Research Article

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The influence of time and distance traveled by bed bugs, *Cimex lectularius*, on permethrin uptake from treated mattress liners

Stephen A Kells* **and Sabrina N Hymel**

Abstract

BACKGROUND: Residual insecticides interrupt the dispersal of bed bugs (*Cimex lectularius*, L.), but one of the issues encountered with residual applications is understanding the uptake of active residues by the insect. This study determined permethrin uptake by bed bugs walking on the *Active*Guard® Mattress Liner product, via a combination of video recording in arenas and gas chromatography analyses.

RESULTS: The best model for estimating permethrin uptake utilized a covariance model ($r^2 = 0.469$) with two factors: time of exposure ($F_{1,55} = 2.44$, $P < 0.001$) and distance traveled ($F_{1,55} = 0.30$, $P = 0.0460$). Bed bug permethrin uptake was 15.1 (95% CI: 10.3–22.1) ng insect^{-1} within 1 min exposure, 21.0 (15.0, 31.0) ng insect^{-1} within 10 min and ≈ 42 (29.8, 60.6) ng insect^{-1} within ≥50 min exposure. Correcting for percentage recovery, these values would be increased by a factor of 1.21.

CONCLUSION: This permethrin-treated fabric provides a surface from which bed bugs begin rapidly to absorb permethrin on contact and within the first 1 cm of travel. Variability in uptake was likely a result of grooming and thigmotaxis, and future work should use quantitative methods to study behaviors and formulations that increase exposure to the toxicant.
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Keywords: behavior; *Cimex lectularius*; Cimicidae; gas chromatography; insecticide-treated fabric; toxicity; video analysis

1 INTRODUCTION

Bed bugs (*Cimex lectularius*, L.) continue to be a problem in areas where reservoirs of available insects permit a high risk of reintroduction and spread to neighboring areas.[1] As a consequence, residual insecticides have become an important tool in managing these insects.[2–5] Heat treatments, contact insecticides, steam, or fumigants can control bed bugs that are present and exposed to the agent at the time of application.[6–9] However, treated areas may be prone to reinfestation from bed bugs 'hitchhiking' into the area on personal goods (e.g. backpacks or clothing) or from bed bugs dispersing from refugia.

Historically, bed bugs were removed from mainstream society not only by the use of synthetic residual insecticides[10] but also by the extent to which these insecticides were used; specifically, broad-spectrum insecticides were applied throughout the entire infested room. Earlier control approaches for societal bed bug infestations were effective in part, because there was a likelihood that insects would be in constant contact with active residues, regardless of where they traveled within the living space. However, during the recent resurgence of bed bug infestations, evolved insecticide risk assessment has changed how insecticides are used; the result is that the sites and total surface area where active insecticide residues may be applied is greatly reduced (e.g. various insecticide labels limit application to spots or cracks and crevices). These application restrictions are in place to prevent direct and extended human contact with active ingredients (AIs). However, such application methods may result in greater potential for bed bugs to reinfest premises, because insects may not contact active residues or there may be insufficient duration of contact.

As a part of an integrated pest management (IPM) program, placing residually active control products closer to sleeping surfaces may better intercept migrating bed bugs, especially those attempting to hide close to a host. One of the issues encountered with residual insecticides, though, is an understanding as to how the uptake of active residues by an insect corresponds to the resulting toxic response. Ultimately, exposure requires contact and transfer of the insecticide to active sites in the insect, which then leads to mortality through the disruption of homeostasis.[11] Many factors can affect an insect's ability to take up and accumulate an active ingredient to the point that a toxic effect is initiated. Such factors include (1) insect morphology and behavior,[3,12] (2) the formulation of the insecticide,[13] (3) the substrate type[14] and (4) the duration of contact.[15] There are also behavioral, physiological and molecular resistance mechanisms in an insect population that can reduce the effectiveness of insecticides by reducing the dose of available insecticide at active sites in the insect.[16]

* *Correspondence to: SA Kells, Department of Entomology, University of Minnesota, 219 Hodson Hall, 1980 Folwell Ave., St Paul, MN 55108, USA. E-mail: kells002@umn.edu*

