Sublethal Effects of *Active*Guard Exposure on Feeding Behavior and Fecundity of the Bed Bug (Hemiptera: Cimicidae)

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ABSTRACT Sublethal exposure to pesticides can alter insect behavior with potential for population-level consequences. We investigated sublethal effects of *Active*Guard, a permethrin-impregnated fabric, on feeding behavior and fecundity of bed bugs (*Cimex lectularius* L.) from five populations that ranged from susceptible to highly pyrethroid resistant. After exposure to *Active*Guard fabric or untreated fabric for 1 or 10 min, adult virgin female bed bugs were individually observed when offered a blood meal to determine feeding attempts and weight gain. Because bed bug feeding behavior is tightly coupled with its fecundity, all females were then mated, and the number of eggs laid and egg hatch rate were used as fecundity measures. We observed that pyrethroid-resistant and -susceptible bugs were not significantly different for all feeding and fecundity parameters. Bed bugs exposed to *Active*Guard for 10 min were significantly less likely to attempt to feed or successfully feed, and their average blood meal size was significantly smaller compared with individuals in all other groups. Independent of whether or not feeding occurred, females exposed to *Active*Guard for 10 min were significantly more likely to lay no eggs. Only a single female exposed to *Active*Guard for 10 min laid any eggs. Among the other fabric treatment–exposure time groups, there were no observable differences in egg numbers or hatch rates. Brief exposure of 10 min to *Active*Guard fabric appeared to decrease feeding and fecundity of pyrethroid-resistant and susceptible bed bugs, suggesting the potentially important role of sublethal exposure for the control of this ectoparasitic insect.

KEY WORDS *Cimex lectularius*, fecundity, feeding behavior, insecticide-treated fabric, permethrin

Insecticide efficacy studies typically focus on death as the primary endpoint of interest; hence, many studies overlook sublethal effects that also may adversely affect the growth of pest populations. Sublethal exposure to insecticides has been shown to alter insect behavioral traits including feeding, egg laying, host searching, movement, and even perception of the insecticide itself (Haynes 1988). These changes in behavior at the individual level can have population-level consequences (Maltby 1999); they are so important that the dual consideration of lethal and sublethal effects is now recommended for impact assessments of insecticides on target as well as nontarget organisms (Stark and Banks 2003). Insecticides typically play a major role in control of the bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae). Hence, a better understanding of the sublethal effects of insecticides may be key to the management of these resurgent ectoparasites.

Bed bugs are obligate blood feeders that preferentially feed on humans. A blood meal is required for molting to the next instar, and bed bugs typically feed every 3 to 7 d, depending on environmental variables and host availability (Reinhardt and Siva-Jothy 2007). A hungry bed bug's feeding behavior includes leaving its refugium at night in search of a host, locating a host, probing the host's skin until a blood vessel is located, feeding to repletion, and finally moving away from the host to a secluded site to digest the blood meal (Reis and Miller 2011). Bed bugs imbibe large volumes of blood relative to their size, with an individual bug ingesting up to 6.1 times its weight in a single meal (Usinger 1966).

Adverse allergic reactions typically occur at the bite site in the majority of humans (Potter et al. 2010). As illustrated in deShazo et al. (2012), the mere act of probing can cause cutaneous reactions. In severe bed bug infestations, blood loss from bug bites can result in anemia (Pritchard and Hwang 2009, Paulke-Korinek et al. 2012). Additional human health effects from bed bugs include secondary bacterial infections, asthma, anaphylactic shock, sleeplessness, agitation, and anxiety (Eddy and Jones 2011).

As for many insects, feeding behavior and fecundity are inherently linked in bed bugs. Their mating behavior is influenced by feeding behavior given that male bed bugs preferentially mate with recently fed females (Usinger 1966, Stutt and Siva-Jothy 2001, Siva-Jothy 2006). Furthermore, a female bed bug typically

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requires a blood meal for egg production, and the size of the blood meal is strongly correlated with the number of eggs that she subsequently lays (Johnson 1941).

Pyrethroid resistance has been implicated as a major factor in the global resurgence of bed bugs (Davies et al. 2012). It is, in part, resistance that makes bed bugs such a difficult pest to control. Bed bugs require multiple integrated pest management strategies such as extremely thorough inspections, sanitation, a wide variety of nonchemical and chemical measures, and follow-up monitoring. *Active*Guard Mattress Liners (550 mg of permethrin per square meter) are marketed as a preventive measure or for inclusion in integrated pest management programs to combat bed bugs. *Active*Guard fabric is nonrepellent to bed bugs, as they readily spend time on the permethrin-impregnated fabric; however, the fabric has sublethal effects on bed bug movement parameters such as velocity and the total distance traveled (Jones et al. 2013). In this study, we investigated the impact of brief exposure to *Active*Guard fabric on feeding responses and fecundity of pyrethroid-resistant and -susceptible bed bugs.

