Household and Structural Insects

Effect of Mating Status and Age on the Male Mate Choice and Mating Competency in the Common Bed Bug, *Cimex lectularius* (Hemiptera: Cimicidae)

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Abstract

We investigated male mate choice and mating competency in the common bed bug, *Cimex lectularius* L., using video tracking for 10 min per experiment. In the male mate choice experiment, when a male was placed with two females of different mating status, males preferred to initiate copulation with the virgin female more quickly than with the mated female, and the mean total copulation duration with virgin females (38.0 ± 3.0 s) was significantly longer than with mated females (14.6 ± 3.0 s). When a male was placed with two females of different age, males initiated copulation more quickly with the old virgin female (29–34 d adult emergence) than with the young virgin one (<7 d adult emergence), and the mean total copulation duration with old virgin females (38.4 ± 4.0 s) was significantly longer than with young virgin females (24.0 ± 3.0 s). In the male mating competency experiment where a female was placed with two males of different mating status or age, the virgin males were more eager to mate than the mated males, and the old virgin males (29–34 d adult emergence) were more eager than the young virgin males (<7 d adult emergence), with eagerness measured by the percentage of first mate selected (first copulation occurred) and the total copulation duration by each group of males. Male mating competency is related to postmating duration (PMD); males mated 1 d earlier were significantly less likely to mate than virgin males. However, males mated 7 d earlier showed no significant difference in mating competency compared to virgin males. In conclusion, mate choice in *C. lectularius* is associated with both male and female mating status, age, and PMD.

Key words: bed bug, mate choice, mating competency, postmating duration

For male insects, their response to females is associated with their expectation of benefits and costs (Bonduriansky 2001). Associated costs include longevity costs (Cordts and Partridge 1996, Martin and Hosken 2004) and increased risk of predation (Magnhagen 1991). Moreover, mating with unsuitable females will result in loss of time and fertilization opportunities (Simmons and Siva-Jothy 1998, Delpuech et al. 2010). Thus, selecting suitable mates is important for fathering more offspring during the males' life span.

To maximize their fertilization success from each mating encounter, males tend to favor female phenotypes associated with high fecundity or low sperm competition (Bonduriansky 2001). Body size or abdomen width of females, which is associated with female fecundity, can be assessed directly by males through visual or tactile mechanisms (Bonduriansky 2001). In *Drosophila melanogaster* Meigen, males preferentially select and spend more time copulating with larger females than with smaller ones (Byrne and Rice 2006, Lüpold et al. 2011, Edward and Chapman 2012). However,

different results were reported by Edward and Chapman (2013), in which male *D. melanogaster* preferentially selected the smaller females than the larger ones. The difference among these reports was related to the different selection pressures between populations (Edward and Chapman 2013). For *Tenebrio molitor* L. (Carazo et al. 2004), *Spalangia endius* Walker (King et al. 2005), and *Asobara tabida* (Nees) (Dufour et al. 2012), the mated females that fertilized most of their eggs during the first copulation event were considered as lower quality selections, thus males usually prefer to select virgin females. Male mate selection is not only affected by female mating status, but also influenced by female age. Shelly et al. (2012) reported that when presented with young (10–15 d old) and old (35–40 d old) *Ceratitis capitata* (Wiedemann) females, males mate significantly more often with the young females; this phenomenon is due to the higher fecundity and fertility of the young females.

In addition, the mating competency of male insects also is influenced by their intrinsic factors, such as the male mating status or

age. When a virgin *S. endius* female was presented to a virgin and a mated male, the virgin male responded to the female first (King et al. 2005). Zhou et al. (2012) reported that the mating rate of virgin male *Ophraella communa* LeSage was significantly higher than newly copulated males and those mated 8 d earlier. Studies have reported that old males were less choosy and more likely to mate than young males (Pérez-Staples et al. 2010, Zhou et al. 2012, Karl et al. 2013). In *Bicyclus anynana* (Butler), old male mating advantage was benefited from higher activity; the older males were significantly more active and spent more time walking and flying than younger males (Karl et al. 2013). Furthermore, the mating investment of male *Drosophila pseudoobscura* Frolova and Astaurov increases with their age; the old males prefer to spend more time copulating than the young males in order to father more offspring (Dhole and Pfennig 2014). In addition, mating duration was reported as positively related to male age in *Colaphellus bowringi* Baly; the prolonged copulation of old males is likely due to their declining ability for transferring sperm (Liu et al. 2011).

Bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae), have a unique method of copulation called "traumatic insemination" (Carayon 1966). During copulation, the female bed bug receives the male intromittent organ through the ectospermalege on her abdominal wall, rather than her vagina (Rivnay 1933, Carayon 1966, Stutt and Siva-Jothy 2001). Based on Reinhardt and Siva-Jothy (2007), the spermalege is "a unique bed bug organ positioned under the area of the female's abdominal exoskeleton that receives sperm". Mating behavior of bed bugs has been described by Rivnay (1933). Because no ovarian development has happened in virgin females, only the mated females could produce eggs, with around 173 normal eggs laid by a female after a fertilization event (Cragg 1923). However, the shortest copulation duration required for successful fertilization is unknown.

