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Possible Removal of Rival Sperm by the Elongated Genitalia of the Earwig, *Euborellia plebeja*

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ABSTRACT—Sperm displacement is a sperm competition avoidance mechanism that reduces the paternity of males that have already mated with the female. Direct anatomical sperm removal or sperm flushing is known to occur in four insect orders: Odonata, Orthoptera, Coleoptera and Hymenoptera. In a fifth order, Dermaptera (earwigs), I found that the virga (the elongated rod of the male genitalia) of *Euborellia plebeja* seems to be used to remove rival sperm from the spermatheca (a fine-tubed female sperm storage organ). In this species, copulation lasted on average 4.6 minutes, during which time the male inserted the virga deep into the spermatheca, and then extracted it ejaculating semen from the opening of the virgal tip. The extraction of virgae (with its brim-like tip) appeared to cause removal of stored sperm in the spermatheca. The virga was as long as the body length of males, and the spermatheca was twice the female body length. The long length of the spermatheca and the possible removal function of the virga may select for virgal elongation.

INTRODUCTION

Sexual selection will favour any adaptation in males that prevents or avoids competition between their own sperm and that of other males. By manipulating the stored sperm of rivals within the female sperm-storage organ(s) and consequently placing those sperm at a competitive disadvantage, a copulating male can reduce, or even avoid, sperm competition. Sperm displacement is a sperm competition avoidance mechanism that reduces the paternity of males that have already mated with the female (Simmons and Siva-Jothy, 1998).

Direct anatomical sperm removal or sperm flushing is known in four insect orders. In Odonata, males of several species of damselflies and dragonflies use their penis to remove rival sperm deposited in the bursa copulatrix and/or spermatheca (e.g. Waage, 1979, 1984; Siva-Jothy, 1987). In Orthoptera, male bush crickets *Metaplastes ornatus* introduce a subgenital plate into a female's genital chamber to elicit sperm evacuation from the spermatheca (Von Helversen and Von Helversen, 1991), and male tree crickets, *Truljalia hibinonis*, flush rival's semen out of the female's spermatheca (Ono *et al*., 1989). Males of two species of Coleoptera, *Psacothea hilaris* and *Aleochara curtula*, remove sperm from a female spermatheca by using the distal portion of penis (Yokoi, 1990) and by explosion of an internally placed spermatophore (Gack and Peschke, 1994), respectively. The spined penis sheath of the tenebrionid beetles, *Tenebrio molitor*, removes sperm from the female bursa copulatrix (Gage, 1992; but see Siva-Jothy *et al*., 1996). In another tenebrionid, *Tribolium castaneum*, Haubruge *et al*. (1999) reported rival sperm removal by showing sperm translocation between females on the spined male genitalia. In Hymenoptera, male *Athalia rosae* use a conical projection on their penis to remove sperm from the spermatheca before ejaculation (Shigemura and Naito, 1999).

In Dermaptera, the members of the family Anisolabididae are known to have a greatly elongated virga, which is the posterior part of the ejaculatory duct (a fine sclerotized tube) of male genitalia (Ramamurthi, 1958; Jamet and Caussanel, 1995). The virga is inserted into the sperm-storage organ (a single fine tubed spermatheca) of his mate at copulation (Giles, 1961; Briceño, 1997). During the course of the study on the functional aspects of this elongated virga in the anisolabidid earwig, *Euborellia plebeja* (Dohrn, 1863), I found that it seems to be used to remove rival sperm. In the present paper, first, male and female genital structures and the processes of insemination are described in detail, based on the observations of fixing each mating stage of the pair with liquid nitrogen. Second, several lines of evidence for sperm removal by the elongated virga are shown.

