

# Behavioral effects of sublethal exposure to a combination of $\beta$ -cyfluthrin and imidacloprid in the bed bug, *Cimex lectularius* L.

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## Abstract

**BACKGROUND:** Bed bugs (*Cimex lectularius*) are blood-feeding insect pests with public health relevance. Their rapid evolution of resistance to pyrethroids has prompted a shift to combination products that include both a pyrethroid and neonicotinoid insecticide. Insecticides have both a direct impact on mortality and an indirect effect on behavior. Thus, we assessed the sub-lethal effects of a widely used combination product containing  $\beta$ -cyfluthrin (a pyrethroid) and imidacloprid (a neonicotinoid), as unexpected behavioral changes after exposure have been known to affect efficacy of insecticides.

**RESULTS:** We found that bed bugs exposed to sublethal doses of a combination product containing  $\beta$ -cyfluthrin and imidacloprid did not feed as effectively as untreated bugs. Their locomotion behavior was also reduced. However, aggregation in response to the presence of conspecific harborages was not affected by sublethal exposure.

**CONCLUSION:** Bed bugs exhibit behavioral changes after sublethal exposure to a combination product that could affect pest management choices and outcomes. A reduction in host-finding efficiency and feeding could complement the lethal effects of the insecticide. Alternatively, reduced locomotion following exposure could limit ongoing contact with insecticide deposits. However, an overall reduction in movement indicates that treatments are unlikely to cause dispersal of bugs to adjacent dwellings.

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**Keywords:** sublethal exposure; bed bugs; insecticide; behavior; pest control

## 1 INTRODUCTION

Understanding the relationship between insect behavior and fitness is crucial in pest management, where changes in behavior after treatment with insecticides may impact behavior, fitness and, ultimately, population control.<sup>1–3</sup> The judicious use of insecticides remains a cornerstone of pest management. This is especially true with household and public health pests, where efficient and affordable results are needed and non-chemical tactics alone may not be an option. Many common classes of insecticides, like the pyrethroids and neonicotinoids, act at the neuronal level to disrupt the normally well-orchestrated behaviors of insects. This disruption can reduce longevity (independent of the direct lethal effects) and alter the reproductive potential for many insect species.<sup>4</sup> Even so, the effects of insecticides on insect behaviors are usually overlooked during laboratory evaluations of chemical products for efficacy.<sup>5</sup> This oversight during laboratory product evaluation could, as a result, misrepresent product performance in the field. Much of the information collected on insecticide efficacy is based on direct observations of mortality following exposure to the active or formulated ingredients in standardized laboratory bioassays.<sup>6</sup> This methodology does not consider the diverse ways that an insecticide could affect fitness and population growth in the field.<sup>6</sup>

Sublethal effects are aberrant physiological or behavioral effects occurring after exposure to an insecticide.<sup>3</sup> Behavioral changes from sublethal exposure to insecticide may include both stimulation of or reduction in oviposition behaviors, alterations in the number of feeding and foraging events, inappropriate migration and dispersal, or the avoidance of insecticide residues.<sup>4</sup> Evaluation of sublethal exposure and the resultant effect on fitness could provide a more comprehensive understanding of the impact of an insecticide than mortality data alone.

One pest commonly managed with insecticides is the bed bug (*Cimex lectularius* L.). Bed bugs are flightless, hematophagous insects with public health relevance, as the adults and nymphs feed on human blood.<sup>7</sup> Bed bugs are cryptic and nocturnal.<sup>8</sup> Their aversion to light and tendency to aggregate in small crevices makes detection very time consuming.<sup>9</sup> Although alternative

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non-chemical treatment methods for controlling bed bugs are available (such as vacuuming, steaming, cold treatment and volumetric heating), surviving individuals often experience no long-term consequences.<sup>10</sup> Thus, utilization of residual insecticides is an important part of most pest management programs.<sup>11</sup> Many studies have focused on the inability of insecticides to induce mortality in bed bugs owing to the widespread evolution of insecticide resistance, especially resistance to pyrethroids.<sup>12,13</sup> There is a real or perceived gap between laboratory documentation of the prevalence of insecticide resistance and continued reliance on these products by the pest management industry.<sup>11</sup> Part of the 'disconnect' with respect to efficacy could be due to non-lethal effects on bed bug behavior. One recent study showed that sublethal exposure to permethrin-impregnated fabric affected both feeding behavior and fecundity of bed bugs.<sup>14</sup> Significantly fewer female bed bugs laid eggs after sublethal exposure to the fabric, and exposure times as short as 1 min were enough to induce behavioral changes. These results indicate that population-level consequences are likely.

