Insect Pheromones

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Bed Bug Aggregation Pheromone Finally Identified**

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Abstract: *Bed bugs have become a global epidemic and current detection tools are poorly suited for routine surveillance. Despite intense research on bed bug aggregation behavior and the aggregation pheromone, which could be used as a chemical lure, the complete composition of this pheromone has thus far proven elusive. Here, we report that the bed bug aggregation pheromone comprises five volatile components (dimethyl disulfide, dimethyl trisulfide, (E)-2-hexenal, (E)-2-octenal, 2-hexanone), which attract bed bugs to safe shelters, and one less-volatile component (histamine), which causes their arrestment upon contact. In infested premises, a blend of all six components is highly effective at luring bed bugs into traps. The trapping of juvenile and adult bed bugs, with or without recent blood meals, provides strong evidence that this unique pheromone bait could become an effective and inexpensive tool for bed bug detection and potentially their control.*

Owing to modern hygienic measures and the common use of insecticides, bed bugs had nearly vanished in many countries in the last century.[1,2] However, over the past 15 years, common bed bugs, *Cimex lectularius* (Hemiptera: Cimicidae), "have re-emerged in increasing numbers, distribution, and intensity of infestation".[1] During the first decade of the 21st century alone, the number of bed bug bite injuries treated in emergency rooms in the United States rose from 21 to 15945.[3] Indeed, bed bugs have become a global epidemic. Their resurgence may be attributed to a rise in travel and sales of used furnishings, adjustments in pest-control tactics,[1] and the development of insecticide resistance.[4,5] Because bed bug infestations have severe adverse effects on the human host, causing diverse dermal reactions, emotional distress, and paranoia,[1,6–8] their rapid detection is paramount. Unfortunately, current bed bug monitoring tools are poorly suited for routine surveillance,[1] in part because the human host cues (CO_2, heat, scent) they deploy are technically challenging or expensive. The cost of bed bug treatment is a particular concern for disadvantaged or low-income people who are disproportionately impacted by infestations, and often serve as reservoirs for further insect spread.[9] Thus, although well more than 500 million USD are spent annually on bed bug management,[9] there is no economic and effective tool for detecting the presence of bed bugs. Furthermore, despite intense research[10] on bed bug olfaction and behavior, and on the aggregation pheromone itself, which could be used as a chemical lure, the composition of the complete pheromone blend has proven elusive. Specifically, a missing "contact" pheromone component has been linked to the failure of existing pheromone lures in field studies.[10h] Here, we report the isolation and structural characterization of the bed bug aggregation pheromone blend, and the potential for developing it as an effective and inexpensive tool for the detection and possibly the control of bed bug infestations.

Our initial search for the bed bug aggregation pheromone focused on bed bug exuviae (cuticle shed during moulting) and feces, which are both present in natural bed bug shelters and have been associated with arrestment behavior.[10f] To narrow in on the aggregation pheromone, we designed two-choice (treatment vs. control), single-insect olfactometer experiments[11] that allowed rapid observations of bed bug responses to various test stimuli. Using these olfactometer assays, we found that both fresh and aged exuviae trigger bed bug arrestment, and that physical contact with the exuviae is required for arrestment behavior (Figure 1a).[11] Together, these results indicate that the active component is not volatile, and instead is sensed by bed bugs only upon contact. In order to isolate sufficient amounts of the pheromone for characterization purposes, we extracted over 18000 shed bed bug exuviae with organic solvents of increasing polarity. Of these, only the methanol extract induced arrestment (Figure 1b).[11] Likewise, only the methanol extract of a large collection of bed bug feces was active in olfactometer bioassays, indicating that the arrestant pheromone was common to both the exuviae and feces, and that it was a polar molecule that had thus far evaded detection because of its atypical pheromonal properties. Analysis of the ^{1}H NMR spectra acquired on bioactive methanol extracts showed the presence of amino acids (valine, alanine), histamine, *N*-acetylglucosamine, dimethylaminoethanol, and 3-hydroxy-kynurenine *O*-sulfate (Figure 1c). Further, we noted that crude methanol extracts and normal-phase flash chromatography fractions of these extracts containing the largest amounts of histamine and dimethylaminoethanol elicited the strongest arrestment responses, suggesting that one or both of these compounds are the key pheromone component(s). When we tested authentic samples of dimethylaminoethanol and histamine