Rivnay (1933) reported that male bed bugs often search for mates through vision; the sight of a moving female could attract a male's attention during the mate-detecting period. Meanwhile, in order to successfully copulate, males preferred to approach females that have a body size similar to fed females; this mate selection preference is due to the decreased resistance of fed females (Siva-Jothy 2006, Reinhardt et al. 2009). Female resistance time was defined as the time interval between male's mounting and intromission (mating); it was used to measure a male's ability of overcoming female resistance (Reinhardt et al. 2009). In addition, chemical communication of bed bugs during the mate-selection period has been found to be important. The alarm pheromone released by nymphs when they are mounted by males, decreases the risk of nymphal mating (Harraca et al. 2010). Stutt and Siva-Jothy (2001) reported that bed bugs have last-male sperm precedence, meaning if two males mate with the female, the last mate will have higher chances of impregnation; when copulated with a mated female, a male could still father its own offspring. However, if a male bed bug was placed with more than one female, a mate choice would occur. Similarly, if two males were placed with only one female, mating competition between the males would occur. Whether intrinsic factors, such as mating status or age, would affect male bed bugs mate choice and mating competency is unclear. Furthermore, whether male bed bugs mate choice behavior is due to the difference in 1) body size or abdomen width, or 2) locomotor activity between females with different mating status or age was not reported.

The objectives of this study were to investigate: whether the mating status, age, or postmating duration (PMD) affects locomotor activity, male mate choice, and male mating competency in bed bugs. Our study aims to provide basic information about bed bugs' mating

behavior. This information provides better understanding of this public health pest and could help design more effective control methods.

Materials and Methods

Insects
Bed bugs were collected in 2009 from multiple infested apartments in Bayonne, NJ. They were maintained in plastic containers (5 cm diameter and 4.7 cm height; Consolidated plastics, Stow, OH) with folded papers as harborages (4 by 3 cm; Universal Stationers Supply Co., Deerfield, IL), held at $25 \pm 1°C$, $40 \pm 10\%$ relative humidity (RH), with a photoperiod of 12:12 (L:D) h. Bed bugs were fed weekly on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA) using a Hemotek membrane-feeding system (Discovery Workshops, Accrington, United Kingdom).

To obtain virgin adults of a similar age, last instar nymphs were placed in plastic Petri dishes individually (3.5 cm diameter and 1 cm internal height; Fisher Scientific, Pittston, PA; one nymph per dish) after feeding. After emergence, males and females were sexed and held in the male or female group. All bed bugs were fed 1 d prior to experiments. Enamel paint (Plaid Enterprises, Inc., Norcross, GA) was used to mark bed bugs. For the adults with different mating status, the virgins were marked white, and the mated with green. For the adults with different age, the young virgins were marked white, and the old virgins with green. Bed bugs that were kept in dishes were introduced into the chamber at least 1 h before experiments to let them acclimate to the environmental conditions. During experiments, one researcher stayed in the room, and a distance was maintained between the researcher and the experimental arenas of around 1–2 m. Plastic Petri dishes were used as experimental arenas for all experiments; the bottom of each dish was covered with a clean filter paper (3.5 cm diameter; Fisher Scientific, Pittsburgh, PA), and the inner wall of each dish was coated with a thin film of talcum powder (Sigma-Aldrich, Inc., Milwaukee, WI). For each observation, new clean filter papers were used with new bed bugs. All experiments were carried out under dim red light conditions (25 -W bulb), in a walk-in chamber at $25 \pm 1°C$, 20% RH.

General Mating Behavior
We first observed the mating behavior of bed bugs to obtain baseline information. Pairs of virgin bed bugs (<7 d adult emergence) were placed in plastic Petri dishes. A total of 76 pairs of bed bugs were observed. A male was introduced first and a female was introduced immediately afterwards. Their mating behavior was recorded with a digital camera (Model SONY HDR-XR550V, Sony Corporation, Japan). The recording started as soon as the female was introduced into the Petri dish and ended when the first mating event finished (naturally terminated) or 10 min after female introduction, if no mating occurred. The duration of copulation (the time interval between male's intromission and withdrawing) was noted for each mating event.

After the first mating event or 10 min after recording, if no mating occurred, female and male bed bugs were separated. The females were transferred into clean plastic Petri dishes individually (one female per dish) and the bottom of each dish was lined with filter paper. The number of eggs laid by each female was counted after 7 d. Then the eggs were incubated for 14 d under $25 \pm 1°C$, $40 \pm 10\%$ RH, with a photoperiod of 12:12 (L:D) h.

