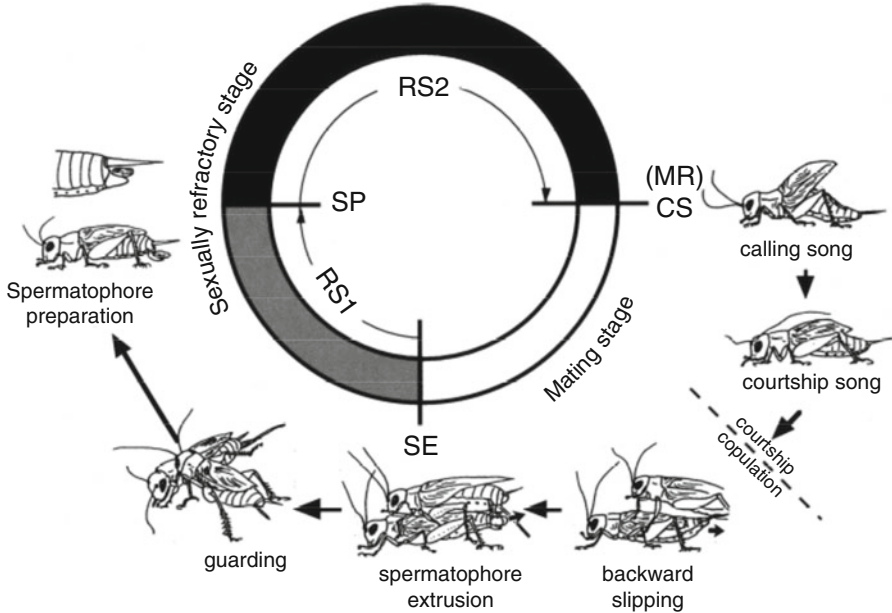
Fig. 16.1 Behavior of the male cricket in the reproductive cycle. The cycle consists of the mating stage, beginning at the onset of the calling song (CS) and ending with spermatophore extrusion (SE), and the sexually refractory stage beginning at SE and ending at CS. The sexually refractory stage is further divided into two stages: the first refractory stage (RS1) between SE and spermatophore preparation (SP), and the second refractory stage (RS2) between SP and CS. The latter interval (SPCS) is time fixed and thus called the time-fixed sexually refractory stage. In the mating stage, the male exhibits courtship and copulation with the female, while in the sexually refractory stage, the male exhibits guarding and aggressive behavior toward the female. The mating response (MR) consists of copulatory actions to a model mimicking the abdomen of the female. The MR can be elicited nearly at the same time as the CS is re-emitted. Thus, the first occurrence of CS or MR after SP indicates the end of the sexually refractory stage or the start of the mating stage

most studies focus almost exclusively on males. Hence, any reference to female crickets is brief and descriptions are included only when necessary.

16.2.2 Mating Stage

Under experimental conditions, when a male and a female are placed in a small space, the male will soon start to call for and court the female. When the female responds to the male by mounting the male's back, the male engages in copulation and transfers the spermatophore to the female. Then, the mating stage ends and the sexually refractory stage starts (Fig. 16.1). However, there are some cases in which the mating stage is terminated without spermatophore transfer (see Sect. 16.2.2.5).

16.2.2.1 Sexual Recognition

The male recognizes the sex of other individuals exclusively by the chemical contact with its antennae; olfactory and visual stimuli are not important (Rence and Loher 1977; Ogawa and Sakai 2009). When the male encounters a female, male courtship of her begins. In contrast, if a male encounters another male, both will engage in aggressive behavior. The ensuing fight is finished rather quickly and its outcome will determine the hierarchical relationship between the males (Adamo and Hoy 1995; Stevenson et al. 2000). Interestingly, if two males are confined to a small space, the dominant male will begin courting the subordinate male (Ogawa and Sakai 2009).

16.2.2.2 Courtship

The details of courtship are described in Fig. 16.2, and they encompass a total of six stages. First, searching males encounter a female (Fig. 16.2(1)). Second, if the male makes antennal contact with the female, he will recognize the other cricket as a *bona fide* female (Fig. 16.2(2)). Third, the male will initiate a large turn in the opposite direction from the female and will spread his antennae wide (Fig. 16.2(3)). Following this, the male then makes several small successive turns in the same direction (lasting 4.5 s). This action allows the male to orient its abdominal end
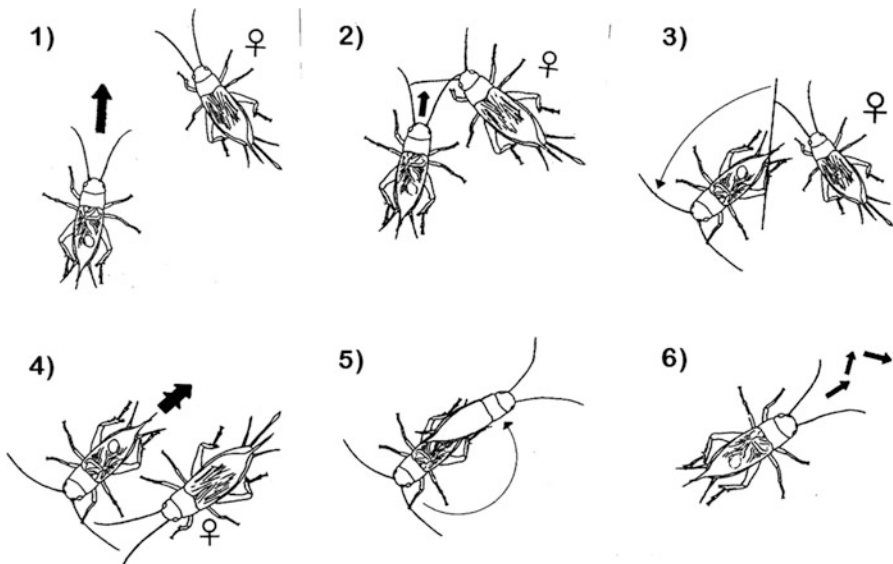
Fig. 16.2 Courtship sequence. (*1*) Search. (*2*) Encounter with the female. (*3*) Turn away from the female. (*4*) Backward walking. (*5*) Quick turn toward posterior direction. (*6*) Search with an intense calling song

toward the female. This, in turn, will provide the female with an opportunity to mount onto the male's back. Fourth, the male walks backward slowly while singing the courtship song (Fig. 16.2(4)). The copulation starts when the female mounts the male (which typically occurs about 15–20 s from the initial contact). Fifth, if the female does not proceed with mounting, the male makes a quick 180° turn to face the female and try to re-engage her (Fig. 16.2(5)). In situations when no female is present, the male will walk in a zigzag manner, singing an aggressive calling for 5–10 s (Fig. 16.2(6)). If the female still cannot be found, the male will cease its calling and walk for 70 s before starting a new search.

All the actions performed by the male are well organized and aimed toward the successful completion of copulation. Note that while engaged in searching (Fig. 16.2 (6)), a male may engage in additional activities, such as spiral walking, an effective search strategy that has been found in desert ants (Muller and Wehner 1994).

