

# Susceptibility of insecticide-resistant bed bugs (*Cimex lectularius*) to infection by fungal biopesticide

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## Abstract

**BACKGROUND:** Bed bugs are a public health concern, and their incidence is increasing worldwide. Bed bug infestations are notoriously difficult to eradicate, further exacerbated by widespread resistance to pyrethroid and neonicotinoid insecticides. This study evaluated the efficacy of the newly developed fungal biopesticide Aprehend™, containing *Beauveria bassiana*, against insecticide-resistant bed bugs.

**RESULTS:** Overall mortality for the Harold Harlan (insecticide-susceptible) strain was high (98–100%) following exposure to Aprehend™ or Suspend SC (deltamethrin). The mean survival times (MSTs) for Harold Harlan bed bugs were 5.1 days for Aprehend™ and 4.8 and 3.0 days for the low and high concentrations of Suspend SC respectively. All three strains of pyrethroid-resistant bed bugs were susceptible to infection by *B. bassiana*, resulting in MSTs of <6 days (median = 4 days) and >94% overall mortality. Conversely, mortality of the three insecticide-resistant strains after exposure to Suspend SC was only 16–40%.

**CONCLUSION:** These results demonstrate that Aprehend™ is equally effective against insecticide-susceptible and insecticide-resistant bed bugs and could provide pest control operators with a promising new tool for control of bed bugs and insecticide resistance management.

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**Keywords:** *Beauveria bassiana*; Aprehend™; Suspend SC; entomopathogenic fungi; insecticide resistance

## 1 INTRODUCTION

Bed bugs, *Cimex lectularius* L (Hemiptera: Cimicidae), are hematophagous ectoparasites that were all but eradicated from the United States and other industrialized nations after World War II. Their disappearance likely was due to the widespread use of dichlorodiphenyltrichloroethane (DDT) and other broad-spectrum insecticides during the second half of the twentieth century.<sup>1</sup> Still, while reports of bed bug infestations declined, evidence of resistance to insecticides, including DDT, were increasing.<sup>2,3</sup>

Over the course of the past decades, bed bugs have re-emerged on the global stage as an important public health pest. The cause(s) of the global resurgence remain unclear, but hypotheses include increased human movement via travel and migration, changes in pest management practices,<sup>1</sup> the unavailability of effective residual insecticides<sup>4–6</sup> and greater resistance to pyrethroid insecticides in reservoir or wild bed bug populations.<sup>4,6,7</sup>

Although pyrethroid insecticides are a mainstay of bed bug control owing to their broad-spectrum activity, persistence in the environment and low cost, their efficacy is on the decline for bed bug control because of resistance.<sup>8</sup> Laboratory studies consistently detect widespread insecticide resistance in

field-collected populations.<sup>4,6,9</sup> Recent reports have provided compelling evidence that many bed bug populations have developed resistance to pyrethroid insecticides, and that resistance may lead to cross-resistance to other classes of insecticides. Recently, high levels of resistance to four neonicotinoids (acetamiprid, imidacloprid, dinotefuran and thiamethoxam) were detected in field populations of bed bugs,<sup>10</sup> including the Jersey City strain used in this study.

Various mechanisms contribute to resistance in bed bugs, and the geographic distribution of resistant bed bug populations is global. Two point mutations, V419L and L925I, have been identified as one mechanism for knockdown resistance (*kdr*) to deltamethrin in bed bug populations in New York.<sup>11</sup>

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Subsequent studies found one or both mutations in bed bug populations throughout the United States,<sup>12</sup> Paris,<sup>13</sup> Central Europe,<sup>14</sup> Australia<sup>15</sup> and Israel,<sup>16</sup> suggesting that *kdr*-mediated resistance is a global phenomenon. Genome-wide analysis of *C. lectularius* has revealed many resistance-associated genes and mechanisms, including upregulation of P450s, esterases and ABC transporters.<sup>17,18</sup> Therefore, the ubiquitous evolution of resistance to pyrethroid and neonicotinoid insecticides in bed bug populations appears to involve multiple mechanisms, which further highlights the need for alternative approaches to control bed bugs.