Locomotor Activity
To determine if the mating status or age affect bed bugs' locomotor patterns, we examined their locomotor activity using EthoVision

XT (Model 10.1, Noldus Information Technology, Wageningen, Netherlands). Six Petri dishes were placed side by side in a 2×3 grid. For each trial, six marked bed bugs were introduced randomly and singly to a Petri dish. In the first test, three virgin and three mated females or males were used, and in the second test, we used three young virgin and three old virgin females or males. The bed bugs' movement was recorded for 10 min. Both males and females of different mating status, virgin and mated, were <7 d adult emergence, and the bed bugs of different ages (young virgin—<7 d adult emergence and old virgin—29–34 d adult emergence) were all unmated; the mated bed bugs were obtained 1 h prior to experiments by holding a virgin female with a virgin male in a plastic Petri dish until copulation was observed.

"Static subtraction" was selected as the detection method for tracking bed bugs. The sample rate (rate at which EthoVision analyzes the images to find the subject) was 25 samples s^{-1}. The "velocity" and "movement" were used as dependent variables. Only the data with <1% missed samples and <1% subject not located (no subject detected by EthoVision) were selected. Velocity or speed (distance moved by the bed bug per unit time) was calculated as cm s^{-1}. Movement was defined as when the bed bug's speed was greater than 0.1 cm s^{-1}. Time spent moving was the duration of movement. The percentage of movement was calculated by dividing the time spent moving by the time spent in the zone or experimental arena (10 min; Weeks et al. 2013). Once the locomotor activity was recorded, the bed bugs were used for studying male mate choice and mating competency.

Preliminary Experiment

This experiment was conducted to determine whether the different marked colors affect locomotor activity of adults, male mate choice, or male mating competency. Male and female bed bugs used in preliminary experiments were <7 d adult emergence and unmated. Each preliminary experiment was replicated 28–33 times. The results indicated that locomotor activity of adults, male mate choice, and male mating competency were not affected by the marked colors. The locomotor activity was recorded when a human host was present; therefore, it was considered "disturbed" activity and might not reflect the activity pattern when a human host was absent.

Experiment I. Male Mate Choice in Relation to Female Mating Status or Age

This experiment was designed to determine whether male bed bugs can discriminate between females of different mating status or age. Each male was placed with two females. In the first test, a virgin male was placed with a virgin and a mated female; male and female bed bugs were <7 d adult emergence; the mated females were obtained 1 h prior to experiments by holding a virgin female with a virgin male in a plastic Petri dish until copulation was observed. In the second test, a male (<7 d adult emergence) was placed with a young female (<7 d adult emergence) and an old female (29–34 d adult emergence); all male and female bed bugs were unmated. All females used were examined for their locomotor activity for 10 min before this experiment. Two females were introduced first in a random order, and then a male was introduced right after. Their behavior was recorded with a digital camera as soon as the male was introduced and ended after 10 min. Each test was replicated 50 times. The exact age of the bed bugs within each age group was unknown. Bed bugs were randomly selected from a pool with known age ranges ("young" or "old"), for each replicate. Each insect was used only once in the experiment. The type of female that first selected for

copulation by the male was recorded; meanwhile, the copulation frequency and total duration of copulation (the total copulation time) of each female was measured for each experiment (replication).

Experiment II. Male Mating Competency in Relation to Their Mating Status or Age

We conducted two tests to investigate the effect of male mating status or age on their mating competency. In the first test, a virgin female was placed with a virgin male and a mated male; all bed bugs (both males and females) were <7 d adult emergence; the mated males were obtained 1 h prior to experiments by holding a virgin female with a virgin male in a plastic Petri dish until copulation was observed. In the second test, a female (<7 d adult emergence) was placed with a young male (<7 d adult emergence) and an old male (29–34 d adult emergence); all male and female bed bugs were unmated. All males used were examined for their locomotor activity for 10 min before this experiment. Males were introduced first, in a random order, and then a female was introduced immediately afterwards. Observation started as soon as the female was introduced, and their behavior was recorded by a digital camera for 10 min. Each test was replicated 50 times. The type of male that first selected the female for copulation, the copulation frequency, and total copulation duration of each male for each experiment (replication) were recorded.

Experiment III. Relationship Between Male Mate Choice and PMD of the Mated Females

This experiment was designed to assess the effect of female PMD on male mate choice behavior. In each test, a virgin male was placed with two females (a virgin and a mated female); all bed bugs (both males and females) were 8–13 d adult emergence. The mated females were obtained at 1, 3, or 7 d prior to experiment, so their PMDs were 1, 3, or 7 d at the time of experiment. The experimental method was the same as Experiment I. Each test was replicated 50 times. The recorded parameters were the type of female that was first selected for copulation by the male, the copulation frequency, and total copulation duration of each female for each experiment (replication).

Experiment IV. Relationship Between Male Mating Competency and Their PMD

Similar to Experiment III, the effect of male PMD on mating competency was evaluated. In each test, one virgin female was placed with two males, both males and females were 8–13 d adult emergence. One of the males was virgin and the other was mated. The mated males were obtained at 1, 3, or 7 d prior to experiment, so their PMDs were 1, 3, or 7 d at the time of experiment. The recorded parameters were the type of male that first selected the female for copulation, the copulation frequency, and total copulation duration of each male for each experiment (replication).

