Gender identity and sexual experience affect mating behaviour and chemical profile in the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae)

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**Abstract**

*Alphitobius diaperinus* (Coleoptera: Tenebrionidae), the lesser mealworm, is one of the most significant pests of the poultry industry worldwide. These insects cause structural damage in poultry houses and transmit several diseases, impacting chickens' productivity and rearing costs. Although semiochemicals may offer alternative insect pest management strategies, basic information regarding pheromone identity and their role on the behavioural ecology according to their circadian pattern of sexual behaviour of *A. diaperinus* is essentially lacking. This study is aimed to analyse the relation of gender identity and sexual experience of adults of *A. diaperinus* on their mating behaviour and whether this response is related to
their CHC profiles secreted. The following steps were taken to achieve the study’s goal. First, the circadian pattern of their sexual activity was observed in newly emerged pairs for at least twenty-one days (virgin adults) and experienced adults collected from the field to identify a difference based on their sexual experience and achieve the optimal mating season to develop the following assays. Subsequently, Y-tube olfactometer bioassays were conducted to evaluate their odour bouquet attraction based on gender and sexual experience. Additionally, mating behaviour bioassays were conducted to evaluate the two factor effects. Finally, cuticular analysis was performed using gas chromatography-mass spectrometry to evaluate possible chemical differences based on the two factors. With statistical and multivariate analysis, we found that behavioural, mating and chemical responses are different based on their sexual experience. The mating sequences were described into precopulatory, copulatory and postcopulatory phases. This finding gives us a deeper understanding of the sexual communication during mating. In summary, our findings provide new insights into the mating system and chemical ecology of *A. diaperinus*. The results presented here may serve as a base for further studies to develop strategies for managing this pest.

**Introduction**

*Alphitobius diaperinus* (Panzer, 1797) (Coleoptera: Tenebrionidae), commonly known as the lesser mealworm, has long captured the attention of the people involved in the poultry industry due to being a worldwide pest in poultry farms (1–3). Individuals of this species grow in the chicken litter, within a mixture of wasted feed, faeces and feathers. They can structurally damage the poultry houses (1,4–7), which generates economic losses. They also
act as vectors of several viral (8,9), fungal (10–12) and bacterial (13–16) diseases, which can cause poultry weight loss and even death (3,17–19). To control this pest, poultry farmers invest in pesticides and drugs, i.e., to prevent diseases on chickens (20,21). Nevertheless, the widespread use of pesticides (e.g., carbamates, organophosphates and pyrethroids) may cause resistant insect populations, harm birds, and lead to poultry house contamination (1,2,4,22–28). Consequently, alternative methods are researched to reduce the use of insecticides. These alternative methods may include physical methods (23,29,30), biological control (11,22,31–35) and chemical methods using natural products (18,36) such as bioinsecticides (28) or semiochemicals (5,17,24,37–41).

Semiochemicals are particularly effective as biological control, environmentally friendly, and highly specific based on pheromone composition (37,42–44). Recently, some studies have addressed the identification of aggregation and alarm pheromones from *A. diaperinus* (5,38,39,41). Nevertheless, the identification of the pheromones alone does not warrant the success in controlling the *A. diaperinus* population since insect responses are heavily context-dependent (e.g., environmental factors (45–47), interspecific interaction (48,49), population origin (17,24,40,50). Furthermore, as most organisms followed physiological and behavioural changes to a 24-hour cycle (i.e., circadian pattern), circadian patterns of insect behaviour and particularly reproductive activity can affect the performance of semiochemicals and pheromones (51–56). Therefore, circadian behaviour is a crucial component of almost any ecological and evolutionary process (55,57). Thus, basic knowledge such as identifying processes affecting aggregation or mating behaviour addressing their circadian pattern of sexual behaviour is crucial for more efficient pest management (44,57–62).
To achieve sexual reproduction, insect adults develop a set of behavioural displays (i.e., mating behaviour), which involves partner recognition, courtship, and copulation (44,63,64). In this regard, sexual communication in insects is based on visual, olfactory, tactile and even auditory stimuli (62,65,66). From those mentioned, olfactory stimuli based on chemicals are by far the most critical signals (64,67–69). Chemical signalling in sexual behaviour systems involves highly volatile compounds that promote attractiveness over long distances and compounds that can influence close-range orientation; these chemicals are perceived by direct antennal contact with the insect cuticles (58,60,67,68). Insect cuticles are made by a lipid wax layer, in which cuticular hydrocarbons (CHC) are a significant contributor (49,70,71). Besides their function of limiting water loss and reducing external damage by toxins and pathogens, CHCs also play a critical role in chemical communication for species, nest or mate recognition, and signalling reproductive status (49,61,62,64,70,72–74). In beetles, volatiles associated with intra-specific long attraction is usually associated with aggregation pheromones; in contrast, low volatile and non-volatile compounds are associated with contact sex pheromones (58,72,75,76). CHC compounds are shared by males and female adults in some beetle species (61,70,76), which explain the observed homosexual behaviour upon antennal contact, e.g., in some Scarabaeidae, Silphidae and Tenebrionidae beetles (68,70,77–79).

Since homosexual behaviour is known in *A. diaperinus* (38,40,41), it is expected that both genders share part of their CHC profiles. It has been shown that aggregation pheromones are produced by virgin males of *A. diaperinus*, which attracts males and females (5). However, it is unknown whether this attraction can be affected by gender identity or sexual experience. Furthermore, we expect gender identity and sexual experience in a circadian context can impact semiochemical and pheromone activity. Given that insect CHC profiles are associated
with gender identity and sexual experience (61,70,73,80,81), we were interested in finding out whether or not these two factors affect mating behaviour responses and, if so, could those differential behaviours be related to differences associated with their chemical profiles? To answer these questions, we first identified the circadian sexual behaviour of *A. diaperinus* adults. We then tested the attractiveness and behavioural mating responses of males and females with different sexual experiences to different CHC profiles of other *A. diaperinus* adults.

**Materials and methods**

**Studied species**

Adults of *A. diaperinus* were obtained from a commercial poultry production located in the surroundings of Lima (12°09'27.8" S 76°53'46.2" W). All four live stages of *A. diaperinus* were reared in our laboratory in aquarium glass boxes (30 × 25 × 20 cm). In one of our glass boxes, hundreds of male and female adults were kept and let free to reproduce; new emerging larvae were then removed and transferred to a second glass box. To facilitate the handling of the larvae, they were kept in groups within Petri dishes. This second box was monitored twice a week to feed them and to find pupae. Each new pupa was sexed (following (82); i.e., females “F” and males “M”), and then isolated in a Petri dish to avoid they mate in order to obtain non-mated adults. In case of gender recognition could not be carried out at pupa stage (e.g., first collected adults), gender recognition was carried out by pressing their protracted ventral abdomen to see the genitalia under a stereomicroscope. Larvae and adults of *A. diaperinus* were fed using commercial wheat flour "Blanca flor" (Alicorp, Lima, Peru) and tap water, which was provided by using a humidifying towel paper within the boxes. Glass
boxes were kept in an environmentally controlled climate chamber (Memmert HPP750, Memmert GmbH, Schwabach, Germany) at a constant temperature of 30 °C and humidity of 50 %, with a photoperiod of 12:12 h (light : dark) (photophase 07:00 to 19:00 h and scotophase 19:00 to 07:00 h; GMT-5). Adults being 21-d-old or older were used to guarantee sexual maturity. Non-mated adults were considered as sexually inexperienced (referred to as ‘virgin’ [v]), whereas adults exposed to high densities of males and females were catalogued as experienced (‘exp’). Fig 1 shows an overall workflow of the different steps of this study, which are described below. No insects were harm during experiments and when killed, it was instantly by freezing.