Alternative approaches to bed bug control vary in complexity, cost of implementation and efficacy. The use of high temperatures to kill bed bugs has increased in popularity over the past decade.<sup>19</sup> While effective, the use of volumetric heating can be expensive, ranging from US \$500 to US \$1000 per room.<sup>20</sup> When alternative methods of control are not possible, placing infested items in a household freezer for several days to kill all life stages remains an option.<sup>21</sup> Other approaches to bed bug control include the use of diatomaceous earth, various essential oils and detergents. Current methods of bed bug control often lack satisfactory efficacy, leaving the public seeking environmentally safe options that are new, innovative and effective.

Entomopathogenic fungi have demonstrated effectiveness against numerous public health pests, including malaria vectors,<sup>22</sup> cockroaches<sup>23</sup> and houseflies.<sup>24</sup> Furthermore, fungal pathogens have been shown to be effective against insecticide-resistant mosquito populations.<sup>25</sup> *Beauveria bassiana* has been identified as a potential candidate for bed bug control.<sup>26</sup> The aim of this study was to compare the efficacy of a new *B. bassiana*-based product on an insecticide-susceptible lab strain of *C. lectularius* and three field-collected strains known to be resistant to pyrethroids. We also compared the mortality of the four bed bug strains after exposure to either a commercial deltamethrin-based insecticide labeled for bed bug control or *B. bassiana*.

## 2 EXPERIMENTAL METHODS

### 2.1 Bed bug maintenance

The four strains of bed bugs used in these experiments were maintained in small plastic containers (Consolidated Plastics, Stow, OH) with plankton netting (BioQuip, Rancho Dominguez, CA) on the bottom for ventilation and through which bed bugs fed on blood. Harborage made of manila folders folded accordion style were used to provide shelter. Insects were maintained in environmental growth chambers at  $27 \pm 1^\circ\text{C}$  on a 12 h light:12 h dark cycle and  $50 \pm 5\%$  relative humidity. Colonies were fed defibrinated rabbit blood (Quad Five, Ryegate, MT) using custom-made glass feeders (Prism Research Glass, Raleigh, NC), and bed bugs were fed 24 h prior to the experiments.

The Harold Harlan strain (= Ft Dix strain) was collected in Fort Dix, New Jersey, in 1973 and has been maintained at North Carolina State University since December 2008; both the V419L and L925I mutations are absent in this strain. The Winston Salem No. 7 (collected in 2008 in North Carolina) and the Jersey City (collected in 2008 in New Jersey) strains are both resistant to pyrethroid insecticides (see Section 3), and have both the V419L and L925I mutations. Campus Courtyard No. 15 (collected in 2009 in North Carolina) has not yet been tested for *kdr* mutations, but is known to be resistant to pyrethroids (see Section 3). The Jersey City strain is also moderately resistant to neonicotinoid insecticides.<sup>10</sup>

### 2.2 Insecticides

Aprehend™ is a formulation of *B. bassiana* (GHA strain) containing 2% (w/v) active ingredient (AI) and a minimum concentration of  $2.4 \times 10^9$  viable conidia mL<sup>-1</sup> (ConidioTec LLC, State College, PA). The ready-to-use formulation was applied at approximately  $2 \mu\text{L cm}^{-2}$  to white, Diamond Double Faced Quilt Fabric (Jo-Ann Fabric and Craft Stores, Hudson, OH), chosen because this fabric is commonly used by manufacturers of box springs (beds). To check the volume applied, surfaces were weighed before and after spray application to determine the actual volume and number of applied conidia per cm<sup>2</sup>. The average volume applied was found to be  $1.72 \mu\text{L cm}^{-2}$ , equivalent to  $4.48 \times 10^6$  viable conidia cm<sup>-2</sup>. The treated fabric was left to dry at room temperature and used for exposure of bed bugs within 2 weeks of the spray application.

