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Mating Behavior, Insemination and Sperm Transfer in the Ground Beetle *Carabus insulicola*

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ABSTRACT—Mating behavior and the processes of insemination and sperm transfer in the ground beetle *Carabus insulicola* were analyzed. *C. insulicola* has elaborate genitalia, in which the strongly sclerotized male copulatory piece is inserted into the female vaginal appendix *in copula*. During mating, I observed pre-copulatory struggles of males and females, as well as delays in ejaculation, suggesting the presence of intersexual conflicts. Insemination was achieved with a spermatophore, which strongly adhered to the openings of the spermatheca, common oviduct, and vaginal appendix. The spermatophore dissolved after copulation, and sperm were transferred into the spermatheca within three hours after copulation. Sperm bundles were contained within the testes and spermatophores, but free spermatozoa were found in the spermatheca.

Key words: Carabidae, genitalia, sexual selection, spermatophore, sperm bundle

INTRODUCTION

Insemination and sperm transfer are important processes that enable male insects to fertilize eggs with their sperm (Parker, 1970; Choe and Crespi, 1997). Although these internal processes are often difficult to observe directly, it is conceivable that males developed strategies to enhance their fertilization success. Many authors have proposed these processes and their evolutionary consequences; e.g., sperm competition causes modification of morphological and behavioral traits in many insect species (Simmons and Siva-Jothy, 1998), intersexual conflict promotes coevolutionary divergence between male and female water striders (Arnqvist and Rowe, 2002). To elucidate the nature of sexual selection that may work in the processes of insemination and sperm transfer, it is vital to have descriptions of these processes.

In Coleoptera, the processes of insemination and sperm transfer have been analyzed in several families (Cicindelidae, Freitag *et al*., 1980; Rodriguez, 1998; Tenebrionidae, Bloch-Qazi *et al*., 1996; De Villiers and Hanrahan, 1991; Bruchidae, Boucher and Huignard, 1987; Staphylinidae, Gack and Peschke, 1994; Chrysomelidae, Dickinson, 1997; Eberhard and Kariko, 1996; Lew and Ball, 1980; Coccinellidae, Katakura, 1985; Kaufmann, 1996; Obata, 1987; Lampyridae, Van der Reijden *et al*., 1997; Rooney and Lewis, 1998; Scarabaeidae, Eberhard, 1993a, b). Generally, male beetles inseminate females with a spermatophore or its equivalent. In some species, the females digest the spermatophores as nutrients (Boucher and Huignard, 1987; Rooney and Lewis, 1998). In the Tenebrionidae, sperm displacement occurs during insemination by subsequent males (De Villiers and Hanrahan, 1991; Gack and Peschke, 1994; Gage, 1992). During mating in some species, males court the female by rubbing her with their legs or other body parts, or by moving their genitalia (Eberhard, 1993a, b; Eberhard and Kariko, 1996; Rodriguez, 1998).

Unlike these Coleopteran families, little has been known for the mating behavior and processes of insemination and sperm transfer in the Carabidae (but see Alexander, 1959, Takami, 2000). *Carabus insulicola* is a medium-to-large (27–31 mm in female body length, Sota *et al*., 2000) ground beetle species belonging to the Carabidae, and is distributed in northeastern Honshu, Japan. Male beetles have a sclerotized, hook-like intromittent organ on the aedeagus, the copulatory piece, and females have a membranous pocket in the vagina, the vaginal appendix, as a counterpart to the copulatory piece (Ishikawa, 1987). The exaggeration of male genitalia may be related to their function during the mating sequence, and the function may be subject to sexual selection (Eberhard, 1985). In this study, I describe and analyze the external and internal process of the mating of *C. insulicola* to find the arena in which sexual selection may operate.