Statistical Analysis

The differences between virgin and mated females or males, or between young virgin and old virgin females or males in the measured variables (moving speed and percentage of movement) were compared using independent samples *t*-test. The speed data were square root transformed and the percentage data were arcsine-square root transformed prior to the analyses, in order to satisfy the assumptions of normality.

In the Experiments I–IV, the differences between virgin and mated females or males, or between young virgin and old virgin

females or males in the mate choice data (the type of female that was first selected for copulation by the male, or the type of male that first selected the female for copulation) were compared using a Chi-square test. Wilcoxon signed-rank test was used for analyzing the copulation frequency. Paired samples *t*-test was used for analyzing the total copulation duration. All data analyses were carried out using SPSS software (SPSS Institute 2004).

Results

General Mating Behavior
All pairs of bed bugs completed their first copulation event during the 10-min period. The mean (min, max) duration of copulation was 42.2 (16, 129) s. The female with the copulation duration of 16 s did not lay eggs. The shortest copulation duration for successful fertilization was 19 s. Within 7 d after mating, the mean reproduction of females was 8.6 ± 0.3 eggs per female; more than 93% eggs successfully hatched after 14 d of incubation.

Effect of Mating Status and Age on Locomotor Activity
The mating status of females had no effect on their moving speed (virgin females: 0.5 ± 0.08 cm s^{-1}, mated females: 0.4 ± 0.04 cm s^{-1}; $t = 1.7$, df $= 42.2$, $P = 0.100$) and percentage of movement (virgin females: $45.5 \pm 5.5\%$, mated females: $34.9 \pm 3.6\%$; $t = 1.7$, df $= 43.3$, $P = 0.101$). However, locomotor activity of females was significantly affected by their age. The old virgin females moved significant faster (moving speed: 0.4 ± 0.04 cm s^{-1}; $t = -3.7$, df $= 97$, $P < 0.001$) and spent more time moving (percentage of movement: $38.6 \pm 2.7\%$; $t = -3.8$, df $= 97$, $P < 0.001$) than young virgin females (moving speed: 0.3 ± 0.02 cm s^{-1}, percentage of movement: $25.0 \pm 2.2\%$). Both male mating status and age had no effect on their moving speed and percentage of movement (all $P > 0.05$). For virgin and mated males, their moving speeds were 0.4 ± 0.05 and 0.5 ± 0.05 cm s^{-1}, respectively; the percentages of movement were 37.6 ± 3.6 and $43.1 \pm 3.3\%$, respectively. For young virgin and old virgin males, their moving speeds were 0.2 ± 0.02 and 0.2 ± 0.02 cm s^{-1}, respectively; the percentages of movement were 22.8 ± 1.9 and $23.1 \pm 2.1\%$, respectively.

Male Mate Choice
When exposed to two females of different mating status, males initiated copulation with the virgin female significantly more quickly than with the mated female ($\chi^2 = 8.0$, df $= 1$, $P = 0.005$; Fig. 1a) . During the 10-min period, the copulation frequency with virgin females was significantly higher than that with mated females ($Z = -2.6$, $P = 0.009$; Fig. 1c); the total copulation duration with virgin females was significantly longer than that with mated females ($t = 4.5$, df $= 44$, $P < 0.001$; Fig. 1e).

When exposed to a young virgin and an old virgin female, males initiated copulation with the old virgin female significantly more rapidly than with the young virgin female ($\chi^2 = 5.1$, df $= 1$, $P = 0.024$; Fig. 1b). During the 10-min period, males spent significantly more time copulating with old virgin females than with young virgin females ($t = -2.5$, df $= 49$, $P = 0.017$; Fig. 1f). However, there was no difference in copulation frequency between young virgin and old virgin females ($Z = -1.7$, $P = 0.083$; Fig. 1d).

