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CHEMICAL ECOLOGY OF BED BUGS (HETEROPTERA: CIMICIDAE) IN THEIR MICROHABITATS

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Abstract Extracts of the exuviae of nymphal bed bugs (*Cimex lectularius*) were analysed for volatile compounds that might contribute to arrestment of adult bed bugs. Four volatile aldehydes, (E)-2-hexenal, 4-oxo-(E)-2-hexenal, (E)-2-octenal, and 4-oxo-(E)-2-octenal were consistently detected in the headspace of freshly shed exuviae. Quantification of the aldehydes in the solvent extracts of homogenized exuviae with varying aging periods indicated that the aldehydes are originally present in the exuviae and dissipate over time, possibly through evaporation. Microscopic observation of the exuviae indicated that the dorsal abdominal glands on the exuviae maintained their reservoirs, within which the aldehydes might be retained. Two-choice olfactometer studies with the volatiles from exuviae or a synthetic blend mimicking the volatiles indicated that adult bed bugs tend to settle close to sources of the aldehydes. Our results imply that the presence and accumulation of bed bug exuviae and the aldehydes volatilizing from the exuviae might mediate bed bugs' interaction with their microhabitats. Some recent studies on the potential functions of the volatile aldehydes within microhabitats of bed bugs are discussed.

Key words *Cimex lectularius*, aldehyde, ketoaldehyde, pheromone, antimicrobial compound

INTRODUCTION

Several recent studies have examined if (*E*)-2-hexenal and (*E*)-2-octenal function as part of the aggregation pheromone blend of bed bugs. In these studies, researchers often detected relatively low concentrations of (*E*)-2-hexenal and (*E*)-2-octenal from the headspace of a bed bug colony or a paper substrate that was used by bed bugs (Siljander et al., 2008; Mendki et al., 2014; Gries et al., 2015; Olson et al., 2017).

Even though the behavioral effects of these chemicals have been investigated in several empirical studies, the potential mechanisms to explain the presence of these aldehydes in the headspace and substrates of the colony have seldom been investigated. The current study examined whether the dorsal abdominal glands on bed bug exuviae function as "dispensers" of the volatile pheromones, allowing the aldehydes to be slowly released from the exuviae. We investigated if the volatile aldehydes could be detected in the headspace of exuviae, and quantities of the aldehydes in the exuviae decreased over time. The morphological characteristics of dorsal abdominal glands on the exuviae are also described. To understand the behavioral significance of the aldehydes, we tested bed bugs' arrestment in a two-choice olfactometer. The potential significances of the volatile aldehydes on bed bug's biology are discussed.

MATERIALS AND METHODS

Insect

Bed bugs used in these experiments were "Earl" strain individuals obtained from Sierra Research Laboratories (Modesto, CA). Six small colonies (nymph only) established from eggs were used to collect 1st, 2nd, 3rd, 4th, and 5th instar exuviae. Exuviae that had fallen to the bottom of the vials were collected using a fine paintbrush.

Chemical Analyses

Headspace volatiles of a group of 25 exuviae (from one instar) were collected with a solid-phase microextraction (SPME) sampler [75 μm carboxen / polydimethylsiloxane (PDMS); Supelco, Inc.] in 2-ml vials (18-hour collection, 25-26°C). Volatiles absorbed on the SPME fiber were analyzed by gas chromatography– mass spectrometry (Agilent 7890A GC / Agilent 5975C MSD).

To determine if quantities of the chemicals decrease as the exuviae age in the open air, freshly collected fifth instar exuviae were aged for 7, 45, and 99 d before extraction. A group of three exuviae were homogenized and extracted in 0.5 ml methylene chloride. One-microliter aliquots of the extracts were analyzed in an Agilent 7890A GC equipped with a flame ionization detector. Calibration curves based on external standards were used for quantification. Simple linear regression equations were calculated to relate amounts of the aldehydes per exuvia (μg) to the aging period (number of days). Quantity values (y) were log-transformed before the regression analyses due to heteroscedasticity. The significance of the regression was tested using analyses of variance (ANOVA) (Zar, 1999).

Gland Morphology

Intact and cut exuviae (from 5th instar) were cleaned in solvents and sputter-coated with a gold-palladium mixture, and observed with a Philips XL30-FEG scanning electron microscope (SEM). To determine if the dorsal abdominal gland reservoirs open externally, we examined whether a colored solvent could be taken up into the gland reservoir by submerging the exuviae in a colored solvent [D-limonene with Sudan Black B (Fisher Scientific)] and gently pressing and releasing the gland reservoirs with a fine probe. The surfaces of the treated exuviae were briefly washed with clean acetone, and observed under a stereomicroscope with bright-field illumination.

