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Research

A Novel Simulated-Use Test for Determining the Efficacy of Insecticides Against Bed Bugs (Hemiptera: Cimicidae)

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Abstract

The common bed bug *Cimex lectularius* L. has undergone a worldwide expansion in recent years, due to increased opportunities for dispersal and development of insecticide resistance. For successful control, efficacy testing of products against bed bugs and determination of insecticide resistance under practical conditions are of outstanding importance. A new test system mimicking the practical use situation of residual insecticides was developed and evaluated. Bed bugs were attracted by CO_2 and heat to cross surfaces treated with alpha-cypermethrin and bendiocarb. In contrast to the complete efficacy of alpha-cypermethrin (less than 1% surviving bed bugs [with one exception of 5%]), only 45.3, 46, and 29% of insecticide-susceptible bed bugs showed lethal damage 7 d after contact with freshly bendiocarb-treated wallpaper or insecticide aged for 1 or 2 wk. Results show that the efficacy of different insecticides can be assessed with this new test system. Moreover, susceptibility to deltamethrin of five bed bug field strains, collected from infested apartments in Berlin, Germany, was determined in a filter paper contact bioassay. Resistance ratios (RRs) ranged between 4.3 and 20.7. In the novel simulated-use test, efficacy of alpha-cypermethrin was tested against the bed bug strain with the highest RR. In contrast to the insecticide-susceptible laboratory strain, alpha-cypermethrin was not effective against the field strain, where 26–50% of the bed bugs survived and even laid eggs. These results provide evidence for the presence of practically relevant pyrethroid resistance in bed bugs in Germany.

Key words: *Cimex lectularius*, simulated-use test, pyrethroid resistance, filter paper contact bioassay

In Germany, increased reports of pest control companies on infestations with the bed bug *Cimex lectularius* Linnaeus (Hemiptera: Cimicidae) are observed (Bauer-Dubau 2009, Kuhn and Vander Pan 2014), which is in accordance with the situation in many other countries (Ter Poorten and Prose 2005, Harlan 2006, Masetti and Bruschi 2007, Kilpinen et al. 2008, Lee et al. 2008, Dang et al. 2017a). Along with globalization, especially increased worldwide travel and second-hand trade, ignorance of bed bugs by people and the evolution of insecticide resistance are considered responsible for the worldwide expansion of the bed bug (Doggett et al. 2004, Potter 2005, Pinto et al. 2007, Romero et al. 2007). Therefore, the use of effective insecticides is a key element for successful control of bed bugs and prevention of resistance.

For the authorization of biocides according to the EU-Biocides Product Directive 528/2012, the proof of efficacy for any biocidal product is mandatory. Moreover, due to the worldwide bed bug resurgence, new effective products need to be developed. For this purpose, typically no-choice residual surface treatments are used to determine the efficacy of products against bed bugs (ECHA 2018). Both the legal requirements as well as the need for research and development of new products require the development of efficacy tests also under practical conditions (simulated-use), which are not available for products against bed bugs at the moment (ECHA 2018).

The objective of this study was to develop a simulated-use test system for efficacy testing of insecticides with residual properties against bed bugs, imitating a typical insecticide barrier treatment under practical conditions. In this test system, bed bugs crossed an insecticide-treated surface attracted by CO_2 and heat which mimicked a potential host. To show the suitability of the simulated-use test for efficacy testing of different insecticide classes, two insecticides containing the pyrethroid alpha-cypermethrin and the carbamate bendiocarb, respectively, were tested against a susceptible bed bug strain.

Due to their low mammalian toxicity and a rapid knockdown effect, pyrethroid-containing insecticides are primarily used for bed bug control (Kilpinen et al. 2011, Davies et al. 2012, Dang et al. 2014). Concurrently, pyrethroid resistance in bed bugs has been described worldwide (Boase 2008, Lilly et al. 2009, Zhu et al. 2010,

Kilpinen et al. 2011, Tawatsin et al. 2011, Durand et al. 2012, Dang et al. 2015, Balvin and Booth 2018), sometimes with resistance ratios (RRs) ranging from thousands to several hundred thousand (Romero et al. 2007, Lilly et al. 2009, Adelman et al. 2011, Koganemaru et al. 2013). In recent years, German pest control companies also report increasing difficulties in controlling bed bugs using only pyrethroids (German Pest Control Associations, personal communications).

Beside methods like topical application (Lilly et al. 2009), contact bioassays are widely used to monitor physiological insecticide susceptibility or resistance in bed bugs under laboratory conditions (Romero et al. 2007, Seong et al. 2010, Durand et al. 2012, Dang et al. 2014). For example, in the filter paper contact bioassay developed by Romero et al. (2007), individual bed bugs are placed in small separate chambers. Contrary to their natural behavior, they are not able to aggregate and thus are in contact to the treated surface during the whole trial period. Therefore, this method is suitable for determination of resistance levels in bed bugs but allows no conclusions on the actual treatment efficacy in practice. The latter might be further modified, e.g., by insecticide avoidance behavior which cannot be monitored in this bioassay. So far, there is no method for determination of phenotypic resistance in bed bugs under practical conditions.

Therefore, the new simulated-use test was also used to obtain data about the occurrence of phenotypic pyrethroid resistance in bed bugs in Germany. For this purpose, RRs for deltamethrin of bed bug field strains collected in infested apartments in Berlin were initially determined in a filter paper contact bioassay. The simulated-use test was then used to ascertain whether the strain with the highest RR for deltamethrin would also show cross-resistance to another pyrethroid. For this purpose, alpha-cypermethrin, the most widely used insecticide against bed bugs in Germany, was applied.

Materials and Methods

Bed Bugs

The insecticide-susceptible laboratory *C. lectularius* strain of the German Environment Agency (UBA) is kept in the laboratory since 1947, with nine supplemental additions to the genetic pool over the past 48 yr. This strain was used for the evaluation of the novel simulated-use test system, the no-choice residual surface test, and as a reference in the 24-well filter paper contact bioassay.