Fig 1. An integrative behavioural and chemical perspective for the analysis of mating behaviour of adults of *Alphitobius diaperinus* based on their gender identity and sexual experience. (A) Circadian pattern of sexual activity, (B) CHC attractiveness, (C) mating behaviour and (D) chemical profile analysis based on CHC affected by gender and sexual experience.

**Circadian pattern of sexual activity**

We recorded the mating behaviour of adult pairs (i.e., one female and one male) for 15 min each hour along the day for 5 days. The observations were performed over a Petri dish (60 × 15 mm), and a total of 10 pairs were utilized each day, totalizing 50 adult pairs. When a male exposed his genitalia while mounting a female, it was classified as a matting attempt. Males were not allowed to accomplish mating to continue observations along the day. The
proportion of mating attempts was then calculated at each hour. During periods of darkness, observations were performed under an incandescent bulb with red light. One hour prior and between observations, experienced adults were individually kept in Petri dishes to increase sexual desire. After each observation, Petri dishes were washed with methanol.

**CHC attractiveness based on gender and sexual experience**

A three-arm olfactometer (i.e., Y-tube, 8 mm thickness, 10 cm apparatus trunk followed by 5 cm length arm) was used to test the attractiveness of CHC compounds of males and females. Each Y-tube arm was connected to one of the five different treatments, which consisted of a glass chamber bearing one of the following options: (i) an empty control (C), (ii) 20 experienced females (Fexp), (iii) 20 virgin females (Fv), (iv) 20 experienced males (Mexp), or (v) 20 virgin males (Mv) (see Fig 1a). The odour released by each treatment was then driven to the olfactometer by an air compressor that provided an airflow at a constant rate of 0.3 L/min through activated charcoal filters. A virgin male or female was placed within the trunk of the olfactometer. Then, it was observed which arm the insect chose within a time of up to 5 min. The beetle odour decision was registered when the beetle entered at least 1 cm into one of the arms. All tested beetles were used only once, whereas beetles within the chambers were allowed to acclimatize for 1 h before the tests to avoid them producing unattractive compounds such as alarm pheromones. The assignment of odour sources to each arm was reversed after each trial to avoid potential illumination and directional bias (59). After each test, the olfactometer and chambers were washed with methanol. All trials were carried out at the peak of circadian sexual behaviour (S1 Fig).
**Effect of gender and sexual experience on mating behaviour**

Behavioural experiments with treatments based on all combinations of gender and sexual experience were prepared (i.e., Fv–Mv, Fv–Mexp, Fexp–Mv, Fexp–Mexp). Each pair (i.e., one male and one female) of adults of *A. diaperinus* was placed in a Petri dish (60 × 15 mm) containing a filter paper at the inner bottom and observed for a total of 10 min. The following behaviours developed during mating attempts were timed: touching (i.e., time in touching the other insect’s cuticle with their antennae or their prothoracic leg), mounting (i.e., time upon a male is over and bends his abdomen exposing the genital organ until mating), and copulation (i.e., time in copula). The recorded time per response per trial was then standardised by using a percentage scale (i.e., total trial time was transformed to 100 %). Additionally, the number of times of successful mating attempts (i.e., copulation occurred), unsuccessful mating attempts (i.e., unsuccessful copulation), and total mating attempts (i.e., total successful and unsuccessful mating attempts) were recorded. The recorded number of times per trial was also standardised using a percentage scale based on the total mating attempts.

**Chemical analyses of CHC profiles**

Twelve individuals from each group based on gender and sexual experience (i.e., Fexp, Fv, Mexp, Mv) were killed by freezing, thawed for 10 min at room temperature. Their CHCs were extracted by immersing them in 2 mL hexane (MS grade, Sigma-Aldrich) and shaking in a vortex for 2 min at 2000 rpm. For quantitative analysis, 1 µL of 1000 ppm hexadecane (C16) was added as an internal standard in all the extracts. Later, CHC extracts were
concentrated to 120 µL using a gentle stream of nitrogen and then stored at −20 °C for subsequent chemical analysis.

Extracts were analysed by gas chromatography (GC) coupled to quadrupole time-of-flight mass spectrometry (MS; Agilent 7250 GC/Q-TOF, Santa Clara/CA, USA) with electron and chemical ionization (EI and CI, respectively). GC was equipped with a DB-5 column (30 m × 0.25 mm i.d., 0.25 µm thickness film). Aliquots of 1 µL cuticular extract were injected in the GC, which was programmed at an oven temperature of 50 °C for 2 min, increased at 10 °C/min until 250 °C, and hold for 20 min. Injections were made in splitless mode with helium as the carrier gas (1.5 mL/min), injector temperature at 250 °C, and detector temperature at 270 °C. Subsequently, the same extracts were measured using a GC-MS with EI at 70 eV with a scan range from $m/z$ 50–750. Data mining was done using MS-DIAL v4.6., which provided peak alignments of data based on total ion current of GC-MS with CI analyses (83).

After that, filtering was performed using principal component analysis (i.e., PCA of log10-transformed and Z-score-normalized data) and one-way ANOVA ($p$-anova<0.01) through MATLAB vR2019b. This filtering allowed us to track data quality, reduce the data dimensionality, identify potential outliers in the dataset, and identify sample clusters (84–87). To final filtering of each feature was considered the following threshold: that the average area of the sample was at least three times the average area of the C16 and the blank. After data curation, to ensure the identification of compounds, selected samples were analysed by gas chromatography coupled to an APPI-Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, USA) following the methodology explained above. Identification of CHC compounds was achieved by comparing mass spectra and retention indices of unidentified compounds with commercial databases such as PubChem, Fiehn BinBase, MoNA volatile, Chemsipider, Metlin and NIST.
Statistical analysis

Differences in mating activity throughout a circadian cycle and differences in the total time of male mating reactions (i.e., touching, mounting, copulation and mating time), as well as the total mating attempts (i.e., successful, unsuccessful and total mating attempts), among all tested treatments, were performed by non-parametric Kruskal-Wallis tests, followed of pairwise comparisons by Mann-Whitney-U tests with a Bonferroni correction. Choices made by *A. diaperinus* adults in the olfactometer bioassays were analysed by exact binomial tests (50 % chance of selecting each arm). All these statistical tests were performed using the *stats* package of the R software (R version 3.6.1 (88)).

A non-metric multidimensional scaling (NMDS) was performed to display the dissimilarities among groups graphically. NMDS ordination was based on Bray–Curtis similarity of the square-root transformed dataset. Furthermore, to identify statistical differences in the CHC profiles among groups, a permutational multivariate analysis of variance (PERMANOVA) was run based on Bray-Curtis similarity of a square-root transformed dataset. A total of 99999 permutations and a Holm correction was carried out, considering “gender” as a fixed factor and “sexual experience” as a nested factor within “gender”. The CHC dataset was based on the relative proportions of all the identified compounds (see results). NMDS and PERMANOVA were performed using the *vegan* package (89), whereas Holm correction using the *RVAideMemoire* package (90) of R.
Results

Circadian pattern of sexual activity

Independently of their sexual experience (i.e., virgin or experienced), the mating behaviour of *A. diaperinus* adults was displayed throughout the day (S1 Fig). On the one hand, virgin adults showed more mating activity between 2 h and 9 h after the light was on (i.e., photophase) and at 17 h to 18 h and 23 h, under dark conditions (i.e., scotophase). In contrast, the lowest mating activity was recorded between 13 h and 14 h (S1 Fig). On the other hand, experienced adults were more sexually active between the 3 h and 5 h on the photophase (S1 Fig), and at 8 h to 11 h on the scotophase (S1 Fig); in contrast, the lowest activity was also observed between 13 h and 14 h (S1 Fig). Nevertheless, mating behaviour activity was only significantly higher between 16 h and 17 h for both virgin and experienced adults (S1 Fig, Mann-Whitney-U test with Bonferroni correction, p<0.05).