In our study, we examine bed bug behavior after sublethal exposure to Temprid, a product that combines a neonicotinoid (imidacloprid) and a pyrethroid ( $\beta$ -cyfluthrin), hereafter referred to as 'the combination product'. This product is the most commonly used insecticide spray in the United States for bed bug management.<sup>11</sup> Insecticide resistance to pyrethroids, neonicotinoids, as well as combination products have recently been reported,<sup>15–18</sup> although some resistance mechanisms carry a fitness cost, which may enable a reversion to susceptibility in the absence of insecticide. A recent study indicated that, after selection for resistance to a combination of  $\beta$ -cyfluthrin and imidacloprid, reversion to susceptibility occurred post-selection, most likely owing to fitness costs associated with resistance.<sup>17</sup> Thus, our present studies were conducted with three populations of bed bugs that varied in the level of resistance to pyrethroids. We examined behaviors critical for bed bug survival and reproduction, including successfully taking a blood meal, locomotion and responses to harborage previously inhabited by conspecifics. We hypothesized that treated insects would have less success taking a blood meal, that they would lose their ability to respond to putative aggregation pheromones, that they would spend a smaller proportion of time moving and that their nocturnal periodicity of locomotion would be disrupted. Each of these behavioral changes would have implications for the efficacy of a widely used combination product for bed bug management programs that has previously not been understood.

## 2 EXPERIMENTAL METHODS

### 2.1 Insect rearing

Three strains of bed bugs were used in all experiments. The progenitors of the CIN-1 colony were collected in Cincinnati, Ohio, in 2005, and while it was initially highly resistant to pyrethroids, it has become more susceptible over time.<sup>15</sup> NY-1 was collected from New York City, New York, in 2007 and is now moderately resistant to pyrethroids. LEX-8 was collected from Lexington, Kentucky, in 2012 and is highly resistant to pyrethroid insecticides.<sup>15</sup> Bugs were housed in an incubator (Percival Scientific, Perry, IA) (27 °C, 70% RH, 14:10 L:D), and weekly blood meals were administered with a blood feeding system.<sup>19</sup> In this system, defibrinated rabbit blood (Quad Five, Rygate, MT) was pipetted into glass mosquito feeders (Kimble Chase Custom Glass Shop, Vineland, NJ) and heated to 39 °C with a circulating water bath. Parafilm lined the bottom of the glass feeder containing the blood. Bed bugs in 59 mL plastic

jars (Consolidated Plastics, Stow, OH) covered with organza (a fine-mesh synthetic fabric) had to pierce the organza and the parafilm membrane to feed. Bed bugs used in experiments were 7 days post adult eclosion.

### 2.2 Residual deposit mortality bioassays

An LT<sub>10</sub> (lethal time of exposure resulting in 10% mortality) was determined independently for each strain using a residual deposit bioassay.<sup>16</sup> Adult bed bugs were held individually in 24-well plates (Costar, Corning, NY), with wells measuring 1.6 cm in diameter. Each well was lined with Whatman No. 2 filter paper (Whatman, Maidstone, UK) cut to a diameter of 1.7 cm. Each filter paper had been saturated with 50  $\mu$ L (0.075% AI, with a 2:1 ratio of imidacloprid and  $\beta$ -cyfluthrin) of the combination product diluted in water at the label rate, or water alone (as a control). Filter papers were given 24 h to air dry prior to the bioassay. At this time, a bed bug was confined to the treated surface in one well and scored for mortality at 5, 15 and 30 min, at 1, 4, 12 and 24 h and at 3, 7 and 14 days (if necessary). Mortality was scored at each time point by gently turning bugs onto their dorsal side with soft forceps. If the insect could not recover by turning over to the ventral side, it was considered to be moribund. Three replicates with ten bugs per replicate were conducted.

