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Research Article

Bed Bug (Hemiptera: Cimicidae) Attraction to Human Odors: Validation of a Two-Choice Olfactometer

Zachary C. DeVries,[1,2,3,4] Ahmed M. Saveer,[1,2] Russell Mick,[1] and Coby Schal[1,2,3]

[1]Department of Entomology and Plant Pathology, North Carolina State University, Raleigh, NC [2]W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC [3]Center for Human Health and the Environment, North Carolina State University, Raleigh, NC [4]Corresponding author, e-mail: zcdevrie@ncsu.edu

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Abstract

Bed bugs (*Cimex lectularius* L.) (Hemiptera: Cimicidae) are obligate hematophagous ectoparasites, and, therefore, must locate suitable hosts to ensure survival and reproduction. Their largely nocturnal activity suggests that chemosensory and thermosensory cues would play critical roles in host location. Yet, the importance of olfaction in host attraction of bed bugs remains unclear. We developed and validated a Y-tube, two-choice olfactometer and tested its suitability for investigating attraction to human odors (from skin swabs). Olfactometer orientation significantly affected the percentage of bed bugs that were activated by human odors, with significantly more bed bugs responding when the olfactometer was oriented vertically (bug introduced at bottom of the olfactometer) compared with all other orientations. Starved (7–10 d) adult males, mated females, and nymphs responded (47–77% moved up the olfactometer and made a choice) when human odors were present in the olfactometer, while starved, unmated females did not respond. Skin swabs from all five human participants elicited high response rates (65–82%), and bed bugs from four different populations responded to skin swabs (40–82% response rate). However, in all assays including those resulting in relatively low response rates, bed bugs exhibited >90% preference for human odors over blank controls. These results provide strong evidence that bed bugs can respond and orient towards human odors, independently of all other host cues. Furthermore, the validated olfactometer should enable rapid and efficient evaluations of bed bug behavioral responses to semiochemicals.

Key words: host odor, host attraction, behavior, olfaction, chemotaxis

Bed bugs (*Cimex lectularius* L.) (Hemiptera: Cimicidae) remain one of the most challenging pests to manage indoors. Since their resurgence in the early 2000s, infestations have been documented from across the globe (Doggett et al. 2004, How and Lee 2010, Levy Bencheton et al. 2011, Potter 2011, Wang and Wen 2011, Faúndez and Carvajal 2014), with high infestation rates especially frequent in low-income communities (Wu et al. 2014, Wang et al. 2016). Bed bugs affect humans physically (bites) (Goddard and deShazo 2009), psychologically (Goddard and de Shazo 2012, Susser et al. 2012), and they produce copious amounts of histamine that may trigger allergies and asthma (DeVries et al. 2018).

Bed bugs are obligate hematophagous insects, requiring a blood-meal for growth, development, and reproduction (Usinger 1966). Thus, host location is pivotal to their survival. Surprisingly, however, the cues that bed bugs use to locate their hosts have not been thoroughly investigated. As in other hematophagous arthropods, major host-derived cues include heat, odors, and CO_2. Bed bugs have been shown to activate, orient, and feed in response to heat, but these responses appear to be limited to short distances of <3 cm in laboratory assays with an artificial heat source (DeVries et al. 2016). Carbon dioxide has also been considered a bed bug attractant (Anderson et al. 2009, Wang et al. 2009, Singh et al. 2012, Aak et al. 2014), but its effect on behavior remains equivocal because attraction has been inferred from trap catches and questing bioassays, not olfactometer-based assays. Host odors have also been considered attractive to bed bugs (Hentley et al. 2017), but their relative importance has varied greatly across studies (Rivnay 1932, Aboul-Nasr and Erakey 1968, Harraca et al. 2012). Studies by Rivnay (1932) and Aboul-Nasr and Erakey (1968) found some bed bug attraction to host odors, but these studies are confounded because they did not separate visual, olfactory, and thermal cues. Based on single sensillum electrophysiological recordings and behavioral assays, Harraca et al. (2012) concluded that human odors attract bed bugs, but they are unlikely to have substantial effects independent of other cues (e.g., heat and/or CO_2). Electrophysiological assays have also shown that bed bugs can detect a greatly reduced repertoire of human odors compared with other hematophagous arthropods (Harraca et al. 2010, Liu and Liu 2015). Indeed, the bed bug genome encodes a substantially reduced assembly of chemosensory

