Journal of Economic Entomology, 114(1), 2021, 265–273
doi: 10.1093/jee/toaa304
Advance Access Publication Date: 9 January 2021
Research

Methods for Testing Repellents Against Bed Bugs (Hemiptera: Cimicidae)

Anne Krüger[1], Erik Schmolz, and Arlette Vander Pan

German Environment Agency, Boetticherstr. 2 Haus 23, 14195 Berlin, Germany and [1]Corresponding author, email: a.krueger@arthroscience.de

Subject Editor: Michael Rust

Received 22 September 2020; Editorial decision 1 December 2020

Abstract

Bed bug repellents should not only prevent humans from being bitten but impede an infestation of personal belongings. Only a few test proposals for evaluating the efficacy of repellents against bed bugs have been published so far. In the present study, two test systems were assessed for efficacy testing with five potential bed bug repellents (cinnamon oil, icaridin, *N*,*N*-diethyl-3-methylbenzamide (DEET), permethrin, and margosa extract). The first test setup was a harborage choice test system that consisted of a crystallizing dish with a treated and an untreated harborage. Sixty minutes and 24 h after treatment, DEET, icaridin, and cinnamon oil showed the highest repellency with a median proportion of at least 99% repelled bed bugs. The second test system was a barrier test. Bed bugs were attracted by CO_2 and heat to cross filter papers treated with the potential repellents. The repellency of substances was significantly lower in comparison to the harborage choice test, except for DEET. The latter showed the highest repellency (97%) against bed bugs 24 h after application compared to controls. Results show that bed bugs are less sensitive to repellents when searching for a bloodmeal than when searching for a shelter.

Key words: repellents, test system, simulated-use test, cinnamon oil, DEET

The bed bug *Cimex lectularius* (Linnaeus, Hemiptera: Cimicidae) is a pest that has spread again worldwide in the last three decades due to globalization and the increase of insecticide resistance (Ter Poorten and Prose 2005, Harlan 2006, Masetti and Bruschi 2007, Davies et al. 2012, Ashbrook et al. 2017, Dang et al. 2017, Cambronero-Heinrichs et al. 2020). Eradication of a bed bug infestation requires integrated pest management (IPM) that considers all available pest control techniques. Bed bug management may include the use of insecticides, desiccant dust, heat, freezing techniques, mechanical removal, and monitoring (Pereira et al. 2009, Kells and Goblirsch 2011, Koganemaru and Miller 2013, Romero et al. 2017, Lee et al. 2018, Ashbrook et al. 2019). Prevention should be an integral part of IPM, which involves education, creating bed bug unfriendly surroundings, informing staff and travelers, specific luggage storage, and in some cases, the use of repellent products (Singh et al. 2014, Romero et al. 2017). Especially long-lasting repellent products might have the potential to serve as an additional IPM method (Wang et al. 2013, Zhu et al. 2018).

Repellents are commonly used to protect humans from bites of mosquitoes and ticks. Active substances like *N*,*N*-Diethyl-3-methylbenzamide (DEET) or icaridin (picaridin; 1-(1-Methylpropoxycarbonyl)-2-(2-hydroxyethyl)piperidine) protect against these arthropods for several hours (Carroll et al. 2005, Syed and Leal 2008, Goodyer et al. 2010, Kulma et al. 2019). Natural repellents like cinnamon oil (*Cinnamomum* sp. (Laurales: Lauraceae)) or margosa extract (*Azadirachta indica* (A.Juss., Sapindales: Meliaceae)) are used as protection against arthropods like mosquitoes or ants (Caraballo 2000, Barnard and Xue 2004, Chang et al. 2006). Permethrin is used to protect against arthropods via sprays or impregnated clothing. Contact damages or repels the target organism or has sublethal effects on bed bug behavior, fecundity, and feeding (Jones et al. 2013, 2015; Tangena et al. 2018). Repellents used for spatial or material treatments can be used as a barrier treatment for personal protection or prevent bed bug dispersal and introduction to uninfested locations. Skin repellents could be used for bite protection. This is supported by recent research on the repellency of several synthetic and plant-based substances against bed bugs (Sõukand et al. 2010, Wang et al. 2013, Liu et al. 2014, Singh et al. 2014, Anderson et al. 2018, Zhu et al. 2018).

Currently, for personal protection against bed bug bites, nonrepellent products like sticky tapes or slippery traps are installed under or around bed legs (Lilly et al. 2009). Also, permethrin-impregnated fabrics like bed linen are used (Jones et al. 2013, 2015; Londono-Renteria et al. 2015). For spatial and surface treatment, some sprays containing several different plant-based ingredients—e.g., essential oils claiming to deter bed bugs—are on the market,

too. The use of natural products is related to the fact that consumers are increasingly aware of substances they consider to be less toxic and harmful to themselves (Caraballo 2000, Barnard and Xue 2004, Nerio et al. 2010, Singh et al. 2014).

According to the EU-Biocides Product Directive 528/2012, repellents are considered as biocidal products. For authorization, applicants must provide a dossier that contains, among other data, evidence that the product is efficient. Up to now, there are no test methods agreed on that are available for the evaluation of repellents against bed bugs (ECHA 2018).

The aim of our study was to develop and describe a test system for bed bug repellents in general. For this, we tested products but did not try to assess complete protection times or other product performance indicators in more detail. Two test systems are described to evaluate the repellency of active substances and products against bed bugs. The first test system is a small-scale harborage choice test offering two harborages and simulates the treatment of potential bed bug shelters (e.g., luggage) to prevent dispersal. The second test system mimics a situation where bed bugs have to cross a treated barrier to reach a simulated host (Vander Pan et al. 2019). This setup simulates the situation in which a repellent barrier has been sprayed, e.g., around a bed, to protect a sleeping person in an infested room. The comparison of data from both test systems provides information about the impact of the test design on the test outcome. To evaluate the suitability of both test systems for repellent efficacy testing, three commercial insect repellent products and two dilutions of plant-based products were tested against a susceptible bed bug strain. Since no authorized repellents against bed bugs are on the EU market at the moment, we tested products that are not intended by their manufacturers for use against bed bugs or were authorized for this purpose but were assumed to have a repellent effect on bed bugs.

Materials and Methods

Test Organisms

Bed bugs of the insecticide-susceptible laboratory *C. lectularius* strain of the German Environment Agency (UBA) have been held since 1947 and fed weekly on rabbits (*Oryctolagus cuniculus* (Linnaeus, Lagomorpha: Leporidae) f.dom). The bed bugs used in the experiments were separated from the rabbit fed strain and fed once a week on a parafilm membrane with defibrinated porcine blood (elocin-lab GmbH, Oberhausen, Germany) in the last two years before the beginning of this study. Between feedings, they were kept in Petri dishes with two filter paper discs (grade 3 hw, 70 mm diameter, 65 g/m^2, Munktell, Ahlstrom-Munksjö GmbH, Bärenstein, Germany) in an incubator (24 h darkness; 26.5 ± 1°C and 45 ± 10% humidity). Only adult bed bugs were used in the experiments. The bed bugs were fed seven times in total from hatching to the adult stage. Since bed bugs were held in Petri dishes with mixed sexes, females' mating status was not examined. Individuals had a maximum difference in age of 7 d. After the last bloodmeal, they had 6–8 d for digesting the blood. Bed bugs were only used once and were discarded after the end of each experiment.