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MATERIALS AND METHODS

Organism preparation

C. insulicola breeds in the spring (Sota, 1985). Overwintered adults mate and reproduce from May to July, larvae mature during the summer, and new adults emerge in late summer and autumn. New adults then overwinter as virgins. Sexually active beetles were caught in pitfall traps during the mating season, from May to July of 1998–2001, at Tsukui-machi, Kanagawa Prefecture, Japan. Males and females were kept separately in plastic containers (12.5 ×12.5×9 cm) with moistened moss, and these were stored in a breeding incubator (20°C and 16L: 8D). Beetles were fed with minced beef and apple every two days to maintain sexual maturity (Sota, 1986). To diminish the effect of previous matings in the wild, beetles were held for at least 2 days after field collection until mating experiments. Virgin beetles were caught in pitfall traps in September of 1998–2000. Virgins were housed in an overwintering incubator (5°C and no light). They were transferred to the breeding incubator the following spring, and sexual maturation was induced for at least 18 days as described over. For all matings in this study, a male and a female were randomly chosen and introduced into a plastic container at 23±2°C under room light conditions. Pairs that did not begin mating within 10 min were returned to the breeding containers for use in later experiments.

External events

To investigate the behavioral sequence of mating, conspicuous behavioral characteristics were recorded during copulation, in which both of mated and virgin beetles were used. Durations from mounting to aedeagal insertion, and of copulation and post-copulatory amplexus were measured. Averages±one standard deviation were calculated.

Internal events

The process of insemination was investigated by fixing mating pairs in liquid nitrogen *in copula*. Fixing time was 20, 40, or 60 min after the beginning of copulation. Then, the male and female genitalia were gently removed from the bodies, and the conditions of genital coupling and ejaculates were examined under a binocular microscope.

The process of sperm transfer and storage and the temporal change in the ejaculates were examined as follows. Pairs of *C. insulicola* were allowed to mate; I used females caught in spring and autumn. Mated females were either frozen immediately or held in the breeding incubator for 3, 6, 12, 18, or 24 hr after copulation before being frozen. The vagina was then dissected. Ejaculate was removed, placed onto a small piece of aluminum foil, and weighed with an electric balance to the nearest 0.1 mg; it was then observed on a slide glass with a binocular microscope. In females that had been virgins prior to the experiment, the spermatheca was removed from the vagina and dissected on the slide glass with Ringer's solution to examine the presence of transferred sperm. For comparison with spermatophore weight, the bodies of males obtained from the field population were weighed.

Ejaculates

Mated females that were used in the study of mating behavior were dissected to examine the ejaculates. For comparison, males were also dissected to examine sperm within the testis. The morphology and location of ejaculates in the vagina were observed in a petri dish filled with Ringer's solution, and photographed with a camera lucida attached to a binocular microscope. Sperm within the ejaculates, spermatheca, and testis were also examined on a slide glass under the binocular microscope (100×), and photographed. For precise observation of sperm morphology, I used Giemsa staining: sperm were spread on a slide glass, dehydrated with 99.5% ethanol for 20 min, processed in Giemsa staining solution for 30 min, washed with water, and dried for microscopic observation. Stained sperm were photographed, and these were scanned to produce digital images. The sperm were measured on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

RESULTS

External events

Seventy-eight trials for mating were performed using 78 males and 69 females, in which 9 females were used more than once, but were actually mated only once. Of 69 females, 24 and 45 were mated and virgin females, respectively.

A. Pre-copulatory behavior

When a male and female were introduced into a plastic container, the male extended his antennae, rubbed them against the floor, and pursued the female. Although there was no barrier between the male and female in the arena, the male did not go directly toward the female, but tracked her with antennal movement.

Mating was initiated by the male attempting to mount the female. Sixty-nine of 78 males actually attempted to copulate (88.5%). The male mounted the back of the female and grasped her body with his legs. Male beetles have robust forelegs with broadened tarsi that have sucker-like structures on the ventral sides; these structures held the constricted part of the female body between the pronotum and anterior part of the elytra. The middle- and hindlegs supported both sides of the female body. The male aedeagus then appeared from the abdominal terminalia, and the apex of the aedeagus was turned forward. The male extended the aedeagus to its maximum length and attempted to insert it into the female vaginal opening.