To determine the RRs of bed bugs from infested locations in Berlin, Germany, bed bugs were collected and reared in the laboratory without insecticide selection pressure. Five field strains (named by the location where they were collected: LB, HO, SK, OB, and AS) were maintained in the laboratory. Bed bugs of the strains LB, AS, OB, and HO originated from individual infested apartments. In contrast, the SK strain consisted of bed bugs pooled from four separate infested apartments located in different districts of Berlin, due to insufficient numbers of bed bugs at the respective infestation sites as starting material for breeding of a laboratory strain. Sufficient numbers of bed bugs for determination of the resistance status were obtained about 1 yr after (about six generations) initial introduction in the laboratory. To detect a possible phenotypic cross-resistance to alpha-cypermethrin bed bugs of the AS strain showing the highest RR for deltamethrin were also exposed to alpha-cypermethrin in the novel simulated-use test system.

All strains were kept in petri dishes with two filter paper discs (grade No. 1, 70 mm diameter, Whatman, Maidstone, United Kingdom) in an incubator (24 h darkness; 25 ± 3°C and 45 ± 10% humidity) and were fed weekly on rabbits (*Oryctolagus cuniculus* f. dom.). Within 1 wk after the last blood meal, eggs were deposited on the filter paper discs. Filter papers with the eggs were then removed and incubated in separate petri dishes (24 h darkness; 32 ± 2°C and 45 ± 10% humidity). Every petri dish was sorted by date and generation. Thus, the time of the last blood meal and age of the bed bugs could be determined. After seven feedings (6 wk) the majority of the bed bugs were adult and individuals that were used for bioassays had a maximum difference in age of 7 d. The filter paper contact bioassay was performed 8 d after their last blood meal while in the simulated-use test with three consecutive replicates bed bugs were used 7, 8, and 9 d after their last blood meal, respectively.

Simulated-Use Test

To simulate a bed bug control situation by insecticides under practical conditions, bed bugs were introduced into a simulated-use test system (Fig. 1). Bed bugs were attracted by a source of CO_2 and heat to cross the insecticide-treated surface. This test system was set up according to the requirements of the Guidance on the Biocidal Products Regulation (ECHA 2018).

The simulated-use test system (Fig. 1) consisted of a cylindrical container standing on a plastic tube (Fig. 1, a). The container was made of acrylic glass with a removable lid (Fig. 1, b). On the back of the cylinder, an acrylic glass pipe (5 cm diameter) was located at a height of 10 cm and closed with a plug (Fig. 1, c). Inside this plug, an extraction hose (1 cm diameter) with a total length of 1 m was connected to a suction pump. Another pipe (5 cm diameter) was located at the front of the same cylinder at ground level (Fig. 1, d). This 10-cm-long acrylic glass pipe was connected also on ground level to an acrylic glass box with a lid (Fig. 1, e). In the ground of this box, a round opening (5 cm diameter) was situated which was closed with gaze and used for easy removal of flexible surfaces treated with insecticide. At the opposite end of the box, a second 20-cm-long acrylic glass pipe (5 cm diameter) was connected to the ground level of the acrylic glass box. This pipe (Fig. 1, f), in turn, was connected to a steel container with lid (Fig. 1, g) and protruded at a height of 20 cm above the ground by 10 cm into the container. Under the open end of this pipe, a catch basin (glass aquarium) with two round filter papers inside was placed (Fig. 1, i). For CO_2 supply (0.75 liter/min), a plastic hose (0.5 cm diameter) was inserted in one of several vent holes on the back (1 cm diameter) of the steel container and fixed 5 cm in front of the open end of the pipe (Fig. 1, h). To warm up the air inside the steel container, 300 ml of water in an Erlenmeyer flask were maintained at 80°C by means of a heating plate (Fig. 1, j).

Wallpaper is often treated with insecticides during control measures since bed bugs frequently cross these surfaces when moving between hiding places and hosts. Therefore, pieces of wallpaper (19 cm × 9 cm, woodchip wallpaper, Erfurt und Sohn KG, Wuppertal, Germany) were treated according to the label recommendations with an alpha-cypermethrin-containing product (Fendona SC, 1.5 µg alpha-cypermethrin per cm^2, BASF, Ludwigshafen, Germany) or a bendiocarb-containing product (Ficam W, wettable powder, 15 µg bendiocarb per cm^2, Bayer Environmental Science, Langenfeld, Germany) commonly used for bed bug control in Germany. Before being glued (double-sided tape) into the acrylic glass box, wallpapers were allowed to dry for 1 h. The simulated-use test was conducted with freshly sprayed wallpaper and then repeated after 1 and 2 wk using the remaining wallpapers to test the residual efficacy of the product. Wallpapers were stored at room temperature in a dark place. Experiments were conducted in three replicates on consecutive days (n = 300 bed bugs). Bed bugs (50 females and males each) were transferred to a pocket made of kitchen towel and tape. The

