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No Sex-Related Differences in Mortality in Bed Bugs (Hemiptera: Cimicidae) Exposed to Deltamethrin, and Surviving Bed Bugs Can Recover

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ABSTRACT Exposure of a pyrethroid-susceptible strain of bed bugs, *Cimex lectularius* L. (Hemiptera: Cimicidae) to varying concentrations of deltamethrin for 24 h indicated no significant difference in mortality between males, females, and nymphs at 24 nor at 168 h postexposure when bed bugs were removed to untreated surfaces at 24 h. In addition, many bed bugs classified as morbid or moribund at 24 h and removed to untreated surfaces at this time, recovered by 336 h (2 wk) and were capable of feeding when given the opportunity. Adult female bed bugs that survived were able to lay eggs and the resulting nymphs blood-fed. By contrast, all bed bugs classified as morbid or moribund at 24 h that remained on deltamethrin-treated surfaces for 336 h either died or were still classified as morbid or moribund at the end of this time. No bed bugs classified as morbid or moribund blood-fed when given the opportunity at 2 wk, regardless of whether they remained on the treated surfaces or were removed to untreated surfaces. A power analysis demonstrated we would have detected even moderate differences in mortality between males and females, had differences existed. Therefore, using males exclusively in efficacy assays is a suitable strategy to preserve females for laboratory colony purposes. Results also indicated there is little reason to assess efficacy beyond 1 wk, even when bed bugs are exposed for only 24 h.

KEY WORDS bed bug, *Cimex lectularius*, deltamethrin, sex-specific mortality

The common bed bug *Cimex lectularius* L. (Hemiptera: Cimicidae) is an obligate, blood-feeding insect that attacks humans, and there is a global resurgence in bed bug infestations (Doggett et al. 2012). The increase in the number of bed bug infestations in the United States, alone, has resulted in bed bugs being deemed a pest of public health importance (Centers for Disease Control and Prevention and U.S. Environmental Protection Agency 2010). One potential cause for this resurgence has been attributed to bed bug resistance to pyrethroid insecticides (Romero et al. 2007, see Davies et al. 2012). Of the over 300 formulations of insecticides approved by the U.S. Environmental Protection Agency (EPA) for use against bed bugs, the vast majority of these formulations are pyrethroid-based (U.S. Environmental Protection Agency 2012a). Despite the reports of widespread resistance of field populations of bed bugs to pyrethroids, compounds belonging to this chemical class are still commonly used, and their apparent misuses have also been documented (Centers for Disease Control and Prevention 2011).

In an effort to make comparisons between products easier to interpret, regulatory agencies such as the EPA are beginning to standardize protocols for the laboratory testing of chemicals and formulations designed to control bed bugs (U.S. Environmental Protection Agency 2012b). In this current article we examine the mortality and survivability of a widely available, pyrethroid sensitive strain of bed bugs exposed to a pyrethroid, deltamethrin. We had three main goals. The first was to determine if males alone can be used in testing protocols, thereby saving and reserving females for laboratory colony maintenance. Our criterion was whether a single 'dose–response' curve adequately represented the effect of deltamethrin concentration on mortality for all bed bug groups. The second was to examine the fate of bed bugs that survived insecticide exposure, though in a morbid or moribund state, and determine if they recovered at a later time. The third was to determine a suitable endpoint, if any, with regard to the duration of efficacy studies.

Materials and Methods

Insects. A colony of *C. lectularius* was established from bed bugs originally obtained from Harold Harlan (Crownsville, MD). The colony was kept at ambient

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conditions (25 ± 5°C and 40 ± 15% relative humidity [RH]) and fed weekly on expired, human red blood cells and plasma using an artificial (in vitro) feeding system (Feldlaufer et al. 2010). These bed bugs, often referred to as the "Harlan strain," are considered susceptible to pyrethroid insecticides (M.F.F., unpublished data; Moore and Miller 2006). For the experiments described below, adult males, adult females, and nymphs (third-fifth instar) had not been fed for 8 d. Females were presumed to be previously mated.