Male Mating Competency
Virgin males were more likely to mate than mated males; virgin males initiated copulation with females significantly more rapidly than mated males ($\chi^2 = 8.5$, df $= 1$, $P = 0.004$; Fig. 2a). Virgin males copulated significantly more times ($Z = -3.0$, $P = 0.003$; Fig. 2c) and had
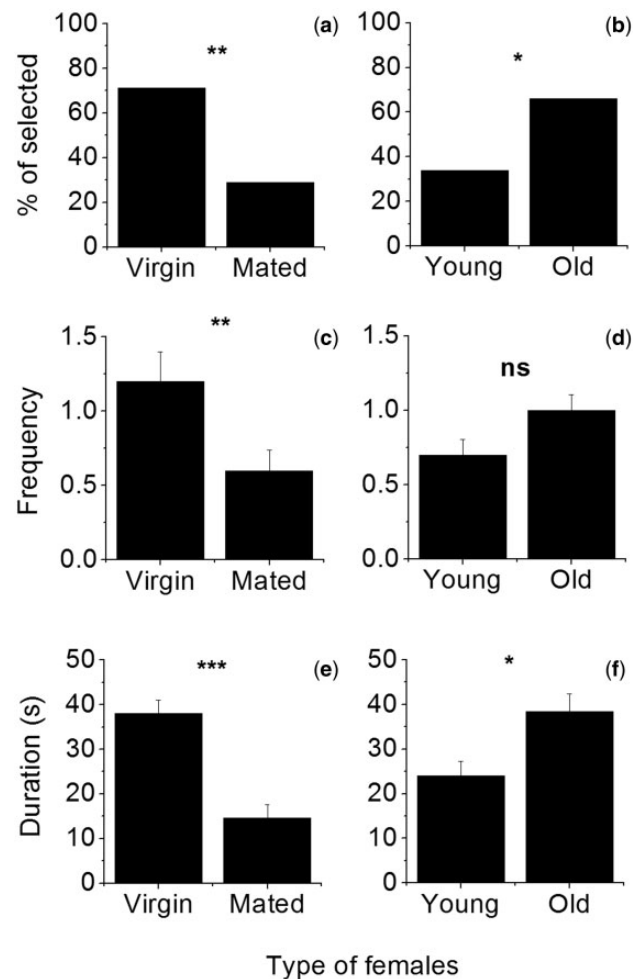

Fig. 1. (a–f) Male mate choice measured by percentage of male first mate selected (the type of female that was first selected by male for copulation), mean copulation frequency, and mean total copulation duration with female bed bugs during 10-min period. Each male was offered two females of different mating status (virgin vs. mated 1 h earlier) or age (young: <7 d adult emergence vs. old: 29–34 d adult emergence).

longer copulation duration ($t = 2.8$, df $= 37$, $P = 0.007$) than mated males in the 10-min period (Fig. 2e). Similarly, old virgin males were more likely to mate than young virgin males; old virgin males initiated copulation more rapidly ($\chi^2 = 5.4$, df $= 1$, $P = 0.020$; Fig. 2b) and copulated significantly more times ($Z = -3.2$, $P = 0.002$; Fig. 2d) and had longer copulation duration ($t = -9.0$, df $= 35$, $P < 0.001$) than young virgin males, in the 10-min period (Fig. 2f).

Relationship Between Male Mate Choice and PMD of the Mated Females
During the 10-min period, males preferred to spend significantly more time copulating with virgin females than with mated females with 1 PMD ($t = 2.3$, df $= 48$, $P = 0.026$; Fig. 3g). However, this preference disappeared between virgin females and mated females with 3 or 7 PMD (all $P > 0.05$; Fig. 3h and i). Males initiated copulation with the virgin female significantly more rapidly than with the mated female with 3 PMD ($\chi^2 = 6.8$, df $= 1$, $P = 0.009$; Fig. 3b), but no significant difference between virgin and mated female with 1 or 7 PMD (all $P > 0.05$; Fig. 3a and c). No significant difference was observed in copulation frequency between the virgin and mated females with 1 or 3, or 7 PMD (all $P > 0.05$; Fig. 3c, f, and i).
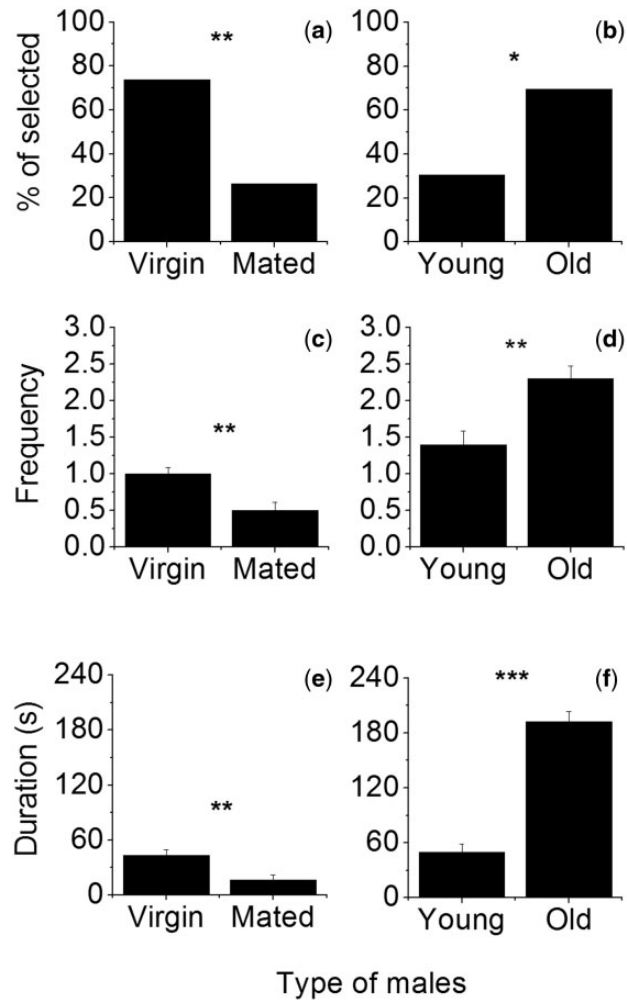
Fig. 2. (**a–f**) Male mating competency measured by percentage of male first mate selected (the type of male that first selected female for copulation), mean copulation frequency, and mean total copulation duration of the male bed bugs during 10-min period. Two males of different mating status (virgin vs. mated 1 h earlier) and age (young: <7 d adult emergence vs. old: 29–34 d adult emergence) were offered to each female.