proteins compared with generalist and phytophagous insects, with 30 ionotropic receptor genes and 48 genes encoding 49 olfactory receptors (Benoit et al. 2016).

A common constraint with most studies on bed bug olfaction is the lack of a simple, robust bioassay for investigating attraction (Weeks et al. 2011a). Instead, many studies have used endpoint metrics such as trap catch or still-air bioassays (Weeks et al. 2011b). The latter bioassay design has proven useful, but it does not directly measure attraction; instead, time spent in a specific area of the assay arena is quantified, rather than behavioral choices in a kinetic assay. In this study, we describe and validate a two-choice Y-tube olfactometer, and document bed bug attraction to human odors alone using multiple human participants, several bed bug populations, and various life stages of bed bugs.

Materials and Methods

Bed Bugs

Four populations of *C. lectularius* were used in this study: Harold Harlan (HH), Winston Salem (WS), Jersey City (JC), and Liberty (LI). The HH population, a known insecticide-susceptible strain, was originally collected in 1973 from Ft. Dix, NJ, and has been maintained in our laboratory since 2008. The other populations were more recently collected from residences as follows: WS from Winston Salem, NC in 2008; JC from Jersey City, NJ in 2008; and LI from Liberty, NC in 2017. The populations collected from residences are highly resistant to pyrethroid insecticides and exhibit varying degrees of resistance to other insecticides.

Bed bugs were reared in the laboratory, as described by DeVries et al. (2017). Briefly, colonies were maintained in 168 cm^3 plastic containers on paper substrates as shelters at 25°C, ~50% relative humidity and a photoperiod of 12:12 (L:D) h cycle. Bed bugs were fed defibrinated rabbit blood using an artificial feeding system that included a heated water bath (B. Braun Biotech Inc., Allentown, PA) to circulate 37°C water through a custom-made water-jacketed glass feeder, with bed bugs feeding through an artificial membrane (plant budding tape, A.M. Leonared, Piqua, OH).

Adult males were used in all bioassays. In addition, adult females (mated and unmated) and fifth instar nymphs were also used to validate the assay. All bed bugs were removed from colony jars immediately after feeding and starved for 7–10 d prior to testing, to ensure that bugs were in a host-seeking state. Mated females were confirmed by production of viable eggs following feeding and separation. Unmated females were separated as fed fifth instars, allowed to molt in isolation, then kept in isolation to ensure that no mating occurred. Unmated females were fed once after molting, starved for 7–10 d, and then tested. Each bed bug was used for only one bioassay and all bioassays were conducted during the scotophase under a single fluorescent light in a red photographic filter, held 1 m above the olfactometer.