Chemicals. Deltamethrin [(S)-α-Cyano-3-phenoxybenzyl (1R,3R)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate, PESTANAL grade] was from Sigma–Aldrich (St. Louis, MO). Spectral grade acetone (Honeywell Burdick & Jackson, Morristown, NJ) was used for all dilutions.

Experimental Design. Dilutions of deltamethrin were prepared in acetone and applied (174 μl) to 47 mm (diameter) filter paper disks (Whatman No. 1) to yield eight concentrations of 0.5, 1, 5, 10, 25, 50, 100, and 200 $\mu g/cm^2$. All filter papers were allowed to dry for 75 min before being placed in Pyrex (glass) petri dishes (60 by 15 mm). Ten bed bugs (males only, females only, and nymphs only) were then placed on each of the treated surfaces. An additional assay was conducted where males and females (five each per dish) were kept together to determine if the presence of both sexes influenced mortality. Bed bugs placed on acetone-treated filter paper acted as controls in all assays. Two replicates were conducted in the assays described above, so a total of 720 bed bugs were used. After 24 h, mortality was assessed based on the behavioral response exhibited by each bed bug when probed with a dissecting needle, similar to that reported by Jones and Bryant (2012). However, we scored bed bugs as either 1) dead (no movement when probed), 2) healthy (ran quickly away when probed), or 3) morbid or moribund ("M/M"). This latter category ranged from bed bugs that were upright, but did not respond to stimuli (morbid), to bed bugs that were on their back, with only a single appendage, twitching (moribund). At 24 h, all bed bugs that were classified as M/M were transferred to petri dishes containing untreated filter paper. These bed bugs were reassessed at 48, 72, 168 (1 wk), and 336 h (2 wk) posttreatment at which time bed bugs scored as healthy or M/M were given the opportunity to feed. We also noted whether or not eggs had been deposited in dishes containing adult female bed bugs, and the fate of any nymphs resulting from these eggs.

Another group of bed bugs (adult males only, adult females only, mixed sexes, and nymphs) were exposed to the same treatment regime, except that after being scored at 24 h, they remained on the deltamethrin-treated surfaces for the duration of the experiment. Controls consisted of bed bugs of each group placed on acetone-treated filter paper. A single replicate consisting of 360 bed bugs were used in this particular experiment. Assessments were made in the same time frame as for the bed bugs removed at 24 h. At the end of 336 h (2 wk) any bed bugs that had not died were given the opportunity to feed on blood. Bed bugs in all experiments were kept at 25 ± 2°C and 65 ± 1% RH.

Data Analyses. Bed bugs were classified at each time period as being in one of three states; thus, the dependent variable is multinomial. However, to establish a dose–response curve, we used a dichotomous variable (dead vs. other) allowing us to model the logit of the proportion dead as a function of concentration of deltamethrin, sex or stage, whether the bed bugs were removed after 24 h, and time using a binomial model (logistic regression). We found that a transformation of concentration to log (concentration + 0.5) spaced the proportions in a way that allowed for smooth quadratic functions to be used in the dose–response models (adding 0.5 to concentration also avoided taking logs of zero for controls). For figures, the results were back-transformed to the data scale (percent dead and concentration in micrograms per square centimeter) for ease of interpretation. Modeling was done with the R function glm (R Core Team 2012), specifying a quasi-binomial family. This allows for statistical tests to be adjusted for over-dispersion, which is a typical feature in biological modeling (Gbur et al. 2012). We found no evidence of a replicate effect, so replicates were pooled for analyses.

