



# Differential responses to aldehyde pheromone blends in two bed bug species (Heteroptera: Cimicidae)

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## Abstract

The behavioral responses of two bed bug species, *Cimex lectularius* L. and *C. hemipterus* (F.), to conspecific or heterospecific nymphal aldehyde blends were examined using a two-choice olfactometer. Volatile cues from exuviae or a synthetic blend containing (*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal were tested. In both species, the adults settled preferentially on the olfactometer treatment side when conspecific volatile aldehyde cues were provided. When tested with heterospecific volatile aldehyde cues, only adult *C. lectularius* preferentially responded to *C. hemipterus* volatile cues. Adult *C. hemipterus* was indifferent to the aldehyde blend of *C. lectularius*. Potential implications of the finding on bed bug biology and practical pest management are discussed.

**Keywords** *Cimex lectularius* · *Cimex hemipterus* · Olfactometer · Hexenal · Octenal

## Introduction

Bed bugs (Heteroptera: Cimicidae) are a group of hematophagous external parasites (Usinger 1966). The common bed bug, *Cimex lectularius* L, and the tropical bed bug *Cimex hemipterus* (F.), are common urban pests. Recent phylogenetic analyses indicated that *C. lectularius* and *C. hemipterus* diverged approximately 47 MYA, descending from bat and bird-associated lineages, respectively (Roth et al. 2019). Their distributions are mostly allopatric; *C. lectularius* mainly in cooler environments, and *C. hemipterus* in tropical and subtropical regions (Usinger 1966). However, these bed bug species can occasionally occur in sympatry. For example, both species are found in Australia (Doggett and Cains 2018), Asia (Lee et al. 2018), Africa (Fourie and Crafford 2018), and more recently in Florida (Campbell et al. 2016) and Hawaii (Lewis et al. 2020), USA.

Due to bed bugs' status as a major urban pest, much research has been done searching for an effective lure for use in a monitor or trap (Weeks et al. 2011a). The investigations

on the chemical ecology of bed bugs have found that *C. lectularius* respond to several conspecific chemical cues, such as those associated with cuticles (e.g., cuticular hydrocarbons), exuviae, and feces (Siljander et al. 2008; Domingue et al. 2010; Weeks et al. 2011b; Choe et al. 2016). In particular, like other Heteropterans, the bed bugs are well known to produce several short-chain aliphatic aldehydes, which are among the major constituents of the typical odors associated with bed bugs (Staddon 1979). A group of related aldehydes [(*E*)-2-hexenal, 4-oxo-(*E*)-2-hexenal, (*E*)-2-octenal, and 4-oxo-(*E*)-2-octenal] are produced by both *C. lectularius* and *C. hemipterus* (Liedtke et al. 2011; Dery et al. 2020). Adult bed bugs produce two of these [(*E*)-2-hexenal and (*E*)-2-octenal] in their metathoracic scent glands, while nymphs have all four in their dorsal abdominal glands (Künckel 1886; Usinger 1966; Staddon 1979; Feldlaufer et al. 2010). In *C. lectularius*, (*E*)-2-hexenal and (*E*)-2-octenal are released in relatively large quantities during the response to a predator (Usinger 1966; Levinson et al. 1974) or unwanted mating attempts (Harraca et al. 2010b; Kilpinen et al. 2012), causing an alarm response. When released at low quantities, the same aldehydes appeared to function as constituents of an aggregation pheromone (Siljander et al. 2008; Gries et al. 2015; Choe et al. 2016; Ulrich et al. 2016).

Studies on these aldehydes found that while all four aldehydes are produced by *C. lectularius* and *C. hemipterus*, there are significant interspecific differences in the quantities

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(i.e., the ratio among compounds) produced (Liedtke et al. 2011; Dery et al. 2020). Qualitative similarity but with a distinct quantitative difference of these pheromonal aldehydes brings about an interesting biological question. Despite not producing viable hybrids (Usinger 1966; Newberry 1988), “interspecific” mating (in the laboratory as well as in the field) between *C. lectularius* and *C. hemipterus* appears to be common in some of the areas where these species are sympatric (Walpole and Newberry 1988; Newberry 1989). Since the mating would occur where the bed bugs congregate (i.e., harborages) (Gershman et al. 2019), *C. lectularius* and *C. hemipterus* might respond to their aldehyde pheromone blends interspecifically as well as intraspecifically. While bed bug aldehydes and their behavioral functions have been previously studied within each species of *C. lectularius* and *C. hemipterus*, their behavioral responses towards heterospecific aldehyde blends have not been investigated. This information may also have important implications for developing novel bed bug chemical lures for commercial traps or monitors. In the current study, a two-choice olfactometer study was conducted to investigate each bed bug species’ behavioral responses to both the conspecific and heterospecific aldehydes. Two different sources of aldehydes were tested, shed exuviae and a synthetic aldehyde blend.