Test Substances

In both test systems, five substances were tested as potential bed bug repellents. Cinnamon oil (Mystic Moments, Fortsbridge, United Kingdom) and margosa extract (Vectrade UG, Penzberg, Germany) were applied with a commercial spray bottle (Dirk Rossmann GmbH, Burgwedel, Germany) in a 1:10 dilution with isopropanol (Chemsolute, Geyer GmbH & Co KG, Renningen, Germany). Autan Protection Plus with 20% icaridin (MCM Klosterfrau Healthcare Group, Köln, Deutschland), Nobite Skin with 50% DEET (Tropical Concept Sarl, Paris, France), and Nobite Clothes with 2% permethrin (Tropical Concept Sarl, Paris, France) were tested as ready-to-use products. These substances were applied using the spray bottle of the respective product. All substances were applied until the filter papers were saturated, which corresponded to 1.73 ± 0.1 mg/cm^2 DEET, 1.19 ± 0.03 mg/cm^2 icaridin, 0.28 ± 0.01 mg/cm^2 permethrin, and 0.48 ± 0.05 mg/cm^2 cinnamon oil and margosa. The papers were subsequently dried for up to an hour and then immediately used in the experiments.

All materials that came in contact with the used substances were cleaned with a laboratory cleaning agent (RBS 35, 2% v/v in tap water, Carl Roth, Karlsruhe, Germany) and then thoroughly rinsed with tap water.

Harborage Choice Test

The first test system represents a laboratory choice test (Fig. 1). It consisted of a crystallizing dish (230 mm diameter, 100 mm height, VWR, Dresden, Germany) with two harborages for the bed bugs, each consisting of two filter papers (grade 3 hw, 70 mm diameter, 65 g/m^2, Munktell, Ahlstrom-Munksjö GmbH). The filter papers were lying on top of each other and were fixed to the bottom with tape (Tesafilm, Beiersdorf, Hamburg, Germany) or in case of oily substances with beeswax (Stockmar, Kaltenkirchen, Germany). One of the harborages was treated with the repellent substance, and the other was untreated. The bed bugs could move to the harborages or stay outside in the dish. The treated and untreated filter papers were swapped for the replicates to avoid side preferences of the bed bugs choosing a harborage. For every substance, seven replicates (n = 700-bed bugs) were tested at the same time.

Untreated controls were conducted to determine bed bugs distribution in the two harborages and the crystallizing dish. The bed bug distribution was documented 24 h after their release. The respective treatment experiments and controls were conducted simultaneously. With a total of n = 1,300-bed bugs, two control replicates each were conducted for treatments with margosa and icaridin, and three control replicates each for treatments with DEET, cinnamon oil, and permethrin.

At the beginning of the experiment, 100-bed bugs (sex ratio 1:1) per replicate were put in the middle of the crystallizing dish

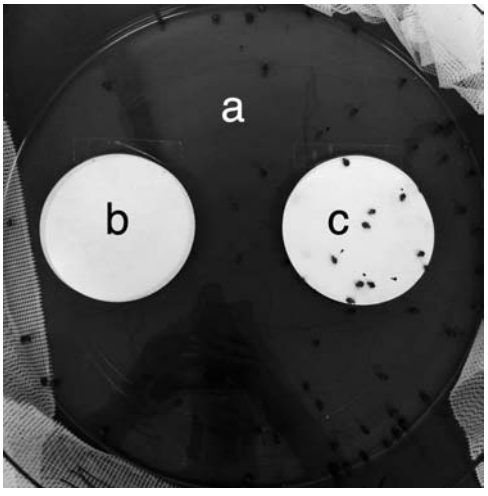

Fig. 1. Top view of the harborage choice test setup. (a) Crystallizing dish with bed bugs; (b) treated, and (c) untreated filter paper harborage.

between the harborages. The number of bed bugs in the treated and untreated harborages or outside in the crystallizing dish was documented every 5 min for 1 h. For each substance, 8,400 contacts (outside, treated, and untreated shelter) were counted in total. During the 1-h observation period, the crystallizing dishes were left open. To minimize the impact of light on bed bug behavior, lights in the room were switched off, and soft twilight from the outside was used for data recording. Afterward, dishes were closed with mesh fixed with a rubber band to prevent bed bugs from escaping, and the room was completely darkened. After 24 h, the position of all bed bugs was counted again.

Since none of the tested substances was authorized as a bed bug repellent, and the effects on bed bugs have not been evaluated yet, possible physical impairment of the bed bugs was tested. Therefore, after 24 h in both test systems, the number of lethally affected bed bugs was determined by forceps stimulation. Individuals were categorized as lethally affected when no or only uncoordinated movement was observed or when bed bugs in the dorsal position could not turn back into the ventral position.

Barrier Test

The second test system (Fig. 2) was a simulated-use test mimicking a human host. The bed bugs were attracted by CO_2 and heat to cross the surfaces treated with the different potential repellents. The systems' design and test procedures are described in detail in Vander Pan et al. (2019). The simulation of a host (heat and CO_2) in the test system lured the bed bugs to cross the filter paper (19 cm × 9 cm, grade 3 hw, 58 cm × 58 cm, 65 g/m^2, Munktell, Ahlstrom-Munksjö GmbH). The host was simulated by heat (Erlenmeyer flask with 300 ml water, kept at 80°C with a heating plate) and a CO_2 source (gas flow rate 0.75 ml/min), both located in the steel container (Fig. 2A and D). Bed bugs that were successfully lured across the filter paper towards the simulated host fell through the open end of the tube and were trapped in a glass aquarium with two filter papers inside for hiding.

As a control, the host-seeking behavior of bed bugs in this test system was determined in seven replicates (n = 700-bed bugs) without a repellent. To factor in natural mortality, 100-bed bugs of an equal sex ratio were placed in Petri dishes in direct proximity to the test system simultaneously to each replicate.

For each test substance, experiments were conducted in three replicates on consecutive days (n = 300-bed bugs). One hundred bed bugs (sex ratio 1:1) were transferred into the kitchen paper towel pocket, which was then closed and fixed at the bottom of the cylindrical container in the test system. Bed bugs had about 1 h to acclimate. Afterward, the treated surface was fixed on the bottom of the test chamber, which was covered with mesh and fixed with a rubber band to allow airflow and prevent bed bugs from escaping. The pocket was opened by two diagonal cuttings, that the bed bugs could crawl to the simulated host. Then, the room was completely darkened to minimize the impact of light. After 24 h, the position of all bed bugs was documented. The bed bugs that either passed the filter paper and fell into the glass aquarium, stayed in the test chamber with the treated filter paper, or remained in the tube, which extended into the steel container, were counted as "not repelled" (Fig. 2).