The female often showed rejection behavior by extending her abdominal segments to keep the vaginal opening away from the apex of the aedeagus (weak rejection), or by bending the abdominal terminalia between the 8th and 9th segments upwards (strong rejection), thereby avoiding deeper insertion of the aedeagus into the vaginal chamber. In thirty-seven copulations with virgin females, 14 (37.8%) and 6 (16.2%) females showed weak and strong rejection posture, respectively. In response to rejection by the female, the male rubbed the forebody of the female, especially her antennae, with his own vibrating antennae and continued his attempts at copulation. Of forty-five trials using virgin females, 8 males (17.8%) gave up and dismounted the female as a result of pre-copulatory struggles. The duration of the pre-copulatory struggle ranged 0 to 49 sec (mean =15.4±11.2, n=37 for copulations in which males successfully inserted virgin females).

B. Copulation behavior

Following the pre-copulatory struggle, the male inserted the aedeagus into the vagina. When the two genitalia were coupled, the female ceased struggling. During copulation, the female often walked around while the male remained on her back, continually vibrating his antennae. The male sometimes showed thrusting movement of the aedeagus into the vagina. In one case, the male released the female, and copulation was terminated. In most cases, the aedeagus was deeply inserted at the onset of copulation. The depth of aedeagal insertion decreased over time, and eventually the basal half of the aedeagus appeared. Copulation was terminated by the withdrawal of the aedeagus from the vaginal opening. The mean copula duration was significantly longer in copulation with virgin females than mated females (96.5±32.8 min [n=37] vs 78.3±40.7 min [n=24], respectively; Mann-Whitney *U* test, P=0.0148).

C. Post-copulatory behavior

Even after the end of copulation, the male often remained on top of the female with his legs holding her body. In most cases, amplexus ceased when the male voluntarily departed; sometimes, however, the male was shaken off. The duration of the post-copulatory amplexus ranged 0 to 180 min (mean=65.7±48.5, n=37 for copulations with virgin females).

Internal events

A. Insemination process

Thirty pairs of *C. insulicola* were frozen *in copula* and dissected. Observation of fixed genitalia revealed that genital coupling and insemination could be divided into 5 stages: stage 1) ejaculation had not begun, and the endophallus with the copulatory piece was not everted from the aedeagus; stage 2) ejaculation had not begun, the endophallus was partly everted, and the copulatory piece was partially inserted into the vaginal appendix; stage 3) ejaculation (spermatophore formation) had begun, the spermatophore was emerged from the gonopore, the endophallus was fully everted, and the copulatory piece was fully inserted into the vaginal appendix (Fig. 1A); stage 4) ejaculation and spermatophore formation were completed, the endophallus with the copulatory piece was returned into the aedeagus, and the genitalia of both sexes were still coupled; stage 5) copulation was over, the genitalia of both sexes were separated from each other. Ejaculation did not occur until at least 20 min after the onset of copulation (Table 1). The earliest ejaculation was observed 40 min after the beginning of copulation, and the rate of ejaculation rapidly increased toward the end of copulation.