Materials and Methods

Bed Bugs. Five bed bug populations were used in the current study. The Pointe, Republic, and EPM populations originated in Columbus, OH, and were collected between 2010 and 2012. The Earl population was collected from Modesto, CA, in 2007 (acquired from Sierra Research Laboratory in 2012). In addition, the Harlan strain, which was initially collected in 1973 from Ft. Dix, NJ, and laboratory reared thereafter, was used as an internal control for our study because it is a pyrethroid-susceptible population (Zhu et al. 2010). Bed bugs were maintained under controlled conditions ($29 \pm 2°C$, 50% relative humidity, and a photoperiod of 12:12 [L:D] h). Each population of bed bugs was housed in one or more glass jars (13 cm in height by 7 cm in diameter; narrow-mouth Mason pint jar, Ball Corp., Broomfield, CO) containing filter paper strips for harborage, with an organza fabric and filter paper covering held in place with a screw-on metal ring.

To obtain virgin adult bed bugs, groups of fifth instar nymphs from each of the five populations were isolated and fed on defibrinated rabbit blood (Hemostat Labs, Dixon, CA) through a Parafilm membrane using the Hemotek 5W1 system (Discovery Workshops, Accrington, United Kingdom). Individual bugs that had fed to repletion were isolated into individual wells of a 24-well plate (surface area 2 cm^2; Falcon, Corning Life Sciences, Corning, NY) and observed daily to determine when molting occurred (date recorded). The resulting adults were kept isolated in the individual wells until the study commenced. Only adult virgin female bed bugs were used for feeding experiments; adult virgin males were used only for mating purposes.

Pyrethroid Resistance Testing. Bed bugs from the five populations were screened for resistance to deltamethrin, a representative pyrethroid, using a residual assay (Romero et al. 2007, 2009). Technical-grade deltamethrin (99% purity; Chem Service, West Chester, PA) was diluted in acetone to obtain a log series of concentrations. Each dilution was applied to a filter paper disc (Whatman no. 1, GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) to provide 0.00005, 0.0005, 0.005, 0.05, 0.5, and 5 mg deltamethrin/cm^2. Filter paper treated with acetone served as the control. Treated filter paper was placed in a fume hood for 30 min to allow the acetone to evaporate. Ten adult bed bugs were placed in direct contact with the treated filter paper in an open petri dish, and their condition was assessed after 24 h. Three replicates were established per concentration with a total of 210 bugs from the Republic, EPM, Earl, and Harlan populations. Because of limited numbers of adult bugs, the Pointe population was screened at only the highest concentration (plus control).

The number of moribund or dead bed bugs at 24 h was used to calculate the median lethal concentration, LC_{50}. Resistance ratios (RR; LC_{50} resistant/LC_{50} susceptible) were used to describe the resistance of the populations relative to the Harlan strain, the most susceptible population (Moore and Miller 2006, Romero et al. 2007). Based on the LC_{50} and RR values, Pointe was categorized as highly resistant to deltamethrin, Republic and EPM were moderately resistant, Earl was borderline susceptible/moderately resistant, and Harlan was susceptible (Table 1).

Feeding Experiments. Each adult virgin female bed bug was weighed to the nearest 0.01 mg and then randomly assigned to a glass petri dish (1.5 cm in height by 9 cm in diameter) lined with *Active*Guard fabric or untreated fabric (identical fabric that was not impregnated with permethrin) for either 1 or 10 min. Based on previous research observations (Jones et al. 2013), we chose a 1-min exposure time to act as a proxy for an immediate effect and a 10-min exposure time as one that would not be acutely toxic. Upon removal from the fabric, each bug was immediately offered a blood meal provided with the Hemotek membrane feeding system. Each individual was continuously observed during a 30-min feeding opportunity, and its feeding attempts (as characterized by probing the feeding interface) and the total time that it spent feeding were recorded. After 30 min, each bug was reweighed to the nearest 0.01 mg to determine the size of the blood meal, if any. Feeding success was defined as a gain in weight exceeding 0.1 mg. Approximately 40 bed bugs per population (range 36–42) were tested in this manner for each fabric–exposure time combination.