Fig. 2. Barrier test system. (A) Overview of the test setup; (a) cylindrical container; (b) kitchen paper towel pocket; (c) plastic tube base; (d) acrylic glass tube closed with a plug with an extraction hose leading to the suction pump; (e) tube connected to the test chamber; (f) test chamber; (g) tube connected to the steel container; (h) steel container; (i) CO_2 flow meter; (j) CO_2 gas cylinder. (B)Top view of the cylindrical container; (b) kitchen paper towel pocket; (d) acrylic glass tube closed with a plug with an extraction hose leading to the suction pump. (C)Top view of test chamber; (e) tube connected to the test chamber; (f) test chamber, (g) tube connected to the steel container. (D)Top view of the steel container; (k) glass aquarium as bed bug trap with two filter papers as harborage; (l) thermometer; (m) plastic hose for CO_2 supply; (n) heating plate with Erlenmeyer flask and water.

The number of lethally affected bed bugs was determined as described in the harborage choice test.

Data Analysis

Side preferences in harborage choice of the bed bugs in controls of the harborage choice test were checked for statistical significance with the Wilcoxon signed-rank test with the theoretical median of 50% distribution in each harborage (null hypothesis: equal distribution). Since it was not possible to count bed bugs during the 60 min observation period in controls and treatment experiments simultaneously, the bed bug positions in controls were only assessed after 24 h. The data of the bed bug positions (outside in the dish or in the treated or untreated harborages) within the 60 min observation period were evaluated using descriptive statistics. The number of 'repelled bed bugs' in treatments after 60 min and 24 h was calculated from the proportion of bed bugs hiding in the untreated filter papers in the harborage choice test. In the barrier test, 'repelled bed bugs' did not cross the filter paper within the 24 h test period. For the data obtained from both test systems, possible relationships of variables (repellency and sex-specific differences) were calculated using the mid-*p* exact test ($\alpha = 0.05$) provided by the free online statistic software OpenEpi (Dean 2013). The resulting p-values were adjusted applying Holm correction p.adjust in R (R Core Team 2013). Graphs and the Wilcoxon signed-rank test were performed with graph pad prism 8.4.1 (GraphPad Software, La Golla, CA, www.graphpad.com) for Mac OS.

Results

Harborage Choice Test

In controls, the bed bugs' activity was high after their release, but it could be observed that it decreased within a short time of the 60 min observation period. After 24 h, only two of 1,300 bed bugs in total were found outside (Fig. 4). With at least 59%, most bed bugs preferred one harborage in each control replicate. Thus, the distribution was unequal. The deviation from the theoretical median of 50% was statistically significant ($P = 0.0002$).

In general, the bed bugs preferred the untreated harborages rather than staying outside in the dish or the treated harborages in the treatment experiments (Fig. 3). In all experiments, at least 60% of bed bugs chose the untreated harborage within the first 15 min except for the margosa experiments. Most bed bugs (55%) hid only after 40 min in the untreated harborage in these experiments. In all treatments, bed bugs avoided the treated harborages within the 60 min observation period. Most bed bug contacts with the treated harborages were counted in the experiments with margosa (1,123 contacts) and permethrin (555 contacts). The fewest contacts with treated shelters were found in experiments with cinnamon oil (four contacts), DEET (34 contacts), and icaridin (55 contacts). In margosa and permethrin treatments, the maximum median proportion of bed bugs in the treated shelter was 16% and 8%, respectively. After 24 h, in experiments with permethrin, the highest number of bed bugs was found outside the harborages (Fig. 3). The median proportion of bed bugs that hid in shelters treated with cinnamon oil, icaridin, and DEET was 0%. More precisely, none, one, and two out of 700-bed bugs per substance did not avoid the repellent, respectively. In margosa-treated and permethrin-treated harborages, the median proportion of detected bed bugs was 0% (23 out of 700-bed bugs in total) and 1% (13 out of 700-bed bugs in total) after 24 h, respectively (Fig. 3a–e). The number of repelled bed bugs regarding each substance was significantly higher in comparison with the number of bed bugs found in the more visited harborages in the controls (for all substances: $P < 0.00001$) (Fig. 4). Between DEET, icaridin, and cinnamon oil, no significant differences in repellency were found (for all comparisons: $P = 1$). The number of repelled *C. lectularius* regarding these substances was significantly higher in comparison with the number of bed bugs repelled by permethrin and margosa (permethrin vs DEET ($P = 0.0356$), icaridin ($P = 0.0108$), and cinnamon oil ($P = 0.0143$); margosa versus DEET ($P < 0.001$), icaridin, and cinnamon oil (for both substances: $P < 0.0001$)). No differences in repellency were found between permethrin and margosa ($P = 1$). Furthermore, repellency at observation time point 24 h compared to 60 min was significantly higher in experiments with permethrin ($P < 0.01$) and margosa ($P < 0.00001$).

After 24 h, in none of the experiments, lethally affected bed bugs were found.

Barrier Test

In seven control replicates ($n = 700$-bed bugs), 58–90% of the bed bugs crossed the untreated filter papers (Fig. 5). Thus, a median proportion of 30% stayed in the initial harborage. There was no natural mortality in control Petri dishes. DEET displayed the highest repellency with 97–99% repelled bed bugs. In tests with icaridin-treated and cinnamon oil-treated filter papers, 55–82% and 71–80% of the bed bugs were repelled, respectively. Margosa extract and permethrin displayed the lowest efficacy with 49–67% and 45–84% repelled bed bugs, respectively. The number of repelled bed bugs with regard to each substance was significantly higher in comparison with the number of bed bugs that did not cross the untreated filter paper in the controls (for all substances: $P < 0.00001$) (Fig. 5). The number of *C. lectularius* repelled by DEET was significantly higher in comparison with the number of bed bugs repelled by the other four substances (for all substances: $P < 0.0001$). Furthermore, the number of repelled bed bugs regarding icaridin and cinnamon oil was significantly higher than the number of bed bugs repelled by margosa (for both substances: $P < 0.0001$).

In all experiments (control group and treatment groups), more female than male bed bugs sought out the simulated host (Table 1). In controls and treatments except for DEET, these differences in host-seeking behavior between male and female bed bugs were statistically significant.

Except for DEET (60 min: $P = 0.14$; 24 h: $P = 0.067$), the differences in numbers of repelled bed bugs between barrier test and harborage choice test at the observation time points 60 min and 24 h were statistically significant with $P < 0.00001$. After 24 h, in none of the experiments, lethally affected bed bugs were found.

Discussion

Our study reveals that with both test systems, a repellent efficacy of different substances can be detected, but the two test designs led to a different repellency regarding the same substances.