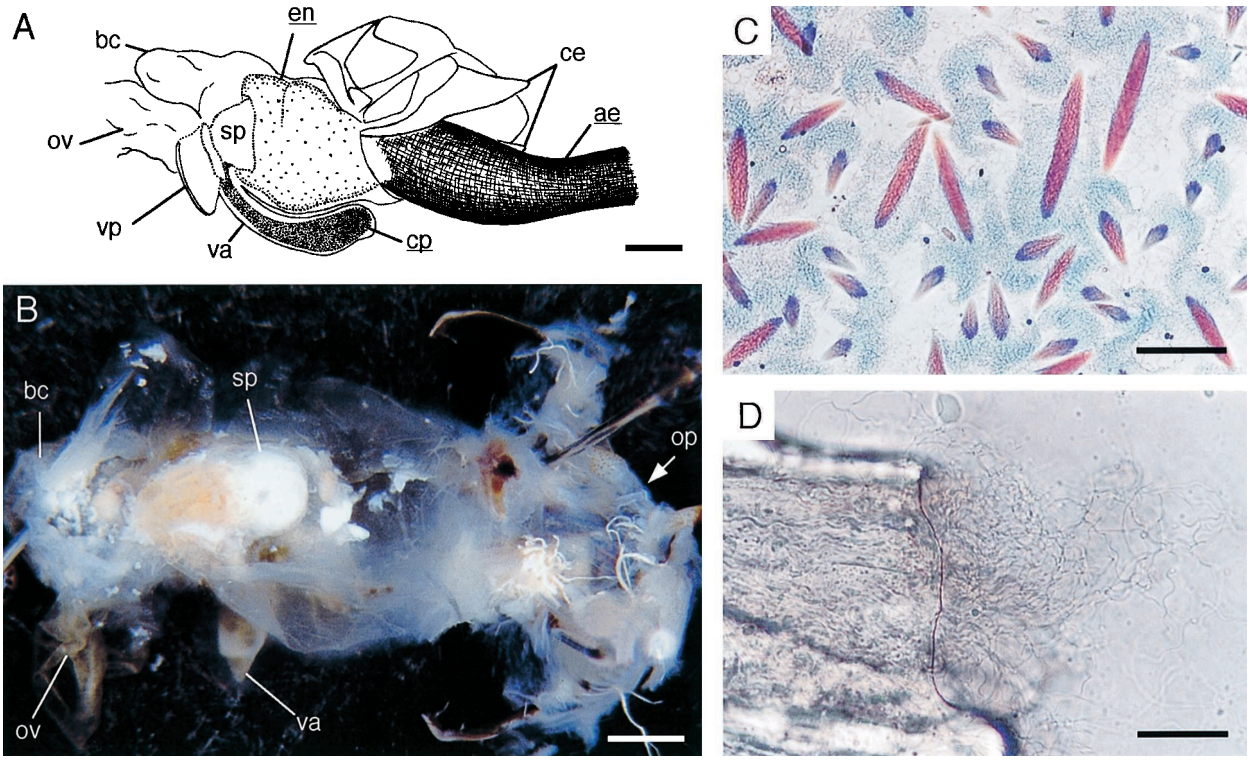

Fig. 1. Genital anatomy, spermatophore, sperm bundles, and free spermatozoa of *Carabus insulicola*. ae: aedeagus, bc: bursa copulatrix, ce: cercus, cp: copulatory piece, en: endophallus, op: vaginal opening, ov: oviduct, sp; spermatophore, va: vaginal appendix, vp: vaginal apophysis. (A) Genital coupling during which formation of the sp begins; underlined letters indicate male organs; the spermatheca is hidden and situated between bc and ov; scale bar=1 mm. (B) Dorsal view of the vagina with sp deposited; the dorsal wall of the vagina and 9th tergite were cut and removed; scale bar=1 mm. (C) Sperm bundles found in the testis with Giemsa stain, scale bar=0.1 mm. (D) Free spermatozoa stored within the tube-like spermatheca, scale bar=0.025 mm.

Table 1. Temporal change of ejaculation and genital coupling in the copulation of *Carabus insulicola*. Numbers of pairs in each insemination stage are shown. Ejaculation does not begin in stages 1 and 2. Spermatophore is formed in the stage 3. Ejaculation and copulation finish in stages 4 and 5, respectively. See results in detail.

Stage of insemination	Time from copulation started (min)		
	20	40	60
1	9	5	–
2	1	1	1
3	–	3	2
4	–	1	3
5	–	–	4
Total	10	10	10

B. Sperm transfer and spermatophore digestion process

Twenty-nine pairs of *C. insulicola* including 5 virgin females were allowed to mate, and spermatophores of 13, 2, 2, 3, 3, and 4 pairs were weighed 0, 3, 6, 12, 18, and 24 hr post-copulation, respectively (Fig. 2). The mean spermatophore weight at 0 hr was 6.3±0.70 mg (range 4.8–7.1), which was 0.61% male body weight (1029.8±92.8 mg, n=30). Spermatophore weight declined significantly with increased time after copulation (Spearman's rank correlation, r_s=–0.872, P<0.0001). Females dissected 18 and 24 hr after copulation had spermatophores that were apparently dissolved. Dissolving was conspicuous at the anterior half of the spermatophore where it faced the openings of the spermatheca and the common oviduct. In the dissolved spermatophores, caps of sperm bundles from which spermatozoa detached were found. Two virgin females dissected immediately after copulation did not contain sperm within the spermatheca, while 2 and 1 virgins fixed 3 and 6 hr after copulation, respectively, had sperm within the spermatheca.