Relationship Between Male Mating Competency and Their PMD

The virgin males were more likely to initiate copulation ($\chi^2 = 6.4$, df = 1, $P = 0.011$), had higher copulation frequency ($Z = -2.7$, $P = 0.008$), and longer total copulation duration ($t = 3.9$, df = 44, $P < 0.001$) than the mated males with 1 PMD, during the 10-min period (Fig. 4a, d, and g). When comparing virgin males with mated males with 3 PMD, only the total copulation duration was significantly different ($t = 2.4$, df = 41, $P = 0.021$; Fig. 4h). All the measured variables (the type of male that first selected the female for copulation, copulation frequency, and total duration of copulation) between virgin and mated males with 7 PMD were not significantly different (all $P > 0.05$; Fig. 4c, f, and i).

Discussion

In our male mate choice experiment, when exposed to two females with different mating status, male bed bugs were more likely to initiate copulation with the virgin female; when exposed to a young virgin and an old virgin female, male bed bugs copulated with the old virgin

female more quickly than with the young virgin female. Because the mated females copulated 1 h prior to experiment, there was no significant difference in body size between the virgin and mated females; in addition, our results indicated that the locomotor activity of females was not affected by their mating status. Therefore, male mate choice between females with different mating status or age was not related to body size, or locomotor activity. In studies about parasitoids' mate selection, males could strongly discriminate between virgin and mated females (McClure et al. 2007, Martel et al. 2008), and this phenomenon was due to a postmating decrease of the sex pheromone (McNeil and Brodeur 1995). However, whether there was difference in sex pheromone between virgin and mated females or between young and old females in bed bugs is unclear.

Male bed bugs can detect the presence of ejaculate in mated females with their intromittent organ and reduce the copulation duration accordingly (Siva-Jothy and Stutt 2003). In order to avoid sperm competition, male bed bugs may spend more time copulating with virgin females, as was observed in our experiment. This mating strategy could help males reduce the sperm loss due to competition with other males and increase chances of fathering more offspring through copulating. In our male mate choice experiment, during the 10-min period, some males could accomplish more than one occurrence of copulation with the virgin females; therefore, during the subsequent copulations, the male was more likely to detect the ejaculate, which was ejected by himself during the first copulation period, with his intromittent organ. Whether this self-ejaculate detection affects male bed bugs' copulation duration during the subsequent copulations is unknown.

In our male mate choice experiment, males' total copulation duration with virgin females was significantly longer than that with mated females. In addition, male bed bugs spent more time copulating with old virgin females than with young virgin females. The longer total copulation duration of males might be associated with their higher copulation frequency.

When the number of mates is limited, competing for a mating opportunity with a female would likely occur among male bed bugs. Male mating competency was associated with both their mating status and age. Male mating status or age not only affects their copulation initiation behavior, but also the mating frequency and total copulation duration. Virgin males initiated copulation more quickly and spent more time copulating than recently mated males. The theory of lower sperm loads in mated males than virgin males may explain this phenomenon (King et al. 2005). After copulation, males have to spend enough time to replenish the accessory-gland fluid and produce additional sperm for the next ejaculation (King et al. 2005). Reinhardt et al. (2011) reported that male bed bug mating frequency increased with their age (days from adult emergence) and leveled off around 15 d postemergence. In bed bugs, from emergence onwards, cumulative sperm and seminal fluid volume of virgin males increase logarithmically (Reinhardt et al. 2011). Whether the prolonged copulation of male bed bugs with old females was due to males' declining ability of sperm transfer (Liu et al. 2011) should be further studied. In our male mating competency experiment, because only one virgin female was exposed to two males, the detection of the ejaculate ejected by the former male was more likely to affect the copulation of the latter male. Compared with the former male, the latter male was more likely to spend less time copulating.