Table 1. Calculated LC_{50}, RR, and resistance category for bed bugs from five populations used in the current study

Population	LC_{50} (mg/cm^2)	RR	Resistance category
Pointe	>5.0000	>3,500.0	Highly resistant
Republic	0.0750	54.6	Moderately resistant
EPM	0.0677	49.3	Moderately resistant
Earl	0.0160	11.7	Susceptible/moderately resistant
Harlan	0.0014	1.0	Susceptible

Fecundity Evaluation. To evaluate the effect of fabric treatment and exposure time on fecundity, each female was transferred into a well plate provisioned with a 15-mm Whatman filter paper pad, and then a fed virgin male from the same population was added. The pair was observed during a single 30-min period until mating and decoupling occurred, and then the male was removed. The female was monitored periodically for egg production and the subsequent egg hatch.

Statistical Analyses. We analyzed the effects of fabric treatment and exposure time on the feeding behavior of an individual bug using binary logistic regression in the statistical platform R 3.0.2 (R Core Team 2013, Vienna, Austria). We first explored the effects of fabric treatment and exposure time on whether an individual had attempted to feed (as indicated by probing). In a second model, we evaluated these factors' effects on whether an individual had fed. Both models had dichotomous response variables, and population, fabric treatment, exposure time, and the interaction between fabric treatment and exposure time were considered as fixed effects. We used Wald Z tests to assess the statistical significance of these fixed effects. We also computed least-squares means differences to explore the interaction between fabric treatment and exposure time using the *lsmeans* function from the *lsmeans* package (Length 2013). *P* values were adjusted using the Tukey method when necessary.

To assess the statistical significance of population on either attempts to feed (model 1) or feeding success (model 2), we performed Wald tests using the *wald.test* function from the *aod* package in R (Lesnoff and Lancelot 2012). The binary logistic regression coefficients give the change in the log odds of the response variable for a one unit increase in the predictor variable. To aid in the interpretation of these results, we created a new variable for fabric treatment–exposure time combination ($N = 4$) and reran both models using population and fabric treatment–exposure time combination as fixed effects. We then exponentiated the coefficients from both models and interpreted them as odds ratios, which are relative measures of effect (UCLA: Statistical Consulting Group 2014b). Odds ratios are a convenient way to illustrate the likelihood that an event will occur in one group compared with a reference group, which R automatically chooses based on alphabetical order. For example, if the ratio of the odds for Group A to the odds for Group B is 1.35, then the odds that an event will occur for Group A are 1.35 times greater than the odds for Group B (UCLA: Statistical Consulting Group 2014a). Note that an odds ratio of 1 indicates that both groups have the same likelihood of an event occurring (Szumilas 2010). We also calculated the predicted probabilities of attempts to feed (model 1) or feeding success (model 2).

For those individuals that successfully fed, we analyzed pooled data to determine whether the gain in weight from prefeeding to postfeeding, a proxy for blood meal size, differed between the fabric–exposure time groups. While the sample size per group was highly variable (range 4–41), neither the homogeneity of variance assumption was violated (Levene's test, $F = 0.49$; $P = 0.69$) nor were assumptions of normality (Kolmogorov–Smirnov Test, $D = 0.12$; $P = 0.10$), suggesting the validity of the analysis. Difference in blood meal size was assessed using analysis of variance and post hoc pairwise Tukey comparisons.

Zero-inflated Poisson regression with the function *zeroinfl* from the *pscl* package (Zeileis et al. 2008) was used to evaluate whether the fabric–exposure time group affected the number of females that laid no eggs. This analysis is commonly used to model count data with many zeroes, and it was appropriate to use here based on our observations that many females in the experiment did not feed. Zero-inflated Poisson regression allowed us to evaluate whether the likelihood that an individual produced no eggs was a result of the fabric treatment–exposure time group, not because of the lack of a blood meal. We then tested whether the zero-inflated Poisson regression model fit the data better than 1) the null model (i.e., the intercept-only model) using a chi-squared test, and 2) an ordinary Poisson regression model using the Vuong Test (using the function *vuong* from the *pscl* package; Zeileis et al. 2008).