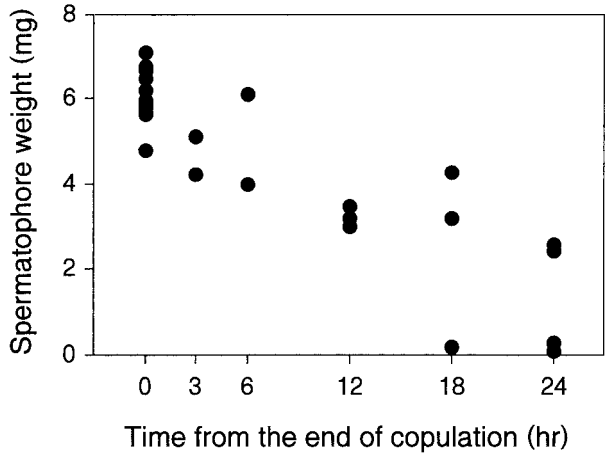

Fig. 2. Post-copulatory change in spermatophore weight in *Carabus insulicola*.

Ejaculates

In all observed copulations, sperm were transferred with a spermatophore, a gelatinous package of sperm. The spermatophore was hemispherical and uniform in the examples examined (Fig. 1B). The spermatophore was deposited in the innermost part of the vagina, and adhered around the inner plate of the vaginal apophysis, covering the openings of the spermatheca, the common oviduct, and the vaginal appendix. Within the spermatophore, semen was retained near the opening of the spermatheca to which the spermatophore was glued.

The spermatozoa of *C. insulicola* were bound together and formed sperm bundles. They were found in the male organs, testis follicle, vas deferens, and ejaculatory duct (see figures in Yahiro, 1998), as well as in the spermatophore deposited into the vagina. The flagella of spermatozoa moved when the spermatozoa were in Ringer's solution, but the sperm bundles did not swim. In sperm bundles processed with Giemsa staining, spermatozoa nuclei were stained bluish purple, suggesting that the head of the spermatozoa was glued to the "cap", which was stained red (Fig. 1C). The length of sperm bundles ranged from 37.0 to 181.5 μm (n=80 from one male), and was bimodally distributed with a significant difference from normality (Kolmogorov-Smirnov test, P=0.010), while their widths ranged from 16.0 to 27.5 μm (n=80 from one male) with a nearly normal distribution (Kolmogorov-Smirnov test, P=0.785) (Fig. 3). The lengths and widths were significantly correlated with each other (Spearman's rank correlation, r_s=0.622, P<0.0001). Another male of which sperm bundles were photographed showed similar variation in sperm bundles. In the spermatheca, only free spermatozoa were found (Fig. 1D). The mean length of spermatozoa was 159.26±6.40 μm (n=28 sperma-

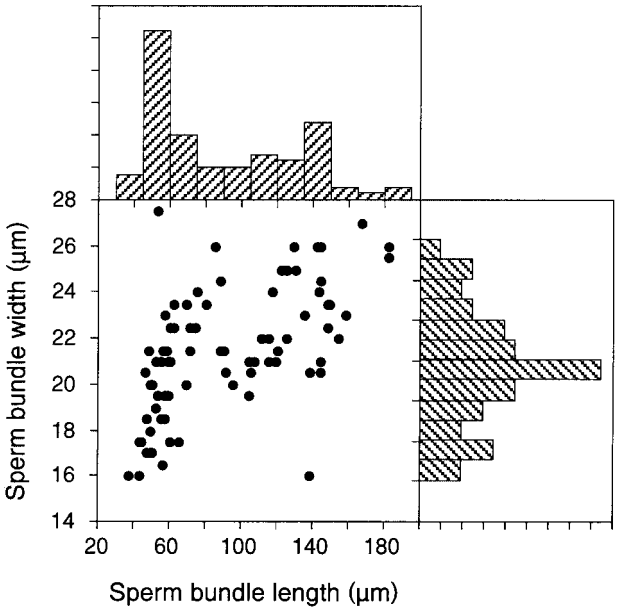

Fig. 3. Variation in the length and width of sperm bundles of *Carabus insulicola*.

tozoa from one male). The size distribution of spermatozoa did not differ from normality (Kolmogorov-Smirnov test, P=0.963).