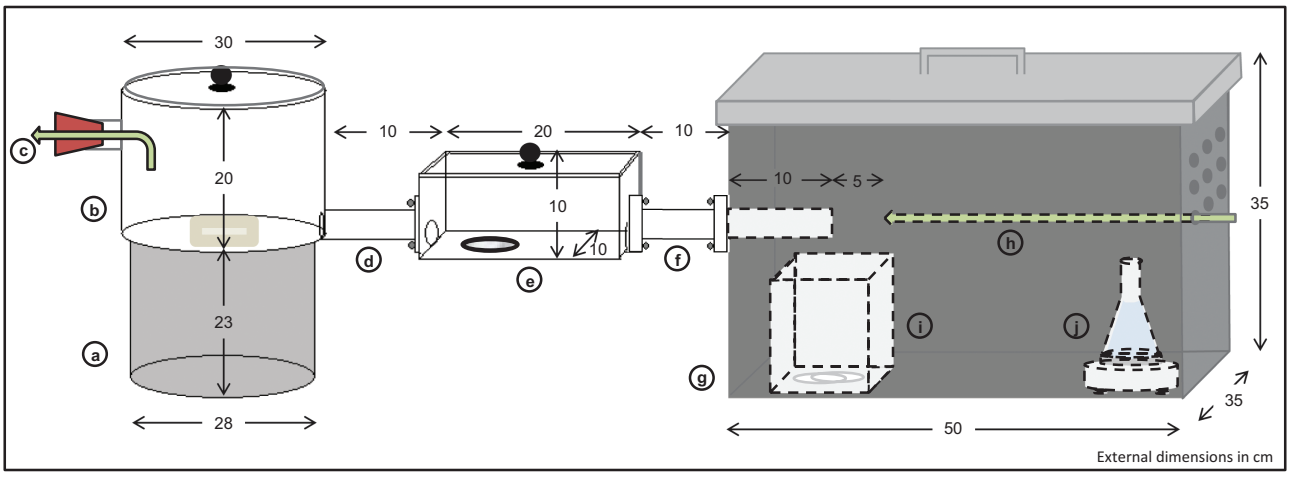

Fig. 1. Schematic representation of the simulated-use test system (not drawn to scale). a, plastic tube, base of the cylindrical container; b, cylindrical container made of acrylic glass with bed bug hiding place inside; c, acrylic glass pipe (5 cm diameter) closed with a plug with an extraction hose (1 cm diameter); d, acrylic glass pipe (5 cm diameter) which connected the cylindrical container and the acrylic glass box; e, acrylic glass box with a hole in the bottom closed with gaze for flexible surfaces (e.g., wallpaper); f, acrylic glass pipe (5 cm diameter) which connected the acrylic glass box and the steel container; g, steel container with lid and vent holes (0.1 cm diameter); h, plastic hose for CO_2 supply (0.5 cm diameter); i, catch basin with two round filter papers inside; j, heating plate with Erlenmeyer flask containing water (300 ml).

closed pocket was fixed with tape on the ground of the acrylic glass cylinder 15–30 min before the test began. To start the test, the pocket was cut open.

Bed bugs were removed 24 h post-exposure and placed into petri dishes for further examination. Since preliminary tests had shown that alpha-cypermethrin and bendiocarb had no repellent activity on bed bugs in the simulated-use test, we assumed that bed bugs, which were found in the pocket, had stayed in the harborage and had not been affected by the insecticide. Therefore, these individuals were excluded and only bed bugs which had crossed the treated surface and thus were found inside the box or the catch basin were included in determination of efficacy. As natural mortality control, 100 bed bugs of the respective strain in an equal sex ratio were placed in petri dishes in direct proximity to the trial system. As efficacy control, the number of lethally affected bed bugs was determined daily by forceps stimulation of the bed bugs for a total of 7 d. Individuals were categorized as vital by showing a normal movement behavior or as lethally affected (lethal damage) when no or only uncoordinated movement was observed and when bed bugs in dorsal position were not able to turn back into ventral position.

No-Choice Residual Surface Test

To confirm results obtained by the simulated-use test, efficacy of alpha-cypermethrin and bendiocarb was evaluated against the UBA strain with a typical no-choice residual surface treatment (Habedank et al. 2011). In contrast to the simulated-use test, wallpaper pieces (10 cm × 10 cm) in the no-choice tests were placed in petri dishes (14 cm diameter). A glass ring (height 2 cm; 8 cm diameter) was placed on top of each wallpaper to prevent bed bugs from escaping.

In three replicates, 12 bed bugs in an equal sex ratio had forced contact to freshly alpha-cypermethrin- and bendiocarb-treated wallpapers or wallpapers aged for 1 and 2 wk after spraying. Treatment of the wallpaper and efficacy control were conducted as described in the section *Simulated-Use Test*. Preliminary tests demonstrated poor efficacy against bed bugs having short contact to bendiocarb-treated wallpapers. For this reason, an exposure time of 2 h was chosen.

24-Well Filter Paper Contact Bioassay

For determination of the RRs, male bed bugs of the five field strains and the UBA reference strain were exposed to deltamethrin on filter papers (1.6 cm diameter round filters [2.011 cm^2] punched out of Whatman grade 589/1 filter paper circles, 15 cm diameter, Whatman, Maidstone, United Kingdom) in 24-well cell culture plates for 24 h (adapted from Romero et al. [2007]). In contrast to other insecticides such as DDT, crystallization of acetone-diluted deltamethrin applied on sorptive surfaces like filter paper has no influence on the lethal damage of bed bugs (Dang et al. 2017b). Based on a preliminary concentration range test, final concentrations of deltamethrin (technical grade, 99% active ingredient, Bayer CropScience, Monheim, Germany) diluted in 50 µl acetone (for analysis, 99.8%, Merck KGaA, Darmstadt, Germany) were 0.012, 0.025, 0.124, 0.249, and 2.486 µg/cm^2. At these concentrations, no crystallization of deltamethrin was observed. Vehicle controls consisted of acetone-treated filter papers only. All filter papers were allowed to dry for 30 min under a laboratory fume hood, before being placed on the bottoms of 24-well cell culture plates with forceps. Each concentration was tested in five replicates on separate plates by exposition of 18 individual bed bugs to deltamethrin and six bed bugs as vehicle controls on one plate (total number of bed bugs: n = 90 insecticide-exposed bed bugs and n = 30 as vehicle controls for each concentration). Plates were closed and stored in darkness at room temperature. At 24 h lethal damage was determined by gently touching each bed bug with forceps. Efficacy control was conducted as described in the section *Simulated-Use Test* (bed bugs did not recover).

Statistical Analyses

For the data obtained in the simulated-use test and no-choice test with alpha-cypermethrin and bendiocarb, possible relationships of variables were initially calculated using the mid-P exact test (α = 0.05) with the free online statistic software OpenEpi (Dean et al. 2013). The resulting P values were adjusted applying Holm correction using p.adjust in R (R Core Team 2013).