The harborage choice test is a small-scale test system mimicking a situation where bed bugs are forced to choose a harborage but should be kept away from one. This is comparable to a spatial treatment in a room where bed bugs should be deterred from a particular harborage. The test setup is simple, can be realized with standard laboratory materials, and our results revealed that it allows the screening of substances and formulations within a short time.

During the 1-h observation period in the harborage choice test, differences concerning the repellent effect of the tested substances were detectable (Fig. 3a–e). After 30 min, 100% of the bed bugs

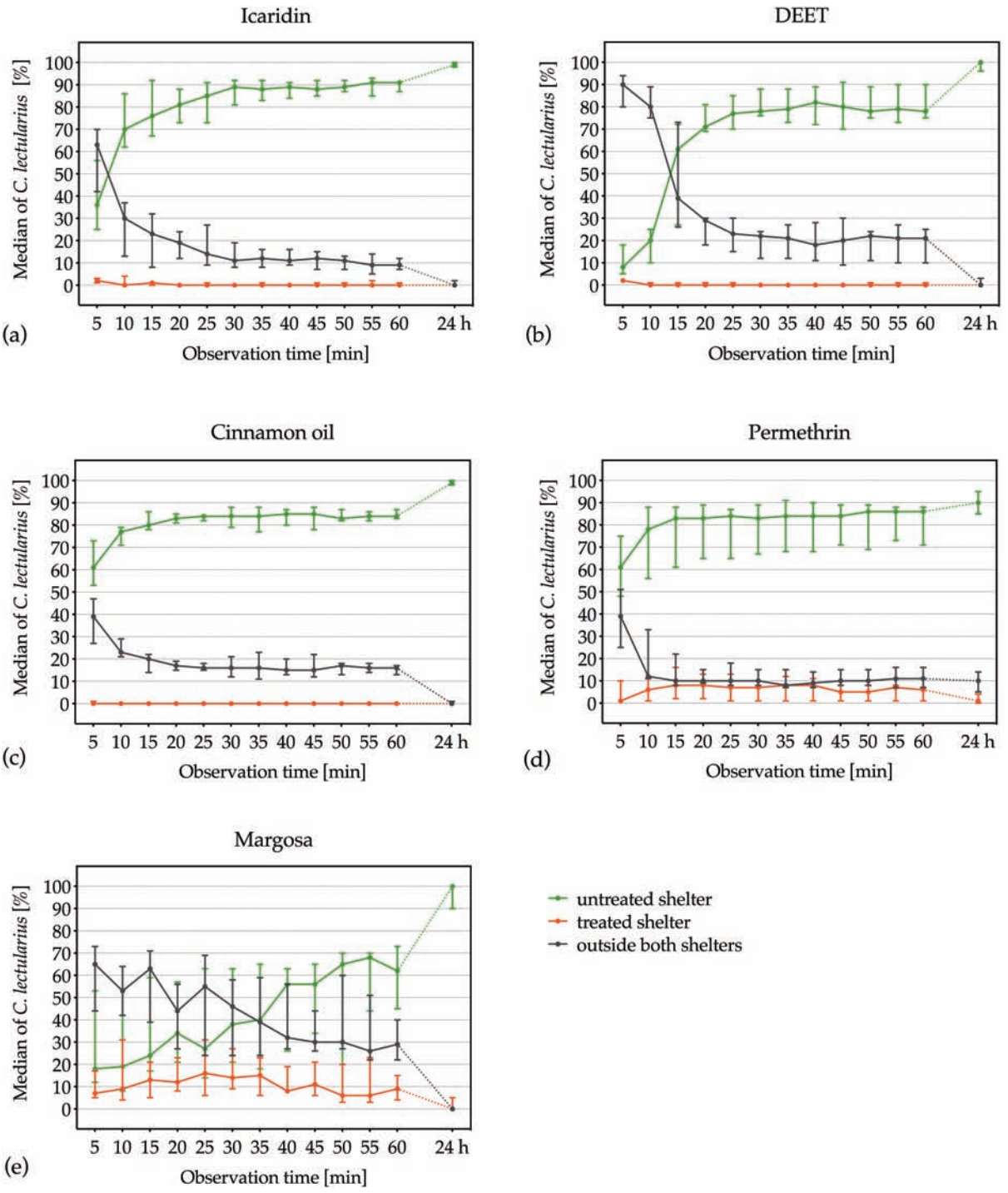

Fig. 3. Distribution of bed bugs in the harborage choice test (untreated shelter, treated shelter, and outside both shelters) at the observation time points within the first 60 min and 24 h after release. Different substances were used for the experiments: (a) icaridin, (b) DEET, (c) cinnamon oil, (d) permethrin, (e) margosa extract. Points indicate the median, and whiskers indicate the interquartile range (Q1 and Q3).

were repelled in the icaridin treatments. In tests with margosa extract, only 84% of the bed bugs were repelled within the same time. By contrast, no differences in repellency between the five substances were detectable after 24 h (all substances: 99–100% repelled bed bugs). These findings suggest that bed bugs that have chosen an untreated harborage within the first hour will not seek another harborage within the 24 h test period without a stimulus. This is supported by the fact that most bed bugs also chose one of the offered harborages in controls and rested there. In the untreated controls, the distribution of the bed bugs was unequal after 24 h. This can be explained by the pheromone-mediated aggregation behavior of bed bugs (Siljander et al. 2008). After feeding or when no host is available (Reis and Miller 2011), bed bugs form dense aggregations, which provide advantages like the reduction of water loss (Benoit et al. 2007), faster development (Saenz et al. 2014), and a higher mating chance (Pinto et al. 2007). Aggregation and

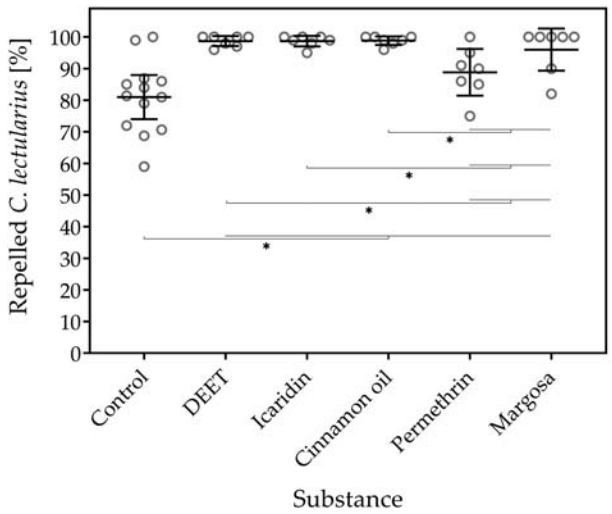

Fig. 4. Repelled bed bugs in the harborage choice test found in the untreated harborages 24 h after release compared to bed bugs found in the more visited control harborages. The circles indicate biological replicates, and the horizontal lines represent the medians. Mid-p exact tests with resulting *p*-values adjusted using Holm correction. Significant differences in repellency of the different substances are marked with an asterisk (*).