### 2.3 Feeding efficiency in an arena

We investigated the effects of sublethal exposure to the combination product on the feeding success of adult bed bugs in an artificial feeding system. Following exposure to either water or strain-specific LT<sub>10</sub>, insects were placed in petri dishes (100 mm × 15 mm; BD Falcon, Corning, NY) with a tent-shaped piece of blotter paper (16 cm<sup>2</sup> on each side) for use as a harborage. These insects were placed in an incubator (27 °C, 70% RH, 14:10 L:D) for 24 h before they were used. Following exposure to either insecticide or water, five live and apparently healthy females and males were randomly selected per replicate.

A harborage with bed bugs was placed in a glass cylinder (25 cm height × 10 cm diameter) sealed on top with organza. The cylinders were placed beneath glass mosquito feeders containing rabbit blood. Blood feeding followed the protocol described above. To feed, the insects needed to walk up filter paper (25 cm length × 2.5 cm width) taped to both sides of blotter paper of the same dimension (for rigidity). This ramp allowed bed bugs the opportunity to move from the bottom of the cylinder to the top to take a blood meal. After an acclimation period of 15 min, bed bugs were permitted access to the feeding system for 30 min. An observer recorded the number of insects that fed, and the time of feeding of each bed bug. Ten replicates with ten insects (five males and five females) per replicate were conducted (200 bed bugs in total, 100 per treatment).

### 2.4 Mass gain after blood meal

We investigated the amount of blood imbibed after feeding in bed bugs exposed to the combination product versus bed bugs exposed to water. Following exposure to either water or strain-specific LT<sub>10</sub>, insects were placed in petri dishes (100 mm × 15 mm; BD Falcon) with a tent-shaped piece of blotter paper (16 cm<sup>2</sup> on each side) for use as a harborage. These insects were placed in an incubator (27 °C, 70% RH, 14:10 L:D) for 24 h before they were used for this experiment. After the 24 h recovery period, eight healthy females (chosen by flipping the female from dorsal to ventral side using soft forceps and assessing ability to

revert back to the dorsal side) were selected and placed individually in Eppendorf tubes (Fisher Scientific, Pittsburgh, PA). Each female was weighed prior to access to a blood meal, and her starting weight was recorded using a balance at a resolution of 0.1 mg. After a starting weight was obtained, each female was placed individually in a 59 mL plastic jar (Consolidated Plastics) and given access to the artificial feeding system described previously. After a blood meal was taken, females were removed and placed back into an Eppendorf tube. The final weight of each female was recorded immediately after feeding occurred. The starting weight was subtracted from this final weight in order to determine the total mass of blood imbibed. Values were compared between the treatment and control groups. Eight replicates of this assay were conducted with females only.

## 2.5 Locomotion assay

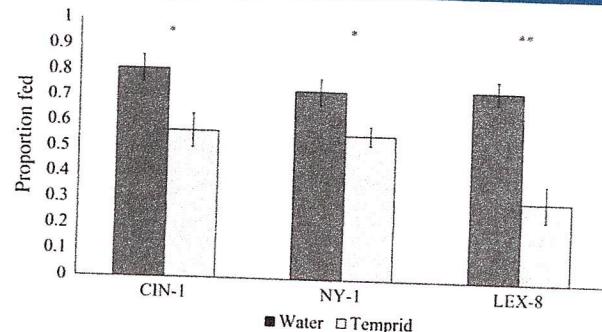
We tested the effects of sublethal exposure to the combination product on locomotor activity of adult bed bugs. Twenty-four hours after exposure, both male and female bed bugs were randomly selected and placed in six-well plates with untreated filter paper (i.e. no further exposure to insecticide) (VWR, Radnor, PA) and housed in an incubator under the laboratory conditions previously described. Movements of these bugs were recorded over a 24 h period using a camera (Sony Cyber-shot DSC H300) programmed to take one picture every 10 min. This interval was based on an earlier study.<sup>20</sup> To record clear pictures during the scotophase, an LED infrared illuminator (Pinecom PN-850) was used and the camera was set to 'nightshot'. After 24 h, pictures were assessed in sequential order and the bed bug in each cell was scored for movement. If the insect's position differed from one frame to the next, we considered this a movement. The proportion of time intervals that a bug moved was determined independently for the day and night. Eleven replicates were conducted (11 insects per treatment per strain).

