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Ovoviviparity and genital evolution: a lesson from an earwig species with coercive traumatic mating and accidental breakage of elongated intromittent organs

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Ovoviviparity or viviparity has evolved independently in animals and involves adaptations in females to accommodate developing embryos for a prolonged duration in their bodies, a condition which has likely to have influenced the evolution of the male genitalia. We aimed to ascertain whether the elongated male genitalia of the ovoviviparous free-living earwig species *Marava arachidis* (Dermaptera: Spongiphoridae) delivers sperm directly to the female ovaries where fertilization occurs. Males mated coercively with females by grabbing the female antenna with their mouth parts. Although females resisted the mating attempts, pairs mated 3.3 times on average over 15 h. The elongated intromittent organ, known as a virga, was inserted into the long-tubed spermatheca during insemination. Surgical ectomy of the spermatheca confirmed that sperm migrated from here to the ovaries with a variable delay. A pair of sclerites in the male genitalia frequently inflicted wounds near the spermathecal opening, while the single, thin virga sometimes broke off during mating. However, unlike earwigs bearing a 'spare' virga, damage was restricted to the tip of the virga, without which the males could still inseminate the females. We discuss the evolution of the genitalia in this insect in the light of sexual selection and sexual conflict over mating and fertilization. © 2016 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2016, **118**, 443–456.

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INTRODUCTION

Although females of most insect species deposit eggs, viviparity or ovoviviparity has been reported in more than ten insect orders, indicating their multiple evolutionary origins (Hagan, 1951; Meier, Kotrba & Ferrar, 1999; Benoit *et al.*, 2015). In (ovo)viviparous species, females exhibit many morphological, physiological and behavioural modifications to accommodate the offspring in their bodies for a prolonged duration (Hagan, 1951). Female insects usually have one or several specialized sperm storage organs (termed spermatheca or seminal receptacle) near the orifice of the common oviduct (Beutel *et al.*, 2014). In response to the descent of a mature egg, females discharge a portion of the stored sperm over the egg as it passes the orifice (Sander, 2012; Beutel *et al.*, 2014). Thus, in many (ovo)viviparous species in which embryos develop directly in the ovaries, this ancestral method must have been altered to ensure fertilization. Females of some (ovo)viviparous insects, such as bat bugs (Heteroptera: Polyctenidae) and several leaf beetles (Coleoptera: Chrysomelidae), completely lack structures for the long-term storage of sperm (Hagan, 1931; Bontems, 1988). In bat bugs, the sperm is believed to be injected into the hemolymph from where it migrates to the ovaries (Hagan, 1931; Bontems, 1988; Tatarnic, Cassis & Siva-Jothy, 2014). In leaf beetles of the genus *Oreina*, males deliver sperm directly to the ovaries where fertilization of the eggs takes place (Bontems, 1988). In some (ovo)viviparous insects, such as in some sarcophagid and tachinid flies, females have an elongated uterus for the incubation of fertilized eggs, and the sperm

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storage organs (spermathecae) are located distant from the female genital orifice (Meier *et al.*, 1999).

Although these specializations in the female morphology likely influenced the evolution of the male genitalia, detailed studies of genital coupling, insemination and fertilization processes are surprisingly scarce for (ovo)viviparous insects. Bat bug males have a specialized, scythe-like intromittent organ to stab the abdomen of their mate and transfer the sperm into the hemocoel (traumatic insemination; Tatarnic *et al.*, 2014). After fertilization in the ovaries, embryos develop and extract nutrition from the pseudoplacental organs (Hagan, 1931). Hemocoelic insemination also occurs in twisted-wing parasites (Strepsiptera), where embryonic development occurs in the hemocoel instead of in the genital ducts (Beani *et al.*, 2005; Pohl & Beutel, 2008 Kathirithamby *et al.*, 2015). In female tsetse flies (Diptera: Glossina), eggs are fertilized by spermathecal sperm, as in oviparous dipterans, while a specialized maternal organ nourishes the hatched larvae in the uterus (adenotrophic viviparity: Benoit *et al.*, 2015). Male flies use their genitalia as internal courtship devices during mating to stimulate the females (Briceño & Eberhard, 2009a, b, 2015), although their relation to viviparity is unclear at present.

Viviparous or ovoviviparous females are believed to have greater opportunities and potential mechanisms for biassing paternity after mating with multiple males (cryptic female choice: Eberhard, 1996; Zeh & Zeh, 2001). On the other hand, the increased migration ability of sperm in a female body, as an adaptation to ovarian fertilization, may broaden male mating tactics to include means such as traumatic insemination. Thus, although scarcely investigated, (ovo)viviparous species provide an excellent opportunity for studying novel evolutionary traits related to sexual selection and sexual conflicts in mating and fertilization.

Female earwigs (Insecta: Dermaptera) display parental care of eggs (Günther & Herter, 1974; Costa, 2006). Pseudoplacental viviparity has been reported for the suborders Arixeniina and Hemimerina, members of which live on mammals [on hamster rats (*Cricetomys* spp.) in Africa and on bats (*Cheiromeles torquatus* Horsfield) in Asia, respectively] (Heymons, 1912; Hagan, 1951; Nakata & Maa, 1974). A recent study has revealed that embryos of *Arixenia esau* were likely to be nourished in the uterus after their initial growth in the terminal ovarian follicles (pseudoplacental-uterotrophic viviparity; Tworzydlo, Kisiel & Bilinski, 2013a). Most typical free-living earwigs, of the suborder Forficulina, are oviparous, but ovoviviparity has also been reported for *Marava arachidis* (Yersin) (Spongiphoridae: Spongiphorinae). Females of this species retain well developed embryos, which are enclosed in a thin but complete chorion until several minutes before hatching (Herter, 1943, 1965; Patel & Habib, 1978). Because the birth product of this species is eggs, some authors have categorized this species as oviparous (e.g. Hagan, 1951). However, nymphs of *M. arachidis* hatch within a few minutes after deposition of the eggs (Herter, 1943), compared with usual oviparous species in which the maternal care of the eggs generally lasts between one and several weeks (Günther & Herter, 1974; Costa, 2006). Based on previous findings (Herter, 1943, 1965; Patel & Habib, 1978; Costa, 2006; Kočárek, 2009), for the purpose of this study, this species is considered ovoviviparous. Kočárek (2009) found developed embryos in the abdomen of fixed samples of *Chaetospania borneensis* (Dubrony) (Spongiphoridae: Labiinae). The most developed embryo lacked a chorion, suggesting viviparity. Other possible examples of (ovo)-viviparous earwigs include *Sphingolabis hawaiiensis* (de Bormans) (Spongiphoridae: Labiinae) (Schneider & Klass, 2013) and *Spongovostox semiflavus* (de Bormans) (Spongiphoridae: Spongiphorinae) (Y. Kamimura, unpublished data). Giles (1963) reported the presence of a spermatheca in the female *Arixenia jacobsoni* Burr (Arixeniina), while females of *Hemimerus vosseleri* Rehn & Rehn (Hemimerina) totally lack a spermatheca (Klass, 2001). A well-developed spermatheca has been also reported for *M. arachidis* and *Spo. semiflavus*, while *Sph. hawaiiensis* completely lacks a spermatheca (Schneider & Klass, 2013). There is no information available on the spermathecal structures of *C. borneensis*.