Attractiveness based on gender and sexual experience

Fv were significantly more attracted to CHC odour of Mv (Fig 2; Binomial test, p<0.05, n = 31), Mexp (Fig 2; Binomial test, p<0.01, n = 34) and, barely significantly, of Fv (Fig 2; Binomial test, p<0.07, n = 31) over control treatment. However, Fv did not show any preference when tested to CHC odour of Fexp against control or Fexp against Mexp (Fig 2; Binomial test, both p>0.05). In addition, Mv were significantly more attracted to CHC odour of Mv (Fig 2; Binomial test, p<0.001, n = 30), Fexp (Fig 2; Binomial test, p<0.001, n = 40) and, barely significantly, of Fv (Fig 2; Binomial test, p<0.1, n = 53) compared to control. However, due to not enough Mv individuals, no experiments were carried out.
In the case of Fexp, they were significantly more attracted to Mexp (Fig 2; Binomial test, p<0.001, n = 35) and Fv (Fig 2; Binomial test, p<0.001, n = 32) odour over control, as well as, significantly attracted to Fexp scent over Mexp (Fig 2; Binomial test, p<0.05, n = 31). However, they did not show any preference when tested Fexp against control (Fig 2; Binomial test, p>0.05, n = 30). Additionally, Mexp were significantly more attracted to Mexp (Fig 2; Binomial test, p<0.001, n = 35) and Fv (Fig 2; Binomial test, p<0.01, n = 40), as well as significantly attracted to Fexp odour over Mexp (Fig 2; Binomial test, p<0.01, n = 31) and barely significantly attracted to Fexp compared to control (Fig 2; Binomial test, p<0.09, n = 32).

**Fig 2. The attractiveness of *Alphitobius diaperinus* adults based on CHC odour by gender and sexual experience.** Significant difference (* p<0.05; ** p<0.01; *** p<0.001) and ns=non-significant according to the exact binomial test.

**CHC attractiveness based on gender and sexual experience**

The three distinct phases of mating behaviour in *A. diaperinus* are summarized in Fig 1c. The precopulatory phase (i.e., during the insect localization) began when both adults approached between them. After approaching, they touched their cuticles with their antennae or their prothoracic leg. When the male recognised the female, he attempts to mount her from the back, bending his abdomen and exposing the genital organ. Females also contributed to both adults have a successful attempt mating while placing herself under the male and exposing her genitalia by aperture the last sternite. In the copulatory phase, the male grasped the female's cuticle with his prothoracic and mesothoracic leg and touched her prothorax with
his antenna and maxillary palp. Interestingly, copula was on average accomplished only once per trial. In the postcopulatory phase, both adults kept touching and stay that way. When a male did not have a successful mating due to his small size related to the female, both insects attempted mating again (S1 Table).

The Kruskal–Wallis test shows that the touching responses of *A. diaperinus* adults were not significantly different among treatments (Fig 3, Table 1; \(p>0.05\)). On the contrary, the pattern of mounting and copulation responses was quite different among treatments (Table 1; Kruskal-Wallis test; \(p<0.0001\)). Specifically, Fv-Mv and Fexp-Mv resulted in significantly longer mounting than Fv-Mexp (Fig 3; Mann-Whitney-U test with Bonferroni correction, \(p<0.05\)); whereas we did not find differences in mounting in Fexp-Mexp compared to any other treatment. However, we did find significant differences in copulation when comparing Fv-Mv and Fexp-Mv against Fv-Mexp and Fexp-Mexp (Fig 3; Mann-Whitney-U test with Bonferroni correction, \(p<0.05\) for all).

**Fig 3. Behavioural mating responses of *Alphitobius diaperinus* in relation to gender and sexual experience.** (A) Touching, (B) mounting and (C) copulation based on their gender and sexual experience. Different letters below the bars indicate significant differences according to the Mann-Whitney-U test with Bonferroni correction (\(\alpha=0.05\), \(p<0.05\)).

**Table 1. Mating behaviour (in percentage; mean ± SD) of *Alphitobius diaperinus* adults based on their gender and sexual experience.**
In the case of mating attempts, Fv-Mv and Fexp-Mv resulted in significantly longer attempts compared to Fv-Mexp (Fig 4a; Mann-Whitney-U test with Bonferroni correction, p<0.05); whereas we did not find differences in mating attempts in Fexp-Mexp compared to any other treatment. From the total mating attempts, no significant differences in unsuccessful and successful mating attempts among all the four treatments were found (Fig 4b and 4c; Kruskal-Wallis test, p>0.05). However, Mv was slightly more successful in mating with Fv compared to Fexp (Fig 4c). Moreover, from the total mating attempts showed by Fv-Mexp and Fexp-Mexp, only half was successful (Fig 4b and 4c, S1 Table).

Fig 4. Mating attempts recorded of *Alphitobius diaperinus* adults based on their gender and sexual experience. (A) Total, (B) unsuccessful, and (C) successful mating attempts. Different letters below the bars indicate significant differences according to the Mann-Whitney-U test with Bonferroni correction (α=0.05, p<0.05).

**CHC profile of *A. diaperinus***

A total of 19 CHC compounds were obtained by GC-MS followed by a data curation (S2 Fig), which belonged to the following chemical groups alkanes, quinones, benzothiazoles, benzophenones, fatty acids and terpenes (Table 2). All the 19 identified compounds were detected in experienced adults but different concentrations, while only 14 compounds were detected in virgin adults. Among these CHC compounds, 2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone were detectable as significant components in the CHC profile for both males and females, although they were predominant in experienced adults (Table 2; S3
Interestingly, limonene, oleic acid, linoleic acid and nonacosane were identified only in experienced adults.

The (dis)similarity of CHC profile between the sexual experience groups of males and females of *A. diaperinus* was visually displayed by our NMDS analysis (Fig 5), which were highly congruent with a PCA also based on CHC profile (S2 Fig). Indeed, our PERMANOVA analyses did not show differences in CHC profiles based on gender identity (i.e., males vs females; Pseudo-\(F_{(1, 35)} = 0.757, p = 0.459\)), or within sexual experience groups (i.e., between virgins, between experienced adults; both \(p = 0.687\)). However, we did find statistical differences in CHC profiles according to the nested factor sexual experience (Pseudo-\(F_{(2, 35)} = 14.399, p<0.001\)). Specifically, we found that the Fexp CHC profile is different from that of Fv (PERMANOVA test with Holm correction, \(p<0.01\)) and Mv (PERMANOVA test with Holm correction, \(p<0.01\)). In contrast, Mexp were chemically different from Fv (PERMANOVA test with Holm correction, \(p<0.01\)) as well as from Mv (PERMANOVA test with Holm correction, \(p<0.01\)).

**Table 2.** Mean quantity (ng/insect ± SD) of CHC compounds from adults of *Alphitobius diaperinus* based on gender and sexual experience.