When mounted by male bed bugs, unfed females could press their right, or entire ventral side of the abdomen flat to the bottom or the wall of experimental arena (Siva-Jothy 2006, Reinhardt et al. 2009). In our experiment, all females were fed 1 d prior to the bioassays; they could not press their right or entire ventral side flat to the
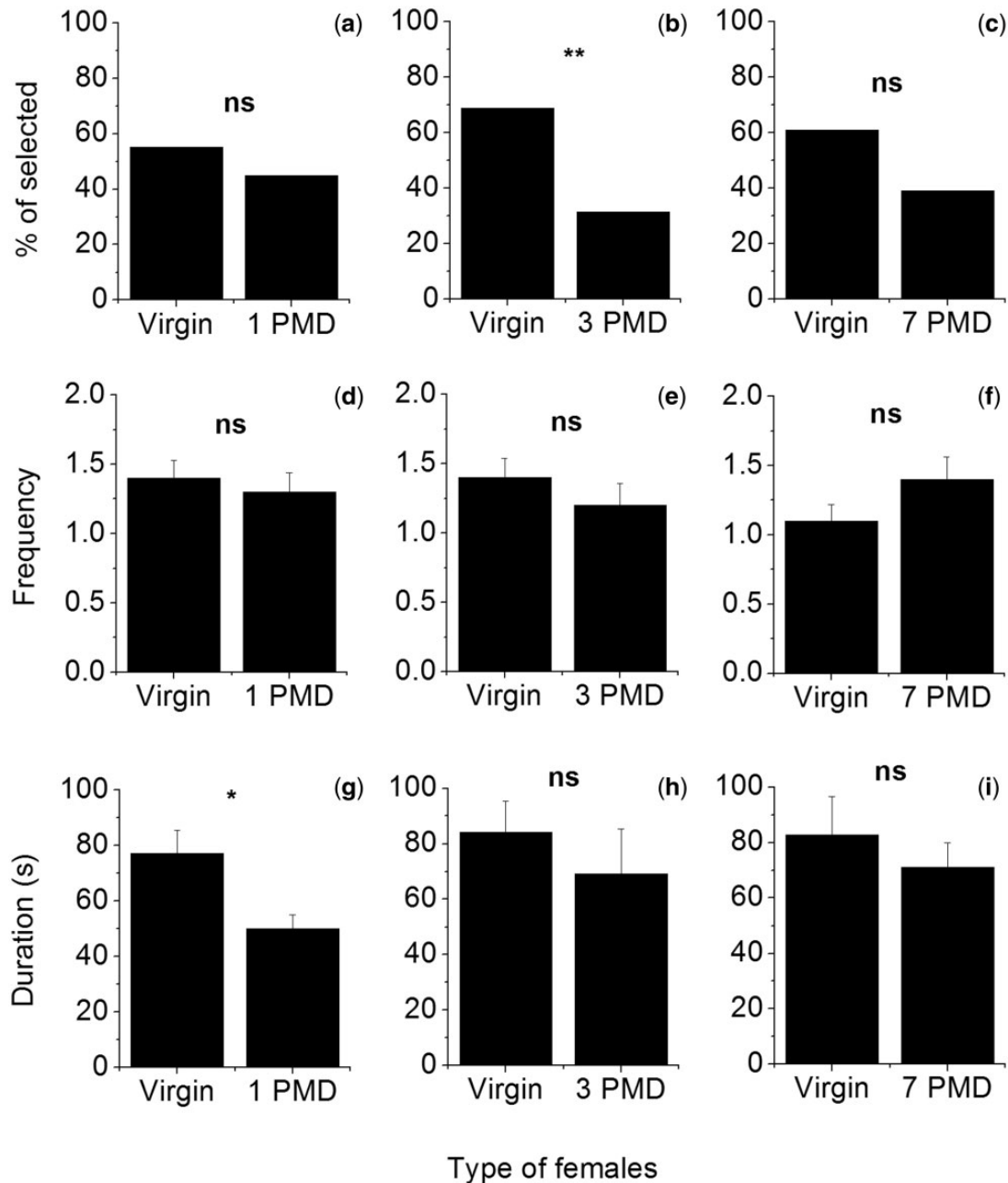
Fig. 3. (a–i) Percentage of male first mate selected (the type of female that was first selected by male for copulation), mean copulation frequency, and mean total copulation duration of female bed bugs during 10-min period. Each male was offered two females of different mating status: a virgin female and a female mated 1, 3, or 7 d earlier (1, 3, or 7 PMD).

bottom or the wall of experimental arena, therefore unfed female resistance was not a confounding variable for our study.

Multiple traumatic inseminations are common in bed bugs, and related to their natural feeding regime (Mellanby 1939, Stutt and Siva-Jothy 2001). After insemination, the male bed bug ejaculate remains localized in the mated females' mesospermaleges for several hours (Carayon 1966). Thus, the male-derived compounds in ejaculates left by former males would be detected by the latter males (Siva-Jothy and Stutt 2003). Our results indicated no significant difference in copulating time between a virgin male and a virgin female or between a virgin male and a mated female at 3 or 7 PMD, but there was a significant difference at 1 PMD. Therefore, it is likely that at 3 or 7 PMD, the male-derived compounds in the ejaculates