## 2.6 Response to harborages with feces from bed bugs

We assessed the ability of bed bugs to detect putative aggregation pheromone after exposure to the combination product. Prior to the experiment, 100 adult bed bugs were placed on 2.5 cm × 2.5 cm filter paper harborages folded down the center to form 'tents'. Recently, fed bugs were permitted to aggregate and defecate on these tents for 3 days. These filter papers should contain compounds that lead to aggregation formation, as shown by previous studies, because bed bug feces are known to contain their aggregation pheromone in addition to other compounds.<sup>21–23</sup> After exposure to the combination product, surviving bugs (as previously described) were placed in Climbug insect interceptors (Susan McKnight, Inc., Memphis, TN). Interceptors were lined with 10.8 cm diameter black filter paper (Ahlstrom, West Carrollton, OH). Each Climbug contained two tents: one control tent (never exposed to bed bugs) and one tent that had previous exposure to other bed bugs for 3 days. Within the Climbug, each bed bug was given 24 h to choose a tent, and tent choice was scored at this time. A single bug that made no choice of either tent (i.e. it was out in the arena) was excluded from the analysis. Twenty replicates of the choice test were conducted.

## 2.7 Data analysis

Probit analysis was used to calculate a strain-specific  $LT_{10}$  value with Minitab 15 for Windows v.2007.<sup>24</sup> All other statistical analyses were conducted using Statistix 10.<sup>25</sup> Wilcoxon rank sum tests



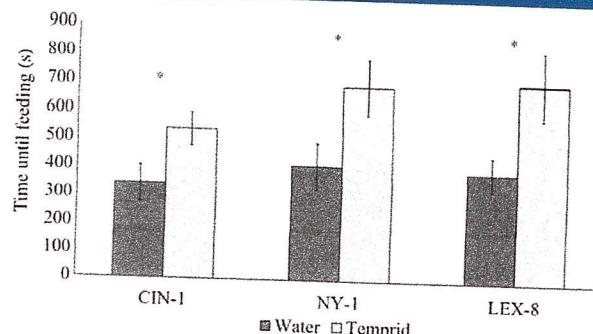
**Figure 1.** Proportion of bed bugs that took a blood meal with and without prior exposure to the combination product. Exposure to the insecticide reduced feeding by 30, 23 and 58% for CIN-1, NY-1 and LEX-8 respectively. Significant differences between treated and untreated groups are denoted by asterisks (\* $P < 0.05$ , \*\* $P < 0.01$ ).

were used in order to examine the effects of sublethal insecticide exposure on the proportion of bed bugs that fed, the time it took for bed bugs to initiate feeding and the mass gained after feeding. A two-way ANOVA was used to examine the impact of photoperiod and insecticide exposure on the proportion of time spent moving over the course of a 24 h period. These raw data were arcsine square root transformed prior to analysis. Binomial tests were used to assess whether bed bugs selected control tents versus tents that had previous exposure to bed bugs for both insecticide-exposed and control bed bugs from each population. Fisher's exact test was used to compare the proportion choosing the tent with exposure to conspecifics in treated versus untreated bugs for each population. All analyses were conducted only within strain because  $LT_{10}$  exposure times were unique to each strain.