16.2.2.3 Copulation

The sequence of copulation behavior has been extensively studied in *Gryllus bimaculatus* and is summarized in Fig. 16.3 (Sakai et al. 1991). First, the male slips under the female in response to female's mounting and walks backward (lasting 3.2 s). Then, the male hangs the epiphallus (hook) onto the female subgenital plate (5.6 s). Following this, the genitalia of the male and female are united (4 s), which leads to spermatophore extrusion. Finally, the spermatophore is pushed up into the female genital chamber where it becomes fixed (9.1 s). On average, the entire copulation is completed in 20 s.
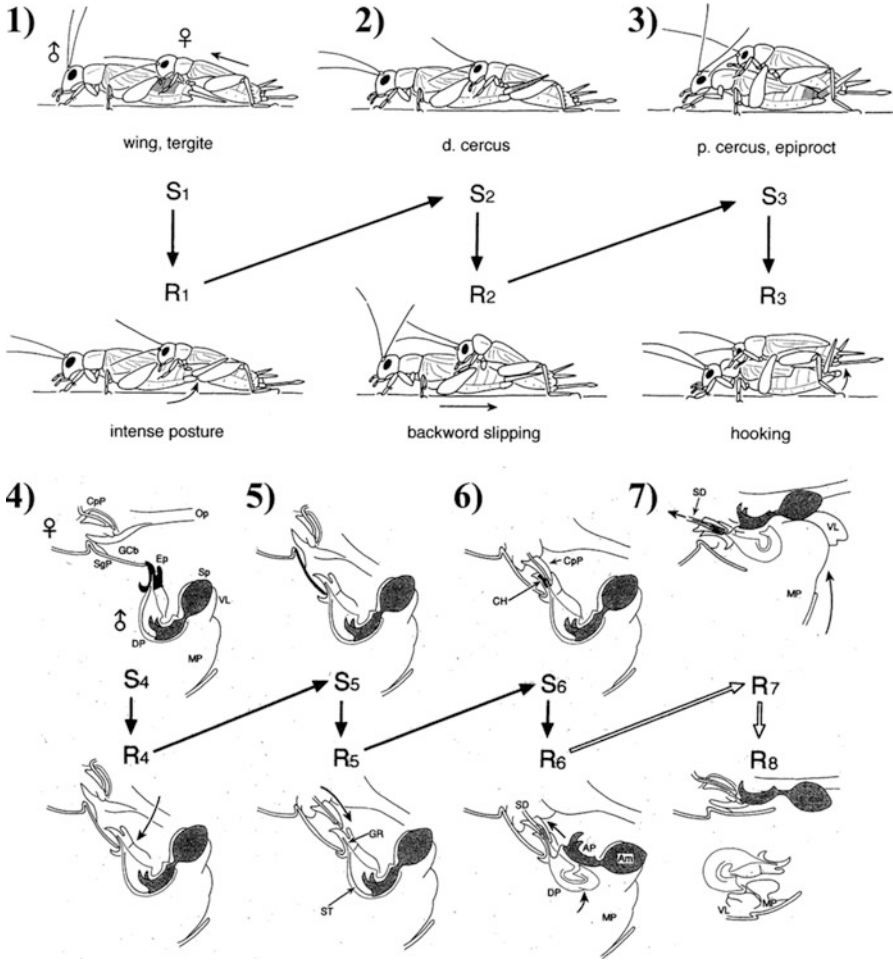
16.2.2.4 Chain Reaction

Every copulatory action of the male cricket proceeds in the manner of a chain reaction as explained for the mechanism of mating behavior in the sticleback by Tinbergen (1951). That is, a stimulation of sensilla in a certain region causes a fixed action response, and as a result of such response, another region will be stimulated. This in turn causes yet another response and so on. The input and output relationships were analyzed by using a model of the female (key stimulus) and surgical ablation of mechano-sensilla on the dorsal surface, i.e., the cerci and the epiphallus (Sakai and Ootsubo 1988). The chain reaction (Fig. 16.4) proceeds as follows: (1) When the female steps on the male dorsal region (S1), the male shows the intense posture with the abdomen slightly raised (R1). (2) The male's posterior cerci make contact with the female ventral region (S2). In response to cercal stimulation, the male walks backward with the cerci vibrating (R2). This cercal vibration seems to be useful not only for the male to help orient his body axis parallel to that of the female but also to make the female quiet. After the start of copulation, the female falls into a thanatotic state due to the vibratory stimulation caused by the male's body thrust. (3) The male stops backward walking when his abdominal end reaches the end of the female's abdomen. The male stops because
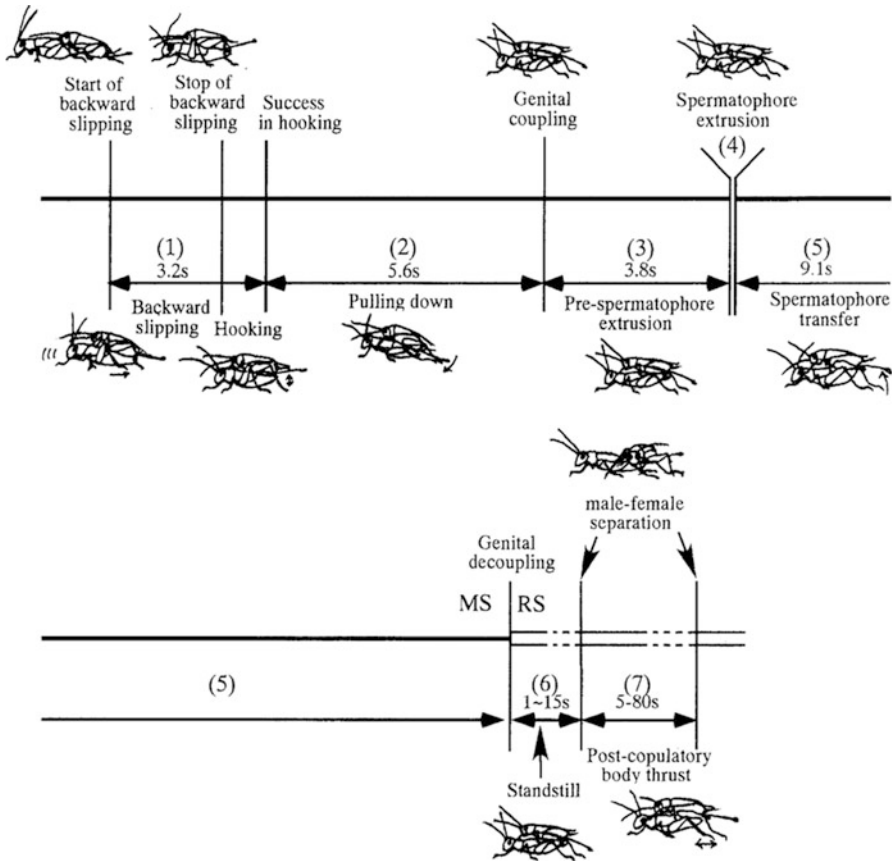
Fig. 16.3 Copulatory sequence and average periods for sequential behavior patterns. Onset at *upper left*. (*1–7*) indicates each step. Time is given in seconds

the posterior region of his cerci loses contact with the female. At that moment, the anterior part of the cerci and the last (tenth) abdominal tergite (see Fig. 16.5) come in contact with the female (S3). This triggers the hooking movement, which is to hang the epiphallus onto the subgenital plate of the female (R3). (4) When the male succeeds in hooking, the sensilla on the hook are stimulated (S4), and it pulls down the subgenital plate of the female (R4). (5) In response to the pulling down of the subgenital plate (S5), the female's copulatory papilla of the genitalia protrudes backward, which naturally enters the epiphallus (R5). Here, the unification of genitalia is attained. (6) If the unification continues at least for 4 s (S6), the dorsal pouch of the male's phallus is suddenly distorted so that the attachment plate of the spermatophore is extruded (R6). (7) Then, the median pouch and ventral lobes inflate with fluid and push up the spermatophore (R7). Finally, the attachment plate is pushed into the genital chamber of the female and fixed onto the convolutions of the upper surface of the genital chamber (R8) (Sakai and Kumashiro 2004a).