Suspend SC (Bayer CropScience, Research Triangle Park, NC) contains 4.75% deltamethrin and is labeled for application at a maintenance rate of 0.03% (w/v) AI and a clean-out rate of 0.06% (w/v) AI in water. These two treatments, referred to hereafter as the low and high concentrations respectively, were applied to white, Diamond Double Faced Quilt Fabric using a potter precision laboratory spray tower (Burkard Scientific, Uxbridge, UK) at a volume of  $15 \mu\text{L cm}^{-2}$ . Air pressure was provided by a carbon dioxide canister (R&D Sprayers, Opelousas, LA) at a pressure of 152 kPa. Treated fabric was permitted to dry overnight and used the following day for bed bug exposure.

### 2.3 Efficacy of *B. bassiana* on four bed bug strains (2014 experiment)

Twenty-four hours after feeding to repletion, adult male bed bugs of unknown ages were randomly placed into either a control group ( $n = 50$ ) or a treatment group ( $n = 50$ ). Each of these groups was further divided into five replicates of ten bugs per strain. Bed bugs were then exposed for 15 min to dry fabric treated with *B. bassiana* in a 9 cm diameter petri dish (VWR, Radnor, PA). Control bed bugs were exposed to fabrics sprayed with water (dried overnight). Following exposure, bed bugs were transferred to clean petri dishes and sealed with Parafilm®. Bed bugs were housed in environmental growth chambers under the same conditions as described above for the duration of the experiment. Bed bugs were checked for mortality 24 h after exposure and then once daily for 14 days. Mortality was defined as the inability of a bed bug to right itself after being flipped on its back, and the lack of any visible muscle twitches over a 1 min period. Dead bed bugs were removed daily and dried over silica gel for 1 week. The dry cadavers were then placed individually into 30 mL plastic diet cups (Dart, Mason, MI) with moist, sterile cotton and allowed to incubate at  $\sim 23^\circ\text{C}$  for 3 days. Each bed bug cadaver was assessed for the presence or absence of mycosis, based on the appearance of white *B. bassiana* conidia around the leg joints and intersegmental membranes.

### 2.4 Comparison of deltamethrin and *B. bassiana* efficacy on four bed bug strains (2015 experiment)

To evaluate the relative susceptibility of the four strains of bed bugs to deltamethrin in comparison with *B. bassiana*, a second bioassay was conducted using high and low concentrations of deltamethrin alongside the *B. bassiana* treatment. Bioassay procedures were identical to those used in the 2014 bioassays, except that Suspend SC was applied to the fabric swatches using a potter spray tower and allowed to dry overnight.

**Table 1.** Proportional hazard tests of the effects of strain and treatment on survival time, with year as a random effect

Effect	Wald $\chi^2$	df	$P > \chi^2$	Adjusted df	Bonferroni adjusted $P > \chi^2$
Strain	95.634	3	<0.0001	2.9999	<0.0001
Treatment	491.252	3	<0.0001	2.9832	<0.0001
Strain $\times$ treatment	277.244	9	<0.0001	8.9997	<0.0001
Year	6.362	—	—	0.8642	0.0092

## 2.5 Statistical analysis

Data for survival analysis consisted of survival times for 50 individual bugs for each combination of year (2014 and 2015), strain (four strains) and treatment (four treatments). The period of observation was 14 days, and bed bugs that survived beyond the period of observation were coded as right censored. Kaplan–Meier non-parametric comparisons between years within treatment indicated that survival differed significantly across years, but the effect was small in comparison with observed treatment effects (see Table 1). Accordingly, random year effects were included in a single proportional hazard regression, or Cox model, to investigate simple factorial effects of the treatment for fixed strains. The model was fitted using PROC PHREG.<sup>27</sup> The Harold Harlan controls exhibited the most survivors and were taken as the baseline in the formulation of the proportional hazard model. The estimated log hazard ratios relative to this baseline were used for statistical separation of treatments within each strain. Where appropriate, mean survival times (MSTs), median survival times and relative log hazard ratios were estimated.