Nothing is known of the insemination process in any of these (ovo)viviparous earwigs. Although absence of a spermatheca (in Hemimerina and *Sph. hawaiiensis*) suggests ovarian fertilization and direct transfer of sperm to the ovaries during or immediately after mating, no empirical studies have confirmed this. On the other hand, in species with a spermatheca (Arixeniina, *M. arachidis*, and *Spo. semiflavus*), it is unclear when and even whether, sperm migrate from the organ to the ovaries. Interestingly, the male *M. arachidis* has elongated genitalia (Ramamurthi, 1956, 1958; Fig. 1C), which may be used to deliver the sperm directly to the ovaries.

This study aimed to describe the specializations of the male and female genital structures in relation to ovarian fertilization of the ovoviviparous species, *M. arachidis*. Based on examination of flash-fixed samples during mating, the coupling of male and female genitalia are described in detail. The significance of sperm stored in the spermatheca was examined using a surgical manipulation technique. We discuss the characteristics of genital evolution in relation to (ovo)viviparity and possible sexual conflict in mating and fertilization of the ova.

MATERIAL AND METHODS

Insect rearing and morphology

Marava arachidis used in this study was identified as an opportunistic scavenger of American cockroach (*Periplaneta americana* Linnaeus) cultures maintained at Universiti Sains Malaysia, Penang Island, Peninsular Malaysia. Nymphs and adults were collected from multiple culture tanks and kept in plastic containers (250 × 180 mm, 130 mm height for stock populations; 110 mm diameter, 30 mm height for temporary accommodation) with plaster of Paris at the base. Similar to the original cockroach cultures, the earwigs were maintained at 26 ± 1 °C under a 12 h light-dark cycle and provided with water and unlimited amounts of commercial cat food. All of the following experiments were conducted under the same laboratory conditions.

To examine the male and female genital morphology and the relationship of genital size to their body size index (pronotum width), adult samples were euthanized by placement in a freezer. After pictures of the prothoracic region were taken under an SZ61 stereo microscope (Olympus, Tokyo, Japan) with an X-cam α CCD camera, the male genitalia (Fig. 1C, D) or the spermatheca (the sperm storage organ in the females) were dissected from samples in insect Ringer solution (0.9 g NaCl, 0.02 g $CaCl_2$, 0.02 g KCl and 0.02 g $NaHCO_3$ in 100 mL water) and mounted on a slide. Male genital length and pronotum width of both sexes were measured on photographs taken under a stereo microscope, using ImageJ Software (Collins, 2007). Photographs of the spermathecal samples were taken under a fluorescent microscope (BX53; Olympus) with a UV filter set (excitation, 330–385 nm; dichroic mirror, > 400 nm; absorbance filter, > 420 nm) without staining. The entire length of the spermatheca was measured using a curvi-meter (COMCURVE-8, KOIZUMI, Tokyo, Japan; to the nearest 3.57–7.69 μm, depending on the magnification: see Kamimura, 2000 for details) for the samples that had been sufficiently spread on a slide ($N = 17$ out of 30 prepared).

Body and genital size measurements were taken by two measurers and were averaged for individuals, except for spermathecal length, which was measured twice by YK, from the base to the end and from the end to the base. A male sample with genitalia shorter than the mean minus 3 SD was considered an outlier and was removed from any subsequent analyses. Repeatability or intraclass correlations (Zar, 2009) were 0.93 ($N = 38$, $F_{37,38} = 25.7$, $P < 0.0001$), 0.94 ($N = 30$, $F_{29,30} = 34.8$, $P < 0.0001$), 0.95 ($N = 38$, $F_{37,38} = 42.9$, $P < 0.0001$) and 1.00 ($N = 17$, $F_{16,17} = 36{,}600$, $P < 0.0001$) for male and female pronotum width, and male and female genital length, respectively.