Results

Feeding Attempts. Feeding attempts of all virgin adult female bed bugs, regardless of whether or not they successfully fed, are shown in Fig. 1. There was no difference among populations ($\chi^2 = 4.2$; df $= 4$; $P = 0.38$), but there was a significant interaction between fabric treatment and exposure time ($z = 3.06$; $P = 0.002$). As shown in Fig. 1, for those individuals on *Active*Guard fabric, bugs exposed for 10 min were significantly less likely to attempt to feed compared with bugs exposed for 1 min (predicted probability of attempted feeding $= 0.17$ and 0.65, respectively; z ratio $= 4.66$; $P < 0.001$). On the other hand, individual bugs on untreated fabric showed no difference in feeding attempts between 10- and 1 min exposure times (predicted probability of attempted feeding $= 0.87$ and
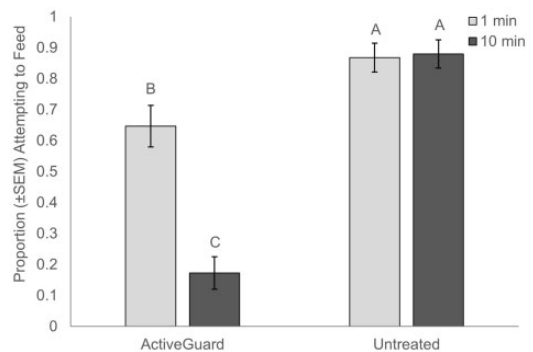
Fig. 1. Proportion (mean ± SEM) of feeding attempts by virgin adult female bed bugs (pooled data) when presented with a blood meal following exposure for either 1 or 10 min to *Active*Guard or untreated fabric. Bars with different letters are significantly different based on post hoc pairwise Tukey comparisons.

Table 2. Odds ratios of feeding attempts and feeding success compared with a reference group (for population, the reference group is Earl, for fabric–exposure time, the reference group is *Active*Guard–10 min)

Focal group	Feeding attempts odds ratio (95% CI)[a]	Feeding success odds ratio (95% CI)[a]
Population: EPM	1.35 (0.44–4.23)	0.75 (0.26–2.11)
Population: Harlan	0.98 (0.32–2.96)	0.75 (0.26–2.11)
Population: Pointe	2.94 (0.89–10.33)	0.84 (0.29–2.43)
Population: Republic	1.04 (0.34–3.17)	0.68 (0.24–1.93)
*Active*Guard fabric– 1-min exposure	9.53 (3.84–25.98)	12.58 (4.32–46.36)
Untreated fabric– 1-min exposure	35.28 (12.48–115.25)	46.19 (15.06–179.96)
Untreated fabric– 10-min exposure	39.1 (13.36–134.64)	25.7 (8.67–96.72)

[a] If the confidence interval (CI) includes 1, it implies that there is no difference between the reference group and the focal group.

Fig. 2. Proportion (mean ± SEM) of virgin adult female bed bugs (pooled data) that successfully fed following exposure for either 1 or 10 min to *Active*Guard or untreated fabric. Bars with different letters are significantly different based on post hoc pairwise Tukey comparisons.

0.88, respectively; z ratio $= -0.17$; $P = 0.86$). Individuals exposed to *Active*Guard fabric for either 1 or 10 min were significantly less likely to attempt to feed when compared with individuals exposed to untreated fabric for either 1 min (z ratio $= -2.59$; $P = 0.048$ and z ratio $= 6.33$; $P < 0.001$, respectively) or 10 min (z ratio $= -2.66$; $P = 0.039$ and z ratio $= -6.27$; $P < 0.001$, respectively).

For all odds ratio comparisons of the fabric–exposure time groups, the *Active*Guard 10 min exposure group was used as the reference group because comparisons with that group were the most biologically relevant. The odds of attempting to feed were 10 times greater for those individuals exposed to *Active*Guard for 1 min compared with those exposed to *Active*Guard for 10 min (Table 2). Compared with those bugs exposed to *Active*Guard for 10 min, the odds of attempting to feed were 35 times greater for those individuals placed on untreated fabric for 1 min and 39 times greater for those individuals on untreated fabric for 10 min.