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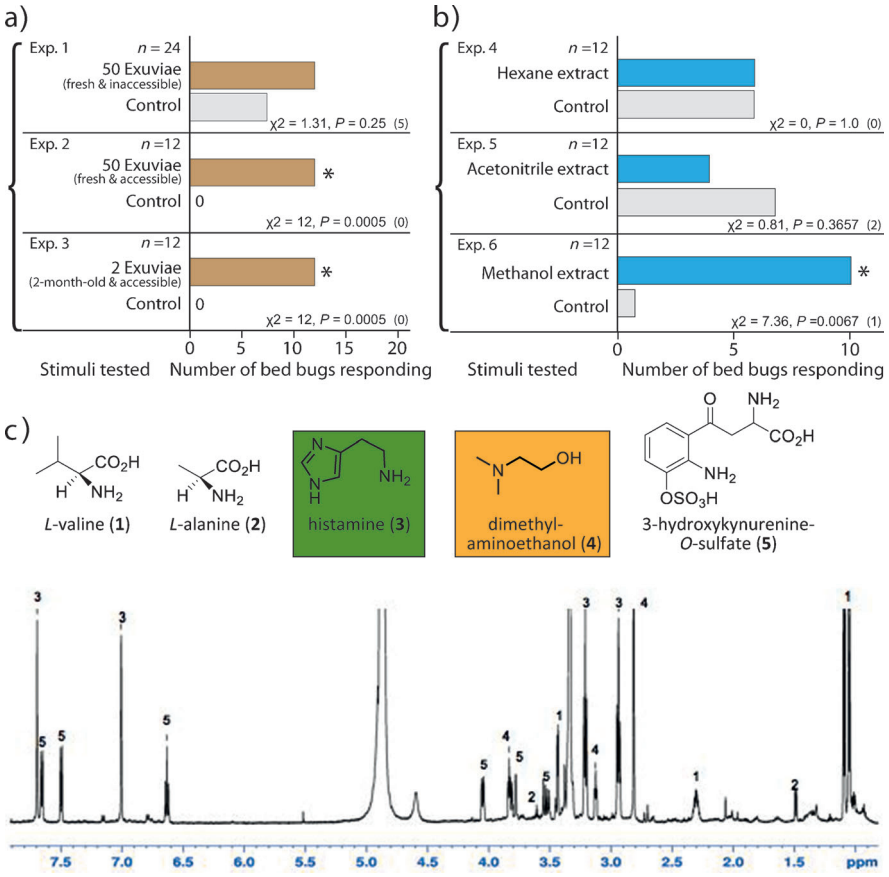

Figure 1. Effect of test stimuli on behavioral responses of bed bugs (a, b), and compounds identified by NMR spectroscopy in bioactive stimuli (c). Effects of a) bed bug exuviae and b) of various organic extracts of six exuviae on behavioral responses of singly tested bed bugs in two-choice, three-dish olfactometers.[11] In each experiment, an asterisk (*) denotes a significant preference ($P < 0.05$) for the test stimulus, *n* indicates the number of bed bugs tested, and numbers in parentheses report the number of bed bugs not responding to either test stimulus. c) Compounds identified by NMR spectroscopy (^{1}H, COSY, HMQC, HMBC) and the ^{1}H NMR spectrum of the crude methanol extract of bed bug feces (CD_3OD, 600 MHz).[11]

at concentrations similar to those found in feces and shed cuticles (20 μg/assay), we found that only histamine as a free base (not as a salt) induced the arrestant behavior.[11] It is noteworthy that the presence of histamine in both bed bug feces and shed cuticle may signal the metabolism of host blood nutrients and thus host presence, and a safe haven for moulting, a vulnerable stage in the life cycle of bed bugs.