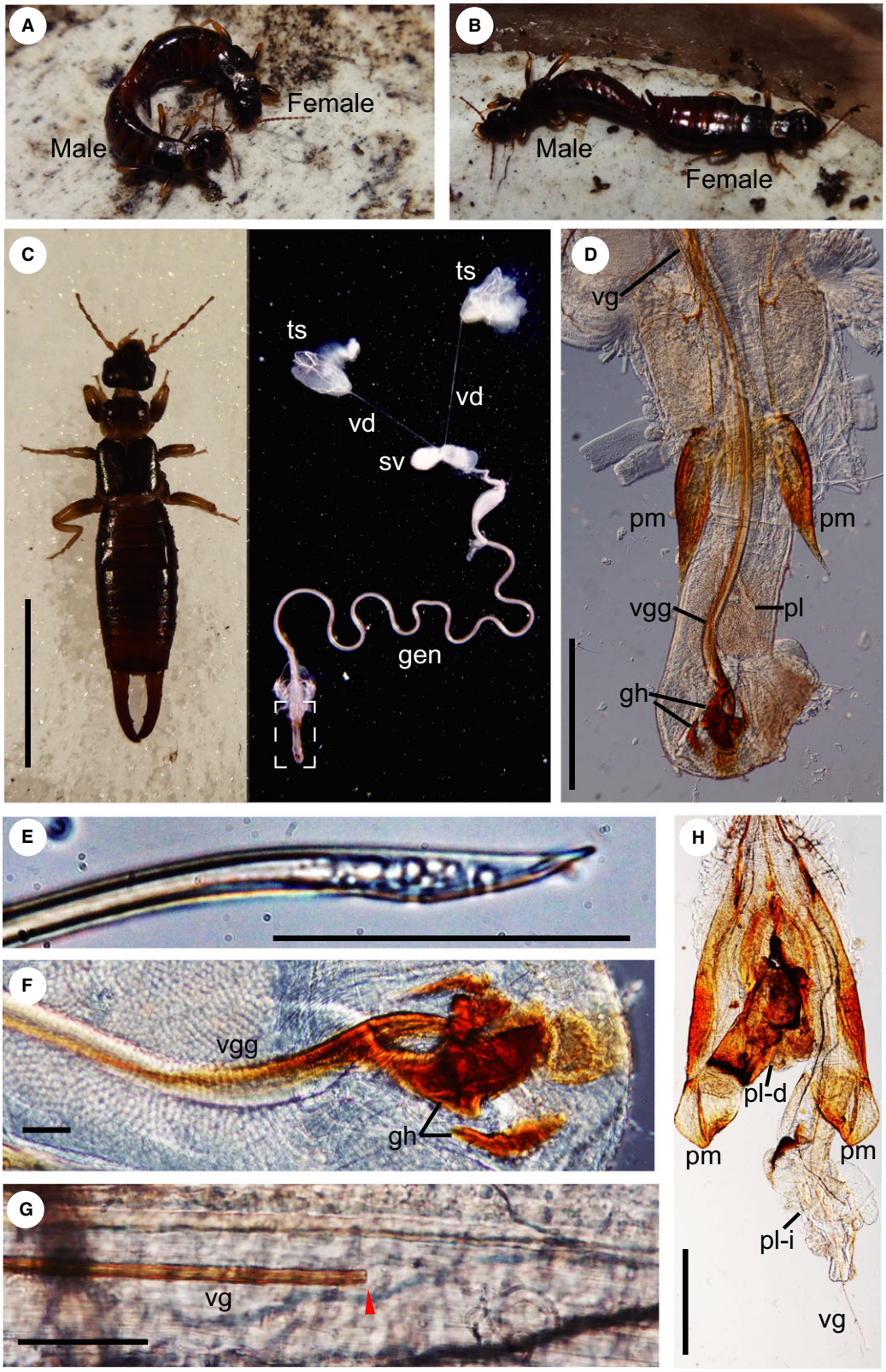
Mating behaviour, genital coupling and damage to the female genitalia

To observe mating behaviour, pairs of virgin males and females (age: 7–20 days after imaginal eclosion) were placed in separate plastic containers (50 × 32 mm, 12 mm height) with plaster of Paris at the base. Virgins of both sexes were obtained by separating newly emerged adults from stock cultures every 3 days. The female adults collected by this method had no sperm in the spermatheca ($N = 43$). Using time-lapse recordings made with a video camera (GZ-MG980S; Victor, Kanagawa, Japan), behaviour was recorded for 15 h for 62 pairs (referred to as the 'mating experiment'). Thirty minutes prior to the start of recording, females were released into the pairing vessel for acclimation, while the males were introduced immediately before the experiments. The 15 h pairings started 1.5 h before the initiation of a 12 h dark phase, during which a dim red light was used to record behaviour. After recording, the male and female samples were euthanized by placement in a freezer and were kept for subsequent observation of insemination status (females), the presence of wounds around the spermathecal opening (females; see Results), and measurement of the prothoracic width (males and females).

Mating pairs were fixed at various times after initiation of mating to directly observe genital coupling. A virgin female (at least 6 days after emergence) and a male (of unknown age) were released into a separate mating arena. Then, 5, 10, 20, or 30 min after the initiation of mating, liquid nitrogen was poured on the mating pairs to flash fix them. Frozen pairs were immediately stored in a freezer (−20 °C) until needed. The meshed male and female genitalia were carefully dissected out in insect Ringer's solution under a stereo microscope and observed under light microscopy (BX53; Olympus).

Experimental removal of spermathecae

To determine the significance of the spermathecae for female reproduction, these were surgically removed from females at various times after their first mating. Virgin females 6–32 days old were individually paired with two males (of unknown age and mating history) in a separate plastic vessel (25 × 20 mm, 10 mm height). Up to 16 triplets (one female plus two males) were simultaneously observed for 2.9–5.8 h using a time-lapse video camera. Females that mated during the video-recording were randomly assigned to one of the following three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treatments: immediate removal (IR) treatment ($N = 20$) where the spermatheca was artificially removed within 1 h (on average 21 min) after the end of mating; 3 days after removal (3R) treatment ($N = 15$) where the spermatheca was removed 3 days after mating; and positive controls (PC) ($N = 5$) where the spermatheca was kept intact until the end of the 10-day observation period. For the surgical treatments, females were lightly anaesthetized (< 15 s) with carbon dioxide gas. By gently lifting the penultimate sternite, the spermatheca was removed from the female samples using sterilized fine forceps. The removed spermathecae were immediately mounted on a slide with a drop of insect Ringer's solution and were checked for the presence of sperm. All the females, and the virgin females that served as negative controls (NC; $N = 4$), were reared for 10 days after mating. Females without detectable sperm in the spermathecae ($N = 7$ of IR and $N = 2$ of 3R) had no developing embryo in the ovaries at 10 days after mating. These females were removed from any subsequent analyses.

After 10 days, all the females were deeply anaesthetized (~1 min) with carbon dioxide gas before removal of the abdomen, which was fixed in FAA (formalin: 95% ethanol: glacial acetic acid = 6:16:1) for 3 days, and then transferred to 70% ethanol. The samples were then washed twice in phosphate-buffered saline (PBS), stained in 2 μg mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI) solution (in PBS) for 24 h, and then washed twice in PBS before observation under a fluorescence microscope (ECLIPSE 80i; Nikon, Tokyo, Japan) with an ultra violet filter set (excitation, 360–370 nm; dichroic mirror, > 400 nm; absorbance filter, > 400 nm).

Experimental breakage of male genitalia

Elongated male intromittent organs of some earwigs occasionally break during mating (Kamimura & Matsuo, 2001). The proportion of females containing broken sections of male genitalia was ascertained from a large number of females, of unknown age and mating history, collected from the original population (cockroach cultures). An experiment was carried out to compare the pattern and extent of damage to the male genitalia between *M. arachidis*, which have a single intromittent organ, termed a virga, and *Euborellia plebeja* Dohrn (Anisolabididae), which have paired virgae (Kamimura & Matsuo, 2001). Mating pairs found in stock cultures of *Marava* were interrupted by gently lifting the male and female abdomens with forceps so that the male became detached from its mate. This manipulation caused virgal breakage, as evidenced by the presence of a broken virgal piece in the spermatheca of the females. For *E. plebeja*, samples were collected from several localities in Kawasaki and Yokohama, Kanagawa Prefecture, central Japan. Field-caught adults or those that had emerged in the laboratory were paired, and matings were interrupted as in *Marava*. Because the mating duration of this species is relatively short (Kamimura, 2000, 2013), matings were interrupted 1.5 min after initiation, at which time the virgae were inserted deeply into the spermatheca (Kamimura, 2000, 2003). The treated males were kept for at least 3 days to allow for the development of detectable melanized patches, which are indicative of wound repair (Kamimura & Matsuo, 2001).