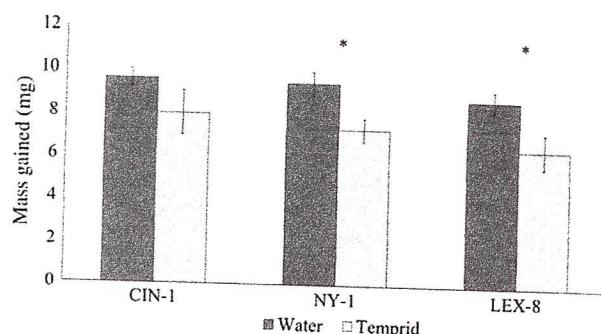
## 3 RESULTS AND DISCUSSION

As expected, the  $LT_{10}$  values for this combination product differed depending on the level of pyrethroid resistance in each of the strains. The  $LT_{10}$  values (in h) were 0.95 (0.46–1.57), 1.14 (0.43–2.11) and 5.0 (1.9–8.93) (95% fiducial confidence intervals) for CIN-1, NY-1 and LEX-8 respectively. All subsequent assays were performed after bed bugs were exposed at these  $LT_{10}$  values. Probit analyses for each strain and all associated data tables are presented in supporting information Table S1.

Regardless of strain, sublethal exposure at the  $LT_{10}$  significantly reduced the proportion of individuals that successfully fed (Wilcoxon rank sum test, CIN-1:  $n = 10$ ,  $Z = 2.4$ ,  $P < 0.05$ ; NY-1:  $n = 10$ ,  $Z = 2.51$ ,  $P < 0.05$ ; LEX-8:  $n = 10$ ,  $Z = 3.29$ ,  $P = 0.001$ ) (Fig. 1). Treated CIN-1, NY-1 and LEX-8 took 58, 68 and 81% longer to initiate feeding than their control groups, respectively (Wilcoxon rank sum test, CIN-1:  $n = 10$ ,  $Z = 2.0$ ,  $P < 0.05$ ; NY-1:  $n = 10$ ,  $Z = 2.08$ ,  $P < 0.05$ ; LEX-8:  $n = 10$ ,  $Z = 2.04$ ,  $P < 0.05$ ) (Fig. 2). NY-1 and LEX-8 imbibed significantly smaller blood meals with insecticide exposure (Wilcoxon rank sum test, CIN-1:  $n = 8$ ,  $Z = 1.31$ ,  $P > 0.05$ ; NY-1:  $n = 8$ ,  $Z = 2.05$ ,  $P < 0.05$ ; LEX-8:  $n = 8$ ,  $Z = 2.27$ ,  $P < 0.05$ ) (Fig. 3). Finding a host is critical to the success of a bed bug because blood allows an individual to develop from juvenile to adult, and it allows adults to produce sperm and eggs.<sup>7</sup> If this process were made more difficult by exposure to insecticides, then a reduction in population growth rate would be expected when bed bugs are not killed, if recovery is slow or absent. These effects may also increase the amount of time bed bugs spend exposed outside harborages. Typically, quick forays from their hidden harborages



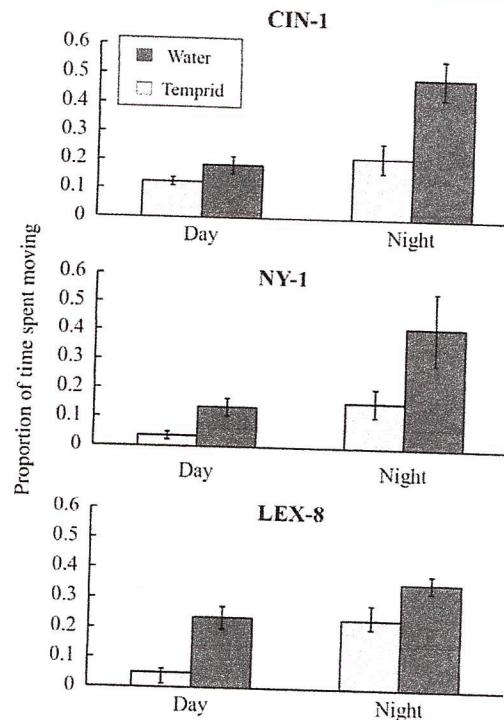
**Figure 2.** Mean time until bed bugs initiated feeding with and without prior exposure to the combination product. Bed bugs that did not feed during the assay were not included in the analysis. Exposure to the insecticide significantly increased the time taken to initiate feeding by 58, 68 and 81% for CIN-1, NY-1 and LEX-8 respectively. Significant differences between treated and untreated groups are denoted by asterisks (\* $P < 0.05$ ).