MATERIALS AND METHODS

Male and female genital structures

About two hundred larvae of *E. plebeja* were collected at two suburban grasslands in the Tama region, Kanto district, central Japan, in 1998 and 1999. Although these two sites are about 10 km apart, all were combined for a laboratory stock. Larvae were reared in plastic pots (8 cm in diameter, 4.5 cm high, floored with plaster of Paris containing a small amount of activated carbon powder) at a

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density of 20–30 individuals per cage, and kept at 23±1°C (14 h light: 10 h dark). They were provided with water and fed commercial cat chow (SMACK, Smack Co. Ltd., Aichi Prefecture, Japan) ad libitum. Newly emerged adults were separated individually into small plastic cages (3 cm in diameter, 5 cm high, filled with leaf litter) within two days of eclosion, and reared in the same way as the larvae. This procedure produced virgin females; the previous mating experiment showed that they never mated in this period (n=10 females). Adults 10–107 days old were used for experiments.

To observe the internal reproductive organs, females were frozen and dissected in insect Ringer (0.9 g NaCl, 0.02 g $CaCl_2$, 0.02 g KCl and 0.02 g $NaHCO_3$ in 100 ml water), after the maximum pronotum width was measured with an ocular micrometer to the nearest 0.026 mm. The spermatheca was dissected out and mounted on a slide, and the total length was measured on a photograph taken under a light microscope (40X), with a curvi-meter (COMCURVE-8, KOIZUMI, Tokyo) to the nearest 0.01 mm. Each spermathecal length was measured twice, from the base to the end and from the end to the base, and these were averaged for individuals. Measurement errors in this procedure were 0.49%±0.33 (mean±SD, n=40).

The males, frozen once, were also dissected in insect Ringer after measuring the pronotum width. The length of virgae was estimated as the total length of a genital sheath, in which virgae are tightly wrapped. It was measured to the nearest 0.1 mm under a binocular microscope (40X). The virgae were also observed by a scanning electron microscope (SEM) (WS-250, ABT, Tokyo) at an acceleration voltage of 15 kV after drying and coating with gold.

Mating experiments

Mating experiments were conducted in dark periods with a dim red light. Sixty-two virgin males were randomly chosen and placed with virgin females in each plastic vessel (same as rearing pots). Forty-one pairs were allowed to copulate without disturbance, and the remaining 21 pairs were frozen by plunging into liquid nitrogen at 0.5 (n=5), 1 (n= 5), 2 (n=6), or 3 (n=5) minutes after the initiation of copulation. The start of copulation was determined by the rigid contact of male and female subgenital plates. Five females of the undisturbed copulation group were also frozen with liquid nitrogen after one day. Later all frozen females were defrosted and the spermatheca was dissected out in insect Ringer. It was slide-mounted to sketch (400X) and photograph (40X), based on which, the lengths of the spermathecal valve, the total spermathecal tube, the section with the inserted virga, and the section containing sperm (the section between the most distally detected spermatozoon and the most proximal one) were measured.

Eighteen females singly mated were remated with another virgin male 1–19 minutes after their virgin-copulation, and the copula durations of virgin- and non-virgin-copulation were compared. To reveal the remating processes, 21 females singly mated were divided into three remating groups. In the first group (control group), five females without remating were frozen with liquid nitrogen 1–4 minutes after copulation. In the second group, ten females were allowed to remate 1–7 minutes later, and the rematings were interrupted after 0.5 minutes by pinching the male abdomen with forceps. Immediately after the dislodgement, the females were frozen. Preliminary observation using virgin females (n=6) confirmed that pinching of the copulating male within one minute never caused ejaculation, so that females of this group had no sperm from the second male. When pairs were undisturbed, sudden movement of the females usually caused the dislodgement of males with no visible behavioural difference to the forced dislodgement of males by abdomen pinching. In the third group, six females were allowed to remate 1–6 minutes later, and were frozen one minute after the beginning of remating without disturbing the pair. Therefore, the females of the third group were fixed with the virga still inserted in the spermatheca, in contrast to the second group females that were fixed just after the inserted virga was extracted. Later all frozen females were defrosted and the spermatheca was dissected out in insect Ringer. Based on the sketches (400X) and photographs (40X) of the slide-mounted spermathecae, the lengths of the spermathecal valve, the sections containing sperm, the sperm-free sections, and the section of inserted virga were measured. The presence or absence of sperm was determined at 0.05 mm intervals along the spermatheca. The inner space of spermathecae in which no spermatozoon was detected was regarded as a sperm-free section.