Fig. 16.4 Chain reaction in cricket copulation. Three steps (1–3) leading to success in hooking. Upper three figures (S1, S2, and S3) show locations (*gray*) of key stimulus detection by male, and lower three figures (R1, R2, and R3) show male responses to respective stimulation. In S1, tergite and wings are stimulated. In S2, distal region of cercus (d. cercus) is stimulated. In S3, proximal region of cercus (p. cercus) and epiproct (last abdominal tergite) are stimulated. *Large black arrows* show stimulus-response chain. Three steps (4–6) occur after success in hooking. Upper figures (S4, S5, and S6) show locations (*black*) of key stimulus detection. Lower figures (R4, R5, and R6) show responses to respective stimulation. *Large black arrows* show stimulus-response chain, while *large white arrows* show sequence of irreversible fixed actions (R6, R7, and R8). See text for labeling in (4)–(7)

The above sequences make up the chain reaction for copulation. Note that this sequence is always caused by the male and that the chain sequence proceeds in a reflexive manner. The female contributes to this sequence of events in only two
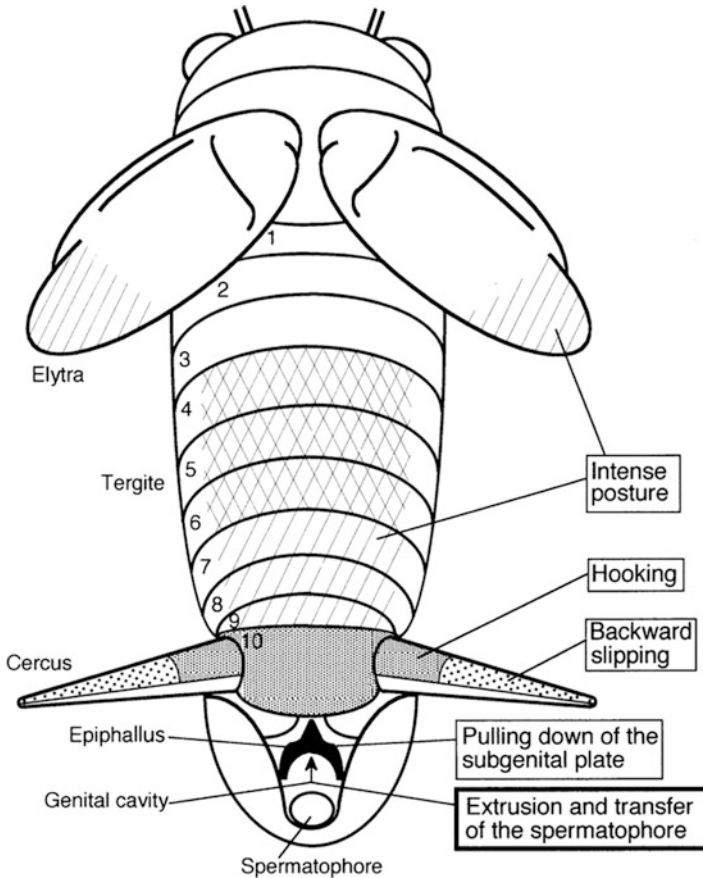
Fig. 16.5 Input and output relationships for copulatory acts in the male cricket. Each region as shown by the different patterns has mechanoreceptors to which contact stimulation elicits a specific motor response. Number (*1–10*) indicates abdominal segments. Reversible acts in early stage are shown in *thin-lined box* and irreversible fixed actions in later stage, in *thick-lined box*. See text for explanation

steps: mounting and copulatory papilla protrusion. The input and output relationships for the response chain are schematically summarized in Fig. 16.5 (Sakai and Kumashiro 2004a).

16.2.2.5 Abnormal Termination of the Mating Stage

While most of the matings result in a successful copulation, in some instances the mating stage ends prematurely due to the unusual ejection of the spermatophore (Sakai et al. 1991). There are three possible scenarios that can lead to this outcome (see their description below for details).

Pseudo-copulation

This scenario occurs when the male and the female are inadvertently separated during the final stage of copulation, genitalia coupling. The male ejects the spermatophore itself exhibiting the action of spermatophore transfer. The posture and movement are essentially the same as those of normal spermatophore transfer. Then, the male enters the sexually refractory stage. The change in the reproductive mode from the mating stage to the sexually refractory stage can be confirmed by observing the occurrence of spermatophore preparation (SP; see below).

Abortion

If the pairing female has a subgenital plate that is artificially closed with wax, the male cannot get genitalia coupling after hook hanging. In this case, the male will re-try the copulatory attempts. A similar behavior is naturally observed when the paired female is sexually reluctant and does not stay quiet on the copulating male. After a number of unsuccessful attempts, the male will eventually stop his activity. The male may recommence copulatory attempts after a long rest. Finally, the male ejects the spermatophore itself and scrapes off the substrate from the genital chamber. Then, the male enters the sexually refractory stage. In this case, however, it is uncertain when the reproductive mode was switched. Abortion certainly saves energy, as otherwise a male can spend unfruitful efforts for an extended period of time.

Spontaneous Cycle Renewal

When a male possessing a spermatophore is kept alone in an isolated condition, it keeps silent and becomes inactive. After a long time (1 or 2 days), the color of the spermatophore becomes brownish and seems to become nonfunctional in the genital chamber as it gets older. The male ejects the spermatophore without any previous sign and enters the sexually refractory stage.

16.2.3 Sexually Refractory Stage

As soon as the male extrudes the spermatophore, the sexually refractory stage begins. In this stage, the male shows a conspicuous behavior called guarding behavior (Fig. 16.1). The male also produces a new spermatophore for the next copulation. To make the spermatophore, the male first cleans the genitalia and then casts the spermatophore material into the phallic complex of the genital chamber. The latter process is called spermatophore preparation. That material soon changes into the complicated structure known as the spermatophore. The period between

spermatophore extrusion and spermatophore preparation is called RS1, while the period between spermatophore preparation and the start of calling song (or mating response) is called RS2 (see Fig. 16.1).

16.2.3.1 Guarding Behavior

During the sexually refractory stage, the male is generally aggressive even when exposed to the female. When the female moves around the male, the male attacks her and exhibits body shaking. This "guarding behavior" effectively protects the transferred spermatophore from being eaten by the female (Khalifa 1950; Loher and Rence 1978). The spermatophore is made of nutritious proteins (Kaulenas 1976) and serves not only as a vessel for sperm but also as a nuptial gift. The first 20 min after spermatophore transfer is critical for the male because it takes that long for the sperm to move into the female's spermatheca. So, the male is highly aggressive in that period to prevent the female from eating the spermatophore.

16.2.3.2 Genitalia Cleaning

As illustrated in Fig. 16.6, a dramatic change occurs in the genital chamber when the spermatophore is extruded from the male genitalia (Kumashiro and Sakai 2001a, b; Kumashiro et al. 2006, 2008). The central portion (median pouch) of the genital chamber floor expands with the body fluid and goes into the empty dorsal pouch serving as the template for the attachment plate of the spermatophore (Fig. 16.6(1–4)). The median pouch wriggles at a frequency of 0.16 Hz. This peristaltic movement is composed of a large shift to right and left, and small crease-like movements (Fig. 16.6(5)). These movements are involved in cleaning remnants of the previous spermatophore material or debris from the inside surface of the dorsal pouch before the spermatophore material is cast into the dorsal pouch. Every remnant or debris in the dorsal pouch is moved away by the cooperative movements of the median pouch and dorsal pouch, and this material is conveyed to the lateral pouch to be stored. This process is essential for the formation of the spermatophore (Kumashiro and Sakai 2016a, b).