In addition, for the resistance studies with alpha-cypermethrin, a multivariate analysis was performed to determine the influence of the explanatory variables *strain* (AS field strain and UBA strain), *age*

of the insecticide on the wallpaper (freshly sprayed, aged for 1 and 2 wk), *sex*, and *observation day* (24 h and 7 d) on the target variable *lethal damage*. For this purpose, a binary logistic regression was calculated using the glm command in R (R Core Team 2013).

To identify significant effects of categorical variables with more than two levels, a Wald test was conducted ($\alpha = 0.05$). Different nested logistic regression models were compared with the likelihood ratio test. Models were optimized by stepwise elimination of variables to minimize the Akaike's Information Criterion (AIC). For a descriptive presentation of effects, odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated.

Mean effective concentrations (EC_{50}) and Hill slopes (HS), a measure for the steepness of a dose-response curve, were calculated using four parameter logistic regression analysis (logit) in GraphPad Prism 7.04 for Windows (GraphPad Software, La Jolla, CA, www.graphpad.com). Resistance ratios (RR = EC_{50} resistant strain/EC_{50} susceptible UBA reference strain) with its 95% CI were calculated using the free web calculator GraphPad QuickCalcs (GraphPad Software 2018). Statistical differences between the EC_{50} values of each field strain and the susceptible UBA strain were calculated by a sum of square *F*-test (GraphPad Prism 7.04 for Windows) and *P* values were adjusted using the Holm correction. The maximum percentages of lethally affected *C. lectularius* at a concentration of 2.486 µg/cm² were tested for statistical differences ($\alpha = 0.05$) with a Kruskal–Wallis test and Dunn's post hoc test (GraphPad Prism 7.04 for Windows).

Results

Evaluation of the Simulated-Use Test With Alpha-Cypermethrin

On alpha-cypermethrin-treated wallpapers, which were freshly sprayed or 1 and 2 wk aged, 80% ($n = 241$), 94% ($n = 281$), and 84% ($n = 253$) of the UBA strain bed bugs, respectively, crossed the treated wallpaper. This indicates that there was no repellent activity of the product which is generally defined as >80% repellency against various insects (ECHA 2018). Bed bugs showed no natural control mortality.

For those bed bugs that crossed the wallpaper, possible relationships within the simulated-use test were calculated using mid-*P* exact tests. Lethal damage was observed 24 h post-exposure for 99.3, 100, and 96% of the bed bugs on wallpaper freshly sprayed with alpha-cypermethrin, aged for 1 or 2 wk, respectively (Fig. 2a). Similar values of 99.7, 100, and 100% were obtained at day 7 (Fig. 2b).

However, statistically significant differences were only found between 1 and 2 wk aged alpha-cypermethrin on wallpaper 24 h post-exposure ($P < 0.05$). Furthermore, statistically significant differences in lethal damage of bed bugs were found on 2 wk aged insecticide between 24 h and 7 d post-exposure ($P < 0.05$).

A difference in lethal damage between male and female bed bugs of the UBA strain was only observed 24 h post-exposure on 2 wk aged alpha-cypermethrin, when significantly more male bed bugs were vital ($P < 0.05$). These results are provided in Supp Table S1 (online only).

Evaluation of the Simulated-Use Test With Bendiocarb

In the simulated-use test with bendiocarb, 71.1% ($n = 212$), 71.7% ($n = 213$), and 54.0% ($n = 162$) of the UBA strain bed bugs crossed the freshly sprayed or 1 or 2 wk aged wallpaper, respectively. This indicates that there was no repellent activity of the product (ECHA 2018). Natural control mortality was below 2%.

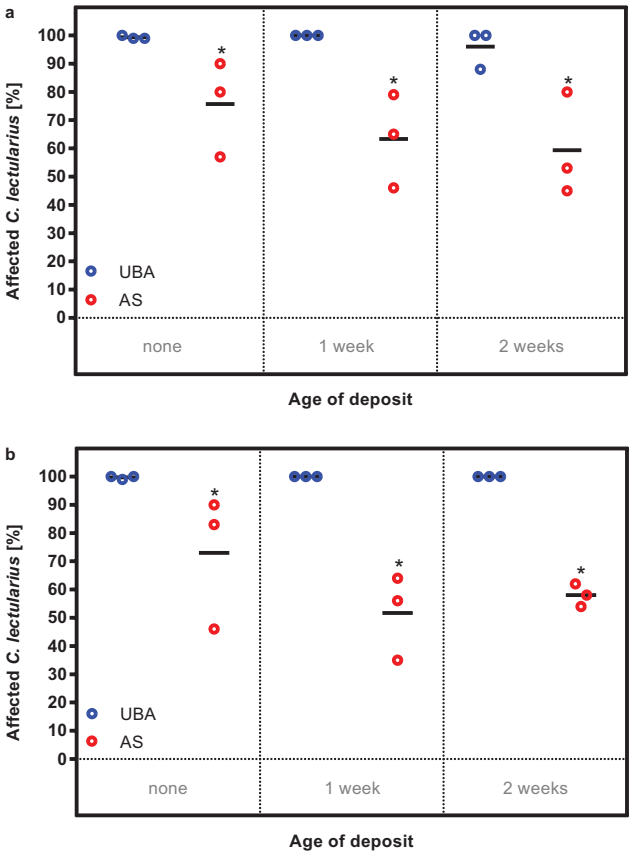

Fig. 2. Effectiveness of alpha-cypermethrin against bed bugs (UBA and AS strain) in a simulated-use test on wallpaper (a) 24 h post-exposure and (b) 7 d post-exposure. The circles indicate biological replicates and the horizontal lines represent the means. Mid-*P* exact tests with resulting *P* values adjusted using Holm correction. *$P < 0.001$.

In contrast to the complete efficacy of alpha-cypermethrin, only 44, 46, and 28% of the UBA strain bed bugs showed lethal damage 24 h after contact with freshly bendiocarb-treated wallpaper or wallpaper aged for 1 or 2 wk, respectively. On day 7, these values were virtually identical ($P > 0.05$) with 45.3, 46, and 29%, respectively. Furthermore, female bed bugs of the UBA strain exposed to bendiocarb-treated wallpapers laid eggs during the 7 d of efficacy control.