In order to verify that rival sperm are displaced under the 'brim-like' end of the virga as suggested by the results of the doubly-mating experiments, the following experiments were conducted. Twenty-six females were placed with males (6–10 females and 3 males in each rearing pot) and allowed to mate freely for two days. The females were paired with another virgin male and the matings were interrupted after 0.5 minutes by pinching the male abdomen with forceps, followed by immediate freezing of the disturbed pairs. Later the frozen females were defrosted and the spermatheca was dissected out in insect Ringer. The presence or absence of the inserted virga and sperm was examined under a light microscope (40–400X).

RESULTS

Genital structure

The spermatheca is a long highly convoluted duct (Fig. 1*a*) with a short muscular valve near the opening. The total length of the spermatheca was 33.6±5.7 mm (mean±SD, n=40). Female pronotum width was 1.65±0.11 mm (n=40). Spermathecal length was not correlated with pronotum width (r=0.215, 0.1<P<0.2, n=40). The virga, 15.8±1.0 mm (n=43) in length, has a brim-like tip (Fig. 1*c*) that fits closely the inner space of the spermatheca when inserted (Fig. 1*b*). Male pronotum width was 1.43±0.11 mm (n=43). Virgal length was not correlated with pronotum width (r=0.064, 0.5<P<0.6, n=43).

Singly-mated Females

Undisturbed copulation lasted 4.6±3.5 minutes (n= 41). When copulating pairs were fixed at 0.5 and one minute after initiation (Fig. 2), the virga was inserted 2.73±1.80 mm (n=5) and 5.45±2.26 mm (n=5) deep into the spermatheca, respectively, but sperm transfer had not occurred. Out of six females fixed after two minutes (Fig. 2), one female had no inserted virga and no sperm (presumably failed copulation). In the other five females, the virgal tip reached 4.12±1.30 mm from the spermathecal opening, and sperm were distributed from there to 9.16±1.87 mm deep from the spermathecal opening. Out of five females fixed after three minutes (Fig. 2), only one female hold the virga, that reaches shallow (0.88 mm from the opening of the spermatheca). All these five females had sperm in the spermatheca throughout the range of 1.02±0.17 mm and 10.07±2.10 mm from the opening. In five females one day after mating, sperm were found along almost the entire length of the spermatheca, from 0.89±0.59 mm to 35.57±5.51 mm (corresponding to 82–100% of the spermathecal length). The lower limit of sperm distribution coincided with the position of spermathecal valves (Fig. 2). The insemination process of *E. plebeja* was thus divided into the two phases: in the first half, males insert the virga deep into the spermatheca, and in the latter half, males ejaculate from a seminal opening