Department of Entomology, University of Minnesota, St Paul, MN, USA

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Permethrin-treated mattress liner products comprise a fabric impregnated with a pyrethroid insecticide (permethrin) in a manner similar to those used for malarial bed nets.[17] However, the fabric product is shaped like a fitted sheet and can be installed on the box spring and/or mattress of a bed. One such product is the *Active*Guard® Mattress Liner (Allergy Technologies, Ambler, PA), which has been demonstrated to affect both susceptible bed bugs as well as bed bugs exhibiting pyrethroid resistance mechanisms (i.e. with *kdr* mutations).[15,18]

Typical bioassay tests only evaluate the endpoint (i.e. mortality) and do not explore the rate or amount of insecticide uptake into the insect resulting from its behavior(s) or exposure time on the treated surface. Considering the myriad of surfaces to which insecticides are applied, endpoint analysis of insecticides through bioassays may be too simplistic. As the treated fabric contains permethrin, an AI to which there are known resistance issues in bed bugs,[18] identifying insecticide uptake characteristics is important to ensure that exposure and dose are maximized and result in continued effective control and prevention. There may be one or several variables that might be illustrated by further quantitative analysis within the pathway from insecticide exposure to death or adverse effects. This study determined the amount of permethrin 'adsorbed' on and 'absorbed' into bed bugs in direct contact with permethrin-impregnated fabric. Permethrin uptake from the treated surface was assessed as a function of time and distance traveled, by using a combination of video recordings (behavioral analysis) and permethrin quantitation via GC-ECD methods (uptake of permethrin by the insect).

2 MATERIALS AND METHODS

2.1 Insect colonies

Bed bugs from the Forest Brook population were used in this study (the laboratory of Dr Susan Jones, the Ohio State University, Columbus, OH). This field population was collected from Delaware, Ohio, on 24 October 2010; the insects were determined to have two *kdr* mutations, and a genotype of V419L/L925I (Jones S, private communication, 2013). A susceptible strain of bed bugs, the ECL-05 strain (Olson J, private communication, 2010), was used during preliminary studies and development of methods. All bed bugs were maintained at 27 °C, 70% RH and 14:10 L:D photoperiod, and provided with human blood through a membrane feeding system.[19] Bed bugs used in the study had been fed 7 days prior to exposure. Adult bed bugs were removed from the rearing jars and held for 24 h in clean jars. This isolation step simplified the handling of individual bed bugs just prior to the exposure period.

2.2 Test arenas and exposure procedure

The fabric used for the study included the commercially available *Active*Guard® Mattress Liner (1.64% permethrin, EPA Reg. No. 82123-1), with the same fabric without permethrin treatment serving as the 'untreated' control. Bed bugs were exposed in arenas consisting of modified petri dishes (9 cm diameter, polystyrene; Fisher Scientific, Waltham, MA) with the bottom floor removed, leaving the lid and a plastic ring. Inverted, the lid became the floor of a circular arena with side walls. Fabric was cut to size and clamped between the floor and plastic ring; all three elements were further secured together with four spots of hot-melt glue.

Ten replicate dishes per treatment were numbered, and a random incomplete block design was used to assign the length of time for exposure (1, 10, 50, 100 and 200 min). This range of exposure times was selected because preliminary experiments with the treated fabric (data not shown) indicated rapid intoxication of the ECL-05 susceptible bed bug strain.

Care was taken to measure accurately each bed bug's exposure time to either the treated or control fabric. The procedure involved placing an individual bed bug into a clean glass test tube that was then carefully inverted onto a piece of filter paper temporarily positioned in the center of the arena. At time zero, the filter paper was carefully removed and the tube lifted, and each insect was permitted to walk onto the fabric in the arena. Insects were held for the assigned time and video recorded (see Section 2.3). As each time interval was completed, insects were carefully removed while ensuring that the forceps did not touch the surface of the fabric or the arena sides. Also, to prevent cross-contamination among insects, forceps were rinsed with acetonitrile (ACN) between each insect transfer. Each insect was sexed, weighed, placed into a new extraction test tube, freeze killed and stored at −25 °C until chemical analyses could be performed.

2.3 Video recording of insects

Recordings of bed bugs in arenas were captured with an overhead camera (Polestar II EQ610; Everfocus, Taipei, Taiwan) under infrared illumination (AT-8S-B; Axton, Salt Lake City, UT). Observers were not present in the recording area except to place and later remove insects. Video files were imported into Ethovision XT (Noldus Information Technology, Wageningen, The Netherlands), which acquired bed bug movement behavior data from the video; exporting the data as a 2010 Excel file (Microsoft, Redmond, WA) enabled further analysis through SAS v.9.4 (SAS Institute Inc., Cary, NC).