DISCUSSION

Male mating tactics to enhance his reproductive success

The male and female reproductive interests often conflict when multiple potential partners that vary in quality are available for either sex (Alexander *et al*., 1997). The intersexual conflict may have obliged males and females to develop a variety of strategies to enhance their reproductive success by overwhelming the 'arms' of mates. Male *C. insulicola* possess devices to accomplish copulation easily. In pre-copulatory struggles, while males attempted to copulate coercively, females often rejected them, which occasionally resulted in the termination of mating. Structures in the forelegs of *C. insulicola* are sexually dimorphic. Males have broadened foretarsi with sucker-like microstructures (Stork and Evans, 1976) and foretibia with angular inner margins. When a male mounted a female, his broadened foretarsi and angular foretibiae effectively grasped her body while she attempted to reject him. In addition, the apex of the aedeagus of *C. insulicola* is more or less pointed or digitate, allowing him to insert the aedeagus easily.

Males of *C. insulicola* show courtship-like behavior during mating. The male frequently touched the forebody of the female, especially her head and antennae, with his own vibrating antennae. The male may stimulate the female to relax her rejection posture, because vibration of the antennae often occurred in response to a struggling female. Similar vibration has been observed in leaf beetles (Dickinson, 1997). In many animals, courtship behavior during mating affects sperm utility pattern by females (summarized in Eberhard, 1996). In this species, however, it is still unclear that male courtship-like behavior enhances his fertilization success.

Males remaining on females in post-copulatory amplexus possibly functions as mate guarding, because sperm transfer into the spermatheca does not occur immediately in *C. insulicola*. Takami (unpublished data) showed that males of *C. insulicola* remained longer on the females that were successfully inseminated and worthy to be guarded than on females incompletely done. However, the efficiency of the male's post-copulatory behavior for mate guarding is not confirmed in the presence of rival males.

Delay of ejaculation

Male-female interaction may affect spermatophore formation during the mating of *C. insulicola*. During copulation, there was a long delay prior to ejaculation. This delay is puzzling, because it results in the elongation of copula duration, which often incurs costs to both sexes, *e.g.*, risk of predation or energetic losses (Dickinson, 1997). The benefits of prolonged copulation, such as increasing certainty of paternity (for males) or acquisition of nutrients from ejaculates (for females), are also considerable, but they do not require the delay of ejaculation. To avoid the cost of prolonged copulation, it would be beneficial for the male to ejaculate just after genital coupling. Thus, ejaculation must be delayed for other reasons, such as intersexual conflict of interests. In this context, the male interest is to minimize the cost of each copulation, whereas the female interest is to evaluate the male during copulation.

One possible scenario that explains ejaculatory delay in terms of intersexual conflict is as follows: the female has a threshold for accepting an ejaculation, such that she can manipulate her vaginal muscles in order to constrict the endophallus and hinder ejaculation; and only males that can overcome this threshold are able to ejaculate (Alexander *et al*., 1997). This scenario is partly supported by the female genital anatomy, in which the vaginal pouch is covered with a thick muscular layer. However, this prediction does not consider that evaluation during copulation may induce a larger cost to the female than evaluation before copulation; these additional costs may include energetic losses, risks of exposure to parasites or pathogens, or genital injuries. Eberhard (1996) suggested that ejaculatory delay resulted from the male need to stimulate females after intromission in order to induce acceptance. This explanation may also be applicable to this species.

In this context, elongation of copula duration in matings with virgin females means that virgin females are more choosy than mated ones. However, possibilities that males employed some additional tactics in matings with virgins, such as courting more carefully or forming larger spermatophores, should be taken into account. Male and female interests that determine copula duration are not fully reviewed and remain to be investigated in *C. insulicola*.