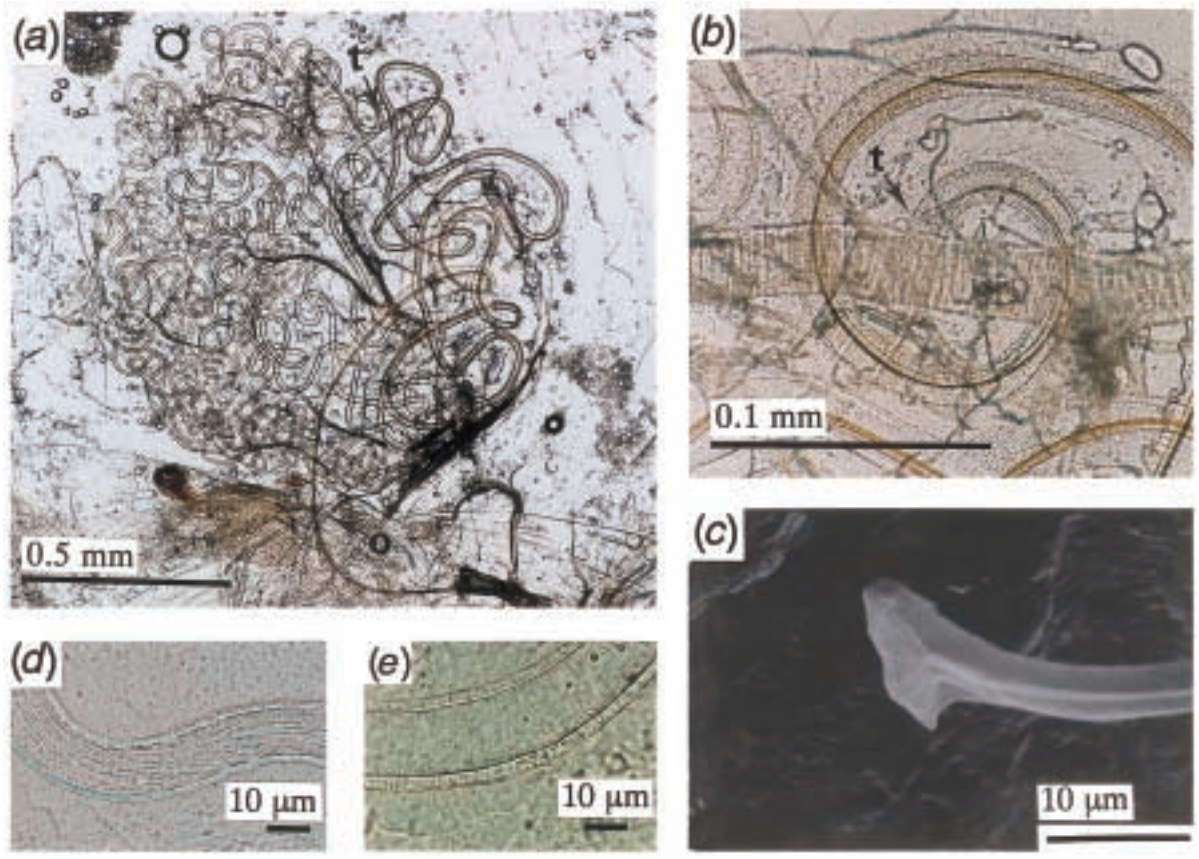

Fig. 1. The virga and spermatheca of the earwig, *Euborellia plebeja*. (*a*) The spermatheca (its opening is indicated by the arrow o) and the inserted virga (its tip is indicated by the arrow t). (*b*)The tip of virga (indicated by the arrow t), inserted in the spermatheca. (*c*) SEM photograph of the brim-like tip of the virga. (*d*) and (*e*) The spermatheca with and without spermatozoa, respectively.