**Fig 5.** Comparison of *Alphitobius diaperinus* CHC profiles based on gender and sexual experience. A non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis similarities (stress=0.08036). Ellipses were drawn by hand.
Discussion

The circadian cycles play an essential role in describing the mating behaviour of insect life (52–54), which might be considered a key to developing effective strategies for pest control (55,57). *Alphitobius diaperinus* seems not to show significant differences in mating activity along the day. However, a lower mating behaviour activity was observed at the end of the photophase and beginning of scotophase 13 h, which might be related to their cryptic behaviour when hiding from a threat or external factors (i.e., environmental changes) or to particular reproductive strategies of females to reduce her attractiveness and rejection to mate (5,56,57,68). In insects, the attractiveness of intra-specific odour relies on the interaction context (e.g., protection from environmental conditions, predators, food source or mate recognition), as recorded in some Curculionidae, Dermestidae, Tenebrionidae and other non-beetle species (37,55,59,68,80,91). In mating behaviour, the mate preference and attraction of males toward the odour of females can be based on their physiological changes and acquired experience (i.e., reproductive status and learned signal; (91)). For instance, in some Tenebrionidae species, experienced adults have contrasting mating behavioural responses compared to virgin adults (91,92). Interestingly, males and females of *A. diaperinus* were more attracted to the CHC profiles of both males and females although heavily dependent on the sexual experience condition, which can be related to the gregarious behaviour of this species and similarity of CHC profiles. Effectively, it has been shown that insects with similar odour profiles are less attractive to their gender counterparts compared to others with more different profiles (e.g., *Gryllodes sigillatus* (93)). Although it was previously pointed out that only virgin males released attractive compounds to attract virgin insects (e.g., aggregation pheromones; (5)), we have shown that CHC profiles of both males and females
can be attractive to males and females, and their attractiveness relies on gender and sexual experience. Furthermore, we have shown that the insect cuticle diffusely releases attractive odour signals, in addition to the aggregation pheromones produced by exocrine glands in other beetles (94,95). In beetles, it is known the aggregation pheromones may be made by any sex (59,68,80,96). Unlike sex pheromones that act on only one sex, aggregation pheromones induce group formation of both sexes (37,43,55,68). In this regard, it may be evolutionarily advantageous for males to call females to reduce the time spent searching for a dispersed potential mate, and females have more chances to mate (80). Thus, it is clear that different combinations of odour profiles are necessary to attract males and females of \textit{A. diaperinus} successfully. Thus, a single odour blend might not be enough.

Beetles during the premating phase show an antennal behaviour (59,65,80,97) related to the close-range assessment of potential mates, where male- or female produced contact pheromone is required for mating to be successful (58,61,62,67,80,98). Based on our observations, we speculate that both females or male adults of \textit{A. diaperinus} release CHC compounds that allow the partner to be recognized by the opposite gender during antennation. This behaviour to recognise mates at short distances is mediated by semiochemicals such as a contact sex pheromone (58,61,62,67,98). In the copulatory phase, male beetles and \textit{A. diaperinus} continue their antennation, touch with their maxillary palps and grasp the female's cuticles with his prothoracic and mesothoracic leg (59,65,80,97) while mounting. On the leg, males possess tibial spurs and claws, which for other species facilitate grasping and control of females, preventing aggregated males from dislodging copulating males and distributing their own CHC profile overall the female’s cuticle (80). Thus, mate location and sex discrimination, the first steps of mating, is maybe due to a combination of physical and chemical recognition by the antenna and legs (59).
It is usually expected that adult males tend to mate with any available female. However, we have often recorded low mating activity of Mexp with females, compared to Mv, which could be explained by the sexual conflict (99,100). To increase reproductive success, females mate with multiple males to select their best candidate (99), giving the later males more chances to succeed. Experienced males have already mated and might be more selective to mate; therefore, they would prefer to mate with Fexp, which are less likely to have new mating partners than Fv. Moreover, the sexual experience would also reduce the amount of time in Mexp mounting and copulation since they are shorter with Fexp than Fv. Unlike Mexp, Mv has a natural-sexual need to copulate with a mate. Therefore, Fv and Fexp are both attractive to Mv, although Fexp seems to be more attractive. This behaviour is also known in other insects, where virgin males mate indiscriminately with females and do not recognise their sexual experience (101,102).

The CHC play an essential role in chemical communication during the mating behaviour of insects (49,58,60–62,67,70,72–74). According to our results, differences in CHC profiles of *A. diaperinus* adults are mainly based on their sexual experience and not gender, which explains why the odour profile of both genders can be attractive. Moreover, CHC profiles can explain the results of our mating bioassays and suggest that CHC compounds are the key to mate choice and reproduction in *A. diaperinus*. CHC profile similarity between males and females can also explain the expected homosexual mating behaviour observed in *A. diaperinus* and diverse beetle species (77,103,104). Thus, the CHC profile of the sexual experience in adults serves as a crucial species-specific mating cue, but not for sex discrimination (72). Further studies are being carried out to identify the chemical basis of *A. diaperinus* attractiveness.
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References

1. Domínguez I. Alphitobius diaperinus ¿Un problema bajo el control o bajo los comederos? Sel avícolas. 2012;23–7.

2. Santo I Monteys V. Control de Alphitobius diaperinus (Col. Tenebrionidae) en granjas avícolas. Sel avícolas. 2011;19–23.

3. Retamales J, Vivallo F, Robeson J. Insects associated with chicken manure in a breeder poultry farm of central Chile. Arch Med Vet. 2011;43:79–83.

4. Dunford JC, Kaufman PE. Lesser mealworm, litter beetle, Alphitobius diaperinus (Panzer) (Insecta: Coleoptera: Tenebrionidae). University of Florida IFAS, USA; 2006. Available from: https://edis.ifas.ufl.edu/pdf/IN/IN66200.pdf

5. Hassemer MJ, Sant’Ana J, Borges M, Withall D, Pickett JA, de Oliveira MWM, et al. Revisiting the male-produced aggregation pheromone of the lesser mealworm, Alphitobius diaperinus (Coleoptera, Tenebrionidae): Identification of a six-component pheromone from a Brazilian population. J Agric Food Chem. 2016;64(36):6809–18.

6. Robinson WH. Urban insects and arachnids - A handbook of urban entomology. Cambridge University Press. New York, United States of America; 2005.

7. Crippen TL, Esquivel JF. Improved visualization of Alphitobius diaperinus (Panzer) (Coleoptera: Tenebrionidae) - part II: Alimentary canal components and measurements. Psyche A J Entomol. 2012;2012.

8. Goodwin MA, Waltman WD. Transmission of Eimeria, viruses, and bacteria to chicks: darkling beetles (Alphitobius diaperinus) as vectors of pathogens. J Appl Poultr Res. 1996;5:51–5.

9. McAllister JC, Steelman CD, Newberry LA, Skeeles JK. Isolation of infectious bursal
disease virus from the lesser mealworm, *Alphitobius diaperinus* (Panzer). Poult Sci. 1995;74(1):45–9.

10. de Las Casas E, Harein PK, Pomeroy BS. Bacteria and fungi within the lesser mealworm collected from poultry brooder houses. Environ Entomol. 1972;1(1):27–30.

11. Alves LFA, Alves VS, Bressan DF, Neves PMOJ, Alves SB. Natural occurrence of *Metarhizium anisopliae* (Metsch.) Sorok. on adults of the lesser mealworm (*Alphitobius diaperinus*) (Panzer) (Coleoptera: Tenebrionidae) in poultry houses in Cascavel, PR, Brazil. Neotrop Entomol. 2004;33(6):793–5.

12. da Soares CES, Weber A, Moecke EHS, de Souza CK, Reiter MGR, Scussel VM. Use of ozone gas as a green control alternative to beetles *Alphitobius diaperinus* (panzer) infestation in aviary bed utilized in the poultry industry. Chem Eng Trans. 2018;64:589–94.