Power Analyses. Because a main objective of the study was to determine if using only males (thereby preserving females for breeding) would be sufficient for testing candidate insecticides, we needed to confirm that we had sufficient power for equivalence testing. We investigated power based on the data where bed bugs were removed from the insecticide after 24 h, as most died (94.0% at 336 h) when not removed from the insecticide-treated surfaces. We used simulation, based on the average dose–response curve at 168 h (we anticipate our results by noting that beyond this time point there was little change). We drew from a binomial distribution with sample size 26 (the average sample size) for each concentration-sex or stage combination. We modified the probability of death given by the dose–response curve averaged over all sexes or stages (based on changing either the intercept or linear slope component) for two of the groups (e.g., males and nymphs) until we found the upper and lower limits beyond which 80% were significant at $\alpha = 0.05$ in contrasts with the third (original parameter) group with 5,000 simulations. These limits correspond to a difference one could detect with 80% power, that is, within these limits we would declare equivalence, and beyond it we would likely find significant differences. Power analyses are usually performed to determine intercept differences (i.e., if one group had higher mortality throughout the concentration range, essentially yielding two parallel lines), but we also investigated slope differences because it is possible that mortality might be similar at low doses but different at high doses. We did not investigate power for the quadratic slope component because (again anticipating results), the quadratic slope parameter was only borderline significant. Modeling was done with the R function glm (R Core Team 2012),

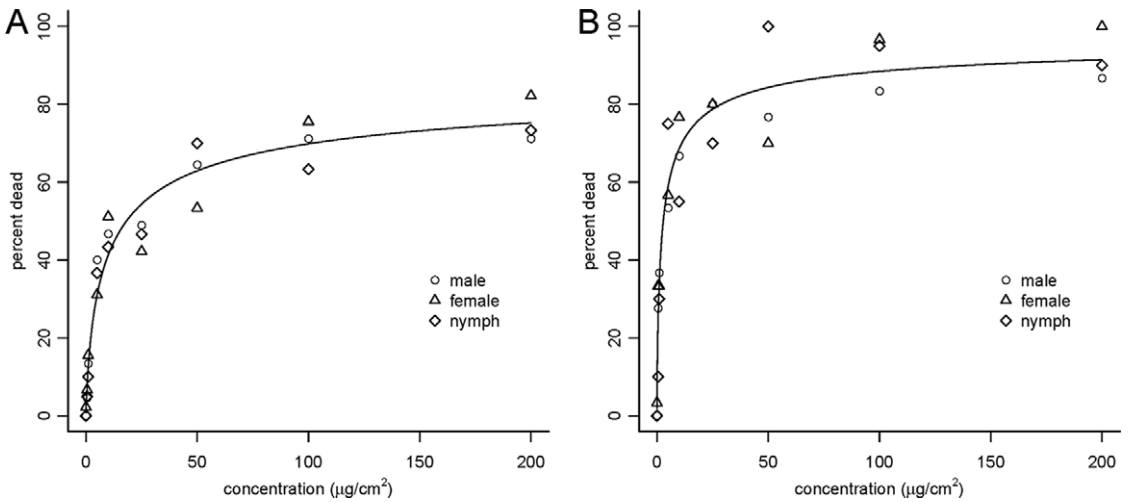

Fig. 1. Percent dead as a function of concentration at (A) 24 h (all bed bugs) and at (B) 168 h (bed bugs removed at 24 h from deltamethrin-treated surfaces to untreated surfaces). The curve represents the model predictions, back-transformed to the original scale for ease of interpretation. Because differences between groups were not significant, groups were combined to establish the curve (logistic regression with linear and quadratic terms, concentration transformed as log concentration + 0.5). Curves (on the model scale) are given by (A) logit $(p) = -1.96 + 0.83x - 0.049x^2$; (B) logit $(p) = -0.78 + 0.78x - 0.037x^2$, where x is log (concentration + 0.5) and p is the proportion dead.

specifying a quasi-binomial family (as in the original data analysis).

Results

All bed bugs in control groups (exposed only to acetone-treated filter paper; $n = 120$) were classified as healthy throughout the experiments. There was no mortality, morbidity, or moribundity in any group of controls, including those adult females that were comixed with adult males. Because our initial concerns regarding mating-induced mortality (Reinhardt and Siva–Jothy 2007) did not occur, results for comixed males and females were added to the data generated for these sexes at the respective concentrations.