While histamine clearly plays an important role as the arrestant pheromone, there was no arrestment behavior when we suspended samples of histamine out of the reach of bed bugs in olfactometer bioassays.[11] This result is not surprising, considering the low volatility of histamine (bp > 200 °C), and inspired us to search for additional volatile attractive pheromone components. Previously,[10h] we identified a ten-component blend of attractive volatiles from the headspace of bed bug colonies containing nymphs, adults, feces, and eggs. This blend was effective in the laboratory, but not in the field. Here, we focused on feces alone as the now proven aggregation pheromone source. To enhance volatile release from dry bed bug feces, we heated (90 °C) feces-stained filter paper and analyzed the collected volatiles by GC-MS. Theses analyses resulted in the identification of a complex blend of 15 oxygen- or sulphur-containing components. As depicted in Figure 2, the blend included six aldehydes (butanal, pentanal, hexanal, (*E*)-2-hexenal, (*E*)-2-octenal, benzaldehyde), one alcohol (benzyl alcohol), three ketones (2-hexanone, acetophenone, verbenone), three esters (methyl nonanoate, ethyl octanoate, pentyl hexanoate) and two sulfides (dimethyldisulfide (DMDS), dimethyltrisulfide (DMTS)). Furthermore, an authentic blend containing less than 4 μg of each component was capable of attracting bed bugs in olfactometer experiments.[11] In order to determine the essential component(s) of this blend, we tested the response of bed bugs to the complete blend and to partial blends that lacked either groups of related organic chemicals (e.g., esters or aldehydes) or specific individual components.[11] Following several iterative rounds of compound deletions, we found that the essential attractive volatile pheromone components (VPCs) in bed bug feces consist of a blend of DMDS, DMTS, (*E*)-2-hexenal, (*E*)-2-octenal, and 2-hexanone. While the VPCs include the two previously known alarm and aggregation pheromone components (*E*)-2-hexenal and (*E*)-2-octenal,[12,10h] the sulfides and 2-hexanone represent new VPCs for bed bugs.

To tease apart the effects of the VPCs and histamine on attraction and arrestment behavior of bed bugs, we repeated our experiments in large bioassay arenas that better reflect the more spacious nature of bed bug habitats. Test stimuli consisted of corrugated cardboard shelters that we baited with histamine, the VPCs, or both, or that we left unbaited (controls). Remarkably, shelters baited with both the VPCs and histamine invariably "captured" nearly all of the bed bugs tested (Figure 3a). In contrast, partial blends containing only the VPCs or histamine were less effective. When we offered bed bugs a choice between histamine- or VPC-baited shelters, bed bugs preferred the former, and when we offered a choice between a histamine-baited shelter or a shelter baited with both histamine and VPCs, bed bugs preferred the latter. Thus, the highly mobile bed bugs are attracted to the VPCs, but adopt a shelter only if histamine is present. In turn, if bed bugs encounter histamine in a shelter with or without VPCs, they adopt that shelter. Together, these results reveal an amazing synergism between the attractive VPCs and the histamine arrestant, which are likely sensed by different types of receptors[13,14] on separate segments of the bed bugs' antennae.[10c]

To determine whether the synthetic pheromone lure was also effective in premises infested with bed bugs, where many