Differences in numbers of lethally affected bed bugs between 24 h and 7 d were not found to be statistically significant in all aging stages of bendiocarb on wallpaper, respectively. Statistically significant differences between the different aging stages of bendiocarb on wallpaper were only found between 1 and 2 wk aged bendiocarb on wallpaper 7 d post-exposure ($P < 0.05$) with 17% less affected bed bugs on 2 wk aged insecticide-treated wallpaper.

24 h and 7 d post-exposure, differences in numbers of lethally affected bed bugs treated with alpha-cypermethrin or bendiocarb were found to be highly statistically significant with $P < 0.0001$ in all aging stages of the insecticides on the wallpaper (Fig. 3), respectively.

In contrast to tests with alpha-cypermethrin, in experiments with bendiocarb lethal damage was significantly different between both sexes. In male bed bugs, lethal damage was observed 24 h post-exposure for 58.3, 70.3, and 37.3% of the bed bugs on wallpaper freshly sprayed with bendiocarb, aged for 1 or 2 wk, respectively. Similar values of 59, 70.3, and 38.7% were obtained at day 7. In contrast, 24 h and 7 d post-exposure lethal damage in female bugs was only 28.7, 28, 16.3% and 30.7, 28, and 16.3%, respectively (Fig. 4).

Proportion of lethally damaged individuals differed highly significantly between male and female bed bugs for all aging stages of the bendiocarb-treated wallpaper 24 h post-exposure ($P < 0.0001$) (Fig. 4a). On day 7, differences were also significant for freshly treated ($P < 0.05$), 1 wk ($P < 0.0001$), and 2 wk ($P < 0.05$) aged wallpaper treated with bendiocarb (Fig. 4b).

Fig. 3. Effectiveness of alpha-cypermethrin and bendiocarb against bed bugs (UBA strain) 7 d post-exposure in a simulated-use test on wallpaper. The circles indicate biological replicates and the horizontal lines represent the means. Mid-*P* exact tests with resulting *P* values adjusted using Holm correction. **P* < 0.001.

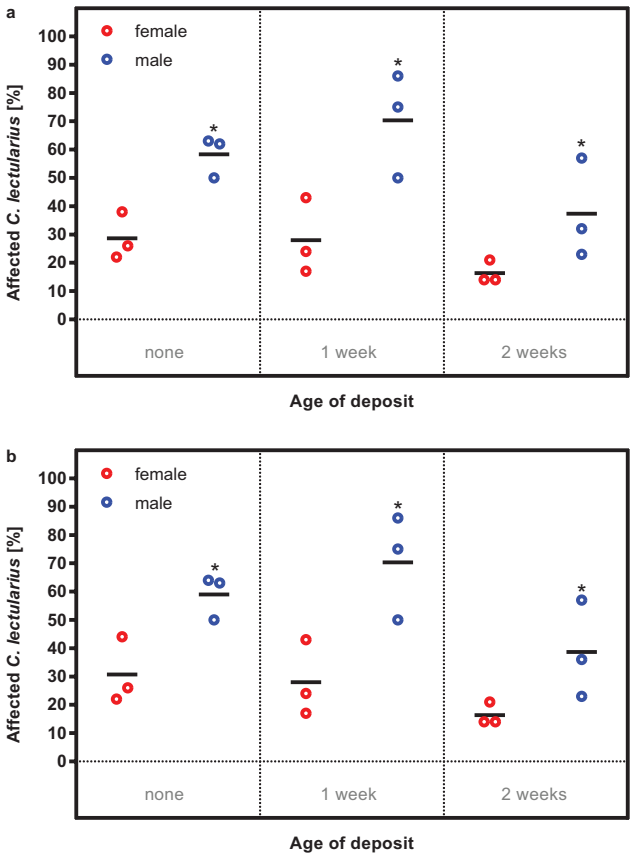

Fig. 4. Effectiveness of bendiocarb against male and female bed bugs (UBA strain) in a simulated-use test on wallpaper (a) 24 h post-exposure and (b) 7 d post-exposure. The circles indicate biological replicates and the horizontal lines represent the means. Mid-*P* exact test with resulting *P* values adjusted using Holm correction. **P* < 0.001.

No-Choice Test to Confirm Simulated-Use Test Results

Lethal damage was observed in 100% of the UBA strain bed bugs, 24 h and 7 d post-exposure on wallpaper freshly sprayed with alpha-cypermethrin or aged for 1 or 2 wk. In contrast to this complete efficacy, 24 h post-exposure only 55.3, 2.7, and 0.0% of the bed bugs showed lethal damage after forced contact to freshly bendiocarb-treated wallpaper pieces or aged for 1 or 2 wk. Similar values of 55.3, 2.7, and 2.7% were obtained at day 7, respectively (Fig. 5). Natural control mortality was below 2% in both tests.

At 24 h and 7 d, the differences in numbers of lethally affected bed bugs treated with alpha-cypermethrin or bendiocarb were statistically significant with $P < 0.05$ on freshly treated wallpapers and $P < 0.0001$ on insecticide-treated wallpaper aged for 1 or 2 wk (Fig. 5).

Differences in numbers of lethally affected bed bugs between 24 h and 7 d post-exposure were not found to be statistically significant in all aging stages of alpha-cypermethrin and bendiocarb on the wallpaper, respectively. Also, comparison of lethal damage between male and female bed bugs revealed no statistically significant differences.

Determination of Susceptibility to Deltamethrin in the 24-Well Filter Paper Contact Bioassay

Since there were no significant differences between all the measured EC_{50} values from the susceptible UBA strain data sets tested in parallel to each field strain, the results for the UBA strain were merged into one data set. There was no mortality in the vehicle controls in any of the bioassays. The EC_{50} value of the insecticide-susceptible UBA strain was low (0.081 µg/cm²). EC_{50} values of the bed bug field strains ranged between 0.352 µg/cm² (LB) and 1.682 µg/cm² (AS). For all field strains, EC_{50} values were significantly higher ($P < 0.0005$) than for the UBA strain (Fig. 6; Table 1). Resistance ratios of the field strains ranged between 4.34 (LB) and 20.73 (AS) (Table 1). Furthermore, Hill slopes of the regression curves for all field strains were also lower than for the UBA strain (Table 1), but differences were only significant for the three strains showing the highest RRs (SK, OB, and AS).