Furthermore, to test whether the broken male intromittent organs were still functional with respect to sperm transfer, three male *Marava*, from which the distal part of the virga had been lost during a preceding mating, were allowed to cohabit with four virgin females for 6 days 9–12 days after the treatment. Three intact males taken from the stock culture served as a positive control. The female and male samples were examined for the presence of sperm in the spermatheca and for damage to the genitalia, respectively.

Statistical analyses

Intraspecific (or static) allometry is usually described in terms of an allometric slope (b), based on the equation $Y = aX^b$, where Y and X are indices of trait and body size, respectively. Generally, male genital measurements are characterized by negative allometry or hypoallometry ($b < 1$), indicating that genital size relative to body size decreases with body size. (Eberhard *et al.*, 1998; Eberhard, 2009). The allometric relationship between genital size and body size

Figure 1. Mating postures (A, B), male habitus (C – left) and male genital structures (C–G) of *Marava arachidis*, together with male genitalia of *Euborellia plebeja* showing damage to the left penis lobe (H). A, A male biting the antenna of the female with his mouthparts before the establishment of genital coupling. B, Mating posture after establishment of genital coupling. C, Male habitus, internal reproductive organs and genitalia; entire view. D, Distal section of the male genitalia, corresponding to the part surrounded by the white broken line in C. E, The tip structure of a virga. F, Genital hooks and a virgal guide in a penis lobe. G, The cut-off point (indicated by the arrowhead) of a virga broken during mating. Abbreviations: gen, male genitalia; gh, genital hook; pl, penis lobe; pl-d, damaged penis lobe; pl-i, intact penis lobe; pm, paramere; sv, seminal vesicle; ts, testis; vd, vas deferens; vg, virga; vgg, virgal guide. Scale bars: 5 mm in C, 500 μm in D and H, 50 μm in E–G.

was estimated using major axis regression, a standard technique used in allometry studies. Confidence limits of the slope were calculated by bootstrapping with 10,000 resampling iterations (Manly, 1997).

We examined factors that affected mating frequency (the mating experiment), the rate of occurrence of copulatory wounds (the mating experiment), the presence/absence of sperm in the spermatheca (the spermatheca-ectomy experiment), and the presence/absence of developing embryos in the ovaries (the spermatheca-ectomy experiment) using generalized linear mixed models (GLMMs). For the analysis of female mating frequency, we adopted a Poisson error structure and a log link function with male and female age, and male and female body size (pronotum width) as the fixed explanatory factors. We also included the interaction term between male and female body sizes (fixed factor) and the block (the date of experiment: random factor). A binomial error structure and a logistic link function were used to analyze the other dependent variables. To analyze the rate of occurrence of copulatory wounds, mating frequency and total mating duration, which were not significantly correlated each other (see Results), were also included in the full model as fixed factors.

Since male age and male and female body size were not recorded in the spermatheca-ectomy experiment, the effects of female age and the duration of mating (fixed factors) on insemination success were tested, incorporating the date of the experiment as a random factor. In addition to these factors, treatment (IR vs. 3R vs. PC) was included to analyze its effect on the presence/absence of developing embryos in the ovaries.

Models were compared using an analysis of deviance to test the significance of the fixed factors in the models, adopting a sequential stepwise removal of terms. The effect of the treatment (IR vs. 3R vs. PC) on embryonic development in the ovaries was further examined by applying the same analyses for the subsamples (either IR vs. 3R, IR vs. PC, or 3R vs. PC) with a sequential Bonferroni procedure for multiple comparisons.

Other standard statistical methods were used where appropriate. All statistical analyses were conducted using R 3.2.0 software (R Core Team, 2015). All data reported are means ± SD, unless otherwise stated.

RESULTS

Morphology of the genitalia

Females showed significantly larger prothoracic width, which acted as a proxy for body size (males: 1.24 ± 0.05 mm, range: 1.12–1.34, $N = 38$; females: 1.41 ± 0.04 mm, range: 1.30–1.51, $N = 30$; $t_{66} = 14.0$, $P < 0.0001$). The male genitalia averaged 23.0 ± 1.0 mm (20.2–24.7) in length, and this was positively correlated with male body size ($r = 0.42$, $P = 0.0087$) with an estimated allometric slope of 1.01 (95% CI, 0.53–2.94; Fig. 2). The virga, which is a single, thin, sclerotized tube that contains the terminal part of the ejaculatory duct, runs the entire length of the male genitalia. The tapering-end tip of the virga, which bears a male gonopore (Fig. 1E), rested in the membranous penis lobe when in repose (Fig. 1D). Adjoining the terminal part of a virga is an elongated, spatula-shaped sclerite (Fig. 1F). This structure has been termed the 'virgal guide', reflecting its function (see below). The penis lobe also encloses a pair of triangular sclerites (Fig. 1F). Ramamurthi (1956) reported a similar triangular sclerite, which he termed a 'genital hook'. It is possible that he failed to detect the smaller structure, which is frequently hidden under the larger one, and henceforth, these sclerites are collectively referred to as 'genital hooks' in this paper.

As described by Schneider & Klass (2013), the spermatheca of *M. arachidis* comprises a single, long, blind tube without internal branching, with an opening located on the venter of the 8th abdominal segment (Fig. 3A). The length of the spermathecal tube, 28.9 ± 4.3 mm on average (range: 19.8–34.8, $N = 17$) was usually longer than the male genitalia and did not correlate with female body size ($r = 0.064$, $P = 0.81$; Fig. 2).