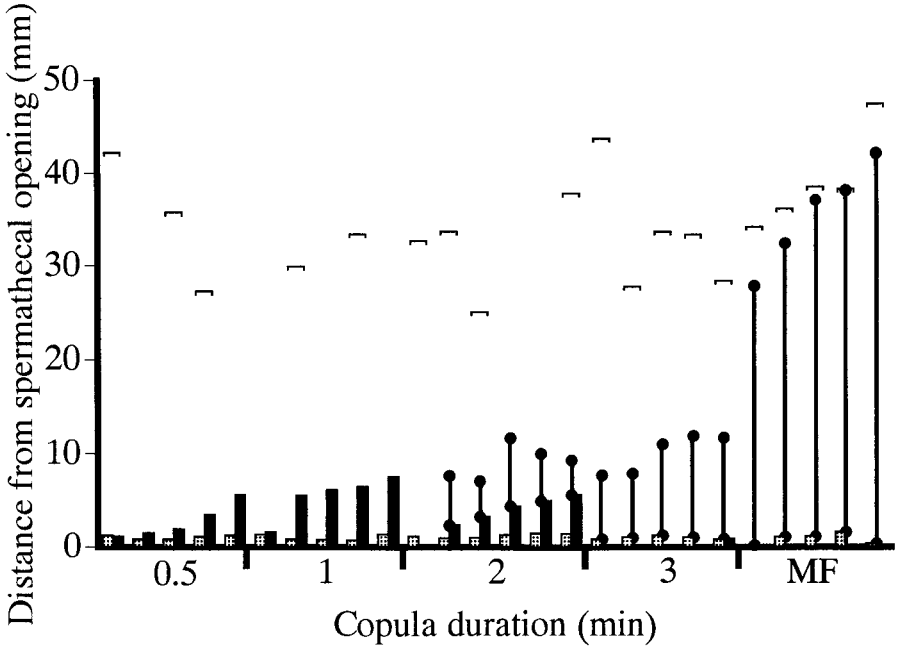

Fig. 2. Lengths of the spermathecal valve (▭), the total spermatheca (⊢⊣), the virga inserted in the spermatheca (▬), and the sperm containing section of the spermatheca (⊷) of individual females (each column) of *Euborellia plebeja*. Mating was interrupted after 0.5, 1, 2, or 3 minutes. The females one day after mating are indicated by 'MF'. The length of the total spermathecae could not be measured for seven individuals. In the groups of females of 0.5, 1 and 2 minutes in copula, they are arranged according to virgal length. In the groups of 3 minutes in copula and MF, female arrangement is based on the length of sperm-containing section in the spermatheca.

at the tip of virgae while pulling out the virga. Sperm spread from the ejaculated site through the spermatheca; for at least one day after ejaculation, they spread up to the whole area.

Doubly-mated Females

In 18 females allowed to mate and remate without disturbance, there was no difference in copula duration between non-virgin mating (4.7±3.1 min) and virgin mating (4.6±3.1 min) (paired *t*-test, $t=0.16$, $0.9>P>0.8$). In 21 females fixed to reveal the remating processes, the previous mate's sperm in the spermatheca reached 11.94±2.73 mm ($n=21$) from its opening (Fig. 3; the reach did not differ among the three experimental groups; ANOVA, $F_{2,18}=1.81$, $P=0.19$). In the control group females (*C* females), which were not allowed to remate, sperm were nearly continuously distributed (Fig. 1*d*) in the spermatheca up to 11–15 mm from the opening (Fig. 3 left). However, in females whose second copulation was interrupted at 0.5 minutes after the initiation by pinching the mate with forceps (with virgal insert and extract but without ejaculation by the second male; *E* females), sperm were distributed irregularly; the spermatheca included many sperm-free sections (Fig. 1*e*) in the basal part (Fig. 3 middle). In females fixed with the virga inserted during remating but before ejaculation by the second male (*I* females), sperm were continuously distributed even in the section with the inserted virga (Fig. 3 right), the same as in *C* females. The cumulative length of sperm-free sections was significantly longer in the spermatheca of *E* females (0.402±0.579 mm, $n=10$) than *C* (0.009 ±0.021 mm, $n=5$) and *I* (0.062±0.130 mm, $n=6$) females (Kruskal-Wallis test, $H=12.42$, $P=0.002$, d.f.=2). Mann-Whitney *U*-values with adjusted error probability of 0.016 are significant for *E* vs. *C* ($U=0$, $P=0.002$, $n_1=10$, $n_2=5$) and *E* vs. *I* ($U=7$, $P=0.012$, $n_1=10$, $n_2=6$), but not for *C* vs. *I* ($U=12.5$, $P=0.56$, $n_1=5$, $n_2=6$). These results suggested that sperm removal occurred when the male extracted the virga.

In rematings disturbed by abdomen pinching, the virga was already extracted from the spermatheca in 23 out of 26 inseminated females that had been allowed to mate freely for two days. In the other three cases, entangled sperm masses were observed under the brim-like end of the inserted virga (Fig. 4).