The mean percentages of lethally affected *C. lectularius* at the highest concentration of 2.486 µg/cm² from the LB, HO, SK, OB, and AS field strains were approximately 8, 10, 22, 28, and 40%

Fig. 5. Effectiveness of alpha-cypermethrin and bendiocarb against bed bugs (UBA strain) 7 d post-exposure in a no-choice test on wallpaper. The circles indicate biological replicates and the horizontal lines represent the means. Mid-*P* exact test with resulting *P* values adjusted using Holm correction. **P* < 0.001.

lower than that of the susceptible UBA strain (Fig. 7). These differences were only significant for the three field strains SK ($P = 0.0023$), OB ($P = 0.0003$), and AS ($P < 0.0001$).

Determination of Phenotypic Resistance With the Simulated-Use Test Using Alpha-Cypermethrin

On alpha-cypermethrin-treated wallpapers, which were freshly sprayed or 1 and 2 wk aged, 71% ($n = 212$), 42% ($n = 126$), and 69% ($n = 207$) of the AS strain bed bugs, respectively, crossed the treated wallpaper. There was no natural control mortality.

For those bed bugs that did cross the wallpaper, possible relationships within the simulated-use test were calculated using mid-*P* exact tests. In contrast to the complete efficacy in the UBA strain, 24 h post-exposure only 75.7, 63.3, and 59.3% of the AS strain bed bugs showed lethal damage after contact with freshly alpha-cypermethrin-treated wallpaper or wallpaper with insecticide aged for 1 or 2 wk (Fig. 2a). The corresponding values for 7 d were 73.0, 51.7, and 58.0% (Fig. 2b). In comparison to alpha-cypermethrin efficacy on freshly sprayed wallpaper, bed bugs from the AS field strain were 24 h and 7 d post-exposure significantly less affected on surfaces treated with insecticide and aged for 2 wk ($P < 0.0001$ and $P < 0.05$).

Statistically significant differences between the insecticide efficacy on freshly sprayed wallpaper and insecticide aged for 1 wk were found 7 d ($P < 0.001$) but not 24 h post-exposure ($P = 0.1057$) in bed bugs from the AS strain. However, no statistically significant differences between 1 and 2 wk aged alpha-cypermethrin on wallpaper were found. Comparison of lethal damage between male and female bed bugs revealed no statistically significant differences.

24 h and 7 d post-exposure, differences in numbers of lethally affected bed bugs between the UBA and AS strain were found to be highly statistically significant with $P < 0.0001$ in all aging stages of the insecticide on the wallpaper (Fig. 2), respectively. Moreover, female bed bugs of the AS field strain exposed to alpha-cypermethrin-treated wallpaper laid eggs during the 7 d of efficacy control.

In addition to the mid-*P* exact tests, the influence of the explanatory variables *strain*, *age of the treated wallpaper*, *sex*, and *examination day* on the target variable *lethal damage* was tested using a binary logistic regression. A generalized linear model without interactions of explanatory variables was calculated. The reference categories were the susceptible UBA strain, freshly sprayed wallpaper, male bed bugs, and the efficacy control 24 h post-exposure (Fig. 8). When comparing both strains, the odds that bed bugs from the AS field strain got lethally affected during the simulated-use test was statistically significantly lower by factor 0.0148 (95% CI: 0.00821–0.0246, $P < 2.0 \times 10^{-16}$) as compared with the susceptible UBA strain. Within this model also the odds that bed bugs which crossed insecticide-treated wallpaper aged 1 or 2 wk got lethally affected was statistically significantly lower by factor 0.469 (95% CI: 0.339–0.647; $P = 4.47 \times 10^{-6}$) and 0.466 (95% CI: 0.349–0.621; $P = 2.1 \times 10^{7}$), respectively. No significant differences concerning bed bug lethal damage between insecticide-treated wallpaper aged for 1 or 2 wk were found (overlapping CIs, Fig. 8). The explanatory variables *sex* and *examination day* had no significant influence on the target variable *lethal damage* within this model (Fig. 8) but were still included since they did at least not increase the AIC which was 1554.3. Inclusion of different interactions between variables increased the AIC value.

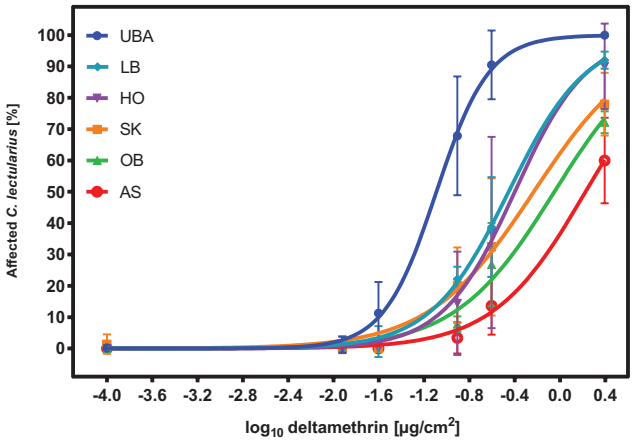

Fig. 6. Dose-response relationship between deltamethrin (µg/cm²) and *C. lectularius* (%) from the susceptible UBA strain and the five field strains determined in the 24-well filter paper contact bioassay. The means and SDs of the number of lethally affected bed bugs are shown for the respective deltamethrin concentration. EC_{50} values and Hill slopes (HS) of the regression curves were calculated by using logistic regression analysis and RRs were determined (Table 1).