within mated females, could not be detected by males. However, with first mate selection, there is no significant difference between virgin and the 1 PMD females, the reason is unclear. In the experiment of male mating competency, under different PMDs, the mating competency of mated males with 1 or 3 PMD was lower than virgin males. However, males with 7 PMD had the same mating competency with virgin males. Thus, we can infer that the mating competency of mated males gradually improves as their PMD increases. We only selected the males or females that mated 1, 3, or 7 d earlier to study the male mate choice and male mating competency in bed bugs. Including shorter PMD (i.e., 2, 4, 8, and 12 h after mating) would have been necessary to determine more detailed information regarding the threshold for PMD.
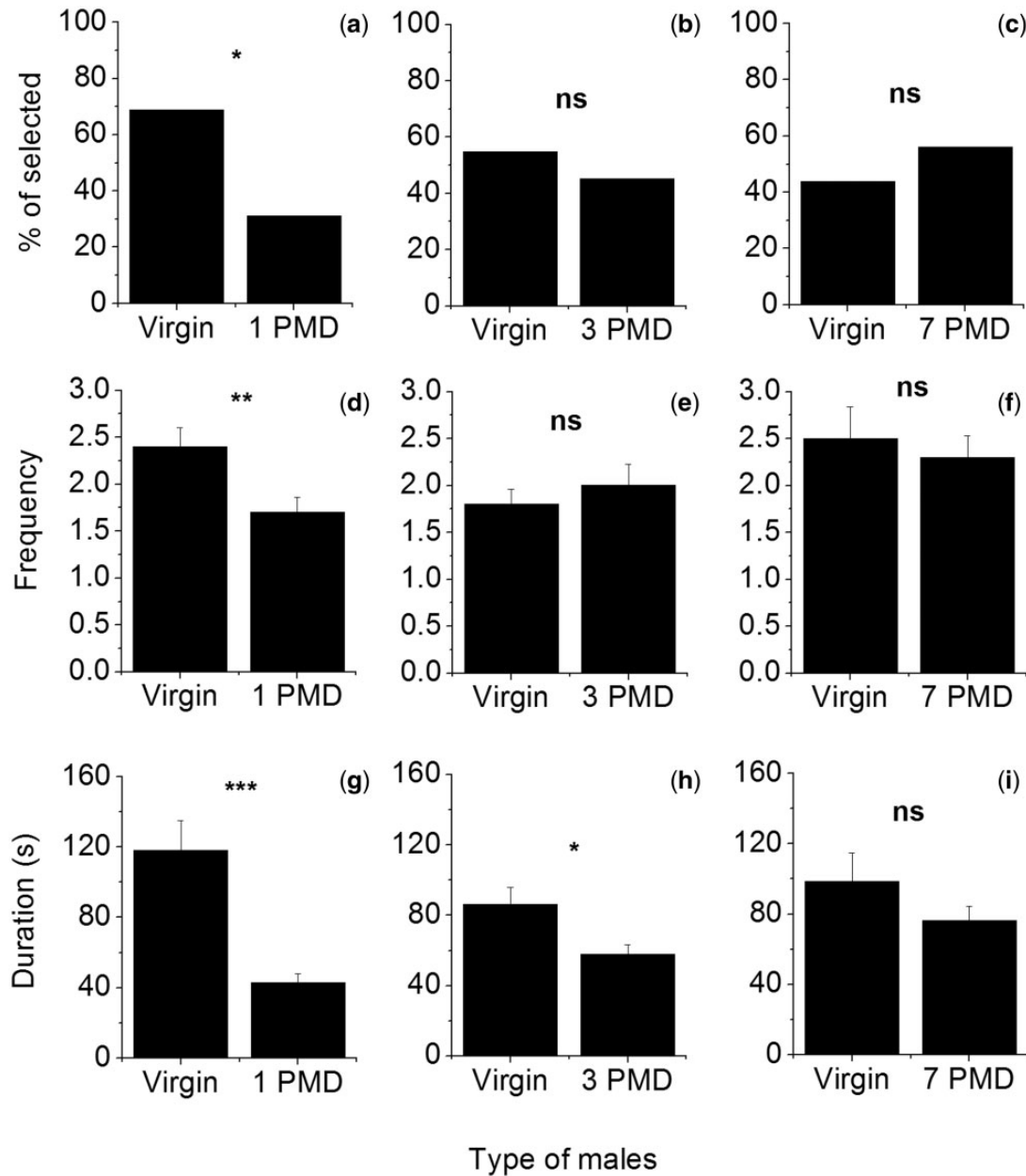
Fig. 4. (**a–i**) Percentage of male first mate selected (the type of male that first selected female for copulation), mean copulation frequency, and mean total copulation duration of male bed bugs during 10-min period. Each female was offered two males of different mating status: a virgin male and a male mated 1, 3, or 7 d earlier (1, 3, or 7 PMD).

In summary, mate choice in bed bugs is associated with male and female mating status, age, and PMD. Compared with the mated bed bugs, the virgins were more likely to mate and invest more time copulating; meanwhile, the old virgin bed bugs initiated copulation more rapidly and spent more time copulating than the young virgins.

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