16.2.3.3 Spermatophore Formation and Preparation

Spermatophore material excreted from the accessory glands and spermatozoa from the testes gradually consolidate in the dorsal pouch and the ventral lobes (Fig. 16.7 (3a-d)). The imnature spermatophore (Fig. 16.7(1)) develops into intricately organized spermatophore within 40 min (Fig. 16.7(2)). The actual mechanism of the spermatophore formation has not been inferred yet (Khalifa 1949; Gregory 1965; Mann 1984; Hall et al. 2000).
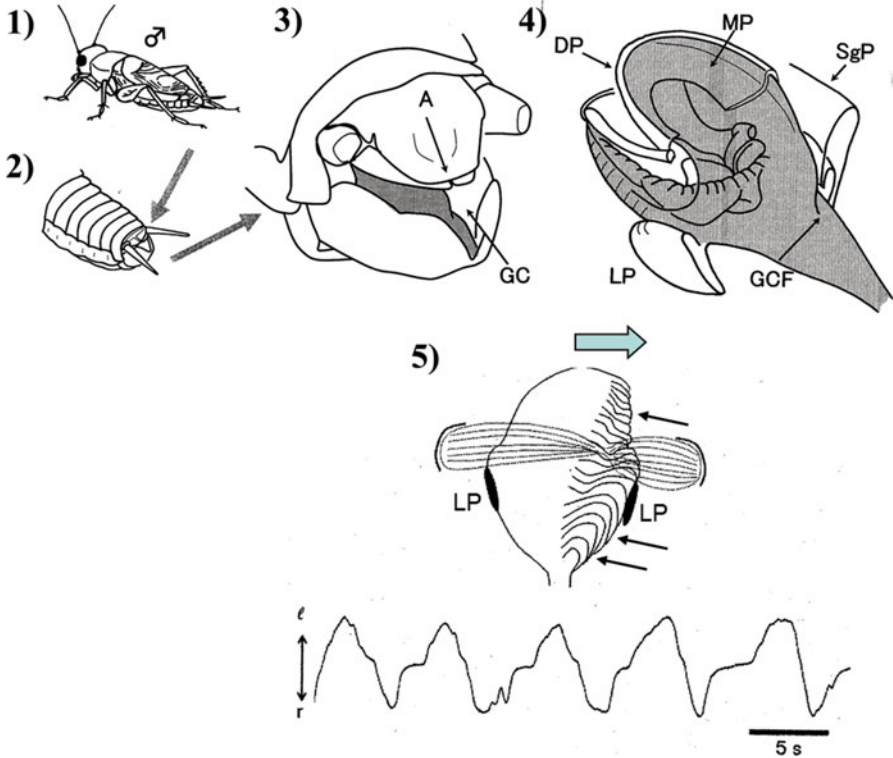
Fig. 16.6 Cleaning of the dorsal pouch following spermatophore extrusion. (*1–3*) The genital chamber (*GC*) below the anus (A) is located in the last abdominal segment of the male. (*4*) The genital membrane consists of the median pouch (*MP*) and genital chamber floor (*GCF*). The central region of the genital membrane in the last abdominal sternite (subgenital plate, *SgP*) forms a sack-like structure, the median pouch. It inflates and occupies the inside of the dorsal pouch (*gray portion at the *bottom*) in the stage between the end of copulation and the start of spermatophore preparation. *A* anus, *DP* dorsal pouch, *GC* genital chamber, *GCF* genital chamber floor, *LP* lateral pouch, *MP* median pouch. (*5*) Movement of the median pouch. The median pouch always shows undulation which consists of a large shift (*thick arrow*) to the right and left with small crease-like movements (*thin arrows*) at about 0.16 Hz. When the pouch leans to the right, a number of crease-like movements appeared on the right side of the median pouch. The *bottom* shows the large movement of the median pouch to the *right* (r) and *left* (l)

Spermatophore preparation is initiated as described by Ootsubo and Sakai (1992). When the male antennae are stimulated by contact with a female, spermatophore preparation occurs within approximately 4 min. It is preceded 45 s by the backward protrusion of the genital pouch (Fig. 16.7(3a)). The white spermatophore material is pushed onto the ventral lobes in the genital chamber (Fig. 16.7 (3b)). If the male does not encounter the female after copulation or the male is under heavy stress, spermatophore preparation does not occur for a long time (>1 h).
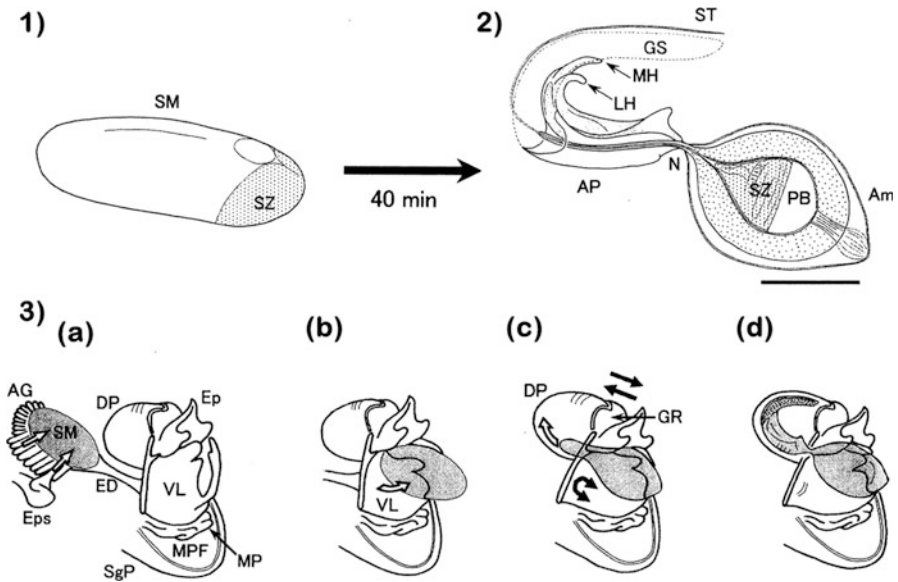
Fig. 16.7 Spermatophore formation. (*1*) Spermatophore material (SM) excreted from the accessory glands (*AG*) and spermatozoa (*SZ*) from the testis into the ejaculatory duct (*ED*). (*2*) Matured spermatophore. *Am* ampulla, *AP* attachment plate, *GS* gelatinous substance, *LH* lateral hook, *MH* median hook, *N* neck, *PE* pressure body, *ST* sperm tube, *Scale* 100 μm. (*3*) Sequence of spermatophore formation. (**a**) Excretion of SM and SZ into ED; (**b**) spermatophore preparation; (**c**) early stage of spermatophore formation; (**d**) matured spermatophore. *DP* dorsal pouch, *Ep* Epiphallus, *GR* guiding rod, *LP* lateral pouch, *MPF* median pouch floor (genital chamber floor)

16.2.3.4 Time-Fixed Interval in the Sexually Refractory Period

The male cannot recommence the calling until the spermatophore is completed. As long as the male is left alone, he does not produce the calling song (CS) even if the spermatophore is matured and the cricket is in the mating mode of the reproductive state. However, when paired with a female, the male starts calling and exhibits courtship behaviors. In the same way, when the male is presented with the mating response (MR) test (see Sect. 16.3.1) instead of a female, he begins copulatory acts to the model (see the legend for Fig. 16.1) at the same timing as CS. Thus, the time necessary to switch the reproductive mode from refractory to mating is defined as either SPMR (the time between spermatophore preparation and mating response) or SPCS (the time between spermatophore preparation and calling song).

It was found that the RS2 was time fixed when measured by SPCS (Nagao and Shimozawa 1987) differently from the first sexually refractory stage (RS1). The RS2 was measured by either SPCS or SPMR and found that it is 50–70 min long, with an average duration of 60 min (Ureshi and Sakai 2001). The RS2 varies among individuals but it is constant within an individual. This result suggested that the male may have a biological timer operating following the hourglass principle (Lees 1973), which starts at the occurrence of spermatophore preparation and stops

around the start of the mating response or the calling song. The RS2 is shortened at higher temperatures and lengthened at lower temperatures. Our 24-h observation indicated that the RS2 did not change throughout 1 day as long as the temperature was kept constant. In fact, one male showed copulation 22 times a day and renewed the male cycle 22 times. Incidentally, the RS2 varies in different species; for example, the length is 116 min in *Loxoblemmus doenitzi* Stein and 136 min in *Loxoblemmus campester* Matsuura.

16.3   Physiology of Reproduction

As demonstrated in multiple different behavioral studies, cricket mating behavior comprises biologically intriguing phenomena including behavioral switching, changes in sexual excitation states, and timekeeping functions. To understand these mechanisms, it is necessary to determine the actual neuronal structure (s) responsible for each function. This has been done by using classical methods such as artificial stimuli combined with ablation and drug application.

16.3.1   Control of Copulatory Actions

When in the mating stage, the male displays the mating response (MR) consisting of body thrusts and hooking (see Sect. 16.2.2.4) in response to the application of the female model to the last abdominal tergite of the male. Sometimes, though, the male shows an evasion response to the same model. This observation suggests that the responsiveness to the model may change depending upon the male's internal state even in the mating stage. In general, the internal state can be described in terms of inhibition or excitation. For example, the MR is suppressed by inhibition when the male is under heavy stress. In contrast, the MR is elicited intesely by excitation when the male is sexually excited.