Variables recorded in the dataset included time stamp (every 0.1 s), position (x, y), distance and velocity. Further filtering of the data was performed within the SAS program to remove outliers and cases where small distances (<0.01 cm) may have been recorded, but during which the bed bug had not actually moved. Data filtering utilized the PROC UNIVARIATE command in SAS to identify outliers; individual videos were then viewed, and tracking data were collected during that time.

2.4 Permethrin extraction procedure

Extraction procedures followed USEPA standard methods for extraction and determination of residual level of insecticides from treated media (soil, meat).[20] In preliminary tests (data not shown), the percentage recovery of residues from permethrin-spiked samples was assessed to determine the simplest method of recovering residues. Unexposed method controls consisted of bed bugs removed from the holding containers but not placed in any arena. These bed bugs were processed to detect any possible cross-contamination during the analytical process. Exposed method controls to calculate the percentage recovery of permethrin consisted of empty tubes and tubes containing unexposed insect samples to which 1 µL of a 5.4 mg kg^{-1} permethrin standard (Sigma Aldrich, St Louis, MO) was applied to the dorsum of the insect. The three types of method control (unexposed insects, exposed insects and blank tubes) were repeated for each experiment; prior to handling the permethrin-treated fabric, sampled insects were placed in clean test tubes and stored at −25 °C.

Preliminary studies with spiked-insect samples from the susceptible ECL-05 strain suggested that the permethrin on the treated fabric was so rapidly absorbed across the cuticle that, under the present test conditions, adsorption and sequential absorption of

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the AI could not be distinguished. Therefore, extraction of residues from the exoskeleton (i.e. adsorbed amount) and internal insect tissues (i.e. absorbed amount) utilized a whole-body extraction rather than separating the external rinse procedure from the internal extraction protocol.

Extraction procedures consisted of manually macerating the insect in 1 mL of acetonitrile (ACN; 99% ACS grade; Fisher Scientific, Pittsburgh, PA) with a prerinsed PTFE tissue grinder in a 5 mL glass test tube. The tube was then covered and soaked for 24 h in a −25 °C freezer. After soaking, the contents were emptied into a glass funnel with a glass-fiber filter that had been prerinsed and wetted with ACN. Rinsing of the residual insect material was done twice more, with approximately 2 mL of ACN per rinse. Rinsate was collected into 20 mL evaporator centrifuge tubes, and the volume was reduced to ~1 mL via nitrogen gas purging. Rinsate was then transferred to a 1 mL class A volumetric flask, and the volume was adjusted with ACN to exactly 1 mL. Each sample was pipetted into a salinized amber vial with PTFE-lined caps and stored at −15 °C until gas chromatography (GC) analyses.

2.5 GC analyses

Analysis of samples was carried out by GC-linked electron capture detection (GC-μECD, Agilent 6890N; Agilent Technologies, Santa Clara, CA). Chromatography conditions were as follows: 1 μL was injected into the injector port (250 °C, splitless, helium: 12.4 psi, 65.1 mL min^{-1}); the volatilized sample was swept onto a capillary column (HP-5, 30 m × 0.32 mm diameter × 0.25 μm film, 2.0 mL min^{-1}; Agilent Technologies). Oven conditions were initially 125 °C held for 5 min, then increased at a rate of 10 °C min^{-1} to 225 °C and held for 3 min, then increased at 25 °C min^{-1} to 300 °C and held for a final 3 min, for a total run time of 24 min. Detector conditions for the μECD were 315 °C with a nitrogen gas make-up flow of 15.0 mL min^{-1}. Control of the GC conditions and data collection and analysis were carried out using Chemstation© software v.B02 (Agilent Technologies).

Standard curves were regularly generated, and samples (≤12 samples × 2 injections each) were bracketed with blank ACN and standard curves, as there was a tendency for the μECD to become more sensitive within each run. An additional lot of method-control and spiked-insect samples was run concurrently, to rule out sample contamination as well as to calculate the percentage recovery of permethrin for the extraction and analytical methods. The average r^2 for standard curves was 0.996 (range 0.992–0.999). Level of blank (LOB) was calculated as 0.5 ng, with level of detection (LOD) at 1.5 ng and level of quantitation (LOQ) at 5.0 ng.[21] Percentage recovery for the method without insect material was 97.4%, whereas percentage recovery from spiked-insect samples was 82.7%. For the purposes of the results, a recovery correction from the spiked-insect samples was not included in the analysis; however, the significance of this correction is addressed in Section 4 below.