**Figure 3.** Weight gain after initiation of feeding with and without exposure to the combination product. Exposure to the insecticide significantly reduced the amount of blood imbibed during a blood meal in NY-1 (23%) and LEX-8 (26%), but not in CIN-1 (17%). Significant differences between treated and untreated groups are denoted by asterisks (\* $P < 0.05$ ).

are expected.<sup>25,26</sup> These findings indicate that sublethal exposure to insecticide might temporarily reduce the number of bites a host would sustain while increasing the vulnerability of bed bugs to other mortality factors (such as mechanical control) and result in gradual population decline. Conversely, rapid recovery would cause these effects to be short lived. Thus, future studies should address recovery time after sublethal exposure. However, there is some initial evidence that recovery to the combination product used here is gradual (Crawley SE et al., unpublished data).

The percentage of time spent moving after a sublethal exposure to the combination of  $\beta$ -cyfluthrin and imidacloprid was significantly reduced in all three strains (ANOVA, CIN-1:  $F_{1,40} = 7.5, n = 11, P < 0.01$ ; NY-1:  $F_{1,40} = 21, n = 11, P < 0.0001$ ; LEX-8:  $F_{1,40} = 22, n = 11, P < 0.0001$ ) (Fig. 4). As expected, bed bugs moved more frequently during the night than the day (ANOVA, CIN-1:  $F_{1,40} = 8, n = 11, P < 0.01$ ; NY-1:  $F_{1,40} = 10, n = 11, P < 0.01$ ; LEX-8:  $F_{1,40} = 15, n = 11, P < 0.001$ ) (Fig. 4). There was no interaction between treatment and time of day, indicating that the insecticide did not affect the periodicity of the response (ANOVA, CIN-1:  $F_{1,40} = 3, n = 11, P = 0.10$ ; NY-1:  $F_{1,40} = 2, n = 11, P = 0.15$ ; LEX-8:  $F_{1,40} = 1, n = 11, P = 0.29$ ) (Fig. 4), but did consistently reduce the movement rate. The propensity to move during part of the night before stimulation by host cues is likely to be an important part of host-finding.<sup>27</sup> A reduction in this movement rate is likely to reduce host-finding success.



**Figure 4.** The proportion of time spent moving during both the day and night with and without exposure to the combination product. As expected, bed bugs from all three populations were more active at night than during the day ( $P < 0.01$ ). Prior exposure to the insecticide significantly reduced movement in all three populations ( $P < 0.01$ ). There was no interaction between treatment and the light cycle (scotophase versus photophase), indicating that the periodicity of movement was not affected by exposure to the insecticide.

Some insecticides cause insects to increase the time they spend moving, or to disperse in ways they normally would not.<sup>22,28</sup> When this effect is quick and acute, the insecticide (e.g. pyrethrum) can be used as a 'flushing agent', which can be useful in detecting the presence of cryptic pests such as cockroaches.<sup>29</sup> Alternatively, stimulating movement of pests can have the negative side effect of dispersing individuals to unoccupied spaces. However, we found that sublethal exposure to a combination product significantly reduced rather than enhanced movement in survivors. These results suggest that treatments with this combination product should not cause increased dispersal of bed bugs into neighboring residential units, a pressing concern for pest management professionals.<sup>26</sup> In addition, we conducted a two-choice test and allowed untreated bed bugs to choose between insecticide-treated tents or control tents. We found no evidence of behavioral avoidance or repellency, which could be an alternative cause of dispersal (supporting information Fig. S1). Thus, it appears that, overall, treatments are not likely to result in increased dispersal after exposure to residual deposits.