## Materials and methods

### Insects

*Cimex lectularius* colonies were started from “Earl” strain individuals collected in Modesto, CA, USA in 2007 and purchased from Sierra Research Laboratories (Modesto, CA, USA). *Cimex hemipterus* were collected from George Town, Penang, Malaysia in 2015 and were reared in the quarantine facility at the University of California, Riverside (CDC PHS Permit No. 03282018–11057 and 20,90426-2698A).

Bed bugs were kept in screened vials with a height of 9 cm and a diameter of 4.5 cm containing a corrugated filter paper cylinder. Bed bugs are fed through the screen with a grafting tape membrane (Aglis & Co., Ltd., Yame City, Fukuoka, Japan) feeder containing defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA) approximately every fourteen days. Colonies of *C. lectularius* were maintained at 24–26 °C and 15–30% RH, with a photoperiod of 12:12 (L:D) hours. Colonies of *C. hemipterus* were maintained at 22–23 °C and 40–60% RH, with a photoperiod of 12:12 (L:D) hours.

### Behavioral assay

The behavioral responses of adult bed bugs to conspecific or heterospecific aldehydes were examined using two-choice

olfactometer assays. The experimental design was modified from Choe et al. (2016), using acetone as a solvent to reconstitute the synthetic aldehyde blend. Mixed-sex adult bed bugs were randomly collected from a colony vial and placed individually in the wells (16 mm × 19 mm) of 24-well cell culture dishes (Corning Inc., Corning, NY, USA) lined with filter paper. These bed bugs were kept in the well plate for at least 24 h before use at the experimental temperature and humidity. A 15-cm section of flexible polyvinyl chloride tubing (ID: 9.5 mm; OD: 12.7 mm; Superflex Ltd., Elizabeth, New Jersey, USA) was used as the body of the olfactometer. Approximately 50 exuviae (third to fifth instar) were collected from colony vials randomly and placed into a 2 ml glass vial (Agilent Technologies, Santa Clara, CA, USA). Ten vials of exuviae were prepared for each species, and these served as the source of volatiles. One vial with exuviae was inserted into the olfactometer tube with a small mesh screen to separate the vial contents from the inside of the tube. An empty screened vial was inserted on the opposite end of the tube, serving as the control. The position of the vials was randomized. One adult bed bug was introduced into the olfactometer via a slit in the center of the tube. The trials were initiated three hours before the start of scotophase, and the position (control or treatment screen) of the bed bug was observed after 18 h. All olfactometer assays were conducted at approximately 22–23 °C and 20% RH. The mesh screens and olfactometer were used only once for each replicate. For each of the four combinations of bed bug species and exuviae source, 50 replicates were conducted.

To determine if the behavioral response of bed bugs is primarily due to the volatile aldehydes from the exuviae, the olfactometer experiment was repeated using blends of synthetic aldehydes in place of the exuviae. (*E*)-2-hexenal and (*E*)-2-octenal were purchased from Sigma-Aldrich (St. Louis, MO, USA), while 4-oxo-(*E*)-2-hexenal and 4-oxo-(*E*)-2-octenal were synthesized using the method described by Moreira and Millar (2005). A blend of the four aldehydes was prepared in acetone, and 20 µl of this acetone preparation was applied to a small piece of cotton ( $32.3 \pm 7.8$  mg; mean  $\pm$  SD;  $n = 70$ ) inside each treatment vial. The synthetic aldehyde blend of each species mimicked the ratio of the four aldehydes quantified in freshly shed fifth instar exuviae by Dery et al. (2020). The amount of each aldehyde applied for *C. lectularius* consisted of (*E*)-2-hexenal: 66.3 µg, 4-oxo-(*E*)-2-hexenal: 73.6 µg, (*E*)-2-octenal: 181.2 µg, and 4-oxo-(*E*)-2-octenal: 15.55 µg. The *C. hemipterus* synthetic blend was comprised of (*E*)-2-hexenal: 73 µg, 4-oxo-(*E*)-2-hexenal: 131.95 µg, (*E*)-2-octenal: 146.1 µg, and 4-oxo-(*E*)-2-octenal: 2.45 µg. The amount of aldehydes applied per vial was equivalent to that of 50 freshly shed fifth instar exuviae (*C. lectularius*: 336.7 µg, *C. hemipterus*: 353.5 µg). As the amount of aldehydes in shed exuviae decreases over time (Choe et al. 2016), the total amount of synthetic aldehyde