Skin Swab Collection

The North Carolina State University Institutional Review Board approved this study (#14173). Before participation, adult participants (>21 yr old) provided informed consent. Participants were asked to collect personal skin swabs following a standard protocol (to reduce controllable variation). Prior to collecting skin swabs, participants were instructed to: 1) not eat 'spicy' food at least 24 h before collecting a skin swab; 2) take a morning shower; 3) not use a deodorant or cosmetics/lotions on the sampled surfaces; 4) not exercise or perform any strenuous physical activity; and 5) take the skin swabs 4–8 h after showering. Participants were then provided with #1 Whatman filter papers (90 mm diameter, Whatman plc, Madistone, United Kingdom) and glass vials (20 ml) and asked to collect skin swabs as follows: 1) rinse your hands with water (no soap) and dry before use; 2) use a single filter paper and swab the left arm from hand to armpit for 12 s using both sides of the filter paper; 3) rub the left leg from the lower thigh to ankle for 12 s using both sides of the filter paper; 4) rub the left armpit for 6 s using both sides of the filter paper; 5) place the filter paper into a glass vial and label the vial; and 6) repeat with a new filter paper swabbing the right side of the body. Skin swab samples were stored in a -30°C freezer for up to 1 mo (>95% used within 2 wk). A total of five participants submitted skin swabs for testing (three males and two females). Participant A's (male) skin swabs were used for all orientation experiments and experiments comparing results of different bed bug life stages. Participant B's (male) skin swabs were used for experiments with different bed bug populations. All other participants' skin swabs were used to further validate the olfactometer.

Olfactometer Bioassays

A glass Y-tube olfactometer was custom-made and used for all behavioral assays (Fig. 1). Air was pushed through the olfactometer at a rate of 300 ml/min, which resulted in a linear velocity of 4.5 cm/s at the distal end of the olfactometer as calculated by converting volumetric flow rate (measured at the downwind end of the olfactometer) to linear velocity. Breathing quality air from a compressed air tank was first passed through a charcoal filter, then a humidifier before entering the olfactometer. The olfactometer was positioned at five orientations using a ring stand—inverted vertical (bed bug introduced on top), inverted 45° (top introduction), horizontal, 45° (bottom introduction), and vertical (bottom introduction)—to optimize bed bug response rate. Response rate was defined as the proportion of bed bugs that made a choice, that is, moved 1.5 cm into either arm of the olfactometer within 5 min of initiating the assay. Because bed bugs do not walk well on glass surfaces, especially in a vertical orientation, a screen walkway (Nitex®, 300 µm, BioQuip Products Inc., Rancho Dominguez, CA) was cut to fit inside the olfactometer, serving as a 'catwalk' that also forced bed bugs to walk in the center of the olfactometer tube.

Prior to entering the olfactometer, bed bugs were placed into introduction tubes and acclimated to the air flow (300 ml/min) for 5–10 min. The introduction tubes were constructed of plastic (polyallomer) centrifuge tubes (i.d. = 13 mm, height = 50 mm; Beckman Instruments Inc., Palo Alto, CA), with the bottom removed and replaced with a screen, so that air could flow through the tube but the bugs could not escape. After the acclimation period, 1/16th of the filter paper used to obtain a skin swab (4 cm^2) was placed into the distal end of one arm of the olfactometer and an identically sized clean piece of filter paper (treated in an identical manner as the skin swab) was placed into the other arm (gloves were worn to ensure no cross-contamination). Next, the introduction tube containing an acclimated bed bug was attached to the downwind end of the olfactometer to begin the assay. Bed bugs were allowed 5 min to freely move in the olfactometer and make a choice (odor vs. control), which was determined when a bed bug moved 1.5 cm into one arm of the olfactometer (Fig. 1). Choice of either the odor or control arm was used to determine preference. Bed bugs that failed to reach this point within 5 min were considered non-responders. The positions of skin swabs were randomized every two replicates to the right or left arm of the olfactometer. The glass olfactometers were cleaned with acetone and the mesh ramp was replaced after every new skin swab was introduced (every 1–2 bioassays).

In total, four olfactometer experiments were conducted to determine the effects of 1) olfactometer orientation (HH bed bugs, participant A's skin swabs), 2) bed bug life stage (HH bed bugs, participant A's skin swabs), 3) odor source (HH bed bugs, all participants' skin swabs), and 4) bed bug population (all bed bug populations, participant B's skin swabs). Also, bioassays with control filter papers on both sides of the olfactometer were conducted to assess (a) baseline response rate without any odors, and (b) degree of symmetry or side-bias of the olfactometers.