Contrary to our hypothesis, insects in the treatment or control groups for all strains selectively rested on tents that had previous exposure to bed bugs rather than control tents (e.g. both treated and control bugs chose marked tents, Fisher's exact test, CIN-1:  $P = 0.23$ ; NY-1:  $P = 1.0$ ; LEX-8:  $P = 0.1$ ), indicating that the insecticide treatment had not affected their ability to respond to aggregation pheromone. Treatment insects chose tents exposed to conspecifics at frequencies of  $0.85 \pm 0.08$ ,  $1.0 \pm 0.00$  and  $0.84 \pm 0.08$  (proportion  $\pm$  SEM) for CIN-1, NY-1 and

LEX-8 respectively. Control insects chose tents previously exposed to conspecifics 100% of the time. Only one insect made no choice (treated, LEX-8) and was omitted from analysis. Independent of other behavioral effects of the insecticide, bed bugs would be expected to continue to aggregate in harborages marked by other bed bugs. Thus, one would expect that treated bugs will not be more likely to leave harborages during the day. Combined with the decrease in movement caused by the insecticide, this would result in bed bugs spending more time in harborages, and thus there would be a premium on treating in those spaces.

Resistance to insecticides is often documented by contrasting insecticide exposures (duration/dosages or dose) necessary to kill individuals from a field population with individuals from a population known to be susceptible. In bed bugs, resistance to pyrethroids is common and widespread, and reduced susceptibility was also reported recently to neonicotinoids.<sup>15–18</sup> The rapid evolution of resistance to a combination product was likewise found in one laboratory study where selection was imposed on three strains of bed bugs.<sup>16</sup> Recently, high levels of resistance to neonicotinoid/pyrethroid combination products were found in field populations.<sup>15,16</sup> The populations that we studied here varied in their susceptibility to combination products, as measured by LT<sub>10</sub> values, suggesting that lethal and sublethal effects co-vary to some extent. It is conceivable that resistance to an insecticide could result from a diminution of the deleterious sublethal effects with no change in lethal effects. However, that is not the case in the populations tested here.

Population-level effects of insecticides may be underestimated by laboratory assessment of lethal effects alone. This can lead to erroneous conclusions about the aggregate effect of treatment. In theory, when the gap (dose or exposure time) between lethal and sublethal effects is small, then little is missed with a mortality-based assay. However, when the gap is larger, lethal assays do not reflect potential behavioral effects on individuals. In this study, the propensity to aggregate in response to aggregation pheromone is likely to have a small gap between sublethal and lethal effects (i.e. our assay did not detect a behavior effect at the LT<sub>10</sub>). However, feeding frequency, time to initiate feeding and movement frequency follow a different pattern, with some variation between populations in the size of the gap between lethal and sublethal effects. It is clear from our study that sublethal effects on a population may be very important if duration of exposure to the insecticide is shorter than required for lethal effects. This may be the case when coverage is incomplete and aggregations are missed (leading to reduced exposure), or when resistance levels are higher, leading to reduced impact of exposure. Here, we studied the impact of only one insecticide product. It is very likely that insecticides with different modes of action will be characterized by distinctive suites of behavioral effects, as they target different elements of the nervous system. A systematic study of the behavioral symptoms associated with insecticides with different modes of action could be used to build predictions about how these classes will affect behavior. Future work should also investigate the effect of individual active ingredients to gain a better understanding of the specific causes of the behavioral effects observed here.

## 4 CONCLUSIONS

Our study found that some bed bug behaviors, such as feeding and locomotion, were adversely affected by sublethal exposure to a combination of  $\beta$ -cyfluthrin and imidacloprid, while other indicators of feeding, e.g. mass gained after initiation of feeding,

were variable. Aggregation behavior and periodicity of movement, however, were not affected. The net effect of this exposure has the potential to be detrimental to populations of bed bugs and thus helpful in managing this pest. Alternatively, there is also the possibility that sublethal effects on movement could cause small 'reservoirs' of treated insects that are temporarily unexposed to ongoing contact with insecticide as they recover. This possibility could impact pest management decisions regarding timing of treatments and the total number of necessary visual inspections. Importantly, the ultimate impact of an insecticide on a population of insects should not be assessed exclusively based on direct lethal effects. Some discrepancies between laboratory evaluations of insecticide lethality and field results could be the consequence of poorly understood behavioral effects.

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## SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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