## 3 RESULTS

The overall model indicated that the estimated variance component for the random effect (year) was statistically significant ( $P = 0.0092$ ) but small (estimated variance component = 0.0308) in comparison with observed strain and treatment effects (Table 1). Therefore, a single proportional hazard regression was implemented with random year effects included to investigate the effects of the treatments for fixed strains. Survival curves for all treatments and bed bug strains are shown in Fig. 1. Estimated MSTs and median survival times for each bed bug strain and treatment are summarized in Table 2.

Two treatments were replicated in two independent experiments, in 2014 and 2015, the control treatment and the *B. bassiana* treatment. Relatively low mortality occurred in the control populations of bed bugs (exposed to fabric treated with water) across the four strains over the 14 day duration of the two experiments, with  $\leq 8\%$  mortality in 2014 (mean survival  $\pm$  SEM:  $95.5 \pm 1.5\%$ ) and  $\leq 44\%$  mortality in 2015 (mean survival:  $83.0 \pm 2.7\%$ ). Overall survivorship across all four strains was  $89.3 \pm 1.5\%$ . The estimation of mean survival time (MST) in Kaplan–Meier survival analysis requires that the majority of subjects die within the monitoring period in order to calculate accurate estimates. As a result, MSTs for each of the control populations could not be estimated (Table 2).

Mortality across all four bed bug strains exposed to *B. bassiana*-treated substrates was  $99.0 \pm 0.7\%$  in 2014 and  $95.5 \pm 1.4\%$  in 2015 by the end of the 14 day monitoring period (overall survivorship:  $2.5 \pm 0.8\%$ ) (Fig. 1). Mean survival times were similar for all four strains, ranging from 4.6 days (Campus Court-yard) to 5.3 days (Winston Salem), with no significant differences among them according to the Wilcoxon test statistics computed from the Kaplan–Meier curves ( $\chi^2 = 3.670$ ,  $df = 3$ ,  $P = 0.2994$ ).

Mortality in the *B. bassiana*-treated bed bugs began on days 2 or 3 after exposure, and bed bugs in all four strains reached between 70 and 80% mortality by day 4 (Fig. 1). Mycosis was confirmed in 100% of cadavers in the *B. bassiana* treatments. Only one bed bug cadaver in the control group was found to have mycosis, and this individual was from the Harold Harlan strain.

The four strains of bed bugs were differentially affected by exposure to fabric treated with deltamethrin. Harold Harlan strain bed bugs were highly susceptible to both high and low concentrations of Suspend SC, with 100% mortality within 7 and 11 days respectively (Fig. 1A), and MSTs of 3.0 and 4.8 days (Table 2). There were no significant differences in survival of Harold Harlan bed bugs between the two deltamethrin treatments and *B. bassiana* (Table 2).