mimicking freshly shed exuviae represents an overall larger amount of aldehydes than were present in the exuviae olfactometer trials. The control vial contained a piece of cotton, to which 20  $\mu$ l of acetone was applied. The vials remained uncapped for 10 min to allow the solvent to evaporate. The remainder of the experimental procedures was otherwise identical with the olfactometer trials with exuviae. Between 50 and 72 replicates were conducted for each combination of bed bugs and aldehyde blend. A total of 464 olfactometer replicates were conducted using either exuviae or synthetic aldehydes. If a bed bug was found nonresponsive (i.e., remaining in the center of the olfactometer or dead) at 18 h after introduction ( $n = 18$ ), the trial was repeated with a different insect and new olfactometer.

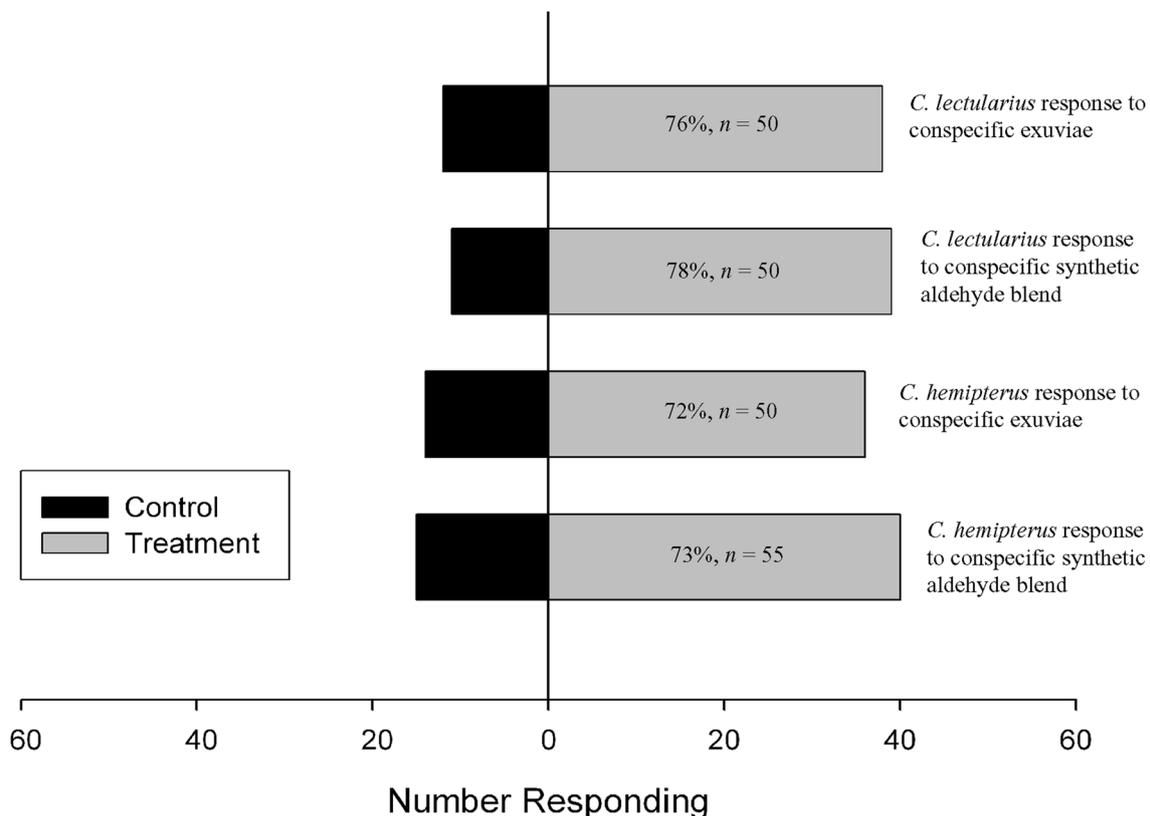
### Statistical analysis

To determine if bed bugs settled on one side of the olfactometer (e.g., the side with a treatment vial) significantly more often than expected from random choice (50%: 50%), the results of the olfactometer experiments were analyzed with chi-square goodness of fit tests using R version 4.0.3 (R Core Team 2020).