16.3.1.1   Inhibition

Classical experiments showed that while decerebrated males (lacking the brain) exhibited an evasion response to the female model, decapitated males (lacking the brain and subesophageal ganglion) showed the MR (Huber 1955). This result led to the conclusion that the inhibition center for the MR is located in the subesophageal ganglion (Huber 1955), similar to the situation in the praying mantis (Roeder 1935). More recent studies, however, exploring the effects of subesophageal ganglion ablation, did not agree to Huber's conclusion: the center for inhibition of the MR is actually located in the brain (Matsumoto and Sakai 2000a). In light of this new

insight, we now recognize three different types of mating inhibition, which are described below.
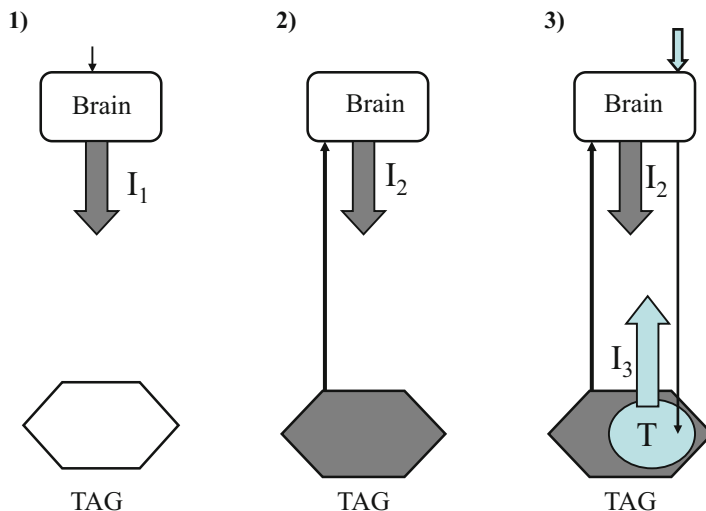
Inhibition in the Mating Stage

In general, the male in the mating stage elicits the MR when stimulated with the female model. If, however, the male receives a noxious stimulus, such as a strong pinch to the leg or an antenna, he ceases to exhibit the MR and instead shows the evasion response (Matsumoto and Sakai 2000a, b). On the other hand, the male which had a leg pinching quickly recovers the ability to perform the MR when his head is cooled, chopped off, or ligated with a thread. To take advantage of such inhibitory and disinhibitory phenomena, male responsiveness was compared between decerebrated males and subesophageal ganglion-ablated males. These experiments showed that decerebrated males can quickly recover the MR after leg pinching. In contrast, the subesophageal ganglion-ablated males continued to display the evasion response after leg pinching. Hence, these results reveal that the inhibition center for the MR is located in the brain and not in the subesophageal ganglion (Fig. 16.8(1)). In addition, when one of the two connectives was cut between the head and thorax, normal mating behavior, including courting and copulation, was observed to the female. These individuals, however, were unable to inhibit the MR after leg pinching, similar to the decerebrate males. Hence, the loss of even half of the descending inhibitory input, mediated by the axons running through the connective, is enough to release the MR.

Inhibition in the RS1

In the first sexually refractory stage (RS1), the intact male never shows the MR. The mating response is elicited after the two connectives are cut at the head-thorax boundary (Matsumoto and Sakai 2000a, b). This fact suggests that brain inhibition on the MR is continuously exerted through the RS1. Furthermore, males with one of the connectives cut also had a difficulty in suppressing the MR. At first, this would appear similar to the situation in males at the mating stage whose connectives were cut following the noxious stimulation. However, males in RS1 also had a difficulty in suppressing the MR when an abdominal connective between the 6th abdominal ganglion and the terminal abdominal ganglion (TAG, 7th to 9th fused ganglia) (Fig. 16.8(2)).

These results reveal that the disinhibition that arises after cutting a connective at the head-thorax boundary is not solely dependent upon descending inhibition from the brain. This descending inhibition is commanded by an ascending signal from the TAG. The combined insight from the above experiments suggests that the reproductive mode in the TAG is switched to the refractory mode at spermatophore extrusion, and this signal is transmitted continuously to the brain through ascending connections which, in turn, inhibits the pattern generator for the MR through

Fig. 16.8 Schematic diagrams of 3 types of inhibition. (*1*) Inhibition of mating response in the mating stage which is induced by noxious stimulation. This inhibition (I_1) of the brain is transient. (*2*) Inhibition of mating response in the sexually refractory stage (RS1) between spermatophore extrusion and spermatophore preparation. This inhibition (I_2) is exerted by the brain. The brain is tonically driven by the tonic input from the terminal abdominal ganglion (TAG) which is in the sexually refractory mode. (*3*) Inhibition of mating response in the sexually refractory stage (RS2) between spermatophore preparation and the recommencement of mating response. In this period, inhibition (I_3) commanded by the timer (T) in the TAG operates in addition to the brain inhibition (I_2). Two downward arrows to the right show the start of spermatophore preparation triggered by female stimulation to the antenna.

descending inhibition (I_2 in Fig. 16.8(2)). This scenario is consistent with the idea that the primary cause of post-copulatory inhibition resides in the TAG (Sakai et al. 1995).

Inhibition in the RS2

In the second sexually refractory stage (RS2), males do not show the MR even after decerebration, which is different from the responsiveness in the RS1. Such a difference suggests a presence of another inhibition system for the MR. Furthermore, males decerebrated just after spermatophore preparation which did not respond to the model began to respond as time elapses (>20 min after decerebration following spermatophore preparation; Sakai et al. 1995). A similar time dependency was observed even in a small isolated body region consisting of a few posterior abdominal segments which contain the TAG only. This body region which had not responded to the model began to show the movement characteristic of the MR 20 min after abdominal transection. These results suggest that the

primary cause of time-dependent inhibition in RS2 is located in the TAG (I3, Fig. 16.8(3)).

16.3.1.2 Facilitation

The male responds more easily and persistently to the model when he is sexually excited. Specifically, the body thrust and hooking actions change depending upon the duration of preceding courtship (Matsumoto and Sakai 2001). The longer the male's courting of the female, the more intense the MR will be. This causal relationship can be further examined by measuring the movement parameters in decerebrate males. The duration of the MR in response to a single stimulus becomes longer, and the number of body thrusts increases as well. In addition, the time per individual body thrust (cycle length) becomes shortened. That is, the frequency of the body thrust per stimulus increases. These results reveal that the excitatory level of the nervous system underlying the MR has been facilitated during courtship.

Pharmacological experiments have elaborated on our knowledge of facilitation. Drugs injected into the abdomen of decerebrate males were tested with the model and the MR was assessed. Injection of octopamine resulted in shortened cycle length. Similarly, octopamine agonists such as synephrine and forskolin also shortened the cycle length. On the other hand, an antagonist, phentolamine, increased the cycle length. A similar facilitatory effect was induced by electrical stimulation of the connectives at the head-thorax boundary. These results suggest that some descending neurons from the head ganglia may release octopamine to facilitate the activity of the pattern generator for the MR, which is located in the thoracic and abdominal ganglia.

16.4 Neural Activity Related to Reproductive Behavior

The previous classical physiological studies indicate that the brain and the TAG are the key structures that control male behavior in the reproductive cycle. Here we review recent advancements in our understanding of how neural activity affects different aspects of this behavior.

16.4.1 Neural Activity Responsible for Spermatophore Extrusion

The spermatophore is extruded when the sensilla of the epiphallus are mechanically stimulated with the model mimicking the copulatory papilla of the female (Sakai et al. 1991). Specifically, there are relatively larger bristle sensilla on the outside of

the epiphallus, and smaller sensilla (cavity hairs) are found on the inside of the epiphallus. Sensory neurons innervating both groups of sensilla project their axons to the TAG. On the motor side, the attachment plate of the spermatophore is ejected by the deformation of the dorsal pouch in the genitalia. This is caused by the contraction of the dorsal pouch muscles, each of which is innervated by a single dorsal pouch motoneuron (mDP) in the TAG (Kumashiro and Sakai 2001a, b).