Data Analysis

Chi-square analysis was used to compare response rates among experimental treatments (e.g., orientation, life stage, participants, bed bug populations), with differences among individual treatments determined by individual chi-square tests with Bonferroni corrections applied. Chi-square analysis was also used to determine bed bug preference for human odor, with the null hypothesis of no directional preference within the olfactometer. All analyses were performed in SAS 9.4 (SAS Institute, Cary, NC).

Results

Olfactometer Orientation

Olfactometer orientation significantly affected the percentage of bed bugs (HH population) that responded (χ^2 = 38.81, df = 5, P < 0.0001), with more bed bugs responding when the olfactometer was oriented vertically (73% responders), so bed bugs walked upward, than any other orientation (Fig. 2A). When bed bugs were required to orient downward (inverted vertical orientation with bed bugs introduced at the top), none of the bed bugs responded. At all other olfactometer orientations, however, there was a significant preference for human odor (Participant A) (P < 0.0254; Fig. 2B), regardless of response rate. Bioassays with control filter papers at both sides of the olfactometer resulted in low response rates (25%, Fig. 2A) and random orientation to the left and right arms of the olfactometer, indicating no apparent side bias (Fig. 2B). These results guided us to proceed with the vertically oriented olfactometers with bottom introductions for all subsequent bioassays.

Different Bed Bug Life Stages

Response rates were significantly different among the bed bug (HH population) life stages tested (χ^2 = 30.35, df = 3, P < 0.0001), all of which were starved for 7–10 d to ensure that they were in a host-seeking state. None of the unmated females responded to skin swabs (Fig. 3). Males, mated females, and nymphs all had significantly higher response rates (>46%), and all three groups showed significant preference for human odor (Participant A) over the control arm of the olfactometer (P ≤ 0.0002 for all assays, excluding unmated females), ranging from 95 to 100% (Fig. 3).

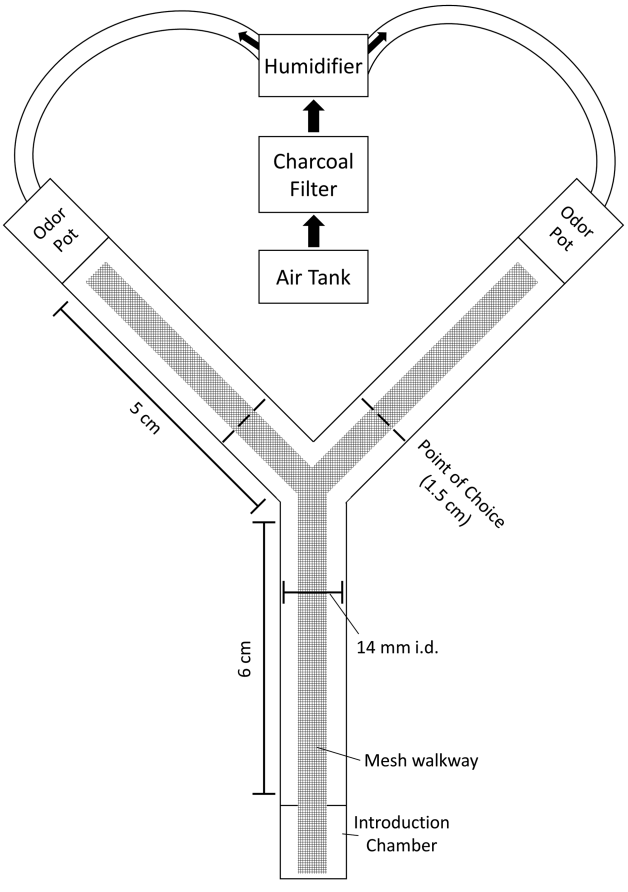

Fig. 1. Schematic of the Y-tube olfactometer used in behavioral assays with human skin swabs. The mesh walkway was positioned in the center of the olfactometer to enable bed bugs to walk toward the odor source at all orientations.