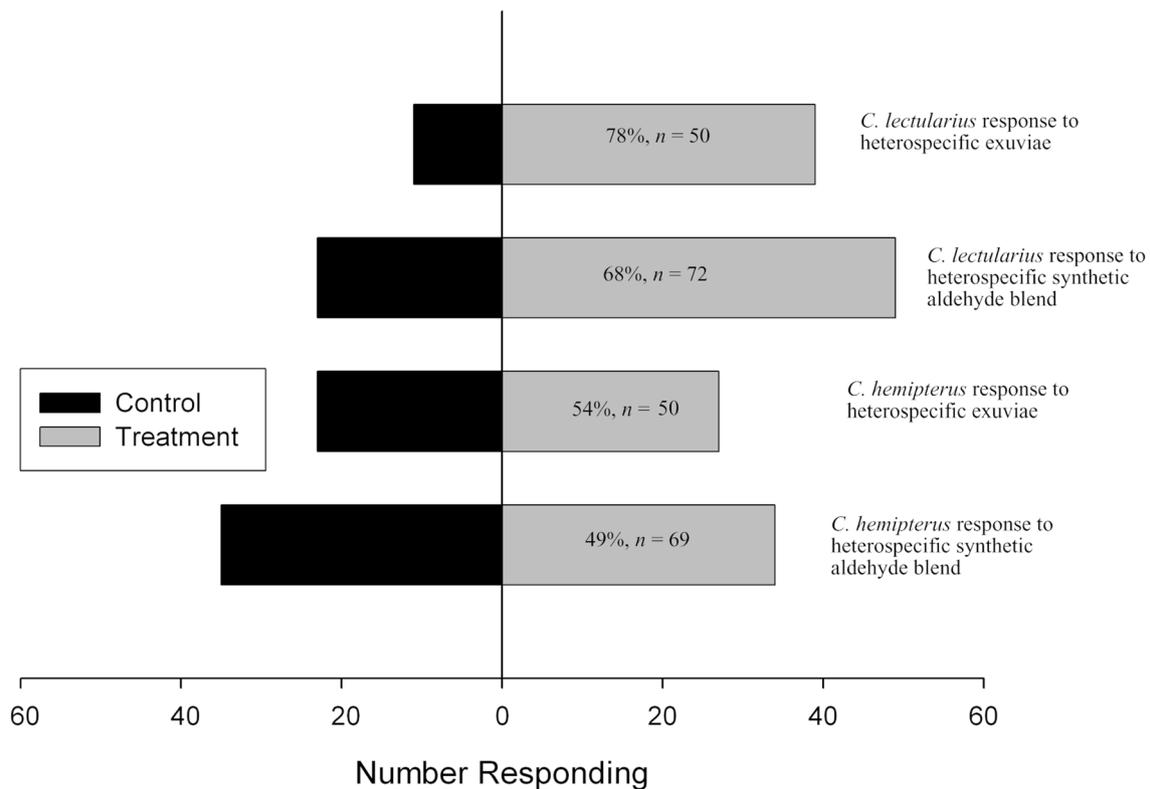
## Results

In both bed bugs species, adult insects settled preferentially on the treatment side of the olfactometer when conspecific volatile cues were provided. In *C. lectularius*, adults responded preferentially to both the volatiles of their own exuviae ( $\chi^2 = 13.52$ ,  $P < 0.001$ ,  $n = 50$ ) or the synthetic aldehyde blend mimicking the conspecific ratio ( $\chi^2 = 15.68$ ,  $P < 0.001$ ,  $n = 50$ ) (Fig. 1). Similarly, adult *C. hemipterus* responded preferentially to the volatiles of their own exuviae ( $\chi^2 = 9.68$ ,  $P < 0.005$ ,  $n = 50$ ) as well as the synthetic aldehyde blend mimicking the conspecific ratio ( $\chi^2 = 11.36$ ,  $P < 0.001$ ,  $n = 55$ ) (Fig. 1).