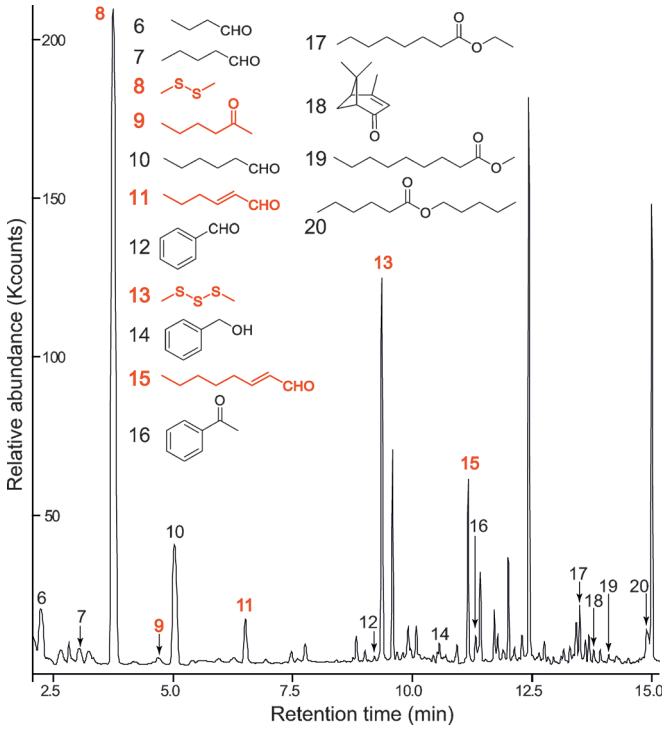

Figure 2. Total ion chromatogram of volatile components released from filter paper stained with feces of 300 bed bugs over four weeks.

competing attractants are present and previously reported pheromone blends[10h] failed to attract bed bugs, we repeated the trapping experiments in a heavily infested residential apartment. Experimental replicates consisted of paired corrugated cardboard shelter traps placed against a wall or furniture, with 30 cm spacing between paired traps and more than 1 m spacing between pairs. Within each pair, we randomly baited one trap with either the complete pheromone blend (VPCs and histamine) or a partial pheromone blend (VPCs or histamine), and kept the other trap unbaited. As highlighted in Figure 3b, traps baited with the complete synthetic pheromone blend "captured" 4.5 times more bed bugs than unbaited control traps, whereas traps baited with the VPCs captured only 2.2 times more bed bugs than control traps. In a second set of two concurrently run experiments, shelter traps baited with the complete synthetic pheromone blend captured 6.7 times more bed bugs than control traps, whereas traps baited with histamine captured only 1.3 times more bed bugs than control traps. Large captures of bed bugs only in traps baited with the complete synthetic pheromone confirm the synergism between the VPCs, which attract bed bugs, and histamine, which causes their arrestment on contact.

During the course of the field studies, we found that the pheromone-baited shelter traps contained all five nymphal instars (fed and non-fed) as well as adult males and females (fed and non-fed). These data show that the synthetic pheromone attracts and retains bed bugs irrespective of their developmental stage, gender, or physiological condition, as does the natural aggregation pheromone.[10c] Pheromone-baited shelter traps were also effective in residential apartments with light to moderate bed bug infestations. In all apartments where at least one live bed bug was observed during careful inspection of bedding, mattresses, and furniture, at least one bed bug was captured. Remarkably, of the 27 bed bugs captured in these apartments, 26 were in the pheromone-baited traps.

The functional role of DMDS and DMTS as bed bug pheromone components seems surprising, given that they are common semiochemicals[15,16] and linked to bacterial decomposition of organic material.[17] Nevertheless, behavioral bioassay data showing that synthetic pheromone blends without these sulfides fail to attract bed bugs,[11] and electrophysiological data showing that specific olfactory sensilla on the bed bugs' antennae respond to DMDS and DMTS,[13] provide integral support for the conclusion that DMDS and DMTS are part of the bed bug aggregation pheromone. The known alarm pheromones[12] (*E*)-2-hexenal and (*E*)-2-octenal as additional components then impart "bed bug specificity" to the pheromone blend. The bifunctional