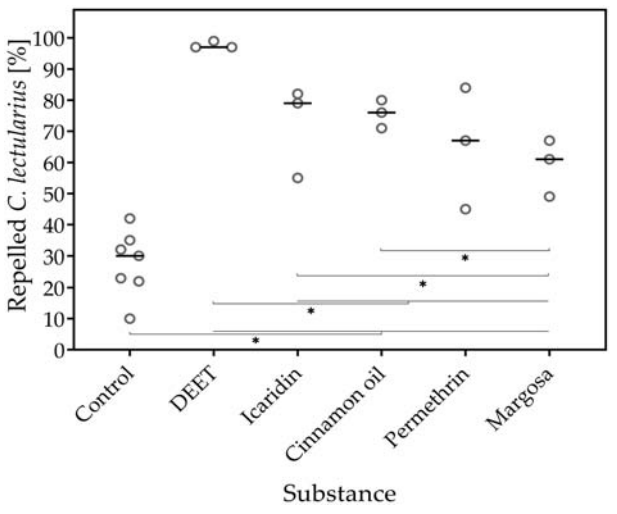

Fig. 5. Repelled bed bugs in the barrier test that did not cross the treated barrier within 24 h after release compared to bed bugs that did not leave the harborage in the controls. The circles indicate biological replicates, and the horizontal lines represent the medians. Mid-*p* exact tests with resulting *p*-values adjusted using Holm correction. Significant differences in repellency of the different substances are marked with an asterisk (*).

Table 1. Sex-specific differences of nonrepelled bed bugs of all experiments in the barrier test. Mid-*p* exact tests with resulting *p*-values adjusted using Holm correction

Substances	*n*	No. of nonrepelled bed bugs	No. of nonrepelled females	*p*-value
Control	700	506	279 (55%)	<0.001
Icaridin	300	84	63 (75%)	<0.00001
DEET	300	7	5 (71%)	0.8724
Cinnamon oil	300	73	51 (70%)	0.002
Permethrin	300	104	65 (63%)	0.0271
Margosa	300	123	75 (61%)	0.0271

associated positive thigmotactic behavior might have influenced the bed bugs distribution after the 1-h observation period more than the repellency of the substances (Benoit 2011, Weeks et al. 2013). Therefore, data recording within the initial observation period seems to be more important for repellent evaluation than later observation periods. This also applies to the controls, but simultaneous counting of bed bugs in treatment and controls was not feasible in our study. However, this should be considered for further experiments. After 24 h, the number of repelled bed bugs regarding each substance was significantly different from the more visited control harborages. This indicates that the number of repelled bed bugs observed after 24 h can lead to overestimated repellency values for the test substances. For regulatory purposes, the observation period should be adapted for gaining data on complete protection time. In this context, the use of cameras for monitoring could be beneficial, and the possible impacts of CO_2 emitted by laboratory staff or light needed for data collection could be minimized. Furthermore, bed bug exposed filter papers containing aggregation pheromone may influence harborage choice. The efficacy of a repellent product in this test setup should be examined in further studies (Olson et al. 2009; Weeks et al. 2011, 2013).

The barrier test was initially developed to provide a new approach for efficacy testing of insecticides against bed bugs mimicking a barrier treatment during a pest management situation. Furthermore, it was successfully tested to detect phenotypic resistance in bed bugs (Vander Pan et al. 2019). In this study, we demonstrated that this system could be additionally used for efficacy assessment of repellents applied to surfaces. The test is designed to simulate a situation where a host is present and should be protected from bites by a repellent treated barrier, e.g., an overnight stay in a bed bug-infested hotel room without any skin treatment needed. In contrast to the simple harborage choice test, the barrier test setup is made of custom-built materials and needs more space. However, the size of the experimental system is still smaller than a semifield test setup recreated in a room and would allow simultaneous replicates in one room. Also, bed bugs can be found easily in contrast to scenarios with an entire room. It was unambiguous how many bed bugs crossed the treated surface since only those that were found in or behind the test chamber were counted. As described for the harborage choice test, the use of cameras could be an advantage for gaining data concerning the complete protection time, which is mandatory for the EU biocide approval. In our study, the bed bug location was recorded after 24 h. Depending on the substance label claim, the data collection can be adapted to shorter or longer efficacy. Wang et al. (2013), e.g., evaluated the repellent efficacy of DEET (25%) for three weeks with a comparable test setup using CO_2 and heat for host simulation.

The repellency of all tested substances in both test systems was significantly different from controls after 24 h. However, this may not be sufficient to prove the efficacy of a repellent product, and for regulatory purposes, the criteria for a minimum repellency should be defined. Nobite Skin (50% DEET) yielded the highest repellency in

the harborage choice test with 100% (after 60 min and 24 h) and with 97–99% in the barrier test and would probably fit authorization criteria. Our results confirm the wide range of repellent efficacy of DEET against arthropods (Goodyer et al. 2010), e.g., mosquitoes (Fradin and Day 2002, Frances et al. 2004), ticks (Jaenson et al. 2003, Carroll et al. 2005, Jensenius et al. 2005, Kulma et al. 2019), cockroaches (Mengoni and Alzogaray 2018), and bed bugs (Wang et al. 2013, Anderson et al. 2018). Products containing DEET can potentially be used to protect personal belongings (e.g., luggage) from being infested and humans from being bitten.

In the harborage choice test, Autan (20% icaridin) and cinnamon essential oil (1:10 dilution) showed results comparable to DEET since they also repelled nearly all bed bugs after 60 min and up to 24 h. By comparison, in the barrier test, DEET showed the highest repellency compared to all other substances. The repellency of icaridin (55–82%) and cinnamon oil (71–80%) in the barrier test was lower than in the harborage choice test. This indicates that the impact of the opportunity of getting a bloodmeal is remarkably high and should be considered in an experimental setup. This effect was also observed by Singh et al. (2014). However, lower DEET concentration yielded lower repellency even without a host simulation, as shown by Wang et al. (2013). Compared to our results, the repellency of icaridin in experiments conducted by Wang et al. (2013) was much lower (about 20–30%). This might be due to a lower concentration of 7% icaridin used by Wang et al. (2013) or the fact that bed bugs were compelled to stay on the treated side for hiding in this described test design. Thus, repellents containing icaridin should contain higher concentrations (over 20%) against bed bugs and other blood-sucking arthropods (Goodyer et al. 2010).

Nobite Clothes (2% permethrin) and margosa (1:10 dilution) displayed the lowest repellency: up to 13% and 26% of the bed bugs were respectively found in the treated harborages after the first hour of the harborage choice test. The repellency increased within the following 23 h of the test period (permethrin 99%; margosa 100%). By contrast, only 45–84% and 49–67% of the bed bugs were repelled by permethrin-treated and margosa-treated barriers, respectively, in the barrier test. Although the repellency was significantly different from the controls, these values can be considered as too low to claim protection from bed bug bites.