In stark contrast, two species of bed bugs responded differently when tested with heterospecific volatile cues. Adult *C. lectularius* still responded preferentially to the volatiles from *C. hemipterus* exuviae ( $\chi^2 = 15.68$ ,  $P < 0.001$ ,  $n = 50$ ) and synthetic aldehyde blend mimicking *C. hemipterus* ratio ( $\chi^2 = 9.39$ ,  $P < 0.005$ ,  $n = 72$ ) (Fig. 2). However, adult *C. hemipterus* preferred neither the volatiles from *C. lectularius* exuviae ( $\chi^2 = 0.32$ ,  $P > 0.05$ ,  $n = 50$ ) nor the synthetic aldehyde blend mimicking the heterospecific ratio ( $\chi^2 = 0.01$ ,  $P > 0.05$ ,  $n = 69$ ) (Fig. 2).



**Fig. 1** Behavioral responses of adult *Cimex lectularius* and *Cimex hemipterus* to conspecific aldehyde sources (exuviae or synthetic blend) in olfactometers. Numbers in bars indicate the percent of bed bugs responding to the volatile cue and sample size



**Fig. 2** Behavioral responses of adult *Cimex lectularius* and *Cimex hemipterus* to heterospecific aldehyde sources (exuviae or synthetic aldehyde blend) in olfactometers. Numbers in bars indicate the percent of bed bugs responding to the volatile cue and sample size

## Discussion

Data from the two-choice olfactometer trials indicated that adult bed bugs of both species preferentially responded not only to volatiles from conspecific late instar exuviae but also a reconstructed synthetic aldehyde blend. These findings are consistent with the observations made by Choe et al. (2016), further supporting the notion that the volatile aldehydes in the exuviae are at least in part responsible for the behavioral response of adult bed bugs towards their exuviae (i.e., attraction and/or arrestment). In contrast, behavioral responses to heterospecific aldehyde blends were completely different between *C. lectularius* and *C. hemipterus*. While adult *C. lectularius* preferentially responded to *C. hemipterus* volatile cues, *C. hemipterus* was indifferent to the aldehyde blend of *C. lectularius* (i.e., no difference when compared with a blank control). The mechanism underlying the observed differential response to the pheromone blends is unknown. Two types of sensilla on adult bed bug antennae are known to detect these four aldehydes (Harraca et al. 2010a, b). While Liedtke et al. (2011) did not find *C. lectularius* and *C. hemipterus* to differ in the number of antennal sensilla, Singh et al. (1996) reported *C. hemipterus* to have more olfactory and mechanoreceptive sensilla in its antennae than

those in *C. lectularius*. Further exploration of how both *C. lectularius* and *C. hemipterus* detect and respond to these aldehydes may give more insight into the observed behavior.

Regardless of the mechanism behind this differential response, the current findings that *C. lectularius* and *C. hemipterus* respond differently to the heterospecific aldehyde blend might provide valuable insights for previously observed interspecific interactions of two *Cimex* species in their habitats. Walpole and Newberry (1988) and Newberry (1989) reported the presence of “interspecific” mating (in the laboratory as well as in the field) between *C. lectularius* and *C. hemipterus*. Interspecific mating between female *C. lectularius* and male *C. hemipterus* has been the focus of several previous investigations. This particular pairing has some serious adverse effects on the female *C. lectularius* (e.g., short lifespan, sterility) (Newberry 1989). Female *C. lectularius* also shows a massive immune response to the heterospecific sperm (Walpole 1988). However, female *C. hemipterus* do not seem to suffer that much adverse effect when mated with male *C. lectularius* (also, it lacks a massive immune response to the heterospecific sperm) (Walpole 1988). Omori (1939) and Newberry (1989) hypothesized that their interspecific mating and its adverse effect on the female *C. lectularius* might be playing an important role in

deciding the abundance and distribution of *C. lectularius* in areas where both species can otherwise coexist.

In many cases, this hypothesis supports the idea that the resident *C. hemipterus* population prevents *C. lectularius* from establishing itself. Indeed, Newberry et al. (1987) reported that some houses in South Africa were initially infested with *C. lectularius* and *C. hemipterus*, but a few months later, only with *C. hemipterus*. Similarly, Prisniy (2020) reported that *C. lectularius* were present in dormitories of Belgorod National Research University in Belgorod, Russia in 2010, but were apparently displaced by *C. hemipterus* in 2020. In contrast, Gbakima et al. (2002) reported that both *C. lectularius* and *C. hemipterus* were found in similar abundances (56.1 and 43.9%, respectively) in internally displaced person camps in Freetown, Sierra Leone in 1999. However, as the research took place in locations where new residents arrived daily from many geographic locations (Gbakima et al. 2002), there is a high likelihood of new introductions of both species to these camps frequently occurring. This aspect, along with the absence of a follow-up study, might explain the apparent discrepancy between Gbakima et al. (2002) and other two reports mentioned earlier [Newberry et al. (1987) and Prisniy (2020)], which found that populations shifted to *C. hemipterus* alone over time.