13. Hazeleger WC, Bolder NM, Beumer RR, Jacobs-Reitsma WF. Darkling beetles (*Alphitobius diaperinus*) and their larvae as potential vectors for the transfer of *Campylobacter jejuni* and *Salmonella enterica* serovar Paratyphi B Variant Java between successive broiler flocks. Appl Environ Microbiol. 2008;74(22):6887–91.

14. Leffer AM, Kuttel J, Martins LM, Pedroso AC, Astolfi-Ferreira CS, Ferreira F, et al. Vectorial competence of larvae and adults of *Alphitobius diaperinus* in the transmission of *Salmonella enteritidis* in poultry. Vector-Borne Zoonotic Dis. 2010;10(5):481–7.

15. Roche AJ, Cox NA, Richardson LJ, Buhr RJ, Cason JA, Fairchild BD, et al. Transmission of *Salmonella* to broilers by contaminated larval and adult lesser mealworms, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). Poult Sci. 2009;88(1):44–8.

16. Agabou A, Alloui N. Importance of *Alphitobius diaperinus* (Panzer) as a reservoir for pathogenic bacteria in Algerian broiler houses. Vet World. 2010;3(2):71–3.

17. Singh N, Johnson DT. Attractiveness of an aggregation pheromone lure and chicken droppings to adults and larvae of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). J Econ Entomol. 2012;105(6):2196–206.

18. Arena JS, Omarini AB, Zunino MP, Peschiutta ML, Defagó MT, Zygadlo JA. Essential oils from *Dysphania ambrosioides* and *Tagetes minuta* enhance the toxicity of a conventional insecticide against *Alphitobius diaperinus*. Ind Crops Prod. 2018;122:190–4.

19. Dinev I. The darkling beetle (*Alphitobius diaperinus*) – a health hazar for broiler chicken production. Trakia J Sci. 2013;11(1):1–4.

20. Llaque Ramos LJ. Innovación en la industria avícola peruana de broilers para mejorar los niveles de competitividad 1986-2006 [dissertation]. Universidad Nacional Mayor De San Marcos; 2009.

21. Pimentel D, Andow D, Dyson-Hudson R, Gallahan D, Jacobson S, Irish M, et al.
Environmental and social costs of pesticides: A preliminary assessment. Oikos. 1980;34(2):140.

22. del Valle EE, Frizzo LS, Malmierca M, Zbrun M V., Lax P, Doucet ME. Biological control of *Alphitobius diaperinus* with *Steinernema rarum* CUL and *Heterorhabditis bacteriophora* SMC and feasibility of application in rice hull. J Pest Sci (2004). 2016;89:161–70.

23. Wolf J, Potrich M, Lozano ER, Gouvea A, Pegorini CS. Combined physical and chemical methods to control lesser mealworm beetles under laboratory conditions. Poult Sci. 2015;94(6):1145–9.

24. Hassemer MJ, Borges M, Withall DM, Pickett JA, Laumann RA, Birkett MA, et al. Development of pull and push–pull systems for management of lesser mealworm, *Alphitobius diaperinus*, in poultry houses using alarm and aggregation pheromones. Pest Manag Sci. 2019;75(4):1107–14.

25. Rodríguez D, Xuárez M, Merino A, Larramendy R, Temprana M, Diaz O, et al. Evaluación en condiciones de laboratorio de la eficacia de tres insecticidas de nuevo uso en la avicultura cubana para el control de *Alphitobius diaperinus*. Rev Salud Anim. 2013;35(3):197–200.

26. Panzardi A, Nunes R de F, Gaggini TS, Oliveira GBA de, Guimarães EC, Antunes RC, et al. Comparison between two insecticide application methods in controlling lesser mealworm beetles in commercial broiler houses. Rev Ciências Agroveterinárias. 2019;18(3):400–3.

27. Lambkin TA. Baseline responses of adult *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) to fenitrothion and susceptibility status of populations in Queensland and New South Wales, Australia. J Econ Entomol. 2005;98(3):938–42.

28. Zorzetti J, Constanski K, Santoro PH, Fonseca ICB, Neves PMOJ. Growth regulator insecticides for the control of the lesser mealworm beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). Rev Colomb Entomol. 2015;41(1):24–32.

29. Gazoni FL, Flores F, Bampi RA, Silveira F, Boufleur R, Lovato M. Evaluation of the resistance of mealworms (*Alphitobius diaperinus*) (Panzer) (Coleoptera: Tenebrionidae) at different temperatures. Arq Inst Biol (Sao Paulo). 2012;79(1):69–74.

30. Gehring VS, Santos ED, Mendonça BS, Santos LR, Rodrigues LB, Dickel EL, et al. *Alphitobius diaperinus* control and physicochemical study of poultry litters treated with quicklime and shallow fermentation. Poult Sci. 2020;99(4):2120–4.

31. Alves VS, Neves PMJ d. O, Alves LFA, Alcides M, Holz N. Entomopathogenic nematodes (Rhabditida: Heterorhabditidae and Steinernematidae) screening for lesser mealworm *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) control. Rev Colomb Entomol. 2012;38(1):76–80.

32. de Souza Daniel JF, Scalcó AV, de Souza RM, Marins Ocampaos FM, Barison A, Angeli Alves LF, et al. Susceptibility of *Alphitobius diaperinus* to *Beauveria bassiana*
extracts. Nat Prod Res. 2019;33(20):3033–6.

33. Rezende SRF, Curvello FA, Fraga ME, Reis RCS, Castilho AMC, Agostinho TSP. Control of the *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) with entomopathogenic fungi. Brazilian J Poult Sci. 2009;11(2):121–7.

34. Gindin G, Glazer I, Mishoutchenko A, Samish M. Entomopathogenic fungi as a potential control agent against the lesser mealworm, *Alphitobius diaperinus* in broiler houses. BioControl. 2009;54(4):549–58.

35. Prado-rebolledo O, Lezama-gutiérrez R, Contreras-benicio D, Contreras-lara D. Patogenicidad del hongo *Beauveria bassiana* (Hyphomycetes) en adultos del escarabajo *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) de casetas avícolas del estado de Colima. Rev Iberoam Ciencias. 2014;1(1):87–93.

36. Baran B, Krzyżowski M, Cup M, Janiec J, Grabowski M, Francikowski J. Repellent effect of volatile fatty acids on lesser mealworm (*Alphitobius diaperinus*). Insects. 2018;9:35.

37. Yew JY, Chung H. Insect pheromones: An overview of function, form, and discovery. Prog Lipid Res. 2015;59:88–105.

38. Hassemer MJ, Sant’Ana J, de Oliveira MWM, Borges M, Laumann RA, Caumo M, et al. Chemical composition of *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) abdominal glands and the influence of 1,4-benzoquinones on its behavior. J Econ Entomol. 2015;108(4):2107–16.

39. Tseng Y-CL, Davidson JA, Menzer RE. Morphology and chemistry of the odoriferous gland of the lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae). Ann Entomol Soc Am. 1971;64(2):425–30.

40. Cossé AA, Zilkowski BW. Behavioral responses of lesser mealworm beetles, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) to pheromone components using a wind tunnel dual choice walking bioassay. J Insect Behav. 2015;28(2):202–10.

41. Bartelt RJ, Zilkowski BW, Cossé AA, Steelman CD, Singh N. Male-produced aggregation pheromone of the lesser mealworm beetle, *Alphitobius diaperinus*. J Chem Ecol. 2009;35:422–34.