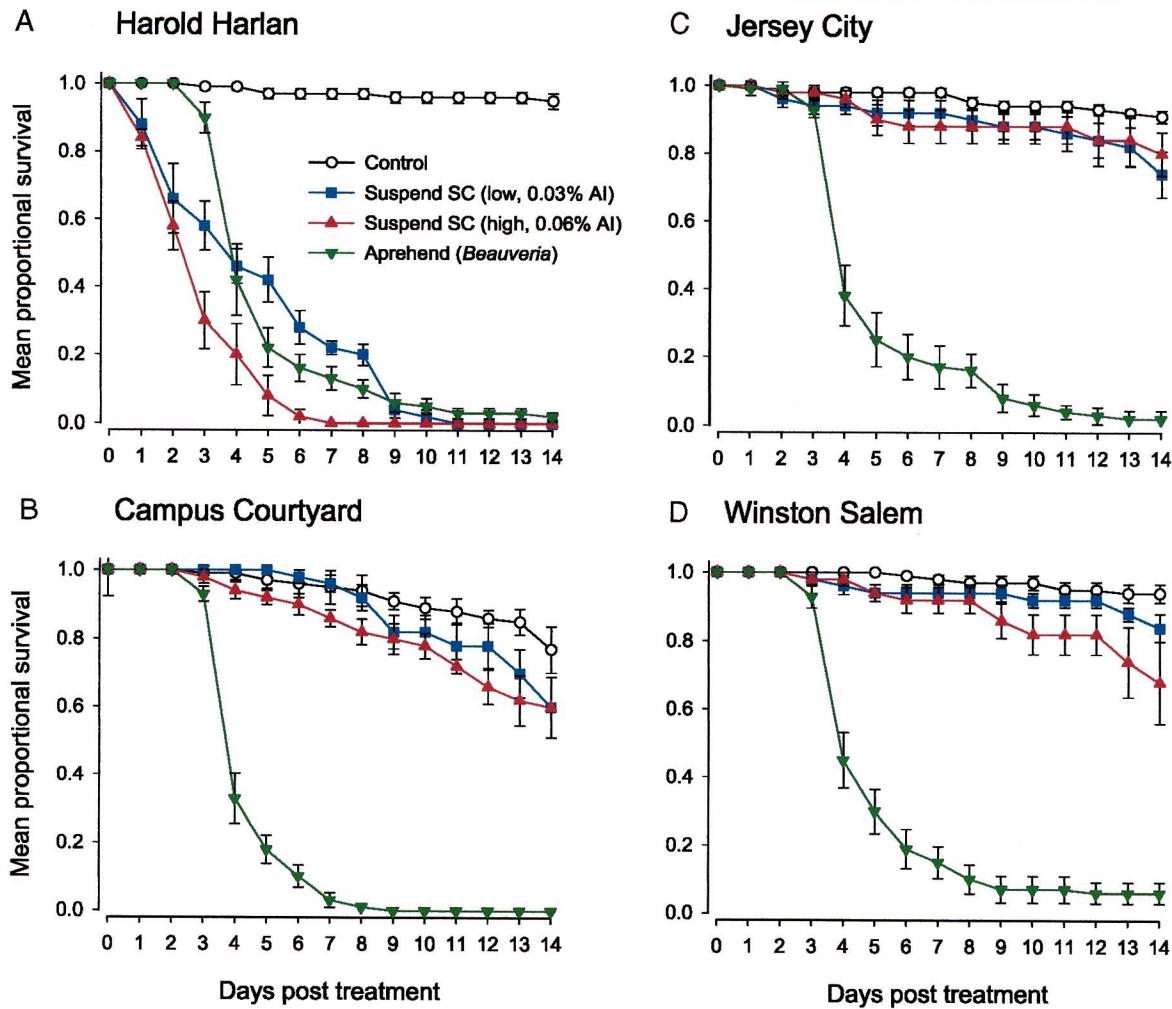
In contrast, the three field-collected strains were highly resistant to deltamethrin, with only 16–40% mortality 14 days after exposure (Figs 1B to D). In two of these strains (Campus Court-yard and Jersey City) the survivorship was not significantly different from the control bed bugs, whereas in the Winston Salem strain only the high concentration of deltamethrin resulted in lower survivorship than in the controls (Table 2). For all three strains, deltamethrin-treated bed bugs survived significantly longer than *B. bassiana*-treated bed bugs (Table 2).

## 4 DISCUSSION

Our evaluations confirmed that the Harold Harlan strain was susceptible to deltamethrin, while the field-collected Campus Court-yard, Jersey City and Winston Salem bed bug strains tolerated treatments with relatively high concentrations of deltamethrin. Although resistance has been shown to decline over time in some bed bug colonies following years of laboratory rearing without pyrethroid selection,<sup>28</sup> these three populations retained sufficiently high pyrethroid resistance after 7–8 years in laboratory culture to experience low mortality after 15 min exposure even to a high concentration of Suspend SC.

Most importantly, this study corroborates that resistance to pyrethroid insecticides does not confer cross-resistance to infection by *B. bassiana*. Similar results have been observed for *Anopheles* spp. mosquitoes, which demonstrated that resistance to permethrin, DDT and bendiocarb did not result in reduced infection by *B. bassiana* or *M. anisopliae*.<sup>25</sup> This study also demonstrated that *B. bassiana*- and *M. anisopliae*-infected mosquitoes with *kdr* mutations displayed increased susceptibility to chemical insecticides.<sup>25</sup> While we have not investigated this combined effect, it is possible that a similar change in susceptibility could occur in deltamethrin-resistant bed bugs and is worthy of further investigation.