The aforementioned hypothetical mechanism explaining the observed population shift to *C. hemipterus* will only make sense if *C. lectularius* is always the numerical minority when these species are found in the same habitat. In addition to a possible scenario in which a few *C. lectularius* are introduced in *C. hemipterus*-dominated habitat, the behavioral observations in the current study might provide some useful insights. In the present study, *C. lectularius* was attracted to their own cues as well as heterospecific cues. However, *C. hemipterus* was only attracted to their own cues but indifferent to heterospecific cues. In a hypothetical space where small aggregations of these species coexist, these differential behavioral responses would generate the following outcomes. When *C. lectularius* return to aggregations after feeding, they would be attracted to either conspecific aggregations or heterospecific aggregations. However, *C. hemipterus* would only join conspecific aggregations when they come back from feeding bouts. This would almost always put *C. lectularius* numerically “minor” members in the “mixture” aggregations even when relatively large populations of *C. lectularius* are introduced in the common habitat.

Some caution is warranted when interpreting these results. Due to the use of only adults from one strain for each species, the current study could not completely rule out a strain-specific or stage-specific effect for the lack of response from *C. hemipterus* to the *C. lectularius* aldehyde blend. Also, we did not directly test the choice response of bed bugs between conspecific and heterospecific aldehyde

blends in one experiment. Instead, we observed the bed bugs’ response to the aldehydes against a control (blank or solvent only). Additional work is required to determine the exact differences in the aldehyde profiles responsible for the observed differential response. Dery et al. (2020) reported that only the ketoaldehydes differed significantly between *C. lectularius* and *C. hemipterus* based on the quantification study with fifth instar exuviae. As such, differences in the quantity or ratio of one or both ketoaldehydes seem a likely candidate for the observed lack of response by *C. hemipterus*. For example, a particularly striking difference is that the amount of 4-oxo-(*E*)-2-octenal in the exuviae is much greater in *C. lectularius* than in *C. hemipterus* for all instars (Dery et al. 2020).

Much work has been done investigating the potential use of various attractants in bed bug monitors and traps (Weeks et al. 2011a). Heat and CO<sub>2</sub> have already been successfully incorporated into traps for *C. lectularius* (Anderson et al. 2009; Wang et al. 2009; Weeks et al. 2011a). The potential use of bed bug aldehyde pheromones for bed bug detection and management has been of additional interest. For example, adult *C. lectularius* were attracted to a 1:1 blend of (*E*)-2-hexenal and (*E*)-2-octenal (Ulrich et al. 2016). Besides, Benoit et al. (2009) found that the addition of (*E*)-2-hexenal and (*E*)-2-octenal to desiccants increased their effectiveness against *C. lectularius*.

Behavioral differences have been reported between *C. lectularius* and *C. hemipterus*, and some of them have direct implications for bed bug management. For example, Kim et al. (2017) found that *C. hemipterus* adults are better climbers than *C. lectularius* and can escape from commonly used pitfall-type monitors. The differential responses of these two synanthropic bed bug species to heterospecific aldehydes might add another layer of complexity in incorporating these aldehydes into pest management tools that will be equally useful for both species. Our findings suggest that *Cimex hemipterus* may not respond to an active monitor that used the *C. lectularius* aldehyde blend to formulate an attractant despite being congeners. Instead, the aldehyde composition of *C. hemipterus* should be investigated for use as a general lure that can be incorporated into the design of active traps and monitors, potentially targeting both *C. lectularius* and *C. hemipterus*. With *C. hemipterus* increasingly being found outside their historical range, for example, in the USA (Campbell et al. 2016; Lewis et al. 2020), Italy (Masini et al. 2020), Russia (Gapon 2016; Prisniy 2020), Sweden (Naylor et al. 2018), and Japan (Komatsu et al. 2018), further research on the differences between these two species (e.g., behavioral difference) is required to ensure effective control of bed bugs in these areas.

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**Availability of data and material** All relevant data are included within the paper.

**Code availability** The code used for statistical analysis is available upon request.

## Declarations

**Conflicts of interest** The authors declare that they have no conflict of interest.

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** All authors approved the publication of this manuscript.

**Research involving Human Participants and/or Animals** No animals requiring ethics approval were involved in this study.

**Informed consent** Not applicable.

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