2.6 Statistical analyses

A series of multilinear regressions and analysis of variance (ANOVA) statistical approaches was used to evaluate bed bug movement with respect to the amount (ng $insect^{-1}$) of permethrin extracted from bed bugs. Through initial model development, an analysis of covariance (ANCOVA) was used to define the uptake of permethrin as affected by the class effects of (1) time of exposure and (2) sex of the adult bed bug. The distance traveled by the insect and the mass of the insect were treated as covariables in these models.

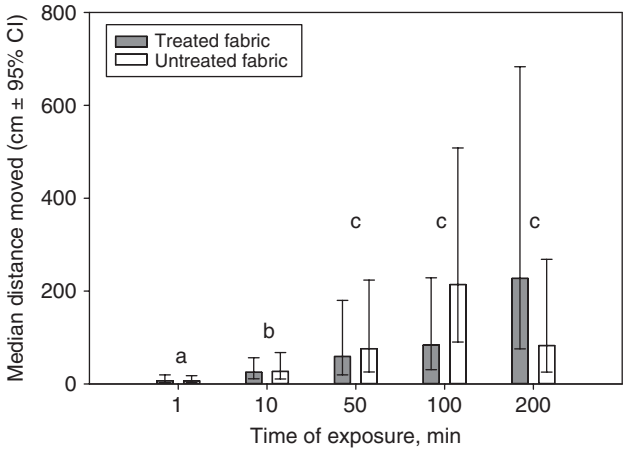

Figure 1. Median distance traveled (cm ± 95% CI) by bed bugs within each time class. Different letters illustrate significant differences in distance moved with duration in arena ($P < 0.05$); there was no significant difference between distance traveled on treated versus untreated surfaces at each exposure time ($P > 0.05$).

As effects were determined to be non-significant ($P > 0.05$), they were removed from the model, and the model was rerun with the remaining effects (or covariables).

3 RESULTS

3.1 The effect of distance and velocity on treated and untreated surfaces

The distance moved by bed bugs was significantly affected by time ($F_{4,139} = 14.22$, $P < 0.0001$), but there was no effect of treatment (treated fabric versus untreated; $F_{1,139} = 0.02$, $P = 0.8812$). The interaction of time and treated surface also was not significant ($F_{4,139} = 1.12$, $P = 0.3514$). Least-squares mean analyses (LSMEANS) indicated that movement significantly increased within the first 50 min in the arena and was stable thereafter, but there was no significant difference between treated and untreated surfaces ($\alpha = 0.05$) (Fig. 1).

3.2 Permethrin uptake

Analysis of permethrin from the method blank and unexposed bed bugs was below LOD, so cross-contamination was unlikely during sample processing. A regression using mass of permethrin regressed on distance indicated a significant effect of distance ($y = 0.0276x + 35.2$; parameter estimate *P*-values were 0.0060 and <0.0001 respectively) (Fig. 2); however, this model was a poor fit ($r^2 = 0.222$). Distance was used as a covariable in subsequent analyses of other effects on uptake of permethrin, including time, body mass and sex.

ANCOVA determined that there was no effect of body mass and sex on permethrin uptake ($F_{1,43} = 0.022$, $P = 0.9820$; $F_{1,43} = 0.020$, $P = 0.9820$ respectively). Upon reanalyzing the ANCOVA by excluding the effects of mass and sex from the model, time of exposure became a significant class effect ($F_{1,55} = 2.44$, $P < 0.0010$), and distance was a significant covariable ($F_{1,55} = 0.30$ $P = 0.0460$). With these two effects in the model, r^2 reached a maximum of 0.469. Separation of LSMEANS indicated that permethrin uptake was between 15.1 (95% CI: 10.3–22.1) ng $insect^{-1}$ and 21.0 (15.0, 31.0) ng $insect^{-1}$ within the first 1 min and 10 min exposure periods, and between 42 (29.8, 60.6) ng $insect^{-1}$ and 55 (38.5, 79.2) ng $insect^{-1}$ within 50 and 200 min of exposure respectively (Fig. 3).