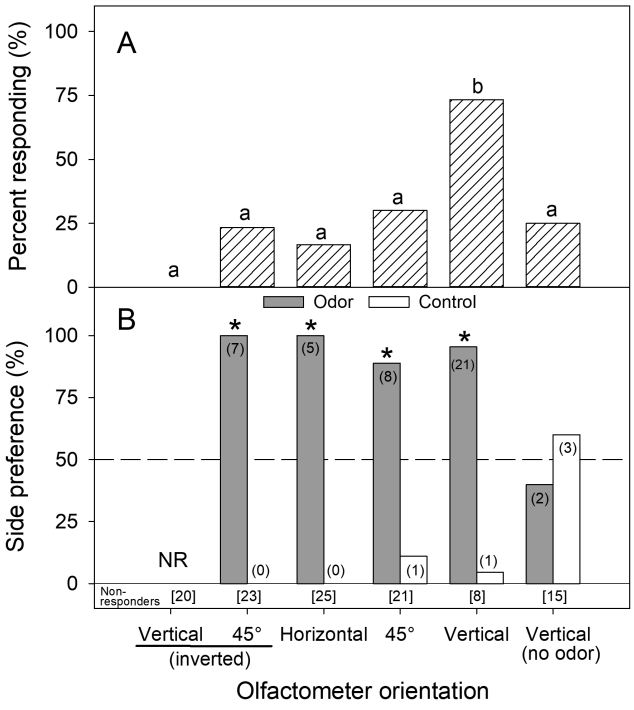

Fig. 2. Effects of olfactometer orientation on bed bug (A) response rate, and (B) preference for human odor (skin swab) versus blank control filter paper. The vertically oriented olfactometer requires bed bugs to walk upwards, whereas in the inverted vertical olfactometer bed bugs must walk downwards to reach the odor source. All bed bugs were fed and then starved for 7–10 d prior to the assays. Numbers in parentheses represent the number of bed bugs that made a choice (odor or control), and numbers in brackets represent the number of bed bugs that did not respond (i.e., did not reach 1.5 cm beyond the bifurcation point). Bars with different lower-case letters are significantly different based on chi-square analysis with a Bonferroni correction. Asterisk above a bar indicates significant preference for that choice (odor or control, chi-square analysis). 'NR' represents no responders of 20 bed bugs tested.

Different Human Participant Odors

Bed bugs (HH population) showed a significant preference for all participant odors over the blank control filter papers ($P < 0.0067$ for all assays), with preference for human odor ranging from 91 to 100% (Fig. 4). Response rates were not significantly different among participants ($\chi^2 = 3.15$, df = 4, $P = 0.5334$), ranging from 65 (Participant A) to 82% (Participant B).