Olfactometer Study

The behavioral responses of adult bed bugs to the volatiles were examined in an olfactometer. A 15-cm piece of flexible polyvinyl chloride (PVC) tubing was used as the body of the olfactometer. Glass vials (2 ml) were attached to each end of the tubing, with a fine fabric screen on the vial openings, serving as treatment and control vials. The piece of fabric screen prevented bed bugs in the olfactometer body from contacting the vial contents. To test exuviae as a source of volatiles, 70-76 exuviae from 3rd, 4th, and 5th instar nymphs were used. Clean empty vials served as controls. To test synthetic aldehydes, blends of test compounds dissolved in paraffin oil (40 μl) were applied to a piece of cotton (≈50 mg) in the treatment vial. Compound ratios were adjusted to match the ratio found in a 7-day old exuviae. Vials containing a piece of cotton treated with clean paraffin oil served as controls. Individual bed bugs were introduced into the olfactometer through a small slit cut at the center of the tubing about 3 hours before the end of photophase, and final location of the bed bug was recorded 18 hours later during photophase. The null hypothesis that adult bed bugs showed no preference for either olfactometer arm (i.e., random choice) was tested using Chi-square goodness of fit tests with the Yates correction for continuity (Zar, 1999).

RESULTS AND DISCUSSION

Chemical Analyses

SPME analyses consistently detected (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal in the headspace of exuviae. Because the exuviae were collected from the bottom of small colonies that were comprised of nymphs only, the compounds detected in the headspace of exuviae likely originated from the exuviae themselves, rather than from contamination associated with alarm / defensive responses of live bed bugs. Based on the linear regression analyses, the quantities of the aldehydes per exuvia measured in micrograms (y) fit a log-linear model with the aging period measured in days (x) (Figure 1). The regression equations with population linearity indicated that average amounts of (*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal decrease by approximately 1.37, 1.83, and 2.28 % per day, respectively. A similar interpretation was not made for 4-oxo-(*E*)-2-hexenal due to its non-linear population regression ($P = 0.003$). This information, together with the presence of the same aldehydes in the headspace, indicates that these aldehydes volatilize from the exuviae.

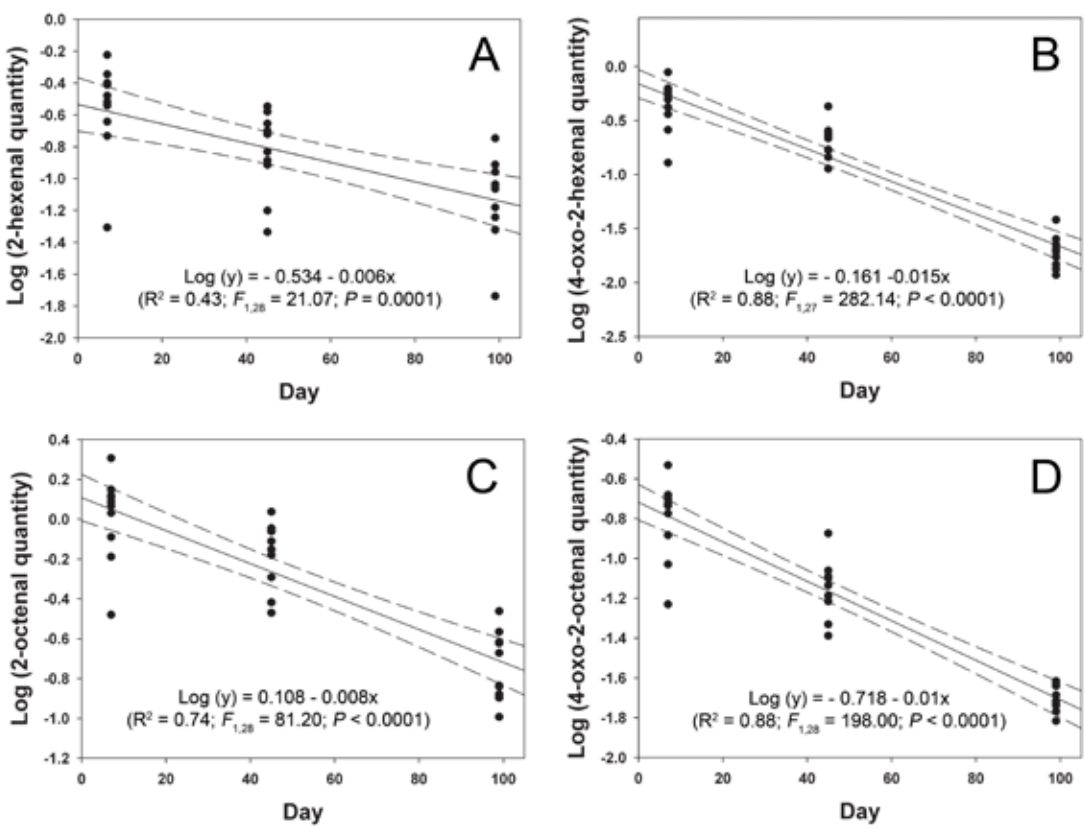

Figure 1. Regression lines of four aldehydes detected from exuviae of 5th instar nymphs. (A) (*E*)-2-hexenal, (B) 4-oxo-(*E*)-2-hexenal, (C) (*E*)-2-octenal, (D) 4-oxo-(*E*)-2-octenal. Units for the chemical quantity per exuvia is mg. Dotted lines indicate 95% confidence intervals.