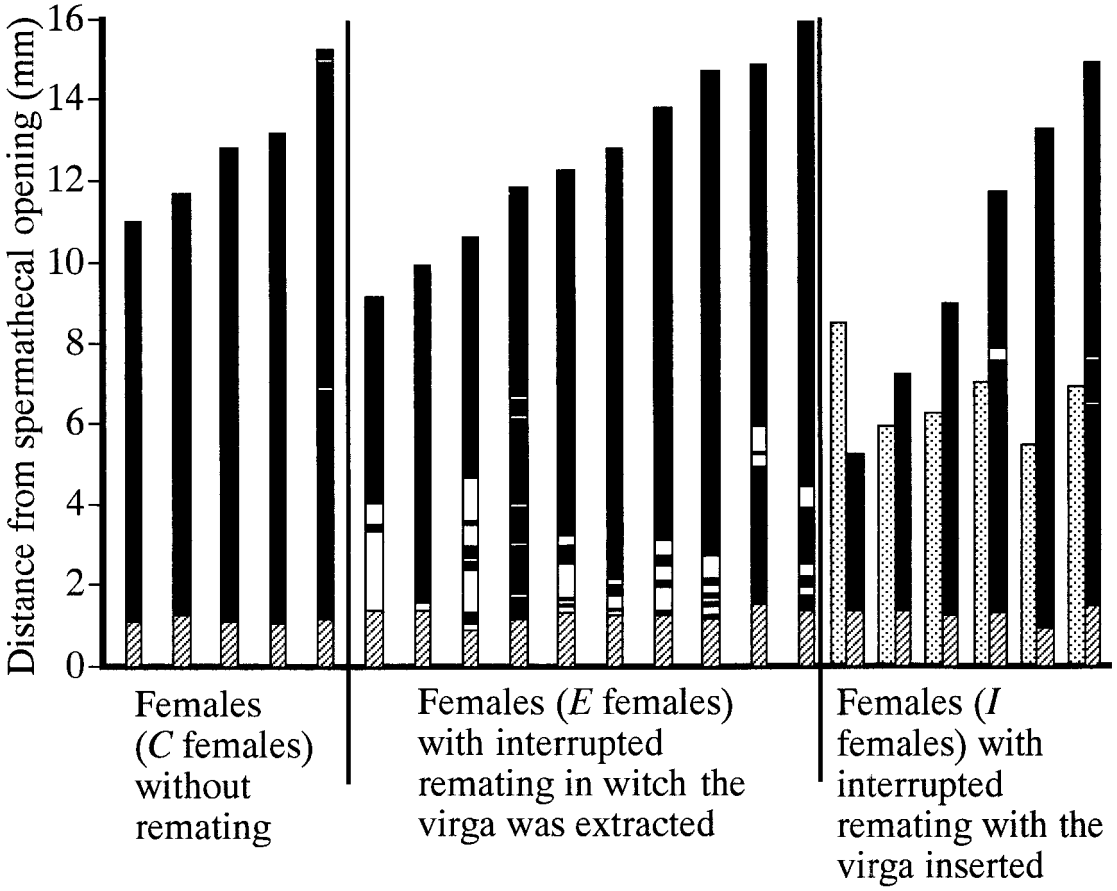

Fig. 3. Lengths of the spermathecal valve (▨), the sperm containing section of the spermatheca (▬), the sperm-free section of the spermatheca (▭) and the virga inserted in the spermatheca (▩) of individual females (each column). Female *Euborellia plebeja* are divided into three groups: females without remating (*C* females), with interrupted remating (without ejaculation by the second male) in which the virga was removed (*E* females) or not (*I* females). Individuals in respective remating groups are arranged according to the length of sperm containing sections in their spermathecae.

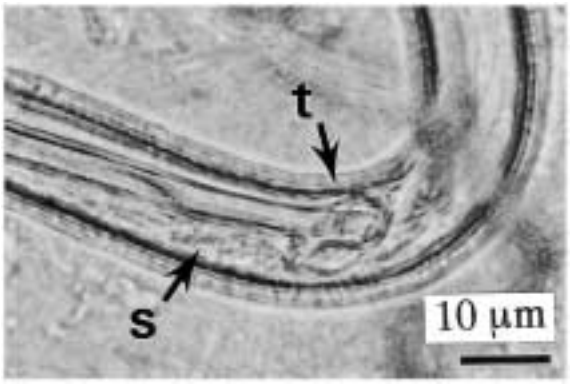

Fig. 4. Entangled sperm mass (indicated by the arrow s) under the brim-like tip (indicated by the arrow t) of the inserted virga being extracted. The photograph was a typical case.