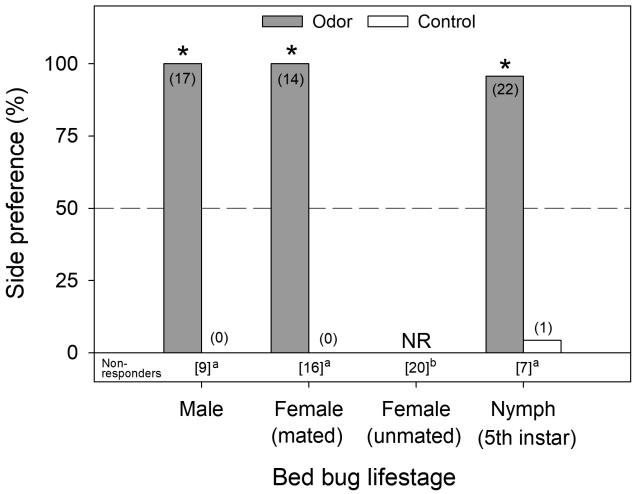

Fig. 3. Responses of different bed bug life stages to human skin swabs. All bed bugs were fed and then starved for 7–10 d prior to the assays. Numbers in parentheses represent the number of bed bugs that made a choice (odor or control). Numbers in brackets represent the number of bed bugs that did not respond (i.e., did not reach 1.5 cm beyond the bifurcation point). Asterisk above a bar indicates significant preference for that stimulus (odor or control, chi-square analysis). Different lower-case letters adjacent to the numbers in brackets (bugs that did not respond) indicate significant difference in response rate among bed bug populations based on chi-square analysis with a Bonferroni correction. 'NR' represents no responders of 20 bed bugs tested.

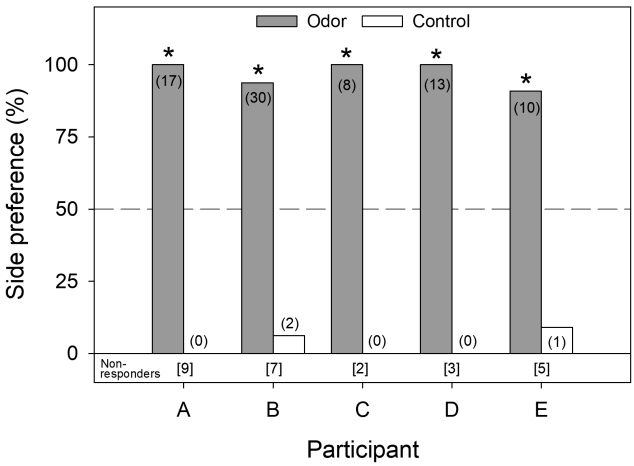

Fig. 4. Responses of bed bugs to different human (participant) skin swabs. All bed bugs were fed and then starved for 7–10 d prior to the assays. Numbers in parentheses represent the number of bed bugs that made a choice (odor or control). Numbers in brackets represent the number of bed bugs that did not respond (i.e., did not reach 1.5 cm beyond the bifurcation point). Asterisk above a bar indicates significant preference for that choice (odor or control, chi-square analysis). No differences were detected among response rates for bugs tested on different participant's skin swabs (chi-square analysis with a Bonferroni correction).

Different Bed Bug Populations

All four bed bug populations tested showed a significant preference for human odor (Participant B) over the control filter paper ($P < 0.0001$ for all assays), ranging from 93 to 100% (Fig. 5). Response rates were significantly different among bed bug populations ($\chi^2 = 16.18$, df = 3, $P = 0.0010$), ranging from 40 (LB) to 82% (HH) (Fig. 5).

Discussion

This paper shows that bed bugs have a clear preference to orient towards human odors, independent of any other stimuli or host cues, while simultaneously providing documentation of a highly sensitive, easy to use, high throughput Y-tube olfactometer for bed bugs. The high response rate and clear preference of bed bugs for skin swabs suggest that human odors play an important role in host location, and can function on their own to elicit host attraction over distance. Bed bug attraction to human odors is not entirely surprising, given that bed bugs have olfactory and ionotropic receptors capable of detecting a wide range of human odors (Harraca et al. 2010, Liu and Liu 2015, Benoit et al. 2016). Despite some differences in the response rate among populations, bed bugs showed a clear preference for human odor over controls, which was consistent across four populations of bed bugs and five human participants. It should be noted that although preference for human odor was present in all bed bug populations, response rates varied from 40 to 82%. We speculate these may be due to time since collection and acclimation to the laboratory environment, although further testing is needed.