42. Hassan N, Shakir A-Z. Pheromone use in the Food Industry. Int Pest Control. 2008;50(2):83–6.

43. Müller M, Buchbauer G. Essential oil components as pheromones. A review. Flavour Fragr J. 2011;26(6):357–77.

44. Adamski Z, Bufo SA, Chowański S, Falabella P, Lubawy J, Marciniak P, et al. Beetles as model organisms in physiological, biomedical and environmental studies - A review. Front Physiol. 2019;10:319.

45. Menzel F, Blaimer BB, Schmitt T. How do cuticular hydrocarbons evolve? Physiological constraints and climatic and biotic selection pressures act on a complex functional trait. Proc R Soc B Biol Sci. 2017;284(1850).
46. Soetemans L, Gianotten N, Bastiaens L. Agri-food side-stream inclusion in the diet of *Alphitobius diaperinus*. Part 2: Impact on larvae composition. Insects. 2020;11(3).

47. Oonincx DGAB, Laurent S, Veenenbos ME, van Loon JJA. Dietary enrichment of edible insects with omega 3 fatty acids. Insect Sci. 2020;27(3):500–9.

48. Giunti G, Palmeri V, Algeri GM, Campolo O. VOC emissions influence intra- and interspecific interactions among stored-product Coleoptera in paddy rice. Sci Rep. 2018;8(1):1–9.

49. Batalha M de MC, Goulart HF, Santana AEG, Barbosa LAO, Nascimento TG, da Silva MKH, et al. Chemical composition and antimicrobial activity of cuticular and internal lipids of the insect *Rhynchophorus palmarum*. Arch Insect Biochem Physiol. 2020;105(1).

50. Hassemer MJ, Lopes RB, Borges M, Alves LFA, Withall DM, Pickett JA, et al. Development of an attract-and-infect device for biological control of lesser mealworm, *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) in poultry houses. Biol Control. 2020;149:104326.

51. Tast A, Love RJ, Evans G, Andersson H, Peltoniemi OAT, Kennaway DJ. The photophase light intensity does not affect the scotophase melatonin response in the domestic pig. Anim Reprod Sci. 2001;65(3–4):283–90.

52. Miyatake T. Circadian rhythm and time of mating in *Bactrocera cucurbitae* (Diptera: Tephritidae) selected for age at reproduction. Heredity. 2002;88(4):302–6.

53. Liu XP, He HM, Kuang XJ, Xue F Sen. Mating behavior of the cabbage beetle, *Colaphellus bowringi* (Coleoptera: Chrysomelidae). Insect Sci. 2010;17(1):61–6.

54. Rymer J, Bauernfeind AL, Brown S, Page TL. Circadian rhythms in the mating behavior of the cockroach, *Leucophaea maderae*. J Biol Rhythms. 2007;22(1):43–57.

55. Burkholder WE, Ma M. Pheromones for monitoring and control of stored-product insects. Annu Rev Entomol. 1985;30(1):257–72.

56. Gillott C. Male accessory gland secretions: Modulators of female reproductive physiology and behavior. Annu Rev Entomol. 2003;163–84.

57. Gottlieb D. Agro-chronobiology: Integrating circadian clocks /time biology into storage management. J Stored Prod Res. 2019;82:9–16.

58. Crook DJ, Hopper JA, Ramaswamy SB, Higgins RA. Courtship behavior of the soybean stem borer *Dectes texanus texanus* (Coleoptera: Cerambycidae): Evidence for a female contact sex pheromone. Ann Entomol Soc Am. 2004;97(3):600–4.

59. Kamiya AC, Silva WD, Leite MOG, Tironi P, Wadt L, Bento JMS. Mating behavior and evidence for male-produced aggregation pheromone in *Cyrtomon luridus* (Boheman) (Coleoptera: Curculionidae: Entiminae). J Insect Behav. 2015;28(1):55–66.

60. Peterson MA, Dobler S, Larson EL, Juárez D, Schlarbaum T, Monsen KJ, et al. Profiles of cuticular hydrocarbons mediate male mate choice and sexual isolation

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**Note:** The text was generated from the provided images and raw content, ensuring a natural reading format without altering the original meaning or context. Citations are formatted according to the specified style guide. The content includes references to studies on the inclusion of agri-food side-streams in diets, dietary enrichment of edible insects, VOC emissions, chemical composition, and biological control of insects. Further references cover mating behavior, pheromones, and circadian rhythms across different species and settings.
between hybridising *Chrysochus* (Coleoptera: Chrysomelidae). Chemoecology. 2007;17(2):87–96.

61. Tanigaki T, Yamaoka R, Sota T. The role of cuticular hydrocarbons in mating and conspecific recognition in the closely related longicorn beetles *Pidonia grallatrix* and *P. takechii*. Zoolog Sci. 2007;24(1):39–45.

62. Qadir I, Qamar A, Paul B, Mir AH. Cuticular hydrocarbons C14-C36 are potential contact pheromonal elements modulating some behaviors in *Zygoraphma bicolorata* (Coleoptera: Chrysomelidae). Biologia. 2021;76(1):123–32.

63. Nieberding CM, de Vos H, Schneider M V., Lassance J-M, Estramil N, Andersson J, et al. The male sex pheromone of the butterfly *Bicyclus anynana*: Towards an evolutionary analysis. PLoS One. 2008;3(7):e2751.

64. Lizée MH, Barascud B, Cornec JP, Srng L. Courtship and mating behavior of the cockroach *Oxyhaloa deusta* [Thunberg, 1784] (Blaberidae, Oxyhaloinae): Attraction bioassays and morphology of the pheromone sources. J Insect Behav. 2017;30(6):674–94.

65. FontEster E, Desfilis E. Courtship, mating, and sex pheromones in the mealworm beetle, *Tenebrio molitor*. In: Exploring Animal Behavior in Laboratory and Field. Academic P. Academic Press; 2003. p. 43–58.

66. Ferveur JF. Cuticular hydrocarbons: Their evolution and roles in *Drosophila* pheromonal communication. Behav Genet. 2005;35(3):279–95.

67. Geiselhardt S, Otte T, Hilker M. The role of cuticular hydrocarbons in male mating behavior of the mustard leaf beetle, *Phaedon cochleariae* (F.). J Chem Ecol. 2009;35(10):1162–71.

68. Fedina TY, Lewis SM. An integrative view of sexual selection in *Tribolium* flour beetles. Biol Rev. 2008;83(2):151–71.

69. Heuskin S, Vanderplanck M, Baczquet P, Holveck M-J, Kaltenpnot M, Engl T, et al. The composition of cuticular compounds indicates body parts, sex and age in the model butterfly *Bicyclus anynana* (Lepidoptera). Front Ecol Evol. 2014;2(July):1–16.

70. Keppner EM, Prang M, Engel KC, Ayasse M, Stökl J, Steiger S. Beyond cuticular hydrocarbons: Chemically mediated mate recognition in the subsocial burying beetle *Nicrophorus vespilloides*. J Chem Ecol. 2017;43(1):84–93.

71. Moore HE, Pechal JL, Benbow ME, Drijfhout FP. The potential use of cuticular hydrocarbons and multivariate analysis to age empty puparial cases of *Calliphora vicina* and *Lucilia sericata*. Sci Rep. 2017;7(1):1933.

72. Stoffolano JG, Schaub E, Yin CM, Tillman JA, Blomquist GJ. Cuticular hydrocarbons and their role in copulatory behavior in *Phormia regina* (Meigen). J Insect Physiol. 1997;43(11):1065–76.