Sex- and Stage-Related Mortality. There was no difference in mortality in intercept or slope (P values of contrasts of group-specific intercept and slope parameters all ≥ 0.74; t-tests; quasi-binomial distribution family) across the concentration range of deltamethrin tested between males, females, and nymphs, when assessed at 24 h after exposure (Fig. 1A). Neither was there a difference in mortality between sexes and stages at 168 h, in bed bugs that were removed to an untreated surface at 24 h (Fig. 1B; all $P \geq 0.34$; t-tests; quasi-binomial distribution family). The quadratic components of the curves were only borderline significant ($P = 0.08$, Fig. 1A; $P = 0.14$, Fig. 1B), so were not used to assess sex and stage differences. However, we retain it in the model because it captures the 'flattening off' effect at the end of the curves (they do not appear to asymptote to one), where there is little difference in mortality among the higher concentrations. The model dose–response curves (sexes and stages combined) are given by logit $(p) = -1.96 + 0.83x - 0.049x^2$, with respective SEs 0.178, 0.123, and 0.023, and over-dispersion parameter 1.222 (Fig. 1A); and logit $(p) = -0.78 + 0.78x - 0.037x^2$, with respective SEs 0.153, 0.103, and 0.024, and over-dispersion parameter 1.246 (Fig. 1B), where x is log (concentration + 0.5) and p is the proportion dead. There was little over-dispersion for our data (<1.5, where values >3.0 are considered high).

Mortality was high and increased over time in all groups of bed bugs that remained on the treated surfaces. At 336 h (2 wk) postexposure, mortality in bed bugs (not classified as dead at 24 h) ranged from 79 to 83% at the two lowest doses, to over 90% in all of the other doses. In addition, no bed bugs in any group at any doses that remained on the treated surfaces were classified as healthy (Table 1). By comparison, mortality at 336 h in morbid or moribund bed bugs removed from the treated surfaces at 24 h, ranged from 23–79%.

Fate of M/M Bed Bugs. Except for five nymphs exposed to the lowest concentration of deltamethrin (0.5 $\mu g/cm^2$), all bed bugs exposed to higher concentrations of deltamethrin (1.0–200 $\mu g/cm^2$; $n = 955$), were classified as either dead ($n = 430$) or M/M ($n = 525$) at 24 h. Bed bugs scored as dead or healthy (the five nymphs) at 24 h did not change class over time, but those bed bugs scored as M/M at 24 h sometimes did, either recovering (scored 'healthy'), or dying, when assessed at a later time (by 336 h). Using the same statistical model as in the previous section, there were no sex- or stage specific differences in the recovery of M/M bed bugs (all $P \geq 0.039$). Table 1 shows the fate of bed bugs at 336 h (2 wk posttreatment) that were initially classified as M/M at 24 h, and either removed from the deltamethrin-treated surfaces or not removed from the treated surfaces. At all concentrations tested, a percentage of bed bugs originally

Table 1. Fate (expressed as a percentage) of morbid or moribund (M/M) bed bugs at 336 h exposed to varying doses of deltamethrin

Dose (μg/cm^2)	Removed				Not removed			
	24 h	336 h			24 h	336 h		
	M/M (n)	Dead	Healthy[a] (%)	M/M[b]	M/M (n)	Dead	Healthy (%)	M/M[b]
200	14	64	21	14	15	100	0	0
100	16	75	13	13	19	95	0	5
50	29	79	7	14	17	100	0	0
25	29	38	28	35	36	92	0	8
10	37	49	46	5	26	100	0	0
5	44	39	55	7	32	97	0	3
1	68	24	76	0	36	83	0	17
0.5	69	23	77	0	38	79	0	21

Data for males, females, and nymphs were combined at each dose. Bed bugs classified as morbid or moribund (M/M) at 24 h were either removed to an untreated surface (n = 306), or remained on the treated surface (not removed; n = 219). At 336 h (2 wk), bed bugs were classified as dead, healthy, or M/M. There was no mortality, morbidity, or moribundity in bugs exposed to acetone-treated papers (data not shown).