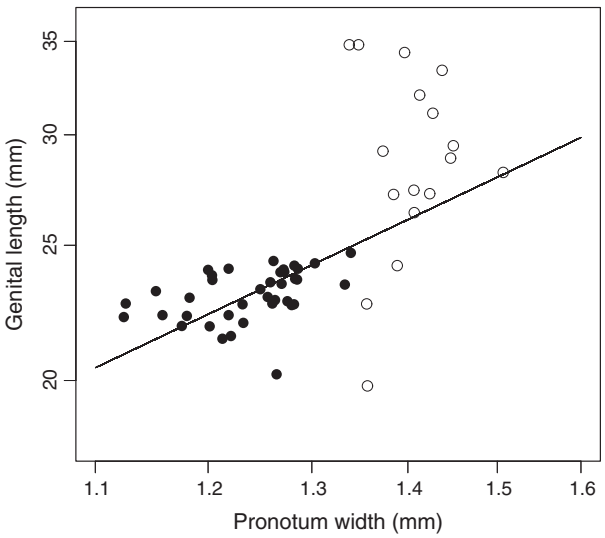

Figure 2. The length of the male genitalia (closed circles) and spermathecae (open circles) in relation to the index of body size (pronotum width). The line shows the estimated allometric relationship for the male trait.

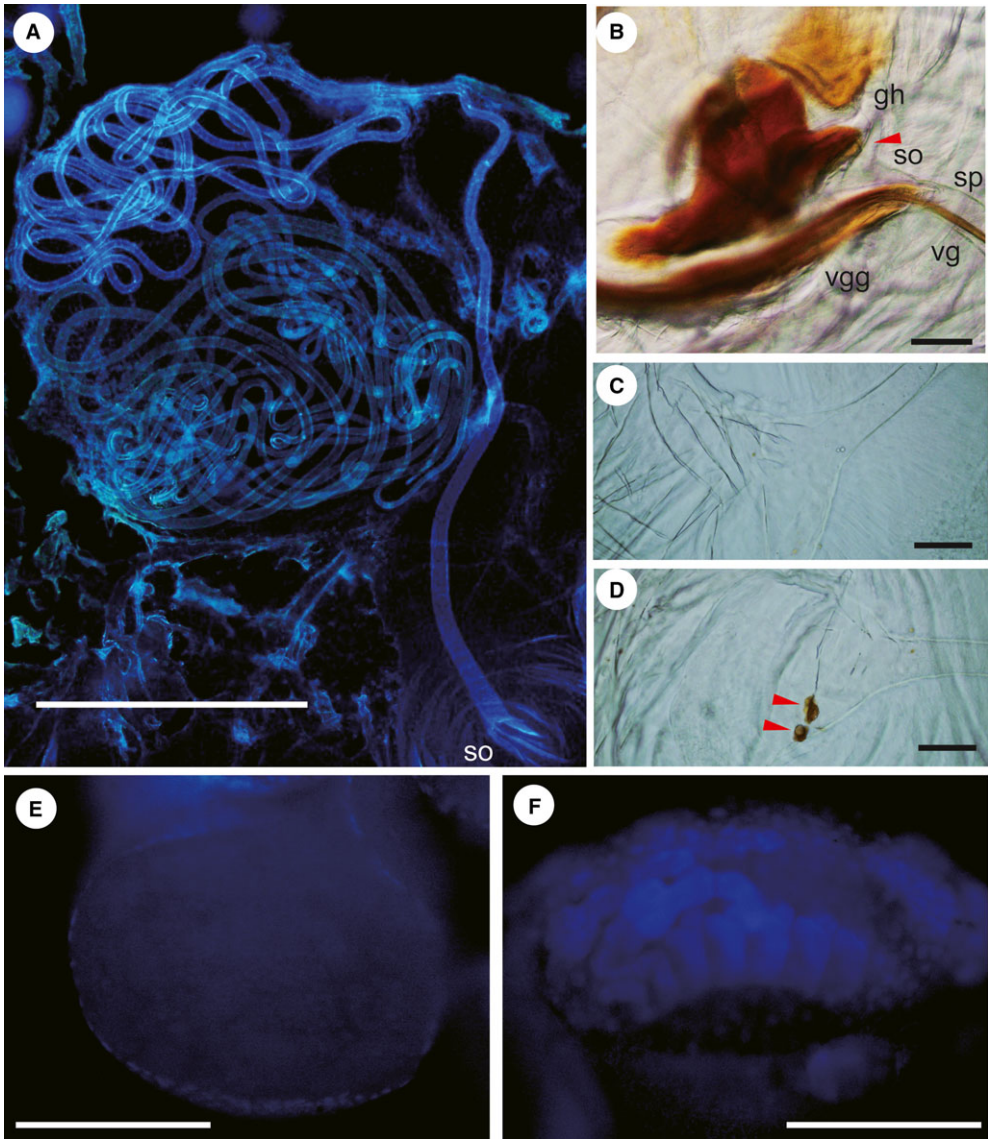

Figure 3. Spermatheca (A), genital coupling (B), the opening region of a spermatheca in a virgin (C) and a mated (D) female, and an undeveloped egg (E) and a developing embryo (F) in the ovary of a female *Marava arachidis*. The arrowheads indicate the contact point of the male genital hook(s) on the membranous spermathecal opening region in (B) and the resultant copulatory wounds in (E), respectively. Abbreviations: gh, genital hook; so, spermathecal opening; sp, spermatheca; vg, virga; vgg, virgal guide. Scale bars: 500 μm in A, E, F, 50 μm in B–D.

Mating behaviour and genital coupling

We recorded the courtship and mating behaviours of 62 virgin pairs for 15 h (mating experiment). Among these, four pairs were discarded from subsequent analyses because of dissection failures in the females. When a male detected a female with his antennae, he frequently tried to grab the female antennae or mouthparts with his mouthparts. Females were usually apparently aggressive toward males, frequently directing their forceps toward them, thereby escaping apparent mating attempts. When a male successfully grabbed a female, it evoked a resistance behaviour in which the female curved her abdomen toward the male in an apparent attempt to pinch him with her forceps. In turn, the male responded to this by curving his abdomen toward the caudal end of the female abdomen to engage in genital coupling, resulting in a circular posture of the pair (Fig. 1A). After the establishment of genital coupling, males released the antennae (or

mouthparts) of their mates, and thus were connected to the females only by their genitalia (Fig. 1B). At this stage, males rotated their abdomen nearly 180° around the anterior-posterior axis, as was generally observed in matings of other earwigs (e.g. Kamimura, 2014), to couple the male and female genitalia, which are located on the ventral side of the abdomen. Later dissection revealed that all females that mated at least once for 10 min ($N = 46$) were inseminated, while those that did not mate ($N = 11$) and a female that mated only for 6 min had no detectable sperm in their spermatheca. Thus, in this experiment, matings (defined as a single event of genital coupling) that lasted over 10 min were counted as successful (however, the results of another experiment showed that matings lasting < 75 min were sometimes infertile; see below).