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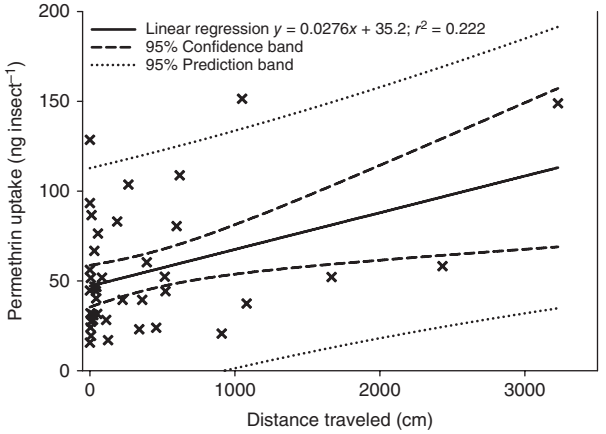

Figure 2. Overall relationship of permethrin uptake by bed bugs with distance traveled. A significant but poor-fitting linear regression model provided the best fit to the data, indicating a permethrin uptake of 35 ng (±) upon contact and 0.03 ng cm^{-1} continued uptake.

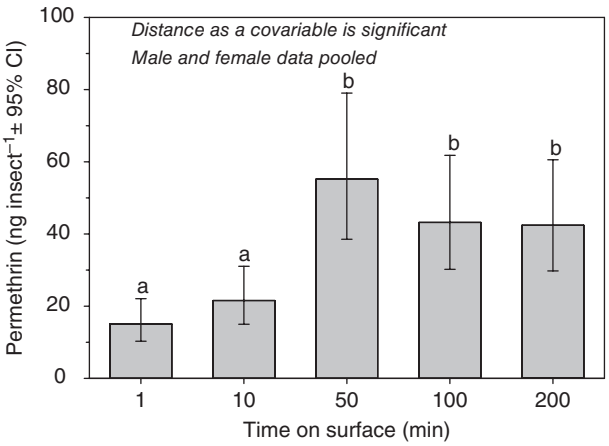

Figure 3. Permethrin uptake by bed bugs with time on surface. Analysis of covariance was carried out, with distance as the covariate. The ANCOVA-adjusted LSMEANS indicate that permethrin uptake occurred in two significant groups: 1–10 min and ≥50 min. Different letters indicate significance at $P < 0.05$. There was no significant ($P < 0.05$) effect of sex or body mass.

4 DISCUSSION

Upon exposure to the treated fabric, uptake (absorption) of permethrin exhibited by bed bugs was significantly influenced by exposure time and distance traveled. Quantifiable residues were detected in whole-body extracts of the insect within 1 min, and the amount of permethrin continued to increase for up to 50 min. Thereafter, no significant change occurred between 50 and 200 min. Correcting for the percentage recovery (i.e. amount quantitated ÷ 82.7%), an average of 25.4 (18.1, 37.5) ng had transferred to the bed bug from the treated fabric within 10 min, and permethrin uptake was 66.5 (46.6, 95.8) ng at 50 min.

Distance traveled was a significant covariable with the time of exposure, and bed bugs traveling even a short distance (<1 cm) exhibited sizeable variations in permethrin uptake (range 5.1–188.0 ng $insect^{-1}$). In addition to time and distance, the thigmotactic behavior of bed bugs[10] may be an important contributor to this variability, given that bed bugs prefer very close contact with substrates. Variability may arise from behaviors such as grooming, resting on surfaces and angling of the body relative to the surface.[10] Video recordings showed that, upon encountering the arena wall, bed bugs were observed pressing against edges or wedging themselves partially into the arena corners; such behavior may be expected to increase the potential for permethrin uptake from the treated fabric. Further study on behavioral variability will be important for determining particular bed bug traits and treatment paradigms that may enable maximum exposure to residual insecticides. For this study, arena surfaces were relatively flat, compared with the uneven surfaces that bed bugs are expected to encounter in living spaces, and these results should be considered to be a conservative measure of the expected uptake by bed bugs contacting treated fabric that is deployed as a covering for the complex shapes of furniture.