Prior to this report, human odors had been described to only exert a weak influence on bed bug behavior when tested alone (Harraca et al. 2012). It is important to note, however, that Harraca et al. (2010) used still air olfactometers (Weeks et al. 2011b), suggesting that the still-air bioassay design might not be sensitive enough to detect responses to some olfactory cues. It should also be noted that

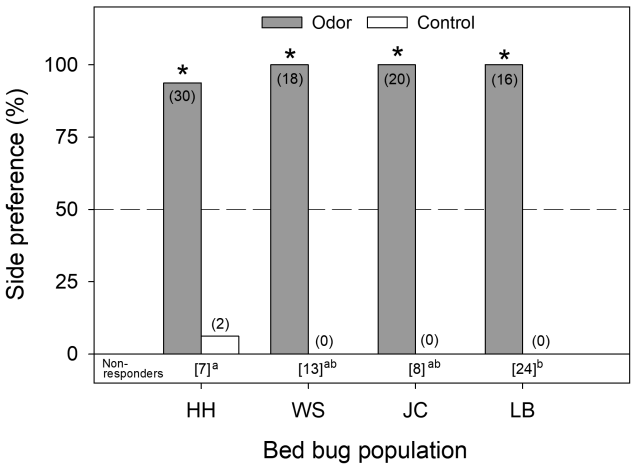

Fig. 5. Responses of bed bug from different populations to human skin swabs. All bed bugs were fed and then starved for 7–10 d prior to the assays. Numbers in parentheses represent the number of bed bugs that made a choice (odor or control). Numbers in brackets represent the number of bed bugs that did not respond (i.e., did not reach 1.5 cm beyond the bifurcation point). Asterisk above a bar indicates significant preference for that choice (odor or control, chi-square analysis). Different lower-case letters adjacent to the numbers in brackets (bugs that did not respond) indicate significant difference in response rate among bed bug populations based on chi-square analysis with a Bonferroni correction.

differences among assays could be due to different odor collection methods used in these studies. Regardless, our results show that bed bugs clearly orient towards human odors and warrant a re-evaluation of bed bug responses to individual odorants and to odor blends using the new olfactometer design.

Interestingly, while all bed bug life stages oriented towards skin swabs, unmated females that were fed once, starved for 7–10 d and then tested, did not respond to human odors. Previous work has documented that unmated females have reduced metabolic rates (DeVries et al. 2013, 2015) and appear to be conserving energy. This is also apparent visually, as unmated females retain their bloodmeal for longer after feeding (Z.C.D., personal observation). Because females do not produce eggs until mated (Usinger 1966), it is possible that 7–10 d of starvation is not sufficient to stimulate host-seeking in unmated females.

The effectiveness of our olfactometer was rooted in two key factors. First, the vertical orientation proved to be critical, as significantly more bed bugs responded (made a choice) in a vertical than in a horizontal olfactometer. Although bed bugs preferred the odor side of the olfactometer regardless of olfactometer orientation, placing the olfactometer vertically, with bed bugs walking upwards, substantially increased the response rate and facilitated much higher throughput. Vertical olfactometers and wind tunnels have been used successfully with other insects, often taking advantage of their positive phototaxis and negative geotaxis to facilitate 'activation' and orientation toward the bifurcation point (Feinsod and Spielman 1979, Visser and Piron 1998, Stelinski and Tiwari 2013). Such olfactometers had not been considered with bed bugs, likely because bed bugs are incapable of climbing smooth vertical surfaces (Hottel et al. 2015). The second feature of the olfactometer that contributed to its effectiveness is the screen walkway spanning the entire length of the olfactometer. This 'catwalk' allowed bed bugs to freely move upwards, and retained them in the center of the olfactometer tube. The latter may be particularly important in future two-choice assays using two odor stimuli, a design that requires symmetry and simultaneous exposure to both odors.

In conclusion, these results provide strong evidence that bed bugs respond to and show preference for human odors, suggesting that human odors play a role in host location. The Y-tube olfactometer design is simple yet robust and should be leveraged to improve our understanding of host attraction in bed bugs and identify specific compounds and blends that elicit behavioral responses. Future experiments should use this olfactometer to evaluate bed bug responses to various semiochemicals and leverage this information to improve bed bug management through detection, monitoring, mass trapping, and baiting.

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