Discussion

The present study describes the first development of a simulated-use test for efficacy testing of products against bed bugs including

Table 1. Evaluation of lethal effects of deltamethrin on the bed bugs of the five field strains (SK, OB, HO, LB, and AS) and the susceptible UBA strain using the 24-well filter paper contact bioassay

Strain	n^a	HS^b ± SE	*P* value^c	$EC_{50}{}^d$ [µg/cm²] (95% CI^e)	*P* value^f	R^{2g}	RR^h (95% CI)
UBA	1,710	1.872 ± 0.134	–	0.081 (0.073–0.090)	–	0.95	–
LB	450	1.262 ± 0.136	0.0818	0.352 (0.292–0.425)	0.0005	0.9634	4.34 (2.616–9.737)
HO	450	1.361 ± 0.322	0.221	0.399 (0.260–0.613)	0.0005	0.8476	4.92 (2.372–11.637)
SK	450	0.939 ± 0.120	0.0005	0.587 (0.413–0.835)	0.0005	0.8802	7.24 (4.288–16.318)
OB	450	0.994 ± 0.074	0.0005	0.892 (0.726–1.096)	0.0005	0.9502	11.00 (7.024–24.279)
AS	450	1.061 ± 0.113	0.0207	1.682 (1.351–2.095)	0.0005	0.9156	20.73 (13.360–45.653)

[a]Total number of insects used.
[b]HS, Hill slope.
[c]Significant differences in Hill slopes compared to the UBA strain.
[d]EC_{50}, half maximal effective concentration.
[e]95% CI, 95% confidence interval.
[f]Significant differences in EC_{50} compared to the UBA strain.
[g]R^2, coefficient of determination. The value R^2 quantifies goodness of fit.
[h]RR, resistance ratio.

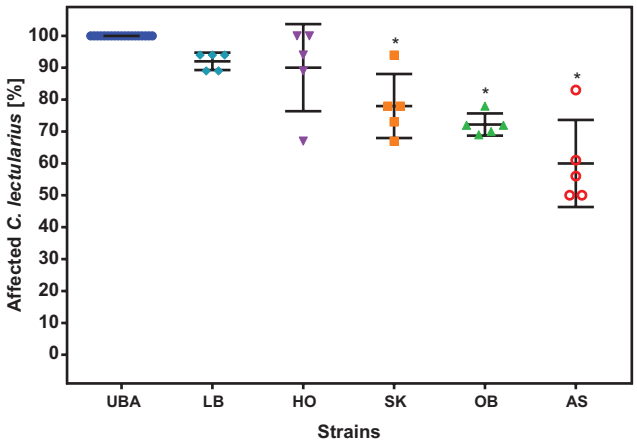

Fig. 7. The percentage of lethally affected *C. lectularius* from the susceptible UBA strain and the five field strains collected in Berlin. Bed bugs were tested in the 24-well filter paper contact bioassay at the highest deltamethrin concentration of 2.486 µg/cm² for 24 h. Results of five replicates were compared with the Kruskal–Wallis test and Dunn's post hoc test. Lines indicate the mean and error bars indicate the SD. $*P < 0.01$.

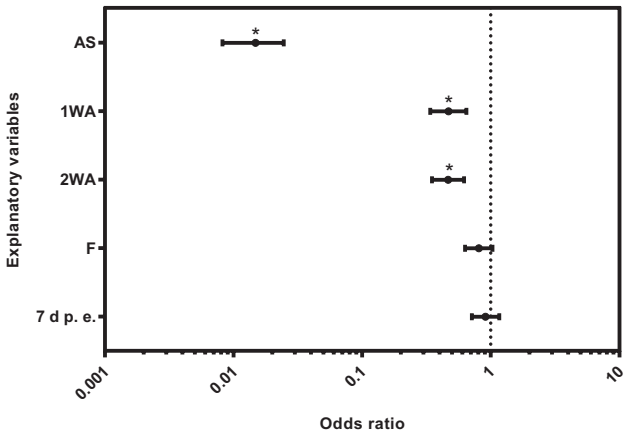

Fig. 8. Comparison of the effectiveness of alpha-cypermethrin applied on wallpaper in a simulated-use test against *C. lectularius* from the susceptible UBA strain (reference strain) and the AS field strain. Here, odds ratios with 95% CIs on the target variable *lethal damage* concerning the influence of the explanatory variables were obtained by binary logistic regression. Wallpapers were either used on the day of insecticide spraying (reference level) or after aging for 1 (1WA) or 2 (2WA) wk. Other variables in the final model were the sex (F = female, reference level male) and the time point of examination after exposure (7 d post-exposure, reference level 24 h post-exposure). Significant differences between levels of the same variable were obtained in a *t*-test as implemented in the regression analysis. $*P < 0.001$.

detection of pyrethroid resistance. With 42–94% of the bed bugs crossing the insecticide-treated surface, the simulated-use test imitates a typical insecticide barrier treatment against bed bugs involving insecticides with residual properties under controlled and reproducible as well as practically relevant conditions.

To evaluate the simulated-use test system, the insecticides Fendona SC (alpha-cypermethrin) and Ficam W (bendiocarb) were chosen due to different properties and modes of action of these residual sprays. The experiments with alpha-cypermethrin and bendiocarb were not conducted to test the efficacy of different types of insecticides, but rather the acquisition of data from different insecticides should evaluate the realistic simulation of a praxis situation in the simulated-use test. The results show that regardless of the age of the insecticide on wallpaper at day 7 significantly more bed bugs were lethally damaged after crossing alpha-cypermethrin-treated wallpaper. The no-choice tests with alpha-cypermethrin- and bendiocarb-treated wallpaper produced similar results and thus substantiate the results of the simulated-use test. To validate the lab technique field tests with these insecticides are needed.