Spermatophore and sperm transfer

Spermatophore and sperm transfer in *C. insulicola* have some elaborate features. Spermatophores were uniformly shaped and strongly adhered to the innermost part of the vagina. Dissection of pairs *in copula* showed that the spermatophore that emerged from the gonopore was surrounded by the membranous wall of the endophallus (Fig. 1A). These results indicate that male beetles molded and shaped the spermatophore within the vagina using the endophallus. Strong adhesion of spermatophores to a particular site are unique among beetle species, suggesting additional functions of spermatophores.

The weight of spermatophores decreased after copulation, probably due to digestion by females. Since the spermatophore is small (0.61% of male body weight), it may be less valuable for females than in other insects that transfer nutritional resources to females with their ejaculates (*e.g.*, 20% of male body weight in dobsonflies: Hayashi, 1992, 3–25% in orthopterans; Gwynne, 1997). On the other hand, females of some insects sometimes discard the sperm of the current male (Eberhard, 1996). In this species, there was no evidence that dissolved spermatophores were dis-

carded. Dissolution of the spermatophore may result from some substance having protease activity, which is possibly secreted from glands or cells around the openings of the spermatheca and the common oviduct because the spermatophore was more strongly dissolved at the portion facing these openings. This substance may also be associated with the degradation of sperm bundles, because caps of sperm bundles, from which spermatozoa detached, were found within dissolved spermatophores, but did not within undissolved spermatophores.

The shape of sperm bundles of *C. insulicola* and their remarkable variation in length are unique and problematic. The presence of sperm bundles has been reported for various insect species, including polyphagan beetles (Jamieson, 1987, summarized in Hayashi, 1997). In adephagan beetles, to which the ground beetles belong, species of the Gyrinidae and Dytiscidae have paired or grouped spermatozoa, whereas *Cicindela campestris* (Cicindelidae) does not have bundled spermatozoa (Werner, 1965, 1976, 1983; Breland and Simmons, 1970; Jamieson, 1987; Simmons and Siva-Jothy, 1998). The principal structure of sperm bundles of *C. insulicola* is similar to those of the Gyrinidae (Breland and Simmons, 1970). However, the length of caps (=rods in Breland and Simmons, 1970) is much longer in Gyrinids than in *C. insulicola*. Breland and Simmons (1970) concluded that variation of sperm bundle length in Gyrinids represented different developmental stages. However, in *C. insulicola*, it is apparent that small bundles are not immature, because they were found within spermatophores that were formed by sexually mature males. Therefore, the significance of the variation in the length of the sperm bundles also remains vague. The functional significance of the non-swimming sperm bundles of *C. insulicola* is also unclear, unlike those of fishflies that swim and move into the spermatheca (Hayashi, 1996). It is unlikely that the caps of the bundles have nutritive value for females, because the caps are hard to dissolve, even within digested spermatophores.

Possible functions of the copulatory piece

Two possible functions of the copulatory piece can be hypothesized from the results of this study, although no direct evidence for either was obtained. First, the male may fix his penis within the vagina using the copulatory piece to form the spermatophore. Possible female control of ejaculation causing ejaculatory delay suggests that the male needs to combine both genitalia rigidly for proper ejaculation and molding of the spermatophore. This function is also suggested by the experimental copulation using *C. insulicola* in which males with artificially removed copulatory pieces did not form spermatophores in the proper site, but did near the opening of the vagina (Takami, 2000). Second, the male may break or remove the spermatophore of a predecessor via the copulatory piece. The spermatophore of *C. insulicola* is positioned along the trajectory of a copulatory piece that is inserted into the vaginal appendix. If a male mates with a previously mated female and struggles to insert the copulatory piece into her vaginal appendix, then a spermatophore covering the opening of the vaginal appendix might be broken by the copulatory piece. Recent microsatellite analyses revealed that 11 of 14 males removed the spermatophores of previously mated males, and the removal resulted in a high P2 value (Takami, unpublished data). To test these hypotheses, direct observation of genital movement and evidence of a correlation between the morphological variation of the copulatory piece and copulation success (rapidity of ejaculation and certainty of spermatophore removal) are needed.

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