Figure 16.9 shows suction electrode recordings of the genital nerve in which spikes of both sensory and motor nerves can be observed (Sakai and Kumashiro 2004b). Stimulation of the outside hairs of the epiphallus (dotted line) was not enough to activate the mDP (Fig. 16.9(1)). In contrast, stimulation of the cavity hairs activated several motor neurons including the mDP (larger spikes below solid bar) with a long latency of several seconds (Fig. 16.9(2)). When the outside hairs and cavity hairs were stimulated simultaneously, the mDP discharged with a shorter latency to elicit spermatophore extrusion (Fig. 16.9(3)). These results indicate that
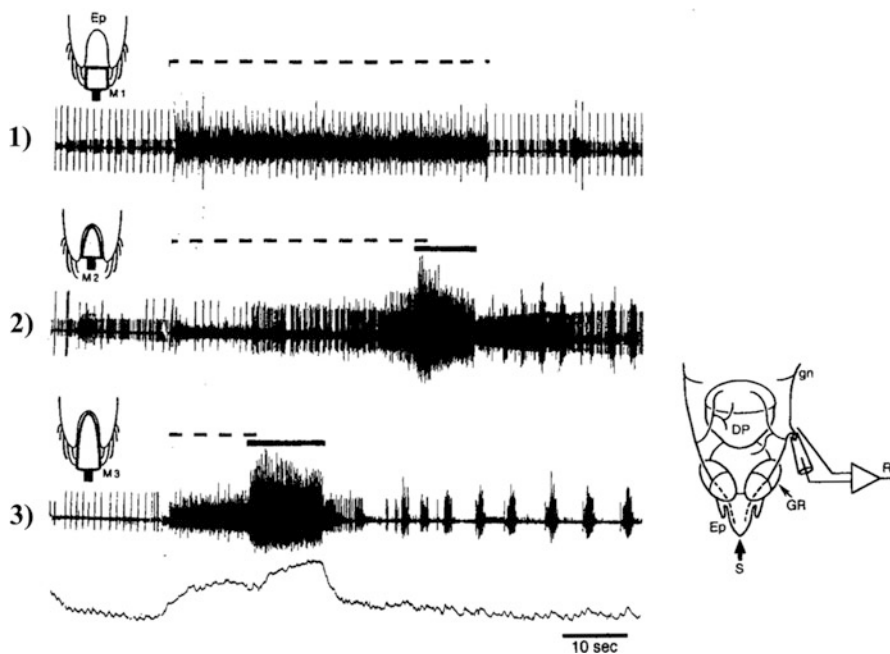
Fig. 16.9 Responses to stimulation of epiphallic sensilla with a model of the female copulatory papilla. (*1*) Response to stimulation (*broken bar*) of the outer hairs (M1) on the epiphallus (Ep) with a model. Sensory component continued to discharge for 50 s but dorsal pouch contraction was not triggered. (*2*) Responses to stimulation of cavity hairs (M2). Spermatophore extrusion and transfer (*solid bar*) was triggered after 39 s as shown by additional responses of genital motoneurons (below *solid bar*). This preparation was the same as in (*1*). (*3*) Responses to stimulation of both cavity hairs and outer hairs (M3). Spermatophore extrusion was triggered 11 s after the onset of stimulation. This preparation was different in (*1*) and (*2*). The *bottom line* is the movement of the genitalia. Right inset shows setups for recording (See Fig. 16.6 legends for abbreviations)

cavity hairs are of primary importance for spermatophore extrusion, while outside hairs play a facilitatory role.

The mDP was morphologically identified (Fig. 16.10(1)) by backfilling with cobalt and nickel (Sakai and Yamaguchi 1983), and its spike activity was singly recorded (Sakai and Kumashiro 2004b; Fig. 16.10(2)). The characteristic feature of this neuron is that the burst discharge occurs abruptly more than 10 s after the start of stimulation. This suggests that some high threshold interneuron(s) may be interposed between the sensory afferent and the mDP. This burst discharge causes the jerky contraction of the dorsal pouch muscle for spermatophore extrusion (see the deflection in the bottom trace).

16.4.2 Neural Activity Responsible for Genitalia Cleaning

After the initial bursting, the mDP firing pattern changes into spontaneous rhythmic activity (Fig. 16.10(2)). The activity continued even after the TAG was isolated by cutting the connectives between the 6th abdominal ganglion and the TAG. These results reveal that rhythmic activity is generated in the neural circuit of the TAG. The ensuing periodical dorsal pouch contractions serve for cleaning the inside of the dorsal pouch in cooperation with the rhythmic movements of the median pouch (Kumashiro and Sakai 2016b).

### 16.4.3 Pattern Generator for Spermatophore Preparation

Spermatophore preparation is a critical event for preparing for a new mating stage. That is, the 1-h timer starts and the RS2 begins. However, the trigger mechanism is totally unknown at the neural level. The chemosensory stimulus of contact with a female activates brain neurons, which send the command to the TAG to trigger spermatophore preparation. Even if both the connectives are cut, at any level, immediately after the pouch protrusion, spermatophore preparation occurs normally 45 s later (Ootsubo and Sakai 1992). This means that the neural circuit for spermatophore preparation is entirely located within the TAG. The brain is only needed to trigger the pattern generator for spermatophore preparation, though this can occur spontaneously, without the trigger stimulation, as well. It is interesting to note that DUM (dorsal unpaired median) neurons in the TAG are also responsible for the excretion of the spermatophore material from the accessory glands to the ejaculatory duct (Yasuyama et al. 1988; Kimura et al. 1989). Thus, the control system of DUM neurons may have a close relation to the pattern generator for spermatophore preparation.
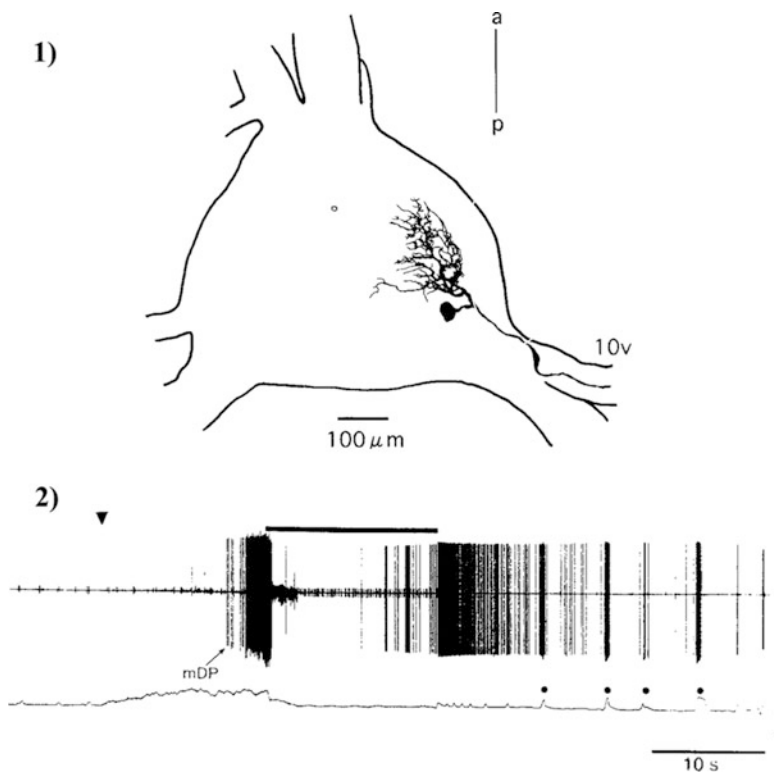
Fig. 16.10 Dorsal pouch motoneuron. (*1*) Morphology of the dorsal pouch motoneuron. *a* anterior, *p* posterior, *10v* the 10th ventral nerve root (cercal motor nerve) of the TAG. (*2*) Dorsal pouch motoneuron (mDP) discharge in normal spermatophore extrusion. The discharge pattern of the mDP (*top trace*) around spermatophore extrusion was elicited by genital stimulation. The start of stimulation is shown by *arrowhead*. The mDP did not respond in the first 13 s, but then exhibited a strong burst at spermatophore extrusion and gradually changed into rhythmic bursts after the spermatophore transfer phase (*horizontal line*). Bottom trace shows movement of the phallic complex in which each upward deflection (*dot*) indicates the rhythmic movement of the phallic complex