Our results show that the use of DEET as a spatial spray can be considered for bite and dispersal protection, and with its long-lasting efficacy shown by Wang et al. (2013), it has the potential as an additional IPM method. Icaridin and cinnamon oil might be potential repellents against bed bugs, too, but further studies with higher concentrations are required. The repellency of permethrin (2%) and margosa is too low for the use as a spatial repellent against bed bugs. In Europe, Nobite clothes (permethrin) is authorized as an insect repellent (not especially against bed bugs), which means that insects should be deterred but not killed. If the repellency is not high enough, as shown in our study, longer contact of bed bugs to permethrin-treated surfaces might lead to intoxication and death. With repellency values obtained in our study, the product would not be authorized as a repellent in Europe against bed bugs, but with data of sufficient mortality with at least 90% after contact, an authorization as an insecticide would be feasible (ECHA 2018). As the repellency of the used concentrations of permethrin and margosa was too low for the use as repellents, higher concentrations of both substances should be tested. Furthermore, the strong and intensive odor of Autan, cinnamon oil, and margosa might not be pleasant for the applicant.

It was notable that the bed bugs acted atypically regarding aggregation behavior in the margosa treatments. Compared to experiments with other substances, they needed more time to choose a harborage, and about 20–40% of the bed bugs stayed outside in the dish within the first 60 min of the experiments. After 24 h, in all experiments, almost no bed bug stayed outside of a harborage except for permethrin treatments (up to 18%). Romero et al. (2009) showed that contact with sublethal doses of deltamethrin increased bed bugs´ activity. Our results indicate that contact with margosa extract and permethrin might have the same effect. In the case of permethrin-treated fabrics, also other behavior patterns like fewer feeding attempts, lower blood intake, and decreased fecundity were observed (Jones et al. 2015). No sublethal effects of margosa extract are reported with regard to the activity of bed bugs or other insects, and further studies are required.

In the barrier test, more females than males crossed the untreated filter papers. The proportion was even higher in the treatment experiments than in the controls. This bias in distribution might be explained by the fact that female bed bugs have higher nutrition demands since egg production depends on the amount of their blood uptake (Mellanby 1939). Furthermore, mainly engorged females are attractive for mating, and attractiveness decreases 36 h after a bloodmeal (Stutt and Siva-Jothy 2001). Females tend to hide in new harborages and therefore are supposed to be responsible for bed bug dispersal (Siljander et al. 2008, Pfiester et al. 2009). Our results support the findings of other researchers that females may leave shelters more often searching for food or new harborages (Pfiester et al. 2009, Aak et al. 2014). Thus, the use of both sexes in bioassays testing the efficacy of repellents is very important. Also, other sex-specific differences, age, or multiple traumatic inseminations might lead to different results between males, females, or juveniles (Abd-Elghafar et al. 1990, Polanco et al. 2011, Benoit et al. 2012, Aak et al. 2014, McNeill et al. 2016, Kulma et al. 2019, Vander Pan et al. 2019). Experiments should be conducted with different life stages in mixed populations to reflect a practical infestation situation. To determine the influence of population composition, further studies are required.

Our results support the findings of Singh et al. (2014) that the response of bed bugs to repellents depends on the respective behavioral context, e.g., looking for shelter to aggregate or searching for a bloodmeal. Furthermore, host cues influence bed bug harborage-seeking behavior. For example, they are especially attracted to luggage containing worn clothes (Hentley et al. 2017, Anderson et al. 2018). This can affect the repellency of products applied on surfaces (Anderson et al. 2018) and lead to a lower repellency in practical application. This impact cannot be modeled in the harborage choice test system. For authorization of a repellent product with the label claim "luggage treatment," an additional modified test system should be used (Hentley et al. 2017, Anderson et al. 2018). Even though the barrier test mimics host cues and is designed for barrier treatment and personal protection against bed bug bites, it is not applicable to test skin protection products. If a product is claimed to be applicable for skin, it could be tested under similar conditions like a test for tick repellents (Dautel 2002, WHO 2009, EPA 2010).

In addition, the behavior of arthropods to repellents can be influenced by insecticide resistance (Deletre et al. 2019). Over the last years, bed bugs worldwide became more and more resistant to insecticides used in pest management (Dang et al. 2017). In different pyrethroid-resistant arthropods like *Blattella germanica* (Linnaeus, Blattodea: Ectobiidae) (Mengoni and Alzogaray 2018), *Aedes aegypti* (Linnaeus, Diptera: Culicidae) (Yang et al. 2020), and *C. lectularius* (Vassena et al. 2019), reduced effectiveness of repellent substances was observed. Based on these findings, repellency testing with resistant strains should be considered.

In conclusion, both test systems evaluated in this study are suitable for repellent efficacy testing for biocide authorization. The harborage choice test enables the screening of different substances and concentrations. It mimics a spatial treatment of potential bed bug shelters in an infested room or a barrier treatment to adjacent rooms. By contrast, the barrier test is more complex and simulates a situation where a host should be protected by a repellent barrier. Our results show that the test outcome depends on the behavioral context, search for hosts, or search for hidings.

Repellents can help to prevent bed bug bites, be very important to minimize dispersal, and long-lasting products might be a part of IPM. DEET (50%) had the most effective repellency and has the potential to be used for spatial treatment and barrier treatment for bite prevention.

Funding

This research received no external funding.

Conflicts of Interest

The authors declare no conflicts of interest.

References Cited

Aak, A., B. A. Rukke, A. Soleng, and M. K. Rosnes. 2014. Questing activity in bed bug populations: male and female responses to host signals. Physiol. Entomol. 39: 199–207.

Abd-Elghafar, S. F., A. G. Appel, and T. P. Mack. 1990. Toxicity of several insecticide formulations against adult German cockroaches (Dictyoptera: Blattellidae). J. Econ. Entomol. 83: 2290–2294.

Anderson, J. F., F. J. Ferrandino, M. P. Vasil, R. H. Bedoukian, M. Maher, and K. McKenzie. 2018. repellency of naturally occurring or related compounds, DEET, and para-menthane-3,8-diol to bed bugs (Hemiptera: Cimicidae). J. Med. Entomol. 55: 666–672.

Ashbrook, A. R., M. E. Scharf, G. W. Bennett, and A. D. Gondhalekar. 2017. Detection of reduced susceptibility to chlorfenapyr- and bifenthrin-containing products in field populations of the bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 110: 1195–1202.

Ashbrook, A. R., M. E. Scharf, G. W. Bennett, and A. D. Gondhalekar. 2019. Bed bugs (*Cimex lectularius* L.) exhibit limited ability to develop heat resistance. Plos One. 14: e0211677.

Barnard, D. R., and R. -D. Xue. 2004. Laboratory evaluation of mosquito repellents against *Aedes albopictus*, *Culex nigripalpus*, and *Ochlerotatus triseriatus* (Diptera: Culicidae). J. Med. Entomol. 41: 726–730.