Feeding Success. As in the previous model, there was no significant difference in feeding success among the bed bug populations ($\chi^2 = 0.61$; df $= 4$; $P = 0.96$). There was a significant interaction between fabric treatment and exposure time ($z = 2.61$; $P = 0.009$; Fig. 2). For those individuals on *Active*Guard fabric, bugs exposed for 10 min were significantly less likely to successfully feed compared with bugs exposed for 1 min (predicted probability of feeding success $= 0.08$ and 0.51, respectively; z ratio $= 4.28$; $P < 0.001$). Individuals on untreated fabric, on the other hand, showed no difference in feeding success between 10- and 1-min exposure times (predicted probability of feeding success $= 0.79$ and 0.68, respectively; z ratio $= 1.29$; $P = 0.20$). Individuals exposed to *Active*Guard fabric for 1 min were significantly less likely to feed compared with individuals exposed to untreated fabric for 1 min (z ratio $= -2.95$; $P = 0.017$), but not significantly different from individuals exposed to untreated fabric for 10 min (z ratio $= -1.73$; $P = 0.31$). Compared with individuals exposed to *Active*Guard fabric for 10 min, individuals exposed to the untreated fabric for 1 min and 10 min were significantly more likely to feed
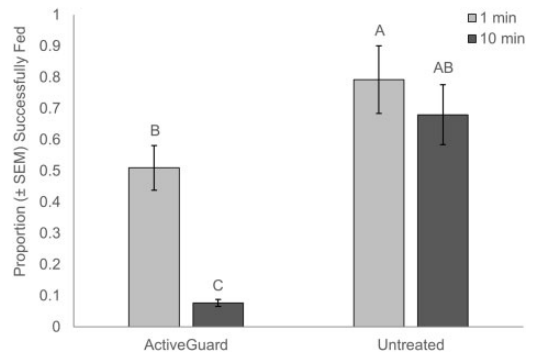
(z ratio $= 6.16$; $P < 0.001$ and z ratio $= -5.38$; $P < 0.001$, respectively).

The odds ratios shown in Table 2 indicate that all five bed bug populations had similar odds of feeding success, but they differed in their feeding likelihoods depending on the fabric treatment and exposure time. For those individuals on *Active*Guard fabric for 1 min, the odds of feeding success were 13 times greater than the odds for those individuals placed on *Active*Guard fabric for 10 min. Furthermore, the odds of feeding for those individuals on untreated fabric for 1 min were 46 times greater than the odds for individuals on *Active*Guard fabric for 10 min, and the odds of feeding were 26 times greater for those individuals exposed to untreated fabric for 10 min when compared individuals exposed to *Active*Guard for 10 min.

Blood Meal Size and Weight Gain. For those individuals that successfully fed, there was a significant interaction between fabric treatment and exposure time for blood meal size ($F = 5.81$; df $= 1$; $P = 0.018$; Fig. 3). Blood meal size was significantly smaller for individuals exposed to *Active*Guard for 10 min when compared with those exposed to *Active*Guard for 1 min ($P = 0.047$) and those exposed to untreated fabric for either 1 min ($P = 0.009$) or 10 min ($P = 0.011$). The average blood meal size did not significantly differ among individuals exposed to *Active*Guard for 1 min, untreated fabric for 1 min, and untreated fabric for 10 min.

Fecundity. Independent of whether feeding occurred, females exposed to *Active*Guard for 10 min were significantly more likely to produce zero eggs when compared with females exposed to *Active*Guard for 1 min ($z = -2.54$; $P = 0.011$), untreated fabric for 1 min ($z = -2.18$; $P = 0.029$), or untreated fabric for 10 min ($z = -2.59$; $P = 0.010$). Modeling the data with zero-inflated Poisson regression fit significantly better than both the intercept-only model ($P < 0.001$) and the ordinary Poisson regression model (Vuong nonnested hypothesis test statistic $= -7.14$, $P < 0.001$).

Of those females exposed to *Active*Guard for 10 min, only a single individual produced any eggs (Table 3).
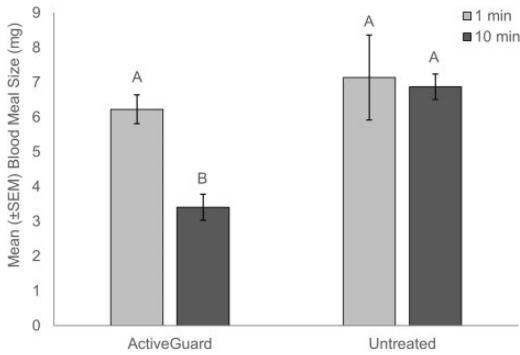
Fig. 3. Mean (mean ± SEM) blood meal size (mg) for virgin adult female bed bugs (pooled data) that successfully fed when presented with a blood meal following exposure for either 1 or 10 min to *Active*Guard or untreated fabric. Bars with different letters are significantly different based on post hoc pairwise Tukey comparisons.