Insecticide efficacy is most often determined by endpoint experiments via bioassays, where the application of an insecticide to the insect or surface in a dose-dependent or time-dependent manner allows for evaluation of mortality (or other affected trait).[22] Insecticide uptake by a pest is seldom studied, except in special cases such as trophallaxis in termites where there is interest in evaluating insecticide transfer between conspecifics.[23] Little has been studied, or at least published, about AI uptake by a pest species in contact with a treated surface. Several steps must be understood, including: (1) exposure to an insecticide; (2) transport of the insecticide to the target site; (3) disruption of homeostasis; (4) irreversible cellular and gross organ injury; (5) eventual death. Also, if an insecticide is prevented from entering the cuticle,[12] physiologically degraded, not compatible for the target site or a combination of any of these mechanisms,[16] an insect could retain homeostasis and/or could repair or guard against injury from the insecticide.

We found that bed bugs were able to take up quantifiable amounts of permethrin after exposure to permethrin-treated fabric in as little as 1 min and 1 cm traversed. Despite the known resistance traits[15,18] of the Forest Brook strain of bed bugs, behavioral changes during the preliminary experiments occurred at approximately 4 h, and included uncoordinated movement and tremors. Comparatively, our initial work with the susceptible ECL-05 strain showed that behavioral changes occurred within 30 min. Our study provides a better understanding of the underlying AI uptake for the results reported, wherein several bed bug strains (regardless of level of resistance) exhibited feeding difficulties and reduced fecundity after 10 min of contact with permethrin-treated fabric.[15] Correcting for the percentage recovery, an average of 25.4 ng is expected to have transferred to the bed bug from the permethrin-treated fabric within 10 min, and this short exposure time provides sufficient permethrin to cause sublethal effects, including feeding inhibition and reduced fecundity.[18]

Direct comparison with this work and other methods of delivering permethrin to estimate lethality are difficult, as there are distinct formulation differences; further work would be necessary to determine permethrin uptake compared with acetone/permethrin mixtures. Upon superficial comparison, however, the amount of insecticide uptake (absorption) was 55 times greater than that necessary to exceed the LD_{50} of a susceptible strain.[24] A more resistant strain would have accumulated only 4–16% of that required to reach the LD_{50} within 10 min of contact.[24,25] Within 50 min, this resistant bed bug strain would have accumulated 12–42% of that required to achieve LD_{50}. Despite a relatively lower ratio of available insecticides compared with the present LD_{50} estimates, there is a time factor not considered in topical bioassays and the toxicity measurement results from a single application and the resulting absorption of the AI to the active sites. Previous studies[26] have shown decreasing

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estimates of LC_{50} between 1 and 12 h with resistant bed bugs exposed to deltamethrin or λ-cyhalothrin.[26] In our study, absorption was measured for the first 200 min of contact, as behavioral changes thereafter were observed to be consistent with permethrin toxicity. To determine whether there is a time-dependent saturation effect, evaluating exposure beyond 200 min may be advisable. While bioassays will be the ultimate expression of the effectiveness of an insecticide, quantitative analysis of insecticide uptake has an important role in understanding the barriers and issues that may affect toxicity. Using quantitative analysis in conjunction with physiological mechanisms (e.g. as enzyme activity[27]) will provide a much clearer understanding of how resistance to insecticides might be better measured and managed in bed bugs.

5 CONCLUSIONS

The fabric used in our study provided a permethrin-impregnated surface formulated in such a manner that bed bugs began rapidly to absorb the AI on contact and within the first 1 cm of travel on the fabric. Permethrin continued to accumulate up to 50 min, at which time the amount taken up by insects stabilized for the duration of the 200 min evaluation period. Correcting for the percentage recovery, an average of 25.4 ng had transferred to the bed bug from the fabric within 10 min, and at 50 min the amount of permethrin uptake was 66.5 ng. This short exposure time (10 min) provided enough permethrin for bed bugs to exhibit sublethal effects.[18] Variability in uptake was likely a result of particular behaviors such as grooming and thigmotaxis causing a greater contact of the insects with the treated surfaces; future work should use quantitative (or qualitative) methods to account for behaviors that might increase contact with the AI. The quantitative methods used in our study have promise in determining the different steps involved from initial exposure through to active site activity. An understanding of the parameters influencing how an AI affects an insect from exposure to intoxication is critical for assessing immediate and sublethal behavioral changes that precede mortality. Moreover, the factors that influence uptake characteristics of residual insecticides will ensure that exposure results in a dose sufficient to accomplish rapid mortality.

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