In cases of at least one successful mating ($N = 46$), pairs mated 3.3 ± 2.3 (range: 1–8) times, yielding 403 ± 289 (range: 10–900) and 174 ± 215 (range: 10–900) min total and mean duration, respectively. Because of the large variation in a single mating bout, the total duration of mating was not significantly correlated with mating frequency ($r = 0.22$, $P = 0.14$). Female body size and its interaction with male body size significantly affected mating frequency, while male body size and age, and female age, and block (random factor) did not (Table 1A). Though difficult to interpret, a post-hoc analysis suggested that larger females tended to mate more frequently, especially when paired with smaller males. None of these factors showed any significant effects on the total and average mating duration (data not shown).

Genital coupling and copulatory wounding

When pairs were fixed at 5, 10, 20, or 30 min after initiation of mating, sperm was detected in all the female spermatheca, except for one female fixed at 5 min. The male virga was deeply inserted in the spermatheca of this female, indicating that it preceded sperm transfer. However, among the inseminated females, an inserted virga was detected in 0, 60, 80 and 60% of the samples fixed at 5, 10, 20, and 30 min, respectively, showing no clear trend with time. Irrespective of the insertion of the virga, the genital hooks were firmly pressed against the opening region of the spermatheca together with the virgal guide, the tip of which was shallowly inserted into the spermatheca (Fig. 3B).

As expected from the genital coupling, 15 out of 46 inseminated females in the mating experiment showed one or several melanized patches, indicative of cured wounds, on the membranous region adjoining to the spermathecal opening (Fig. 3D). None of the virgin controls ($N = 43$) and females that did not mate during the pairings ($N = 11$) showed such melanized patches (Fig. 3C). The probability of being wounded increased with total mating duration, while mating frequency, male and female age, male and female body size, and the interaction term showed no significant effects (Table 1B).

Spermatheca-ectomy experiment

Examination of the spermatheca removed from the IR ($N = 20$) and 3R ($N = 15$) females confirmed successful sperm transfer in 13 cases for each treatment. All five PC females were successfully inseminated. The probability of insemination increased as a function of mating duration, while female age had no significant effect (Table 1C). However, as shown in Figure 4, eight females that mated for > 10 min (up to 75 min) had no detectable sperm in the spermatheca, while an inseminated female mated for only 7 min, indicating a large variation in the timing of sperm transfer. The females with no detectable sperm in the spermatheca had no developing eggs in the ovaries, as was the case with the neg-

Table 1. Mixed model analyses to test the effects of fixed factors on female mating frequency, occurrence of copulatory wounds, insemination and embryonic development in the ovaries

	χ_1^2	*P*-value
A. Female mating frequency (mating experiment)		
Male age	0.215	0.643
Female age	1.955	0.162
Male pronotum width (MPW)	3.168	0.075
Female pronotum width (FPW)	5.237	0.022
MPW × FPW	9.378	0.002
B. Occurrence of copulatory wounding (mating experiment)		
Male age	0.003	0.958
Female age	1.568	0.211
Male pronotum width (MPW)	0.482	0.488
Female pronotum width (FPW)	0.285	0.593
MPW × FPW	0.170	0.680
Mating frequency	0.164	0.686
Total mating duration	6.022	0.014
C. Presence of sperm in the spermatheca (spermatheca-ectomy experiment)		
Female age	0.528	0.468
Mating duration	10.71	0.001
D. Embryonic development in the ovaries (spermatheca-ectomy experiment)		
Female age	0.322	0.570
Mating duration	0.007	0.934
Treatment (IR, 3R, or PC)	17.66	0.0001

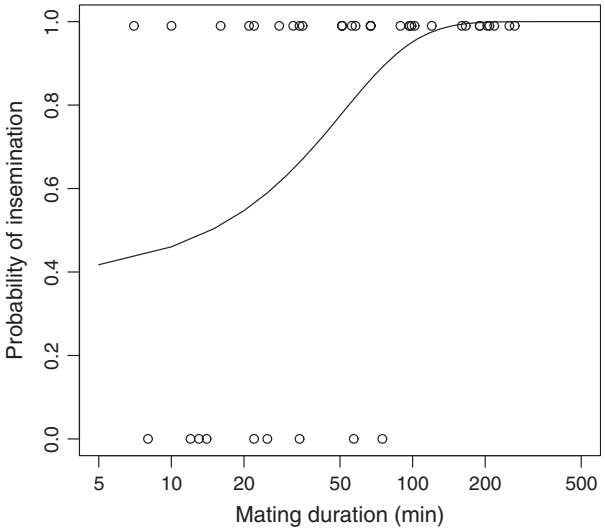

Figure 4. The relationship between the occurrence of copulatory wounds and total mating duration in *Marava arachidis*. The curved line shows predictions of the probability of wounding from logistic regression (Table 1C).

ative controls. These females were removed from subsequent analyses.

Of the inseminated females, 7 out of 13 3R and 4 out of 5 PC females had at least one developing egg in the ovaries. The developmental stages of the embryos varied among the females, but not within an individual female, ranging from those with only cleavage nuclei beneath the egg surface to those with well-developed appendage rudiments (Fig. 3F). In contrast, no IR females contained any developing embryos, as in the negative controls (Fig. 3E). The 3R and PC treatments resulted in a significantly higher probability of the development of offspring compared with IR (GLMM; $\chi^2_1 = 12.5–14.1$, $P < 0.001$), while female age and duration of mating had no significant effects (Table 1D).

Breakage of male genitalia during mating

In several earwig species, the elongated male intromittent organs (virgae) sometimes break off during mating, and the broken pieces remain in the spermatheca (Kamimura & Matsuo, 2001). Among the inseminated female samples of *M. arachidis* collected from the stock cultures ($N = 398$), pieces of broken virgae (2.9, 4.8 and 7.3 mm, respectively) were found in the spermatheca of three females, clearly indicating breakage of the virga during mating.

When mating pairs were artificially and forcefully dislodged, breakage of the virga occurred in eight out of 45 male *M. arachidis*. Three of the eight males, each of which was paired with four virgin females, successfully inseminated four ($N = 1$) or at least two ($N = 2$) females within 6 days. The insemination success was similar to that of the positive controls (all four females, $N = 1$, and three out of four females, $N = 2$), showing the ability of sperm transfer from virgae lacking a distal section. Later scrutiny of the male genitalia confirmed the loss of the distal part of the virgae (Fig. 1G), corresponding to 16.5–21.3% of the total length, while no damage was detected in any other male genital structures, including the membranous penis lobe and the associated sclerites.