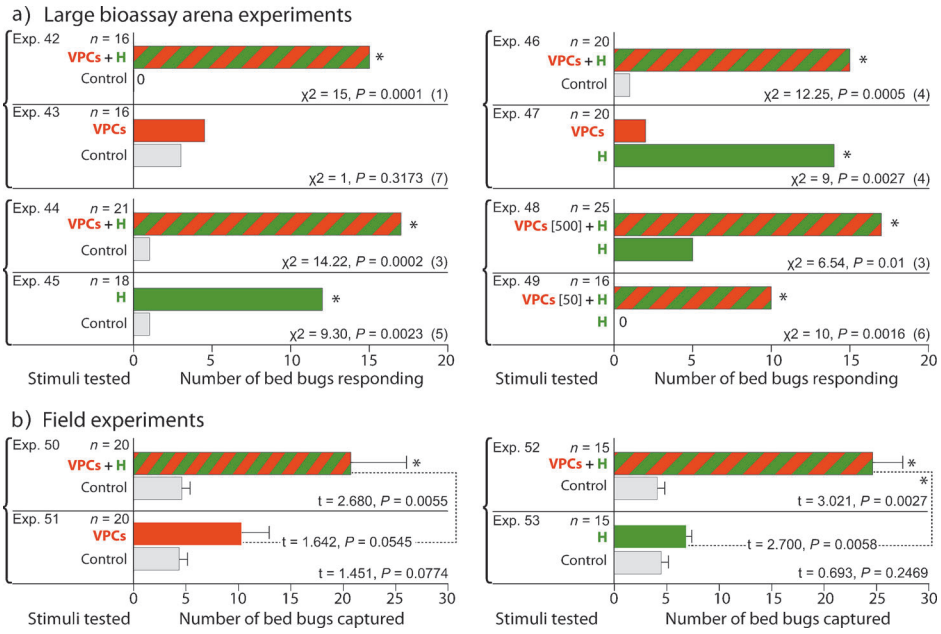

Figure 3. Effect of complete or partial pheromone blends on behavioral responses of male adult bed bugs in a) large bioassay arenas or b) in a residential apartment. The volatile pheromone components (VPCs) were tested at equal amounts for a total of 500 μg in experiments 42–48 and 50–53, and 50 μg in experiment 49. Histamine (H) was tested at 2 mg per assay. An asterisk (*) denotes a significant preference ($P < 0.05$) for a test stimulus, *n* indicates the number of single bed bugs tested in experiments 42–49, or the number of replicates run in field experiments 50–53, and numbers in parentheses report the number of bed bugs not responding to either test stimulus.

nature of these aldehydes as repellents at high concentration[12] and attractants at low concentration[10h] is reminiscent of bifunctional pheromones in stink bugs[18] and bark beetles,[19] where pheromone concentration determines attractive or repellent activity.

The biosyntheses of DMDS, DMTS, 2-hexanone, and histamine may be linked to the bed bugs' diet of human blood, which contains the amino acids histidine and methionine.[20] These could serve as biosynthetic precursors for histamine as well as DMDS and DMTS, respectively.[21] Similarly, free fatty acids in human blood[20] could be converted to 2-hexanone through a few well-established biosynthetic steps, as shown for other methyl ketone pheromones.[22] Predicting that bed bugs may alternatively acquire histamine from the immunological response of fed-on human hosts, we searched for, but did not detect, histamine in human blood withdrawn from bed bugs just after their blood meal. It follows that bed bugs produce histamine de novo or through decarboxylation of the host-derived amino acid histidine.

In light of the striking synergism between the attractive and arrestant components of the bed bug aggregation pheromone (Figure 3), access to the arrestant must be considered in the design of commercial pheromone lures and traps. Because histamine-impregnated shelters are capable of retaining the bed bugs that have entered, there is no longer a need for the commonly used sticky traps for bed bug capture. In fact, sticky trap surfaces would prevent bed bugs from reaching the histamine arrestant and thus interfere with optimal capture of bed bugs. This expectation was confirmed when pheromone-baited shelters were tested with or without an adhesive-coated perimeter, the latter capturing significantly more bed bugs.[23]

In summary, we have determined that the bed bug aggregation pheromone comprises a unique blend of oxygen-, sulfur-, and nitrogen-containing components. The five volatile components and the less volatile arrestant histamine synergistically mediate both attraction and arrestment of bed bugs. Of note, the total chemical cost of the aggregation pheromone employed effectively in residential apartments was less than 10 cents per lure, suggesting that this lure may significantly impact future directions in detection and possibly control of bed bug infestations. This affordable technology may also remove socioeconomic barriers that presently prevent bed bug monitoring in many urban settings.

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[23] In a paired-trap experiment (Exp. 54) run in residential apartments, with VPCs and histamine as trap bait and with one trap in each pair bearing an adhesive-coated perimeter, the mean (± SE) captures of bed bugs in traps with or without adhesive-coated perimeter were 7.125 (±1.697) and 26.68 (±11.78), respectively (15 replicates, $t = 1.9536$, $P = 0.031$), clearly showing an adverse effect of the adhesive on trap captures.