[a] All bed bugs classified as healthy at 336 h blood-fed when given the opportunity.

[b] No M/M bed bugs blood-fed when given the opportunity.

classified as M/M at 24 h and removed to an untreated surface at that time, recovered and were classified as healthy by the 336 h assessment time. Importantly, those bed bugs classified as healthy at this time were able to blood-feed when given the opportunity. This included males, females, and nymphs. In assays where bed bugs remained on the deltamethrin-treated surfaces, the majority of M/M bed bugs died, and no remaining bed bugs were classified as healthy. M/M bed bugs in either group (removed or not removed from treated surfaces) did not blood-feed when given the opportunity.

Eggs/Nymphs From Eggs. Throughout all the experiments, dishes containing adult female bed bugs (females only or comixed with males) were inspected for eggs and/or nymphs. All control (acetone-treated paper) dishes with females contained eggs after 1 wk. In deltamethrin-treated groups where females were removed to an untreated surface at 24 h postexposure, we found eggs present after 1 wk in all concentrations below 100 μg/cm^2 (0.5–50 μg/cm^2). Nymphs resulting from these eggs were evident at the 2 wk postexposure assessment, and all of these nymphs engorged on blood when given the opportunity.

For adult female bed bugs that remained on the treated surfaces, we observed a different outcome. At 168 h (1 wk posttreatment) eggs were found only at the two lowest concentrations, 0.5 and 1 μg/cm^2, presumably because of high mortality at the higher doses. At 1 μg/cm^2 all of the nymphs that hatched had died by the 2 wk assessment. However, eggs present in the 0.5 μg/cm^2 treatment at 168 h subsequently hatched and the nymphs blood-fed when given the opportunity at 2 wk. Particularly noteworthy is that these eggs (and the resulting nymphs) were the offspring of several females classified as M/M.

Endpoint (Duration of Efficacy Studies). We observed only small changes (decreases) in the percentage of bed bugs classified as either M/M or healthy between the 168 h and 336 h assessment (Fig. 2). Most M/M bed bugs that were going to either die or recover (and scored healthy) had done so by this time. Figure 2 follows the percent M/M and healthy bed bugs over time for three concentrations (high, low, or mid) of deltamethrin; other concentrations followed the same pattern of little change (slopes are close to zero) between the 168 and 336 h assessments.

Power Analyses. Our equivalence analyses indicated that we would be able to detect differences in sex- and stage-specific mortality on the order of 10% for differences that were consistent throughout the doses tested (Fig. 3A; if curves differed in intercept) or 15% or less for proportions that deviated in a linear way, for example, if differences became larger with

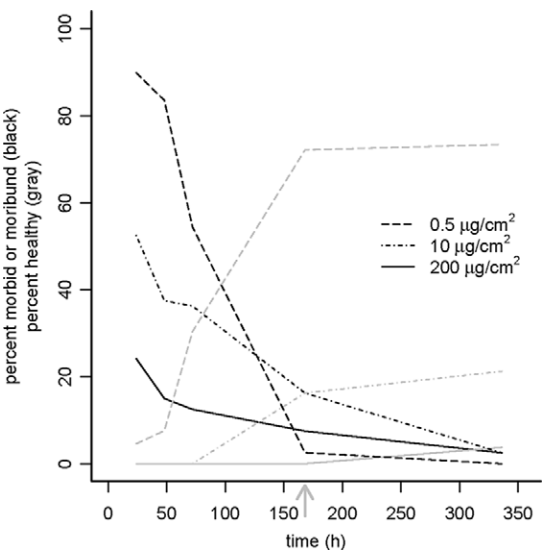

Fig. 2. Percent morbid or moribund bed bugs (black lines) and percent healthy bugs (gray lines) over time for three concentrations of deltamethrin. Data for males, females, and nymphs combined. Bed bugs were transferred at 24 h from deltamethrin-treated surfaces to untreated surfaces. Except for five nymphs classified as healthy at the deltamethrin concentration of 0.5 μg/cm^2, all bed bugs were either dead or classified as M/M at the 24 h assessment. The gray arrow under the x-axis indicates 168 h postexposure, beyond which there was little change.