Compared to *Marava*, genital breakage occurred significantly more frequently in *Euborellia plebeja*, with 31 out of 45 pairs disrupted during mating showing damage (Fisher's Exact probability test, $P < 0.0001$). Although this difference may be due to the timing of mating disruption of the two species (we could not control the timing of mating disruption in *Marava*), in 17 *E. plebeja* males with virgal destruction, necrosis was also detected in the penis lobe (Fig. 1H). This type of damage to the penis lobes was not observed in *Marava* males with a broken virga (Fisher's exact probability test, $P < 0.006$).

DISCUSSION

Insemination and sperm migration

We tested the hypothesis that the elongated male genitalia of *M. arachidis*, which is longer than the body, represents an adaptation for delivering sperm directly to the ovaries of ovoviviparous females. Observation of genital couplings failed to detect male virga directly inserted into the oviduct. Instead, it was inserted into the well-developed spermatheca, which is usually longer than the male genitalia. This type of genital coupling has been reported in many earwig species, all of which are oviparous (Popham, 1965; Briceño, 1997; Kamimura, 2000, 2006; Kamimura & Matsuo, 2001; Kamimura & Lee, 2014a, b). Additionally, even when the virga was not inserted into the spermatheca, the tip of the virgal guide was shallowly inserted into the spermatheca, indicating that insertion of the virga into the oviduct was physically impossible during this stage. Although mating pairs were fixed only at the initial stages (up to 30 min after initiation), the spermatheca-ectomy experiment clearly showed that fertilization of eggs did not occur when the spermatheca was removed immediately after the first mating. In contrast, many females whose spermatheca was removed 3 days after mating, as well as the positive controls, possessed developed embryos in the ovaries, unless the mating was infertile. This result strongly supports the view that *M. arachidis* males transfer sperm

only to the spermatheca during mating as occurs in oviparous earwigs, and that a portion of sperm migrates to the ovaries to fertilize the eggs at a later stage. The exact timing of the sperm migration is not known. Because the spermathecae of IR females were removed up to 53 min after the end of their first mating, lack of fertilization in these females suggests that sperm migration did not take place at least not within this time frame. No developing embryo was detected in 1 PC and 6 3R females that had been successfully inseminated. In addition, the developmental stages of the embryos varied among the 3R and PC females at 10 days after mating. These results suggest a large variation in the timing of sperm migration from the spermatheca to the ovaries, or in the timing of fertilization. Consistent with this view, Herter (1943) observed a large variation (12–26 days after the first mating) in the timing of the appearance of nymphs among females maintained under the same conditions after their first mating. There is increasing evidence of the ability of females to choose sperm after mating (Eberhard, 1996; Peretti & Aisenberg, 2015; see Kamimura, 2013, 2014, 2015 for earwigs). Eberhard (1996) indicated that viviparous or ovoviviparous females have many more opportunities to bias paternity among multiple mates, because of the prolonged interaction between a mother and the developing embryos in the mother's body. However, the most likely mechanism of cryptic female choice in *M. arachidis* is control over the timing of fertilization. Fixation of the mating pairs revealed sperm in the spermatheca in 80% and 100% of the samples fixed at 5 or 10–30 min after the initiation of mating, respectively. In contrast, no sperm was detected in 11% of females in the spermatheca-ectomy experiment that had mated for > 30 min (Fig. 4). The most notable difference between these two experiments was the number of males per female: only one male was present in the former and two in the latter experiment. Hence, this variation suggests a possible control of sperm acceptance by females in relation to the availability of mates, which should be investigated in the future.

Why do females of *M. arachidis* retain and use the spermatheca for storing sperm while females of some (ovo)viviparous insects have completely lost their spermathecae? One possible reason is a shorter history of ovoviviparity in *M. arachidis* compared with other examples of (ovo)viviparous earwigs that lack a spermatheca (*Sphingolabis hawaiiensis* and Hemimerina; see Introduction). Although no clear estimates are available for the phylogenetic relationships of (ovo)viviparous earwigs, several studies have suggested that both Hemimerina and Arixeniina are in-group members of the Forficulina (Klass, 2001; Haas & Klass, 2003; Jarvis, Haas & Whiting, 2005; Kocarek, John & Hulva, 2013; Tworzydlo *et al.*, 2013b). However, because females of the suborder Arixeniina, which show many adaptations for a phoretic-epizoic life and pseudoplacento-uterotrophic viviparity, retain a well developed spermatheca, the extent of the specialization for (ovo)viviparity is unlikely to be the sole explanatory factor for the presence/absence variation of a spermatheca among the taxa. In a group of chrysomelid beetles, all four conditions, i.e. ovoviviparous and oviparous species with and without a spermatheca, are observed, indicating the rapid evolution of a reproductive mode independent of changes in the sperm storage organ (Bontems, 1988). Alternatively, along with sperm choice, the ecology of *M. arachidis*, which requires the long-term storage of sperm, may explain the evolutionary retention of the spermatheca. Under laboratory conditions, females of this species repeat oviposition up to four times without additional matings (Y. Kamimura, unpublished data). This species is frequently found to invade temporary unstable habitats in artificial environments such as merchandise and stored products (Lucas, 1920; Herter, 1943; Ramamurthi, 1956; Marshall & Haes, 1988). While oviparous females have reduced migration ability during their egg care periods, female *Marava* can easily translocate while carrying embryos inside their bodies. The long-term sperm storage and iteroparity (i.e. production of multiple clutches during a lifetime) may also contribute to the establishment of a population in a new environment following successful invasion by a single inseminated female.

Premating struggle and prolonged mating

A notable characteristic of the mating of *M. arachidis* is the conspicuous premating struggle initiated with biting of the female antennae (or mouth parts) by the male. Females respond aggressively to this by directing their forceps at the males. These behaviours have also been reported by Herter (1943) in a German population of this species. To our knowledge, similar behaviour has been reported for only one other species of earwig, *Pseudomarava prominens* Steinmann, which belongs to the same subfamily Spongiphorinae (Briceño & Eberhard, 1995). In other earwigs, female quiescence is usually necessary to establish genital coupling, and thus male earwigs cannot coercively mate with an unwilling female, suggesting a female initiative in sexual conflict over mating frequency (Kamimura, 2014).