16.4.4 Neural Activity Associated with the 1-h Timer

For the time-fixed RS2, the previous experiments suggested that the center for time-dependent inhibition is located in the TAG. To obtain further evidence, reversible inactivation was carried out by cooling the nervous system. During the RS2, cooling either the brain, thorax, or abdomen for 30 min resulted in local inactivation of the central nervous system. Cooling at any of these locations did not lengthen the RS2. However, when the abdominal segment containing the TAG was cooled for 30 min, the RS2 was lengthened by 30 min. This demonstrates that the timer is located within the TAG (Ureshi and Sakai 2001). This is also in line with the
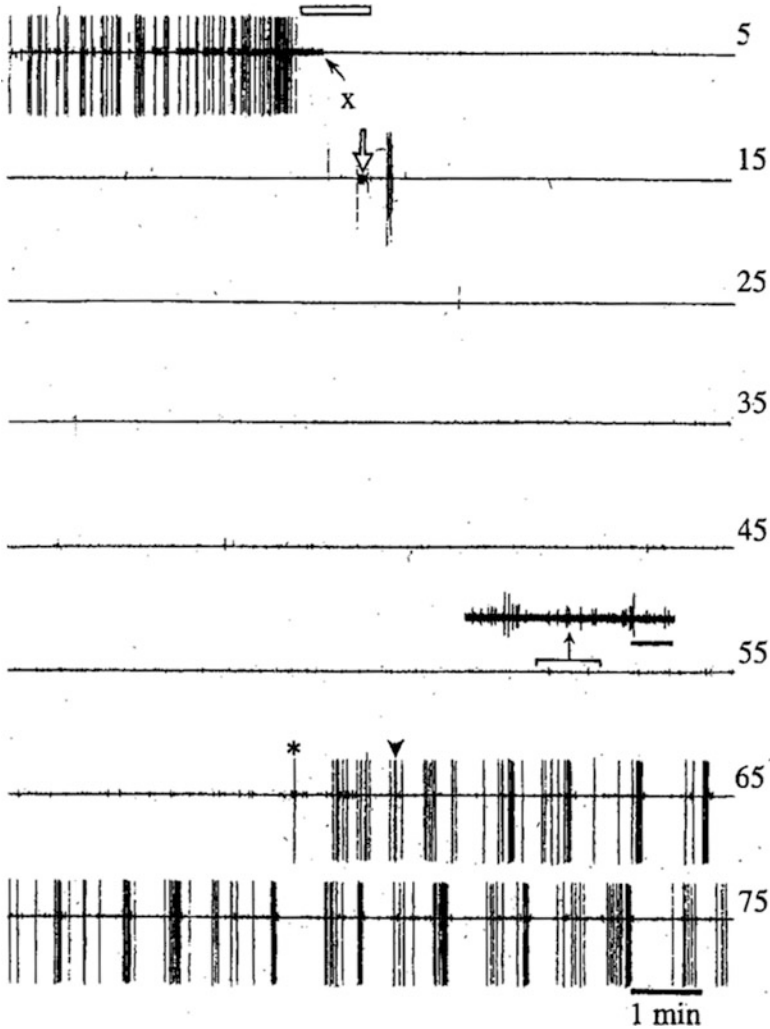
Fig. 16.11 Time-dependent activity of mDP in males with the isolated terminal abdominal ganglion. *Numbers on the right* indicate time (min) after spermatophore preparation. *White horizontal* bar on the first line indicates period between subgenital plate opening (*left end*) and spermatophore preparation (*right end*). *x* indicates small-sized spikes. The *open arrow on the second line* indicates the time of connective cut. The *asterisk* indicates the first spike of the mDP after the long silence. *Arrowhead* indicates the onset of auto-spermatophore extrusion. Inset between lines labeled 45 and 55 shows an enlarged recording of the indicated portion in which tiny spikes with at least three different amplitudes are discerned. Scale bar for *inset*, 10 s. See text for explanation

observation that the dorsal pouch motoneuron (mDP) showed time-dependent activity in the isolated TAG. Furthermore, the mDP, which is silent just after spermatophore preparation, automatically started to discharge one hour later (Fig. 16.11; Kumashiro and Sakai 2003). While it is currently unknown how the

time-dependent activity of the mDP is controlled by the neuron(s) responsible for the timekeeping, there is some indication that serotonin may be involved in this process (Ureshi and Sakai 2002).

16.5   Development of Mating Behavior

While studying mating behavior was traditionally focused exclusively on adults, very little is understood about how postembryonic development might contribute to mating. In general, most of the behavioral repertoires in the larvae of hemimetabolous insects are similar to those of adults, except for flying and mating. Hence, there is a possibility that cricket larvae may already be equipped with the pattern generators for flight and mating, but they cannot exhibit such behavior because the command system is not yet operational. This scenario is supported by the classical studies in *Teleogryllus*, in which nymphs gain the ability to display mating behavior only after the ablation of the mushroom body of the brain, which is involved in inhibition (Bentley and Hoy 1970). However, as this was not the case in *Gryllus bimaculatus*, there may be some species-specific differences in this regard.

16.5.1   Mating Behavior in Nymphs

In contrast to the previous study in *Teleogryllus* (Bentley and Hoy 1970), it was reported that intact nymphs of *Gryllus bimaculatus* can exhibit mating-like actions, i.e., courtship-like behavior (CSLB) and copulation-like behavior (CPLB). The males become adults (ninth instar) after eight rounds of ecdysis. Nymphs at the eighth instar showed both CPLB and CSLB when they were paired with an adult female or male (Sakai et al. 1990). Both actions occurred at higher frequency in the middle stage of the eighth instar than in the beginning and the end. Nymphs at the seventh instar showed CSLB exclusively and much less frequently than in the eighth instar. Those at the sixth instar showed neither behavior. The movement patterns of their mating-like actions are essentially the same as those seen in adults. However, the frequency of their occurrence was less and their duration was shorter than in adults. Nymphs are very sensitive to environmental noises, and they easily stop mating-like actions and do not recommence for a long time. Since the nymph genitalia are immature, the spermatophore preparation does not occur. Thus, the male cycle or the time-fixed sexually refractory stage cannot be detected. The observations that adult-type mating actions can occur in nymphs reveal that the command system for mating behavior is already operational in nymphs. Instead, it suggests that though the mushroom bodies in the brain certainly exert inhibition, this can be canceled by the gradual increase in sexual excitation during development.

16.5.2   Mating Behavior in Fresh Adults

Adult males do not show mating-like or mating actions for a few days following the final molt. Afterward, the mating responsiveness of fresh adult males develops gradually day by day. For example, only 30 % of fresh adults exhibited some copulatory actions on the day of the final molt (day 1). In contrast, most individuals display the body thrust under the female, and hooking appears on day 2. Similarly, spermatophore extrusion and transfer becomes fully successful on day 3. Following this, mating behavior further develops to reach a mature level after 1 week. When combined with the observations in nymphs, we can now postulate that the sexual excitation gradually increases to facilitate mating behavior after the final molt.

For future studies of insect reproduction, it would be interesting to compare every aspect of male behavior between *Gryllus bimaculatus* and other cricket and orthopteran species. The nature of the sexual refractory period, especially the time-fixed RS2, should be examined for its length and consistency in a comparative context. Since the RS2 is associated with the process of spermatophore production, it should also be correlated with the structure and function of the spermatophore. Finally, the neural mechanism of the male sexual state and its switching in the reproductive cycle should be cleared.

To do so, is important to understand the nature of the communication between the brain and the terminal abdominal ganglion, which could be accomplished by recording descending and ascending spike activity. In addition, the neuron (s) responsible for the one-hour timer should be identified in the terminal abdominal ganglion. The molecular mechanism in these neurons should be elucidated, as has been done in the study of circadian clocks. With the advancement of new functional and genomic tools in *Gryllus*, such as those described in this book, these questions can soon start to be addressed.