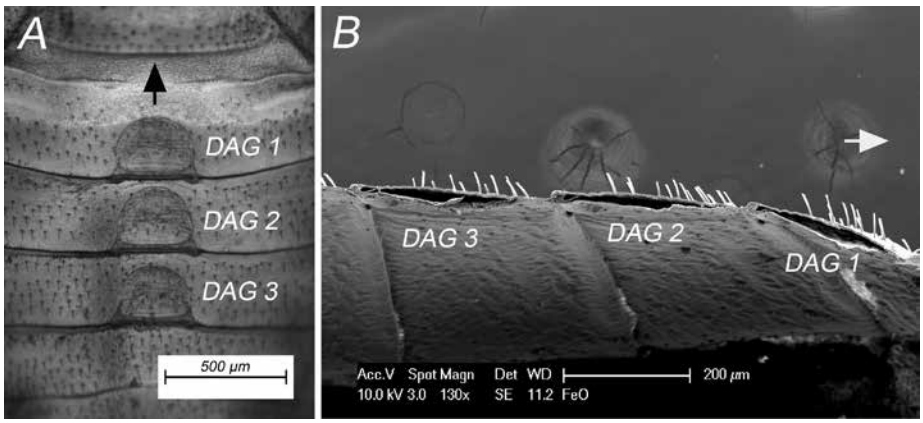

Figure 2. Dorsal abdominal gland morphology study with 5th instar exuviae. (A) Dorsal abdominal glands on an exuvia. (B) Scanning electron microscope image showing the longitudinal cut section of dorsal abdominal glands. The arrow indicates the anterior direction of the exuvia. DAGs 1-3 indicate dorsal abdominal glands on the 3rd, 4th, and 5th abdominal tergites, respectively.

Gland Morphology

Three bell-shaped dorsal abdominal glands were located at the 3rd, 4th, and 5th tergites of the exuviae (Figure 2A). SEM observations of the cut exuviae revealed that the dorsal area of the exuviae contained three reservoirs associated with dorsal abdominal glands (Figure 2B, three hollow spaces). The black colored solvent penetrated the dorsal abdominal gland reservoirs of intact exuviae, suggesting that there were openings and channels connecting the internal space of the gland reservoirs to the cuticular surface. Overall, these evidences support the notion that the compounds retained inside the dorsal abdominal glands of the exuvia slowly evaporate through the small orifices.

Olfactometer Assay

When left as individuals in the olfactometer over 18 h, adult bed bugs showed significant arrestment at the source of the volatile compounds. When the exuviae were used as a source of volatiles, 26 of 32 (81.3%) male bed bugs were found on the treatment vial of the olfactometer ($c^2 = 11.28$, $P < 0.001$). In the same experiment, 17 of 20 (85%) female bed bugs were found on the treatment vial ($c^2 = 8.45$, $P = 0.004$). When the cotton ball with the synthetic blend of aldehydes was used as the source of volatiles, ≈83 % (19 of 23 for male, $c^2 = 8.52$, $P = 0.004$; 20 of 24 for female, $c^2 = 9.38$, $P = 0.002$) of individuals were found on the treatment vial.

CONCLUSIONS

Bed bugs' tendency of settling near conspecific exuviae might have important implications for their biology in their "natural" habitats. Bed bug harborages in residential settings can accumulate numerous exuviae from developing bed bugs. In general, bed bugs are believed to typically return to these harborages after feeding forays, forming dense aggregations, where eggs and fecal materials also accumulate (Usinger 1966; Reinhardt and Siva-Jothy 2007). Attraction / arrestment to the source of aldehyde pheromones would allow the bed bugs to direct their movement towards existing harborages where other conspecific individuals are developing.

However, there are substantial amount of inconsistency and discrepancy in the research literature regarding the behavioral effect of these aldehyde pheromones (Levinson and Bar Ilan, 1971; Gries et al., 2015; Olson et al., 2017). (*E*)-2-Hexenal and (*E*)-2-octenal are known to influence bed bugs' behavior in a concentration-dependent manner (Siljander et al., 2008; Ulrich et al., 2016). Slow and controlled release of the compounds also might be critical to cause attraction / arrestment of bed bugs to the odor source.

In addition to providing information associated with the established harborages, the aldehydes might play a role in modifying biological environments in bed bugs' microhabitats. For example, Ulrich

et al. (2015) reported that (*E*)-2-hexenal and (*E*)-2-octenal inhibit growth of an entomopathogenic fungus targeting bed bugs by direct contact and via indirect exposure (''fumigation''), indicating these compounds may play a role in disinfecting the microenvironment of bed bug harborages. Based on research on several species of alydid, coreid, and pentatomid bugs, Noge et al. (2012) and Noge (2015) reported that 4-oxo-(*E*)-2-hexenal, (*E*)-2-hexenal, and (*E*)-2-octenal showed dose-dependent antibacterial activities against four bacterial species. The potential function of volatile aldehyde pheromones in shaping microbial environments associated with bed bug harborages is currently under investigation.

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