DISCUSSION

Ensuring that rival sperm cannot fertilize eggs by physically removing them from the female sperm-storage organ(s) is an elegant solution to avoid sperm competition. It occurs in species with essentially similar mating systems where females are highly likely to remate (Simmons and Siva-Jothy, 1998). Female *E. plebeja* also remate repeatedly with any courting male (Baijal and Srivastava, 1974; Y. Kamimura, unpublished data). In all species known that males use direct anatomical sperm removal for sperm competition avoidance, ejaculation occurs after rival sperm have been removed thereby avoiding the removal of self sperm (Simmons and Siva-Jothy, 1998; Shigemura and Naito, 1999). In *E. plebeja*, deep insertion of the virga into the spermatheca preceded ejaculation, possibly to avoid removing their own sperm by the following extraction of virgae. Since the virga occupies a substantial portion of the volume of the spermatheca and sperm density could not be determined, the possibility that a portion of sperm is compressed by the inserted virga can not be excluded. The virgal extraction accompanied with the occurrence of many sperm-free sections (Fig. 3) and the entangled sperm masses under the brim-like tip (Fig. 4), however, suggest sperm removal as the major function of the elongated virga. It must be noted that in remating experiments, the mating males were disturbed soon after the beginning of insertion of the virga (the rationale for this procedure is to prevent the second male from ejaculating). Therefore, the cumulative length of sperm-free sections may give only a conservative estimate of the amount of sperm removed. Although the place where the lost sperm have gone has not yet been detected, the tip-brim of the virga may be responsible for sperm removal: it may be functionally equivalent to penis spines and/or projections in insects where anatomical sperm removal has been reported (see Introduction).

Several species of insects exhibit elongated male genitalia, being sometimes longer than the body length (reviewed by Eberhard, 1985). *E. plebeja* is such a case in that the male's virga (15.8±1.0 mm) is as long as his body length (8.5–10.5 mm: Baijal and Srivastava, 1974). The function of such elongated genitalia of insects has been poorly studied. In the chrysomelid beetle, *Chelymorpha alternans*, female sperm uptake was apparently related to the length of male genitalia (Rodriguez, 1995). The underlying mechanism(s), however, are not understood thoroughly. If the virga is used to remove rival sperm in the spermatheca, the longer virga may be able to remove more sperm, which would select for elongation of the virga. Lock and key mechanisms and run-away evolution by cryptic female choice are other possible explanations for the elongation of male (and female) genitalia (reviewed by Eberhard, 1985, 1996). These hypotheses are not mutually exclusive. To evaluate the question of paternity gain by sperm removal, more detailed studies on male and female reproductive interests are required. In *E. plebeja*, the spermatheca is about twice the female body length, and such a long spermatheca allows only partial sperm removal by the male. Positive intraspecific allometry (the tendency for larger individuals to have relatively larger morphological traits) is thought to be more likely for sexually selected traits than naturally selected traits (e.g. Simmons and Tomkins, 1996 on earwig forceps). In *E. plebeja*, however, the lengths of both virgae and spermathecae showed no significant correlation with male and female body size (pronotum width), respectively. The evolutionary causes of the long spermatheca and the consequences of the relatively low proportion of sperm removed by the virga will be examined in the future from the view point of female reproductive strategies.

When mating pairs of *E. plebeja* are strongly disturbed, the long and fine virgae were easily broken with the pieces remained in the mate's spermatheca. Such handicapped males, however, can inseminate further mates with the aid of the remaining counterpart of paired virgae, namely the spare virga (Y. Kamimura, unpublished data). The presence of the spare may compensate for the risk of destruction of virgae and allow elongation to remove sperm from the spermatheca. In Dermaptera, the members of some families (e.g. Pygidicranidae, Diplatyidae, Labiduridae and Anisolabididae) have paired virgae, while the members of the other families (e.g. Cherisochidae, Forficulidae and Spongiphoridae) have a single virga (Burr, 1915a, b,1916; Popham, 1965, 1969; Ramamurthi, 1958). To clarify the evolutionary relationship of these diverged male genitalia, further phylogenetic studies are required.

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