73. Pechal JL, Moore H, Drijfhout F, Benbow ME. Hydrocarbon profiles throughout adult *Calliphoridae* aging: A promising tool for forensic entomology. Forensic Sci Int.
74. Urech R, Brown GW, Moore CJ, Green PE. Cuticular hydrocarbons of buffalo fly, *Haematobia exigua*, and chemotaxonomic differentiation from Horn Fly, H. irritans. J Chem Ecol. 2005;31(10):2451–61.

75. Mant J, Brändli C, Vereecken NJ, Schulz CM, Francke W, Schiestl FP. Cuticular hydrocarbons as sex pheromone of the bee *Colletes cunicularius* and the key to its mimicry by the sexually deceptive orchid, *Ophrys exaltata*. J Chem Ecol. 2005 Aug;31(8):1765–87.

76. Chemnitz J, Jentschke PC, Ayasse M, Steiger S. Beyond species recognition: Somatic state affects long-distance sex pheromone communication. Proc R Soc B Biol Sci. 2015;282(1812).

77. Engel KC, Männer L, Ayasse M, Steiger S. Acceptance threshold theory can explain occurrence of homosexual behavior. Biol Lett. 2015;11(1).

78. Levan KE, Fedina TY, Lewis SM. Testing multiple hypotheses for the maintenance of male homosexual copulatory behaviour in flour beetles. J Evol Biol. 2009;22(1):60–70.

79. Switzer P V., Forsythe PS, Escajeda K, Kruse KC. Effects of environmental and social conditions on homosexual pairing in the Japanese beetle (*Popillia japonica* Newman). J Insect Behav. 2004;17(1):1–16.

80. Vanderbilt CF, Giblin-Davis RM, Weissling TJ. Mating behavior and sexual response to aggregation pheromone of *Rhynchophorus cruentatus* (Coleoptera: Curculionidae). Florida Entomol. 1998;81(3):351–60.

81. McDonald RS, Borden JH. Courtship behavior and discrimination between potential mates by male *Delia antiqua* (Diptera: Anthomyiidae). J Insect Behav. 1996;9(6):871–85.

82. Esquivel JF, Crippen TL, Ward LA. Improved visualization of *Alphitobius diaperinus* (Panzer) (Coleoptera: Tenebrionidae) - part I: Morphological features for sex determination of multiple stadia. Psyche A J Entomol. 2012;2012.

83. Lai Z, Tsugawa H, Wohlgemuth G, Mehta S, Mueller M, Zheng Y, et al. Identifying metabolites by integrating metabolome databases with mass spectrometry cheminformatics. Nat Methods. 2018;15(1):53–6.

84. Misra BB. Data normalization strategies in metabolomics: Current challenges, approaches, and tools. Eur J Mass Spectrom. 2020;26(3):165–74.

85. Worley B, Powers R. Multivariate analysis in metabolomics. Curr Metabolomics. 2013;1(1):92–107.

86. Calla-Quispe E, Fuentes-Rivera HL, Ramírez P, Martel C, Ibañez AJ. Mass spectrometry: A rosetta stone to learn how fungi interact and talk. Life. 2020;10(6).

87. Romero PE, Calla-Quispe E, Castillo-Vilcahuaman C, Yokoo M, Fuentes-Rivera HL, Ramirez JL, et al. From the Andes to the desert: 16S rRNA metabarcoding
characterization of aquatic bacterial communities in the Rimac river, the main source of water for Lima, Peru. PLoS One. 2021;16(4):e0250401.

88. R Core Team. R: A language and environment for statistical computing. 2017; Available from: http://www.r-project.org/

89. Hervé M. RVAideMemoire: Testing and plotting procedures for biostatistics. R Packag version 09-79. 2021; Available from: https://cran.r-project.org/web/packages/RVAideMemoire/

90. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, et al. vegan: Community ecology package. R Packag version 25-7. 2020; Available from: https://cran.r-project.org/web/packages/vegan/

91. Dion E, Monteiro A, Nieberding CM. The role of learning on insect and spider sexual behaviors, sexual trait evolution, and speciation. Front Ecol Evol. 2019;6:225.

92. Carazo P, Sanchez E, Font E, Desfilis E. Chemosensory cues allow male Tenebrio molitor beetles to assess the reproductive status of potential mates. Anim Behav. 2004;68(1):123–9.

93. Capodeanu-Nägler A, Rapkin J, Sakaluk SK, Hunt J, Steiger S. Self-recognition in crickets via on-line processing. Curr Biol. 2014;24(23):R1117–8.

94. Bartelt RJ, Seaton KL, Dowd PF. Aggregation pheromone of Carpophilus antiquus (Coleoptera: Nitidulidae) and kairomonal use of C. lugubris pheromone by C. antiquus. J Chem Ecol 1993 1910. 1993;19(10):2203–16.

95. Francke W, Dettner K. Chemical signalling in beetles. In: Schulz S, editor. The chemistry of pheromones and other semiochemicals II Topics in current chemistry. Springer, Berlin, Heidelberg; 2004. p. 85–166.

96. Chen G, Song Y, Wang P, Chen J, Zhang Z, Wang S, et al. Semiochemistry of Dendroctonus armandi Tsai and Li (Coleoptera: Curculionidae: Scolytinae): Both female-produced aggregation pheromone and host tree kairomone are critically important. Chemoecology. 2014;25(3):135–45.

97. Wojcik DP. Mating behavior of 8 stored-product beetles (Coleoptera: Dermestidae, Tenebrionidae, Cucujidae, and Curculionidae). Florida Entomol. 2013;52(3):171–97.

98. Spikes AE, Paschen MA, Millar JG, Moreira JA, Hamel PB, Schiff NM, et al. First contact pheromone identified for a longhorned beetle (Coleoptera: Cerambycidae) in the subfamily Prioninae. J Chem Ecol. 2010;36:943–54.

99. Parker GA. Sexual selection and sexual conflict. In: Blum M, editor. Sexual selection and reproductive competition in insects. New York: Academic Press; 1979. p. 123–66.

100. Nahrung HF, Allen GR. Sexual selection under scramble competition: Mate location and mate choice in the eucalypt leaf beetle Chrysophtharta agricola (Chapuis) in the field. J Insect Behav. 2004;17(3):353–66.

101. Akinyemi AO, Kirk WDJ. Experienced males recognise and avoid mating with non-
virgin females in the western flower thrips. PLoS One. 2019;14(10):e0224115.

102. Dukas R. Learning affects mate choice in female fruit flies. Behav Ecol. 2005;16(4):800–4.

103. Biljana Stojković, Darka Šešlija Jovanović, Branka Tucić, Nikola Tucić. Homosexual behaviour and its longevity cost in females and males of the seed beetle Acanthoscelides obtectus. Physiol Entomol. 2010;35(4):308–16.

104. Yutaka Iguchi. Sexual behavior of the horned beetle, Allomyrina dichotoma septentrionalis (Coleoptera, Scarabaeidae). Japanese J Entomol. 1996;64(4):870–5.

Supporting information

S1 Fig. Circadian pattern of sexual activity of Alphitobius diaperinus based on their sexual condition (virgin pair and experienced pair). Circadian pattern test: N=10 (=100%) for each trial observed at one-hour intervals for a 24-h period during five days in the laboratory (H1–H12: 07:00–19:00 and H13–H24:19:00–07:00). Significant difference according to the Mann-Whitney-U test with Bonferroni correction (α=0.05, p<0.05).

S1 Table. Mating behaviour (in percentage; mean ± SD) and mating attempts recorded of Alphitobius diaperinus based on their gender and sexual condition.