References

Adamo SA, Hoy RR (1995) Agonistic behavior in male and female field crickets, *Gryllus bimaculatus*, and how behavioural context influences its expression. Anim Behav 49:1491–1501

Alexander RD (1961) Aggressiveness, territoriality, and sexual behavior in field crickets (Orthopera: Gryllidae). Behaviour 17:130–223

Alexander RD, Otte D (1967) The evolution of genitalia and mating behavior in crickets (Gryllidae) and other Orthoptera. Misc Publ Zool Univ Mich 133:12–18

Beck R (1974) The neural and endocrine control of mating behavior in the male house cricket, *Acheta domesticus*. L. Ph.D. thesis University of Nottingham

Bentley DR (1969) Intracellular activity in cricket neurons during the generation of song patterns. J Insect Physiol 15:677–699

Bentley DR, Hoy RR (1970) Postembryonic development of adult motor pattern in crickets: a neural analysis. Science 170:1409–1411

Choperd L (1951) Les divisions du genre *Gryllus* basees sur l'etude de l'appareil copulateur (Orth. Gryllidae). Eos 37:267–287

Evans AR (1988) Mating systems and reproductive strategies in three Australian Gryllid crickets: *Bobilla victoriae* Otte, *Balamara gidya* Otte and *Teleogryllus commodus* (Walker)(Orthoptera: Gryllidae: Nemobiinae; Trigonidiinae; Gryllinae). Ethology 78:21–52

Gregory GE (1965) The formation and fate of the spermatophore in the African migratory locust, *Locasta maigratoria migraroides* Reiche and Fairmaire. Trans R Entomol Soc Lond 117:33–36

Hall MD, Beck R, Greenwood M (2000) Detailed developmental morphology of the spermatophore of the Mediterranean field cricket, *Gryllus bimaculatus* (De Geer)(Orthoptera: Gryllidae). Arthropod Struct Dev 29:23–32

Hörmann-Heck S (1957) Untersuchungen uber den Erbgang einiger Verhaltensweisen bei Grillenbastarden (*Gryllus campestris* L., *Gryllus bimaculatus* DeGeer). Z Tierpsychol 14:137–183

Hsu YC (1929) Crickets in China. Peking Soc Nat Hist Bull 3:5–41

Huber F (1955) Sitz und bedeutung nervöser Zentren für Instinkthandlungen beim Männchen von *Gryllus bimaculatus* DeGeer. Z Tierpsychol 12:12–48

Huber F, Moor TE, Loher W (1989) Cricket behavior and neurobiology. Cornell Univ Press, Ithaca, pp 340–363

Iwasaki M, Delago A, Nishino H, Aonuma H (2006) Effects of previous experience on the agonistic behavior of male crickets, *Gryllus bimaculatus*. Zool Sci 23:863–872

Kaulenas MS (1976) Regional specialization for export protein synthesis in the male cricket accessory gland. J Exp Zool 195:81–96

Khalifa A (1949) The mechanism of insemination and the mode of action of the spermatophore in *Gryllus domesticus*. Q J Microsc Sci 90:281–292

Khalifa A (1950) Sexual behavior in *Gryllus domesticus* L. Behaviour 2:264–274

Kimura T, Yasuyama K, Yamaguchi T (1989) Proctolinergic innervation of the accessory gland in male crickets (*Gryllus bimaculatus*): detection of proctolin and some pharmacological properties of myogenically and neurogenically evoked contractions. J Insect Physiol 35–3:251–265

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

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

Kumashiro M, Sakai M (2003) Auto-spermatophore extrusion in male crickets. J Exp Biol 206:4507–4519

Kumashiro M, Sakai M (2016a) Genital autocleaning in the male cricket Gryllus bimaculatus (1): Structure and function of the genital membrane. Zool Sci 33 in press.

Kumashiro M, Sakai M (2016b) Genital autocleaning in the male cricket Gryllus bimaculatus (2): Rhythmic movements of the genitalia and their motor control. Zool Sci 33 in press.

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

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

Lees AD (1973) Photoperiodic time measurement in the aphid *Megoura viciae*. J Insect Physiol 19:2279–2316

Loher W, Rence B (1978) The mating behavior of *Teleogryllus commodus* (Walker) and its central and peripheral control. Z Tierpsychol 46:225–259

Loher W, Weber T, Huber F (1993) The effect of mating on phonotactic behavior in *Gryllus bimaculatus* (De Geer). Physiol Entomol 18:57–66

Mann T (1984) Spermatophores. Springer-Verlag, Berlin, pp 107–111

Matsumoto Y, Sakai M (2000a) Brain control of mating behavior in the male cricket *Gryllus bimaculatus* DeGeer: the center for inhibition of copulation actions. J Insect Physiol 46:527–538

Matsumoto Y, Sakai M (2000b) Brain control of mating behavior in the male cricket *Gryllus bimaculatus* DeGeer: brain neurons responsible for inhibition of copulation actions. J Insect Physiol 46:539–552

Matsumoto Y, Sakai M (2001) Brain control of mating behavior in the male cricket *Gryllus bimaculatus* DeGeer: excitatory control of copulatory actions. Zool Sci 18:659–669

Muller M, Wehner R (1994) The hidden spiral: systematic search and path integration in desert ants, *Cataglyphis fortis*. J Comp Physiol A175:525–530

Nagao T, Shimozawa T (1987) A fixed time-interval between two behavioral elements in the mating behavior of male crickets, *Gryllus bimaculatus*. Anim Behav 35:122–130

Nishino H, Sakai M (1996) Behaviorally significant immobile state of so-called thanatosis in the cricket *Gryllus bimaculatus* DeGeer: its characterization, sensory mechanism and function. J Comp Physiol 179:613–624

Ogawa Y, Sakai M (2009) Calling and courtship behaviors initiated by male-male contact via agonistic encounters in the cricket *Gryllus bimaculatus*. Zool Sci 26:517–524

Ootsubo T, Sakai M (1992) Initiation of spermatophore protrusion behavior in the male cricket *Gryllus bimaculatus* DeGeer. Zool Sci 9:955–969

Palka J, Levine R, Schubiger M (1977) The circus-to-giant interneuron system of crickets. I. Some attributes of the sensory cells. J Comp Physiol 119:267–283

Pollack GS, Hoy RR (1989) Evasive acoustic behavior and its neurobiological basis. In: Huber F, Moor TE, Loher W (eds) Cricket behavior and neurobiology. Cornell Univ Press, Ithaca, pp 340–363

Rence R, Loher W (1977) Contact chemoreceptive sex recognition in the male cricket, *Teleogryllus commodus*. Physiol Entomol 2:225–236

Roeder DK (1935) An experimental analysis of the sexual behavior of the praying mantis (M.L.). Biol Bull 69:203–220

Sakai M, Katayama T, Taoda Y (1990) Postembryonic development of mating behavior in the male cricket *Gryllus bimaculatus* DeGeer. J Comp Physiol 166:775–784

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

Sakai M, Kumashiro M (2004b) Auto-spermatophore extrusion reveals that the reproductive timer functions in the separated terminal abdominal ganglion in the male cricket. Acta Biol Hung 55 (1-4) 113–120

Sakai M, Matsumoto Y, Takemori N, Taoda Y (1995) Post-copulatory sexual refractoriness is maintained under the control of the terminal abdominal ganglion in the male cricket *Gryllus bimaculatus* DeGeer. J Insect Physiol 41:1055–1070

Sakai M, Ootsubo T (1988) Mechanism of execution of sequential motor acts during copulation behavior in the male cricket *Gryllus bimaculatus* DeGeer. J Comp Physiol A 162:589–600

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

Sakai M, Yamaguchi Y (1983) Differential staining of insect neurons with nickel and cobalt. J Insect Physiol 29:393–394

Shimozawa T, Kanou M (1984) Varieties of filiform hairs: range fractionation by sensory afferents and cercal interneurons of a cricket. J Comp Physiol A 155:485–493

Simmons LW (1986) Female choice in the field cricket *Gryllus bimaculatus* (De Geer). Anim Behav 34:1463–1470

Stevenson PA, Hofmann HA, Schoch K, Schildberger K (2000) The fight and flight responses of crickets depleted of biogenic amines. J Neurobiol 43:107–120

Tinbergen N (1951) The study of instinct. The Clarendon Press, Oxford

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

Ureshi M, Sakai M (2002) Serotonin precursor (5-hydroxytryptophan) has a profound effect on the post-copulatory time-fixed sexually refractory stage in the male cricket, *Gryllus bimaculatus* DeGeer. J Comp Physiol A 188:767–779

Weber T, Thorson J, Huber F (1981) Auditory behavior of the cricket. I. Dynamics of compensated walking and discrimination paradigms on the Kramer treadmill. J Comp Physiol 141:215–232

Yasuyama K, Kimura T, Yamaguchi T (1988) Musculature and innervation of the internal reproductive organs in the male cricket, with special reference to the projection of unpaired median neurons of the terminal abdominal ganglion. Zool Sci 5:767–780