Cuticular thickening appears to contribute to insecticide resistance in bed bugs and other insects, presumably by impeding insecticide penetration. This mechanism has been reported in insecticide-resistant Triatomine kissing bugs,<sup>29</sup> the housefly *Musca*



**Figure 1.** Proportional survival of fed adult bed bugs of four bed bug strains after 15 min exposure to fabric treated with *B. bassiana* (Aprehend™) at the recommended rate and deltamethrin (Suspend SC) at recommended high (0.06%) and low (0.03%) label rates. Control bed bugs are from the same strain as the respective treated bed bugs and were exposed to water-treated fabric for 15 min. Bars represent SEM.

*domestica*,<sup>30</sup> the German cockroach *Blattella germanica*,<sup>31</sup> *Anopheles* mosquitoes<sup>32</sup> and bed bugs.<sup>33</sup> Given that the mode of infection of *B. bassiana* is via germination of the conidia and direct penetration of the appresorium through the cuticle of the host insect, thickening of the cuticle might be expected to impede *B. bassiana* infection. Nevertheless, studies have shown that *B. bassiana* was effective on insecticide-resistant insect populations, for example *Anopheles* mosquitoes,<sup>25</sup> and even on pyrethroid-resistant *Triatomina infestans* with thicker cuticles and greater amounts of cuticular lipids.<sup>29</sup> Therefore, even if cuticle thickening is associated with insecticide resistance in any of our three bed bug strains, it would appear that *B. bassiana* infection was unaffected. It is important to identify the mechanisms that permit entomopathogenic fungi effectively to infect insects with a thicker cuticle. One mechanism, identified in *T. infestans*, involves degradation of cuticular hydrocarbons.<sup>29</sup> Notably, however, detailed descriptions of the infection process by entomopathogenic fungi identify the mouthparts, intersegmental folds and spiracles as the primary sites of invasion.<sup>34</sup> As such, generalized cuticular thickening might not interfere with infection unless it is accompanied with changes in

thinner and softer areas of the cuticle, where conidia preferentially germinate and penetrate.

Aprehend™ is a ready-to-use oil formulation that has been developed to permit application at ultralow volume rates without the addition of water. Oil formulations of fungal conidia have been demonstrated to enhance efficacy<sup>35,36</sup> and facilitate the movement and accumulation of conidia into protected recesses on the insect body.<sup>37–39</sup> Oil formulations create a favorable microenvironment for germination and infection and enhance the efficacy of mycoinsecticides at low (<50%) humidity,<sup>40</sup> and certain oil-based formulating components may disrupt the protective layer of epicuticular lipids, facilitating host penetration.<sup>38</sup> Unlike most chemical insecticides, where the duration of exposure is key to adsorption of the active ingredient through the cuticle, *B. bassiana* relies on movement of the bed bugs over the surface to pick up spores on the tarsi and other body parts. Bed bugs that remain quiescent in one place collect fewer spores on their body surface, resulting in slower time to death, and in some cases survival because a lethal dose was avoided. We expect that longer exposure beyond 15 min

**Table 2.** Kaplan–Meier estimates of mean survival time  $\pm$  standard errors (not given in cases where substantial bias was caused by mortality below 50%), median survival times and log hazard ratio  $\pm$  standard errors relative to baseline (Harold Harlan control). Fed adult males of four strains of bed bugs were exposed for 15 min to control (water-treated), deltamethrin (Suspend SC)-treated or *B. bassiana* (Aprehend™)-treated surfaces. A proportional hazard model was applied to investigate factorial effects of the treatment, separately for each strain, using SAS PROC PHREG