In contrast to no-choice tests, the advantage of the new test system is that bed bugs do not aggregate on the treated surface. Thus, it is impossible for single individuals to avoid the treated surface by sitting on top of each other. Furthermore, contact time is more realistic, as it corresponds to the time a bed bug needs to cross a typical insecticide barrier until they have located their potential host. It could be observed that some bed bugs crossed the surface within a few seconds and others spend a longer time period walking back and forth over the treated wallpaper. An additional practical advantage of the novel assay is the small scale of the experimental system, as it allows simultaneous replicates. In particular, the system is an important contribution for efficacy assessment of type 18 products (insecticides, acaricides, and other biocides against arthropods) against crawling hematophagous arthropods in the framework of the Biocidal Product Regulation (BPR, Regulation (EU) 528/2012). For this purpose, further studies using different classes of biocides (e.g., Chlorfenapyr) and different target species are necessary. The design of the simulated-use test system (Fig. 1) suggests that it can also be used for efficacy assessment for product type 19 (repellents) using a different readout (number of individuals that crossed the barrier). However, future experiments including products with known repellent activity will be required to evaluate this. Overall, the addition of a simulated-use assay that can in principle simultaneously detect changes in insecticide susceptibility and insecticide avoidance would be a huge improvement.

It is noticeable that male bed bugs in the simulated-use test 24 h and 7 d post-exposure on bendiocarb-treated wallpapers in all aging stages suffered significantly more lethal damage than the females. In contrast, only on 2 wk aged alpha-cypermethrin-treated wallpaper 24 h post-exposure significantly more male bed bugs were vital. Moreover, in the no-choice tests with both insecticides, no statistically significant differences in lethal damage between male and female bed bugs were revealed. The latter might be explained by the long exposition period of 2 h.

Alpha-cypermethrin showed in both test systems a very strong knockdown effect on both sexes so that possible differences in lethal damage between male and female bed bugs are difficult to detect. The application of lower doses might identify possible differences in lethal damage between males and females. However, differences in lethal damage between both sexes seem to depend on the respective insecticide and might also involve sex-specific detoxification mechanisms known to occur in other insects (Pruett et al. 2001, Le Goff et al. 2006).

The present study provides first evidence for the occurrence of phenotypic pyrethroid resistance in bed bugs collected in Berlin. The filter paper contact bioassay revealed that differences in the EC_{50} values for deltamethrin between the susceptible UBA strain and the five field strains LB, HO, SK, OB, and AS were statistically highly significant. In addition, the Hill slopes of the three strains with the highest EC_{50} values (SK, OB, AS) were also significantly lower compared to the UBA strain. Genetic diversity concerning resistance mechanisms of strains can strongly affect the Hill slope. While on one extreme end clonal organisms would exhibit no biological variation and all show lethal damage at the same concentration, resulting in extremely steep concentration-response curves, strains with high genetic diversity will show lethal damage of some individuals at low

and others at high concentrations, resulting in flat concentration-response curves. Thus, low Hill slopes might reflect high genetic diversity and it is therefore not surprising that the strain with the highest Hill slope has the longest history of laboratory rearing.

Regarding EC_{50} values, it has to be noted that the RRs between 4.3 and 20.7 observed in our study are considerably lower than those reported from the United States, amounting, e.g., to 5,200 (Adelman et al. 2011) and about 12,800 (Romero et al. 2007) or even higher than 432,000 in Australia (Lilly et al. 2009). To obtain valid data from bioassays in the present study, the use of high numbers of bed bugs was essential, but it was not possible to obtain sufficient individuals from the infested collection sites. Thus, a rearing period of 1 yr was unavoidable. It can be reasonably assumed that the actual resistance levels of the different field populations were higher at the time point when the bed bugs were collected. Another reason for low RRs obtained in the present study might be that deltamethrin-containing insecticides are not the primary choice to combat bed bugs in Germany. However, deltamethrin was chosen in the initial filter paper contact bioassay since it is one of the most widely used pyrethroids to determine RRs between bed bug strains, while there are few reports (Boase 2006, Kweka et al. 2009) on RRs for bed bugs obtained by exposition to alpha-cypermethrin. Therefore, with regard to the comparability of the tests, it was decided to use deltamethrin as an active substance for determination of the RRs in the filter paper contact bioassay. In contrast, an alpha-cypermethrin-containing product (Fendona SC) was used in the simulated-use test, since this is the product which is primarily used to control bed bugs in Germany. The results show that even a relatively low deltamethrin RR may be a hint for resistance under practical conditions against alpha-cypermethrin. For the simulated-use test with alpha-cypermethrin, the binary logistic regression confirmed that the odds that bed bugs from the AS field strain—in contrast to bed bugs of the UBA strain—were lethally affected was statistically significantly lower by factor 0.0148 (95% CI: 0.00821–0.0246, $P < 2.0 \times 10^{-16}$). The chance that bed bugs of the AS strain got lethally affected when exposed to 1 and 2 wk aged insecticide was on day 7 post-exposure even statistically significantly lower than by exposure to freshly sprayed insecticide. With regards to the general definition of insecticide resistance as the heritability of traits selected by a long-term or repeated contact to insecticides leading to a lower susceptibility of insects to these insecticides, which consequently leads to practical control problems (Sawicki 1987, Onstad 2014), the results of the simulated-use test reveal that the AS field strain is resistant to pyrethroids. Moreover, these findings suggest that the eradication of bed bug strains even with relatively low deltamethrin RRs of 20 in the filter paper contact bioassay may not be achievable in practice using pyrethroids like, e.g., alpha-cypermethrin. This is also supported by the fact that females of the AS field strain exposed to alpha-cypermethrin reproduced during the efficacy control.

In conclusion, the novel simulated-use test system provides a suitable method for efficacy testing of insecticides against bed bugs mimicking a practical bed bug control situation, which has not been available so far. Additionally, it is suitable to determine phenotypic resistance under practical conditions.

The new test described here is an important step toward regular use of such tests at least in research and here exemplified for both, alpha-cypermethrin and bendiocarb with different modes of action.

Furthermore, results confirmed the presence of pyrethroid resistant bed bugs in Germany. Therefore, resistance might be a cause for problems or failures in the control of bed bug infestations using pyrethroids, as reported by German pest control companies (German Pest Control Associations, personal communications). To prevent the development and spread of resistant bed bugs, an integrated control approach, which may also consider insecticide-free methods (e.g., heat treatment) for bed bug control, is necessary.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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