Also characteristic to *M. arachidis* is the long (at least up to 15 h) mating duration and large variability among pairs. Herter (1943) and Patel & Habib (1978) also reported a range in mating duration of

9–405 min ($N = 6$ pairs) or 11–350 min (average = 114 min for an unknown sample size) for German and Brazilian populations, respectively. The female *M. arachidis* is polyandrous (Herter, 1943). Thus, prolonged matings of *M. arachidis* may represent post-insemination, in-copula mate guarding behaviour. In support of this view, in many pairs fixed during mating (26.3% in total), the male virga was not inserted into the spermatheca, which had already been filled with his sperm. Although the results of this study strongly suggest that sperm migration does not occur during mating, such guarding behaviour can be still effective in enhancing the paternity of the male if it reduces the possibility of the female remating with other males before fertilization of the ova.

Females of *M. arachidis*, though polyandrous, usually show aggression toward courting males, possibly to avoid the costs of mating caused by prolonged holding with the genital hooks. The resultant copulatory wounds also likely impose a cost on females. Future studies should quantify this although it may be low due to counter-adaptations in female immune systems (Reinhardt, Anthes & Lange, 2014). As observed in this study and by Herter (1943), virgin females of *M. arachidis* frequently escaped from male mating attempts, resulting in ~20% of females remaining infertile at the end of a 15 h cohabitation period with a male. These observations suggest that strong female choice also works in the precopulatory stages, although the target male traits are presently unclear.

Evolution of fragile male genitalia

Possibly due to struggles that continue or recur after the establishment of genital coupling, the thin male virgae sometimes break inside the spermatheca of their mate, as evidenced by the presence of broken sections in the females. This type of breakage of a virga under natural conditions has also been reported for males of two anisolabidid species, *Euborellia plebeja* and *Anisolabis maritima* (Bonelli), which are also characterized by male genitalia being longer than the body (Kamimura & Matsuo, 2001; Kamimura, 2003). In the anisolabidids, the elongated virga and a flange-like projection at its tip are considered to be an adaptation for removing rival sperm from the elongated spermatheca of polyandrous females (Kamimura, 2000, 2003, 2005, 2013). Although the virgal tip of *M. arachidis* lacks any special structures (Fig. 1E), the following genital characteristics are shared by the two groups of earwigs: (1) the length of the spermathecae varies considerably among females and shows no significant correlation with female body size, but is usually longer than the male genitalia (Fig. 2; Kamimura, 2000); and (2) male genital length is also variable, with a negative or almost isometric allometric slope (Fig. 2; Kamimura & Iwase, 2010). These similarities suggest the elongated male genitalia of *M. arachidis* also represents an adaptation related to sperm competition.

The rate of females possessing a broken piece of virga in their spermatheca was similar between *M. arachidis* (three out of 398 females examined) and the two anisolabidids (two of 359 for *A. maritima* and zero of 26 for *E. plebeja*; Kamimura & Matsuo, 2001). In *E. plebeja*, a broken piece of virga does not function as a mating plug and usually has no detrimental effect on female remating and oviposition behaviours (Kamimura, 2003). It is unclear whether this is also the case for *M. arachidis*. However, the extent of damage to the membranous penis lobes differed between these two groups of earwigs. As demonstrated in this study, breakage of the virga in *E. plebeja* and other anisolabidid earwigs was frequently accompanied by necrosis of a penis lobe (Fig. 1H; Kamimura & Matsuo, 2001). Similar damage has been reported for male *Labidura riparia* Pallas (Labiduridae), which also possesses two functional penises (Kamimura, 2006). In these species, although the damaged penises are apparently rendered incompetent, the males are still able to mate with females using the intact spare penis (Kamimura & Matsuo, 2001; Kamimura, 2006). Interestingly, no such damaged penis lobe was detected in male *M. arachidis* when mating was artificially disrupted. In this species, damage was restricted to the thin virgae. The penis lobes of *E. plebeja*, *A. maritima*, and *L. riparia* are devoid of any conspicuous sclerites for holding on to their mates (Kamimura & Matsuo, 2001; Kamimura, 2006). Therefore, the prominent genital hooks of *M. arachidis* likely function to protect the fragile, membranous parts of the male genitalia, by securely holding on to the female genitalia during mating. Instead of having a spare intromittent organ, the specialized virga, which is still competent after the loss of the distal section, and the protective function of the genital hooks, likely have enabled the elongation of the single, fragile genitalia in this ovoviviparous insect.

Traumatic mating and its possible significance

The observation of genital coupling of *M. arachidis* confirmed that the genital hooks are firmly pressed against the membranous region of the spermathecal entrance, where copulatory wounds were frequently found. Increasing evidence shows many male animals inflict wounds on the female through the use of their genital structures during mating (Lange *et al.*,

2013; Reinhardt *et al.*, 2014; Tatarnic *et al.*, 2014). To date, this mode of mating, termed traumatic mating, has been reported in only one other species of earwig, *Echinosoma denticulatum* Hincks (Kamimura & Lee, 2014a).

The functions of copulatory wounding are much debated and may differ among taxa (Lange *et al.*, 2013; Reinhardt *et al.*, 2014; Tatarnic *et al.*, 2014). Because the copulatory wounds of *M. arachidis* do not come into direct contact with male ejaculate during mating, they are not likely to function as entrances for sperm and/or seminal fluids to the female hemocoel (traumatic insemination or traumatic secretion transfer; *sensu* Lange *et al.*, 2013). Copulatory wounding of this species may retard remating of the female (see Discussion in Kamimura & Lee, 2014a), or may represent a side effect of mate-anchoring or stimulatory functions of male genitalia during mating. The occurrence rate of copulatory wounds was positively correlated with the total duration of mating. Possibly as a form of in-copula mating struggle, females sometimes walk and drag the male mate during mating while some of his legs are lifted off the substrate, indicating that the coupling of the genitalia suffices to keep the mating pairs together. The anchoring power of the genital hooks is likely enhanced when they penetrate deeply into the membranous region of the spermathecal entrance, resulting in longer mating duration. Alternatively, it is possible that longer mating results in greater risk of accidental injury caused by the genital hooks. Neither explanation contradicts the view that copulatory wounding in *M. arachidis* represents a side effect of mate-holding. In addition, as discussed above, secure holding of the female genitalia likely also functions to protect the fragile parts of the male genitalia from disturbance during mating. With the unique combination of coercive traumatic mating, breakage of elongated male genitalia during mating and possible control of sperm migration by females, this species provides an excellent opportunity to study sexual conflicts in (ovo)viviparous animals.

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