Strain	Treatment <sup>a</sup>	Grouping <sup>b</sup>	Mortality	Mean survival time $\pm$ SE (days) <sup>c</sup>	Median survival time (days)	Relativelog hazard ratio $\pm$ SE
Harold Harlan	Control	A	5/100 (5%)	–	≥14	0.00 $\pm$ 0.00
	Suspend SC low	B	50/50 (100%)	4.8 $\pm$ 0.45	4	4.62 $\pm$ 0.48
	Suspend SC high	B	50/50 (100%)	3.0 $\pm$ 0.22	3	5.56 $\pm$ 0.48
	Aprehend™	C	98/100 (98%)	5.1 $\pm$ 0.23	4	4.42 $\pm$ 0.46
Campus Courtyard	Control	A	23/100 (23%)	–	≥14	4.42 $\pm$ 0.46
	Suspend SC low	A	20/50 (40%)	–	≥14	4.42 $\pm$ 0.46
	Suspend SC high	A	20/50 (40%)	–	≥14	4.42 $\pm$ 0.46
	Aprehend™	B	100/100 (100%)	4.6 $\pm$ 0.12	4	4.42 $\pm$ 0.46
Jersey City	Control	A	9/100 (9%)	–	≥14	0.61 $\pm$ 0.56
	Suspend SC low	A	13/50 (26%)	–	≥14	1.86 $\pm$ 0.53
	Suspend SC high	A	10/50 (20%)	–	≥14	1.59 $\pm$ 0.55
	Aprehend™	B	98/100 (98%)	5.3 $\pm$ 0.25	4	4.38 $\pm$ 0.46
Winston Salem	Control	A	6/100 (6%)	–	≥14	0.18 $\pm$ 0.61
	Suspend SC low	AB	8/50 (16%)	–	≥14	1.33 $\pm$ 0.57
	Suspend SC high	B	16/50 (32%)	–	≥14	2.08 $\pm$ 0.51
	Aprehend™	C	94/100 (94%)	5.3 $\pm$ 0.27	4	4.28 $\pm$ 0.46

<sup>a</sup> The active ingredient in Suspend SC is deltamethrin, and in Aprehend™ it is *B. bassiana*. Low and high refer to the labeled rate for application at a maintenance rate of 0.03% (w/v) deltamethrin and a clean-out rate of 0.06% (w/v) deltamethrin respectively.

<sup>b</sup> Pairwise comparisons of survival of the four treatments within each strain with Bonferroni-adjusted P-values. Within a strain, treatments with the same letter do not differ significantly, based on a statistical comparison of log hazard ratios ( $P > 0.05$ ).

<sup>c</sup> A dash denotes that estimation could not be performed.

would increase the likelihood of bed bug movement and hence greater efficacy of *B. bassiana*.

There are few effective classes of insecticides labeled for bed bug control, and pyrethroid insecticides, alone or in combination with neonicotinoid insecticides, have become a mainstay in bed bug interventions.<sup>41</sup> However, the overuse of pyrethroid- and neonicotinoid-based products and cross-resistance have selected for the evolution of resistance in many bed bug populations.<sup>10</sup> Pest management practices traditionally used in agricultural systems, including monitoring insecticide efficacy and managing resistance, are largely ineffective for bed bug management because (1) few active ingredients with different modes of action are available for use in rotations, and (2) reservoirs of insecticide-susceptible bed bugs do not persist under the strong selection pressure and relatively closed spatial and genetic structure of bed bug populations.<sup>42</sup> Alternative approaches such as whole-building heat treatment and fumigation are helpful tactics when pyrethroid resistance is high. These approaches are expensive, however, and because they leave no residual insecticide, re-infestations are likely from within the building or from outside sources. *B. bassiana* has a unique mode of action with no known resistance or cross-resistance in bed bugs, and it is highly effective on pyrethroid-resistant bed bugs, making it an excellent candidate for use in bed bug management programs. Entomopathogenic fungi are often considered slow acting compared with neuroactive insecticides. Although death due to *B. bassiana* infection was indeed slower in the insecticide-susceptible bed bug population, within 4 days after exposure its efficacy was similar to that of deltamethrin. Furthermore, the tendency of bed bugs to aggregate is likely to increase the dissemination of the fungus within the harborage and enhance overall population control.<sup>26</sup>

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