Benoit, J. B. 2011. Stress tolerance of bed bugs: a review of factors that cause trauma to *Cimex lectularius* and *C. Hemipterus*. Insects. 2: 151–172.

Benoit, J. B., N. A. Del Grosso, J. A. Yoder, and D. L. Denlinger. 2007. Resistance to dehydration between bouts of blood feeding in the bed bug, *Cimex lectularius*, is enhanced by water conservation, aggregation, and quiescence. Am. J. Trop. Med. Hyg. 76: 987–993.

Benoit, J. B., A. J. Jajack, and J. A. Yoder. 2012. Multiple traumatic insemination events reduce the ability of bed bug females to maintain water balance. J. Comp. Physiol. B. 182: 189–198.

Cambronero-Heinrichs, J. C., L. S. Sánchez-Portilla, Ó. Calderón-Arguedas, and A. Troyo. 2020. *Cimex lectularius* Linnaeus, 1758 (Hemiptera: Cimicidae) in costa rica: first case report confirmed by molecular methods in Central America. J. Med. Entomol. 57: 969–973.

Caraballo, A. J. 2000. Mosquito repellent action of Neemos®. J. Am. Mosq. Control Assoc. 16: 45–46.

Carroll, J. F., J. A. Klun, and M. Debboun. 2005. Repellency of deet and SS220 applied to skin involves olfactory sensing by two species of ticks. Med. Vet. Entomol. 19: 101–106.

Chang, K. S., J. H. Tak, S. I. Kim, W. J. Lee, and Y. J. Ahn. 2006. Repellency of *Cinnamomum cassia* bark compounds and cream containing cassia oil to *Aedes aegypti* (Diptera: Culicidae) under laboratory and indoor conditions. Pest Manag. Sci. 62: 1032–1038.

Dang, K., S. L. Doggett, G. Veera Singham, and C. Y. Lee. 2017. Insecticide resistance and resistance mechanisms in bed bugs, *Cimex* spp. (Hemiptera: Cimicidae). Parasit. Vectors. 10: 318.

Dautel, H. 2002. In vivo test method for repellents against the hard tick *Ixodes ricinus* (Acari: Ixodidae) main vector of Lyme borreliosis and TBE in Europe, pp. 387–398. *In* S. C. Jones, J. Zhai and W. H. Robinson (eds.), 4th International Conference On Urban Pests, Charleston, SC.

Davies, T. G., L. M. Field, and M. S. Williamson. 2012. The re-emergence of the bed bug as a nuisance pest: implications of resistance to the pyrethroid insecticides. Med. Vet. Entomol. 26: 241–254.

Dean, A. G. 2013. OpenEpi: open source epidemiologic statistics for public health, version 3.01 www.OpenEpi.com.

Deletre, E., T. Martin, C. Duménil, and F. Chandre. 2019. Insecticide resistance modifies mosquito response to DEET and natural repellents. Parasit. Vectors. 12: 89.

ECHA. 2018. Guidance on the Biocidal Products Regulation, volume II efficacy - assessment and evaluation (parts B+C). European Chemicals Agency, Helsinki, Finland.

EPA. 2010. Product Performance Test Guidelines OPPTS 810.3700: insect repellents to be applied to human skin. United States Environmental Protection Agency, Washington, USA.

Fradin, M. S., and J. F. Day. 2002. Comparative efficacy of insect repellents against mosquito bites. N. Engl. J. Med. 347: 13–18.

Frances, S. P., D. G. Waterson, N. W. Beebe, and R. D. Cooper. 2004. Field evaluation of repellent formulations containing deet and picaridin against mosquitoes in Northern Territory, Australia. J. Med. Entomol. 41: 414–417.

Goodyer, L. I., A. M. Croft, S. P. Frances, N. Hill, S. J. Moore, S. P. Onyango, and M. Debboun. 2010. Expert review of the evidence base for arthropod bite avoidance. J. Travel Med. 17: 182–192.

Harlan, H. J. 2006. Bed bugs 101: the basics of *Cimex lectularius*. Am. Entomol. 52: 99–101.

Hentley, W. T., B. Webster, S. E. F. Evison, and M. T. Siva-Jothy. 2017. Bed bug aggregation on dirty laundry: a mechanism for passive dispersal. Sci. Rep. 7: 1–5.

Jaenson, T. G., A. Lindström, and K. Palsson. 2003. Repellency of the mosquito repellent MyggA® (N,N- diethyl-3-methyl-benzamide) to the common tick *Ixodes ricinus* (L.) (Acari: Ixodidae) in the laboratory and field. Entomol. Tidskr. 124: 245–251.

Jensenius, M., A. M. Pretorius, F. Clarke, and B. Myrvang. 2005. Repellent efficacy of four commercial DEET lotions against *Amblyomma hebraeum* (Acari: Ixodidae), the principal vector of *Rickettsia africae* in southern Africa. Trans. R. Soc. Trop. Med. Hyg. 99: 708–711.

Jones, S. C., J. L. Bryant, and F. S. Sivakoff. 2015. Sublethal effects of *Active*guard exposure on feeding behavior and fecundity of the bed bug (Hemiptera: Cimicidae). J. Med. Entomol. 52: 413–418.

Jones, S. C., J. L. Bryant, and S. A. Harrison. 2013. Behavioral responses of the bed bug to permethrin-impregnated *Active*Guard™ Fabric. Insects. 4: 230–240.

Kells, S. A., and M. J. Goblirsch. 2011. Temperature and time requirements for controlling bed bugs (*Cimex lectularius*) under commercial heat treatment conditions. Insects. 2: 412–422.

Koganemaru, R., and D. M. Miller. 2013. The bed bug problem: past, present, and future control methods. Pestic. Biochem. Physiol. 106: 177–189.

Kulma, M., O. Kopecký, and T. Bubová. 2019. Nymphs of *Ixodes ricinus* are more sensitive to deet than adult females. J. Am. Mosq. Control Assoc. 35: 279–284.

Lee, C. Y., D. M. Miller, and S. L. Doggett. 2018. Chemical control, pp. 285–310. *In* S. L. Doggett, D. M. Miller and C. Y. Lee (eds.), Advances in the biology and management of modern bed bugs. John Wiley & Sons Ltd., Hoboken, NJ.

Lilly, D. G., S. L. Doggett, M. P. Zalucki, C. J. Orton, and R. C. Russell. 2009. Bed bugs that bite back, confirmation of insecticide resistance in Australia in the common bed bug, *Cimex lectularius*. Prof Pest Manager: 22–26.

Liu, F., K. F. Haynes, A. G. Appel, and N. Liu. 2014. Antennal olfactory sensilla responses to insect chemical repellents in the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 40: 522–533.

Londono-Renteria, B., J. C. Patel, M. Vaughn, S. Funkhauser, L. Ponnusamy, C. Grippin, S. B. Jameson, C. Apperson, C. N. Mores, D. M. Wesson,

et al. **2015**. Long-lasting permethrin-impregnated clothing protects against mosquito bites in outdoor workers. Am. J. Trop. Med. Hyg. 93: 869–874.