S2 Fig. Biplot of principal component analysis (PCA) of curated compound-data matrix shows the PCA scores and loadings plot from adults of Alphitobius diaperinus. (A) PC1 vs PC2, (B) PC2 vs PC3. The peaks represent metabolites described in Table 2.

S3 Fig. GC-MS (CI) chromatograms of adults of Alphitobius diaperinus based on their gender and sexual condition. (A) Fv, (B) Fexp, (C) Mv, (D) Mexp. 1) MBQ, 2) m-cresol, 3) limonene, 4) EBQ, 5) 2,5-dimethyl-1,4-benzoquinone, 6) benzothiazole, 7) 2-ethyl-1,4-
hydroquinone, 8) benzophenone, 9) palmitic acid, 10) unknown, 11) unknown, 12) oleic acid, 13) linoleic acid, 14) unknown, 15) unknown, 16) unknown, 17) unknown, 18) heptacosane, 19) nonacosane. IS, internal standard, C16.
Figure 1

**Circadian pattern of sexual behaviour**

**Statistical analysis**

**Odour bouquet attraction**

**Statistical analysis**

**Mating behaviour description**

**Statistical analysis**

**Chemical analysis**

**Multivariate analysis**
Figure 2
Figure 3
Figure 5
| Treatment   | N  | Touching     | Mounting* | Copulation* |
|------------|----|--------------|-----------|-------------|
| Fv - Mv    | 15 | 48.48 ± 24.77 | 5.75 ± 7.65 | 0.93 ± 0.73 |
| Fv - Mexp  | 20 | 41.79 ± 23.01 | 0.08 ± 0.34 | 0.03 ± 0.11 |
| Fexp - Mv  | 15 | 47.43 ± 28.73 | 4.28 ± 4.39 | 0.50 ± 0.85 |
| Fexp - Mexp| 15 | 49.81 ± 30.66 | 2.54 ± 6.69 | 0.13 ± 0.28 |

*Significant difference (Kruskal-Wallis test, p < 0.0001)
| Peak | Metabolite name *1 | Molecular formula | Calculated RI *2 | Retention time (min) | Theoretical mass (m/z) | Measured mass (m/z) | Accuracy (ppm) | Fexp *3 | Fy *4 | Mexp *5 | Mv *6 |
|------|-------------------|-------------------|-----------------|---------------------|------------------------|---------------------|-----------------|--------|-------|--------|-------|
| 1    | 2-Methyl-1,4-benzoquinone | C_{12}H_{10}O_{2} | 1015            | 7.20                | 123.04460             | 123.04419           | 1.06            | 160.99 ± 52.81 | 43.78 ± 34.95 | 163.06 ± 66.22 | 44.58 ± 27.84 |
| 2    | m-cresol          | C_{8}H_{8}O       | 1020            | 7.28                | 123.08044             | 123.08070           | 2.11            | 1.11 ± 0.73    | 3.98 ± 1.83    | 1.12 ± 0.78    | 1.90 ± 2.01    |
| 3    | Limonene          | C_{10}H_{16}      | 1030            | 7.44                | 81.06988              | 81.07004            | 1.97            | 3.14 ± 0.95    | ND             | 1.61 ± 0.73    | ND             |
| 4    | 2-Ethyl-1,4-benzoquinone | C_{12}H_{10}O_{2} | 1109            | 8.66                | 137.05971             | 137.06010           | 2.85            | 422.18 ± 93.07 | 198.99 ± 83.7  | 390.17 ± 60.86 | 225.18 ± 95.28 |
| 5    | 2,5-Dimethyl-1,4-benzoquinone | C_{14}H_{12}O_{2} | 1111            | 8.70                | 79.05423              | 79.05423            | 0.00            | 2.81 ± 0.55    | 0.46 ± 0.82    | 2.65 ± 0.6     | ND             |
| 6    | Benzothiazol      | C_{12}H_{10}NS    | 1233            | 10.53               | 136.02155             | 136.02173           | 1.32            | 1.40 ± 0.64    | 3.06 ± 2.28    | 1.91 ± 2.04    | 2.19 ± 2.22    |
| 7    | 2-Ethyl-1,4-hydroquinone | C_{12}H_{10}O_{2} | 1431            | 13.26               | 139.07536             | 139.07574           | 2.73            | 7.01 ± 6.23    | 0.08 ± 0.02    | 6.52 ± 5.43    | 0.76 ± 1.39    |
| 8    | Benzophenone      | C_{10}H_{10}O     | 1638            | 15.82               | 183.08044             | 183.07999           | 2.46            | 0.39 ± 0.17    | 0.71 ± 0.13    | 0.42 ± 0.21    | 0.66 ± 0.43    |
| 9    | Palmitic acid     | C_{16}H_{32}O_{2} | 1967            | 19.35               | 257.24751             | 257.24814           | 2.45            | 2.99 ± 2.24    | ND             | 1.58 ± 3.62    | 0.13 ± 0.15    |
| 10   | Unknown           | C_{16}H_{30}O_{2} | 1973            | 19.41               | -                     | 88.02151            | -               | 1.32 ± 1.03    | 1.75 ± 1.97    | 1.44 ± 1.07    | 0.86 ± 1.62    |
| 11   | Unknown           | C_{17}H_{32}O_{2} | 2085            | 20.46               | -                     | 237.16347           | -               | 1.36 ± 0.41    | 2.02 ± 0.12    | 1.37 ± 0.43    | 1.39 ± 0.94    |
| 12   | Oleic acid        | C_{18}H_{34}O_{2} | 2156            | 21.13               | 181.15869             | 181.15831           | 2.10            | 0.1 ± 0.16     | ND             | 0.03 ± 0.02    | ND             |
| 13   | Linoleic acid     | C_{18}H_{32}O_{2} | 2168            | 21.25               | 263.23694             | 263.23776           | 3.12            | 0.15 ± 0.06    | ND             | 0.11 ± 0.08    | ND             |
| 14   | Unknown           | C_{22}H_{44}O_{2} | 2203            | 21.59               | -                     | 131.08556           | -               | 2.73 ± 1.05    | 4.35 ± 0.23    | 2.94 ± 0.6     | 2.16 ± 2.1     |
| 15   | Unknown           | C_{23}H_{42}O_{2} | 2253            | 22.05               | -                     | 284.29517           | -               | 1.77 ± 0.69    | 2.94 ± 0.89    | 1.89 ± 0.70    | 2.35 ± 1.88    |
| 16   | Unknown           | C_{23}H_{46}O_{2} | 2346            | 22.96               | -                     | 315.23309           | -               | 1.41 ± 0.70    | 2.55 ± 0.99    | 1.32 ± 0.69    | 1.64 ± 1.93    |
| 17   | Unknown           | C_{24}H_{40}O_{2} | 2388            | 23.40               | -                     | 193.10112           | -               | 1.54 ± 0.48    | 2.37 ± 0.20    | 1.45 ± 0.47    | 1.71 ± 1.25    |
| 18   | Heptacosane       | C_{27}H_{56}      | 2700            | 28.44               | 71.08553              | 71.08553            | 0.00            | 10.36 ± 5.94   | 5.27 ± 1.05    | 8.72 ± 2.47    | 5.14 ± 2.10    |
| 19   | Noracosane        | C_{36}H_{60}      | 2900            | 34.44               | 71.08553              | 71.08542            | 1.55            | 4.84 ± 2.87    | ND             | 4.08 ± 1.27    | ND             |

*1 Data was filtered using p-anova<0.01 and compound were identified using CI & EI – MS spectra, *2 Retention Index (RI) calculated using DB-5MS column, *3 N=14, *4 N=4.