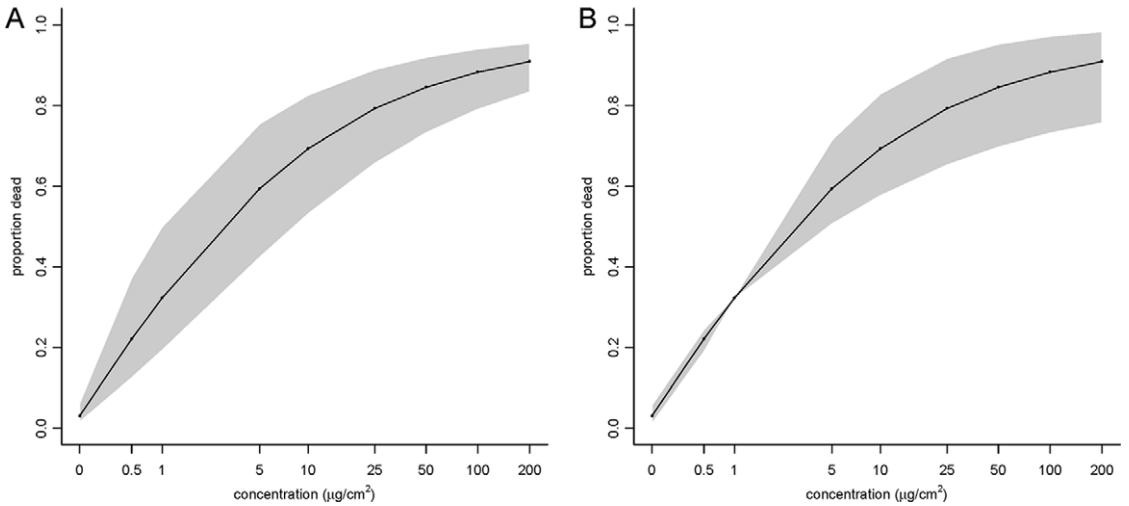

Fig. 3. Power analyses results for (A) intercept and (B) linear slope. Shaded areas represent the regions of equivalence (at 80% power, proportions dead outside those limits would be detectable, by either an intercept or slope difference) based on logistic regression models at 168 h postexposure to varying concentrations of deltamethrin. In the model used for analysis and simulations, the independent variable, concentration, was transformed to log (concentration + 0.05). Changes for the quadratic term were not assessed because that term was only borderline significant. Upper and lower limits were calculated through simulation ($n = 5{,}000$) by distorting the appropriate term (intercept or linear slope) for two of the three groups (e.g., males and nymphs) until significance was reached in 80% of the simulated data sets in contrasts with the third group. The results were back-transformed to the original scale for ease of interpretation. At a concentration of deltamethrin near one, the transformed value is near zero. When this value is multiplied by the linear coefficient (whether estimated or distorted), it also produces a value near zero. This can be seen in (B), where the region of equivalence becomes very narrow near one.

increasing concentration (Fig. 3B; if curves differed in slope). Because the data are binomial in nature, they have the property that SEs are a function of the proportion, with the widest SEs at 0.5. SEs narrow as the proportion approaches 0 or 1 (because a hard limit is being approached, that is, a CI on the proportion can never include 0 or 1). Thus, the detectability limits change among concentrations, as the proportion "dead" changes. The limits are symmetric on the model (logit) scale, but asymmetric when back-transformed to the original scale. For proportions >0.5, the limits are shorter above the proportion than below it, and, for proportions <0.5, they are shorter below the proportion than above it. This explains, in full, the changes in detectability limits in Fig. 3A (intercept). The detectability limits seen in Fig. 3B includes an additional component associated with slope effects, and is explained in the figure legend.