Table 3. Female bed bug fecundity measures (mean ± SEM) for each fabric treatment and exposure time

Fabric	Exposure time	N^a	No. of females that fed	No. of females that laid eggs	Mean no. of eggs per female (SEM)	Mean no. of eggs hatched (SEM)
Active	1 min	49	24	18	8.33 (0.91)	7.56 (0.93)
Guard	10 min	52	4	1	5.00 (NA)	5.00 (NA)
Untreated	1 min	52	41	25	8.00 (0.77)	7.12 (0.72)
	10 min	49	33	24	9.00 (0.73)	8.38 (0.66)

[a] Number of females offered a blood meal.

As such, we were unable to rigorously assess the effects of fabric treatment or exposure time on either differences in the number of eggs produced per female or differences in the hatch rate among groups. When analyzed as fabric treatment–exposure time groups (excluding *Active*Guard 10-min exposure data), we found that neither the number of eggs produced per female ($F = 0.44$; df = 2; $P = 0.64$) nor the hatch rate of those eggs ($F = 0.77$; df = 2; $P = 0.47$) differed among groups.

Discussion

Short-term exposure to *Active*Guard fabric was observed to alter bed bugs' feeding behavior, which may help to limit bed bugs' impact on humans. Significantly fewer bed bugs attempted to feed, as characterized by probing the feeding interface, after being exposed to *Active*Guard fabric compared with untreated fabric, regardless of whether bugs were exposed for 1 or 10 min (Fig. 1). Given that bed bug bites often result in painful allergic skin reactions in humans, reduced bed bug feeding attempts following brief exposure to the *Active*Guard liner may translate to humans experiencing fewer bed bug bites.

Bed bug feeding behavior is a critical factor for population growth since a blood meal acquired from a host provides the females with nutrients for egg production. Exposure to *Active*Guard fabric for 10 min significantly reduced females' abilities to successfully feed compared with those exposed to *Active*Guard for 1 min. Ten minute exposure to *Active*Guard resulted not only in a five-fold decrease in feeding success (Fig. 2), but these females subsequently were significantly less likely to lay eggs (Table 3). In fact, in our study, only a single female laid eggs. This effect was independent of whether or not a female had fed. Although newly molted adult female bed bugs typically require a blood meal to lay eggs, autogeny occasionally occurs and is presumably associated with residual nutrition

remaining from the previous instar (Usinger 1966). Reduced female fecundity after 10-min exposure to *Active*Guard fabric would result in slower population growth, which would have real-world consequences. Exposure time to the *Active*Guard fabric was an important factor, as 1 min was insufficient to significantly alter feeding success and the resulting fecundity. Future research is needed to determine if these sublethal effects persist over time.

Whether the reduction in fecundity observed in those females exposed to *Active*Guard was a direct effect or simply a result of reduced feeding success was not explicitly tested in our study. Although there were striking differences in the numbers of females that laid any eggs for the fabric treatment–exposure time groups (Table 3), the hatch rate of eggs was not significantly different among the three groups (*Active*Guard 10-min group excluded). This suggests that the observed reduction in fecundity likely is an indirect effect of altered bed bug feeding behavior, but further work is needed to clarify this mechanism.

Our study demonstrates the sublethal effects of *Active*Guard exposure on the feeding behavior and fecundity of bed bugs, regardless of their pyrethroid resistance level. Bed bug susceptibility to pyrethroids, as represented by deltamethrin, varied widely among the populations tested in our study; the most resistant population, Pointe, was >3,500 times more resistant to deltamethrin than Harlan, the most susceptible population (Table 1). In contrast, acute toxicity of the *Active*Guard fabric was found to vary depending on pyrethroid resistance level, with only susceptible and moderately resistant bed bugs succumbing by 1 d (Jones et al. 2013). Three of the bed bug populations (EPM, Earl, and Harlan) used in the Jones et al. (2013) study and in the current study showed a decline in RRs from 2012 to 2014. Declining resistance status has been observed in other bed bug populations maintained in the laboratory (Polanco et al. 2011, Potter et al. 2012). This is a factor that requires consideration when rearing bed bugs in the laboratory.

*Active*Guard exposure for 10 min altered the feeding behavior in female bed bugs across populations, such that exposed females were less likely to attempt to feed and, of those that fed, only a minority did so successfully. While some target insects alter their behavior to minimize contact with insecticides, *Active*Guard is non-repellent (Jones et al. 2013) and can be positioned in

areas where bed bugs likely harbor such as on the mattress and box spring, suggesting that bugs that come in contact with the fabric may spend sufficient time on it to amass sublethal effects. Just a 1-min exposure to the *Active*Guard fabric was sufficient to reduce the bugs probing attempts. These findings demonstrate the potential of *Active*Guard fabric to have sublethal effects on bed bugs after very short exposure times.

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