Masetti, M., and F. Bruschi. 2007. Bedbug infestations recorded in Central Italy. Parasitol. Int. 56: 81–83.

McNeill, C. A., R. M. Pereira, P. G. Koehler, S. A. McNeill, and R. W. Baldwin. 2016. Behavioral responses of nymph and adult *Cimex lectularius* (Hemiptera: Cimicidae) to colored harborages. J. Med. Entomol. 53: 760–769.

Mellanby, K. 1939. Fertilization and egg production in the bed-bug, *Cimex lectularius* L. Parasitology 31: 193–199.

Mengoni, S. L., and R. A. Alzogaray. 2018. Deltamethrin-resistant German cockroaches are less sensitive to the insect repellents DEET and IR3535 than non-resistant individuals. J. Econ. Entomol. 111: 836–843.

Nerio, L. S., J. Olivero-Verbel, and E. Stashenko. 2010. Repellent activity of essential oils: a review. Bioresour. Technol. 101: 372–378.

Olson, J. F., R. D. Moon, and S. A. Kells. 2009. Off-host aggregation behavior and sensory basis of arrestment by *Cimex lectularius* (Heteroptera: Cimicidae). J. Insect Physiol. 55: 580–587.

Pereira, R. M., P. G. Koehler, M. Pfiester, and W. Walker. 2009. Lethal effects of heat and use of localized heat treatment for control of bed bug infestations. J. Econ. Entomol. 102: 1182–1188.

Pfiester, M., P. G. Koehler, and R. M. Pereira. 2009. Effect of population structure and size on aggregation behavior of *Cimex lectularius* (Hemiptera: Cimicidae). J. Med. Entomol. 46: 1015–1020.

Pinto, I. C., R. Cooper, and S. K. Kraft. 2007. Bed bug handbook: the complete guide to bed bugs and their control, Pinto & Associates Inc., Mechanicsville, MD.

Polanco, A. M., D. M. Miller, and C. C. Brewster. 2011. Reproductive potential of field-collected populations of *Cimex lectularius* L. and the cost of traumatic insemination. Insects. 2: 326–335.

R Core Team. 2013. R: a language and environment for statistical computing. Computer program, Version 3.1.3 computer program, version by R Core Team, Vienna, Austria.

Reis, M. D., and D. M. Miller. 2011. Host searching and aggregation activity of recently fed and unfed bed bugs (*Cimex lectularius* L.). Insects. 2: 186–194.

Romero, A., A. M. Sutherland, D. H. Gouge, H. Spafford, S. Nair, V. Lewis, D. H. Choe, S. Li, and D. Young. 2017. Pest management strategies for bed bugs (Hemiptera: Cimicidae) in multiunit housing: a literature review on field studies. J. Integr. Pest Manag. 8: 1–10.

Romero, A., M. F. Potter, and K. F. Haynes. 2009. Behavioral responses of the bed bug to insecticide residues. J. Med. Entomol. 46: 51–57.

Saenz, V. L., R. G. Santangelo, E. L. Vargo, and C. Schal. 2014. Group living accelerates bed bug (Hemiptera: Cimicidae) development. J. Med. Entomol. 51: 293–295.

Siljander, E., R. Gries, G. Khaskin, and G. Gries. 2008. Identification of the airborne aggregation pheromone of the common bed bug, *Cimex lectularius*. J. Chem. Ecol. 34: 708–718.

Singh, N., C. Wang, and R. Cooper. 2014. Potential of essential oil-based pesticides and detergents for bed bug control. J. Econ. Entomol. 107: 2163–2170.

Sõukand, R., R. Kalle, and I. Svanberg. 2010. Uninvited guests: traditional insect repellents in Estonia used against the clothes moth *Tineola bisselliella*, human flea *Pulex irritons* and bedbug *Cimex lectularius*. J. Insect Sci. 10: 150.

Stutt, A. D., and M. T. Siva-Jothy. 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. Proc. Natl. Acad. Sci. U. S. A. 98: 5683–5687.

Syed, Z., and W. S. Leal. 2008. Mosquitoes smell and avoid the insect repellent DEET. Proc. Natl. Acad. Sci. U. S. A. 105: 13598–13603.

Tangena, J. A., P. Thammavong, S. Chonephetsarath, J. G. Logan, P. T. Brey, and S. W. Lindsay. 2018. Field evaluation of personal protection methods against outdoor-biting mosquitoes in Lao PDR. Parasit. Vectors. 11: 661.

Ter Poorten, M. C., and N. S. Prose. 2005. The return of the common bedbug. Pediatr. Dermatol. 22: 183–187.

Vander Pan, A., E. Schmolz, J. Krücken, and C. Kuhn. 2019. A novel simulated-use test for determining the efficacy of insecticides against bed bugs (Hemiptera: Cimicidae). J. Econ. Entomol. 112: 2345–2353.

Vassena, C. V., M. Cáceres, and P. L. Santo-Orihuela. 2019. Pyrethroid resistance associated with a decreased DEET repellency in the common bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 112: 997–1000.

Wang, C., L. Lü, A. Zhang, and C. Liu. 2013. Repellency of selected chemicals against the bed bug (Hemiptera: Cimicidae). J. Econ. Entomol. 106: 2522–2529.

Weeks, E. N., J. G. Logan, S. A. Gezan, C. M. Woodcock, M. A. Birkett, J. A. Pickett, and M. M. Cameron. 2011. A bioassay for studying behavioural responses of the common bed bug, *Cimex lectularius* (Hemiptera: Cimicidae) to bed bug-derived volatiles. Bull. Entomol. Res. 101: 1–8.

Weeks, E. N., J. G. Logan, M. A. Birkett, J. A. Pickett, and M. M. Cameron. 2013. Tracking bed bugs (*Cimex lectularius*): a study of the effect of physiological and extrinsic factors on the response to bed bug-derived volatiles. J. Exp. Biol. 216: 460–469.

WHO. 2009. Guidelines for efficacy testing of mosquito repellents for human skin, WHO/HTM/NTD/WHOPES/2009.4. World Health Organization, Geneva, Switzerland.

Yang, L., E. J. Norris, S. Jiang, U. R. Bernier, K. J. Linthicum, and J. R. Bloomquist. 2020. Reduced effectiveness of repellents in a pyrethroid-resistant strain of *Aedes aegypti* (Diptera: culicidae) and its correlation with olfactory sensitivity. Pest Manag. Sci. 76: 118–124.

Zhu, J. J., S. C. Cermak, J. A. Kenar, G. Brewer, K. F. Haynes, D. Boxler, P. D. Baker, D. Wang, C. Wang, A. Y. Li, *et al.* 2018. Better than DEET repellent compounds derived from coconut oil. Sci. Rep. 8: 14053.