These results assured us that large, or even moderate, true differences (see Cohen 1992) in sensitivity to deltamethrin among the groups (sexes and stages) would have been detected had differences existed. Thus, it is reasonable to model a single dose–response curve for the different groups, as well as use only males in subsequent efficacy testing.

Discussion

Mortality studies in insects regarding sex- and stage-specific differences to pyrethroids and other chemical classes have been reported, with varying results. For instance, German cockroach males were found to be more sensitive than females to several pyrethroid insecticides (Abd–Elghafar et al. 1990). However, no significant differences in toxicity between sexes were reported in three species of noctuid pests of agriculture exposed to cypermethrin and permethrin (Usmani and Knowles 2001). A recent study with the tropical bed bug, *Cimex hemipterus* (F.), indicated stage-specific sensitivities to a variety of formulated insecticides (How and Lee 2011). Our current study with bed bugs indicates there are at most only minor differences in mortality between sexes and stages of a pyrethroid-sensitive strain exposed to various concentrations of deltamethrin. While we have not examined other classes of insecticides, their formulations, or other strains of bed bugs, our results suggest that adult male bed bugs can be used exclusively in laboratory efficacy studies if using methodologies similar to ours. This would preserve adult females, and nymphs (presumable 50% females) for colony expansion and maintenance.

Deltamethrin, like many pyrethroid insecticides, acts quickly (generally referred to as "knockdown") and affected insects exhibit tremor-like symptoms (Schleier and Peterson 2011). This characteristic was observed in many bed bugs that we classified as M/M after exposure to deltamethrin-treated surfaces. However, our results indicate that many bed bugs considered as M/M at 24 h can recover at a later time if removed to an untreated surface. At 2 wk postexposure, these bed bugs could feed, females

had laid eggs, and the nymphs resulting from these eggs could also feed when given the opportunity. The likelihood of M/M bed bugs moving to or finding an untreated surface under field conditions is admittedly open to discussion. However, a treated (sprayed) bed bug might become dislodged and fall to an untreated surface, or untreated bed bugs might cross a treated surface to an untreated surface, thereby being exposed to a pesticide for a relatively short time. Conversely, our data indicate that when bed bugs remain on the deltamethrin-treated surface, no bed bugs recover, and those that do not die remain M/M and are unable to feed when given the opportunity.

It is not uncommon in toxicity assays to record M/M individuals as dead. While convenient, this practice is questionable because 1) M/M individuals are not dead, and 2) not all M/M individuals eventually die. For instance, while >99% (955/960) of the bed bugs exposed to deltamethrin in our study were classified as either dead or M/M at 24 h, less than half of these bed bugs were actually dead ($n = 430/960$), the majority being classified as M/M. More importantly, almost 53% ($n = 161/306$) of those bed bugs classified as M/M at 24 h and removed to untreated surfaces, were able to recover and feed. The recovery we observed may be, in part, because of our using a technical-grade active ingredient rather than a formulated product, where components other than the active ingredient may facilitate or synergize the insecticidal action of the active ingredient (see Barile et al. 2008). Recovery after treatment with technical-grade pyrethroids has also been previously reported and quantified in another true bug, *Triatoma infestans* Klug (Alzogaray and Zerba 1997). Recent studies with bed bugs (Jones and Bryant 2012) have used additional behavioral responses, other than dead and healthy, to assess the condition of bed bugs after exposure to a toxicant. These authors used three terms ("sluggish," "ataxic," and "moribund") to capture what we called M/M. Despite the added effort involved, we believe this is a sound practice that more accurately reflects the efficacy of a particular compound and possibly a formulated product. An alternative albeit conservative approach is to record M/M bed bugs as alive in toxicity studies.

Finally, we assessed mortality, morbidity and moribundity, and recovery in bed bugs exposed to deltamethrin-treated surfaces over a 2-wk period. Our results indicate that there is little, if any, need to conduct assessments beyond 1 wk, because there is little change (slope in Fig. 2) in the status of morbid or moribund bed bugs after this time with insecticides such as deltamethrin.

Acknowledgments

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