Fate of Dermally Applied Miticides Fluvalinate and Amitraz Within Honey Bee (Hymenoptera: Apidae) Bodies

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ABSTRACT Varroa mites, *Varroa destructor* Anderson & Trueman, are economically important pests of honey bees. Varroa mites are principally controlled within honey bee colonies using miticides. However, despite their importance in managing mite populations for apiculture, potential effects of miticides on honey bees are poorly understood. Using gas chromatography-flame ionization detection, we investigated concentrations, over variable time frames and within different body regions, of two commonly used miticides, tau-fluvalinate and amitraz, after dermal exposure to honey bees. We also quantified mortality of honey bees exposed to each miticide at both a low and high dose. Significant differences were observed in distributions of miticides among body regions. Within honey bee body parts, tau-fluvalinate was more readily absorbed and decreased in concentration more rapidly than amitraz. Mortality increased with higher dosages of miticides, and at higher dosages mortality was greater from fluvalinate than from amitraz. For individual honey bees, our results for rate of breakdown suggest that fluvalinate may be the preferred miticide for apiculturists, whereas our mortality results suggest that amitraz may be preferable. Either choice must be weighed against geographic variation in varroa resistance to each pesticide and attendant costs of parasitism.

KEY WORDS miticides, amitraz, tau-fluvalinate, honey bees, *Varroa destructor*

Honey bees, *Apis mellifera* (L.) are important agricultural assets, both for the direct production of commodities such as honey and beeswax, and for vital pollination services provided to support a wide range of wild and cultivated crops (Klein et al. 2007). In recent years, declines in colony health have come to the forefront of apicultural research. Although Colony Collapse Disorder (CCD) has been a highly publicized cause for hive losses throughout North America and Europe, its contribution to declines may have been overemphasized (Williams et al. 2010). In many cases, losses to honey bee colonies have been attributed to identifiable causes such as: poor weather, parasitic *Nosema ceranae* microsporidian fungi, and most significantly, parasitic *Varroa destructor* Anderson & Trueman mites (De la Rúa et al. 2009, Neumann and Carreck 2010). Human impacts on honey bees may also play a considerable role in colony vitality, particularly from the input of agrochemicals for protecting crops, or even the honey bees themselves.

Honey bees may be exposed to a wide range of pesticides, particularly insecticides, when foraging for pollen and nectar from agricultural crops (a typical scenario in North America because most colonies are maintained for pollination services). As a consequence of this contamination, there is a potential for bioaccumulation of residues over time within the hive (Albero et al. 2004, Rossi et al. 2004, Totti et al. 2006). Recently Mullin et al. (2010) reported 121 different pesticide and pesticide metabolite residues in wax, comb, pollen, and honey bees from North American hives. Furthermore, evidence by Smodiš Škerl and Gregorc (2010) has demonstrated that treatment with coumophos and imidacloprid induce heat shock protein stress response and apoptosis in the hypopharyngeal gland of *Apis mellifera carnica* Pollmann. This presents a concern for human health, because of the contamination of honey bee products, and accordingly may affect health and survivorship of honey bees.

Applications of chemical controls are frequently required to control *V. destructor* mites and maintain colony health. Miticides such as Apistan (the synthetic pyrethroid tau-fluvalinate; hereafter 'fluvalinate') and Apivar (the foramidine amitraz) are among several control products for management of *V. destructor* (Martel et al. 2007, Smodiš Škerl et al. 2010). Application of these miticides generally occurs during the summer, before adding honey supers, via the installation of strips impregnated with the active ingredient (i.e., 10% fluvalinate, or 800 mg/colony) that provide a slow, long-term release into the colony (Tsigouri et al. 2004). During the recommended 4- to 8-wk treatment period, this results in a 5–10% release of fluvalinate (0.5–1 mg/wk/colony; Bogdanov et al. 1998). Amitraz is applied at a rate of 6.25 g/strip, and presumed to release at a similar slow rate (Medici et al.

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2012). Spread of these compounds may occur via vaporization into the air of the hive, impregnation and exposure in beeswax or honey, and by direct contact with honey bees, so specific concentrations that individual honey bees may be exposed to during their lives may be highly variable. Numerous studies have reported sublethal effects of pesticides on honey bees (Mamood and Waller 1990, Decourtye et al. 2005, Desneux et al. 2007). Furthermore, successive generations may also be exposed through contact with residues in honey and beeswax (Korta et al. 2001, Bogdanov 2006). These chemicals' relatively low toxicity to honey bees has been attributed, in part, to detoxifying enzymes (Johnson et al. 2009) that allow them to metabolize fluvalinate and/or amitraz. However, little is known about the build-up, temporal movement, or detoxification of these compounds within honey bee body parts.

A more complete understanding of miticide movement in honey bee body parts could suggest ways to reduce harmful effects of such pesticides. Objectives of this study were to document distribution and concentration of miticides in honey bees after dermal exposure, and to quantify honey bee mortality from miticides.

Materials and Methods

Honey Bee Collection. Forager honey bees were captured near the hive entrance of a colony in Berwick, Nova Scotia, Canada (45° 4′26″ N, 64° 44′55″ W). Honey bees were individually captured in plastic scintillation vials outside the hive and transported to the lab. Honey bees were cooled in a freezer until immobile, and then treated with miticide or control. Treated honey bees were placed into ventilated plastic dishes, where sugar water was available ad libitum.

Reagents and Chemicals. Analytical standards of tau-fluvalinate ([Cyano-(3-phenoxyphenyl)methyl] 2-[2-chloro-4-(trifluoromethyl)-5–niline]-3-methylbutanoate; molecular weight: 502.91), amitraz (N,N′-[(methylimino)dimethylidyne]di-2,4-xylidine; molecular weight: 293.41), and all solvents (hexane, acetone, and isopropanol) were purchased from Sigma–Aldrich (St. Louis, MO). Pesticide standards were all PESTANAL grade and all solvents were HPLC grade (99%).

Miticide Application. Separate treatments of fluvalinate and amitraz were applied using a previously established protocol for standardized dermal delivery (Johnson et al. 2009). Briefly, compounds were dissolved in 1.25 μl of acetone, individually applied dermally to the notum (dorsal thorax) using a 10-ul Hamilton syringe, and delivered at two separate loadings of 0.125 μg (estimated to be the daily exposure per honey bee in treated hives; Johnson et al. 2009) and 1.25 μg (a 10-fold increase). Apivar loadings were matched to this concentration for direct comparison. Control honey bees were treated with an equal volume of acetone without miticides. After treatment, honey bees were placed in the dark in ventilated petri dishes at room temperature for 1, 6, or 24 h and subsequently frozen to halt possible detoxification (i.e., defecation, metabolism).

Sample Preparation. Residues of miticides in honey bee body parts were measured using gas chromatography and whole body levels of each miticide were assessed at 1, 6, and 24 h posttreatment to evaluate rate of detoxification in high-dosage honey bees (preliminary testing indicated that device sensitivity was insufficient to reliably quantify residues within low dosage treatments). Honey bee bodies were dissected and body parts analyzed for miticide accumulations in the head, thorax, and abdomen. For each individual, wings and legs were removed using iridectomy scissors. Equipment was washed and rinsed in hexane between dissections. Heads, thoraces, and abdomens were individually weighed on an analytical balance, placed in separate vials, and stored at −20°C until residue analysis.

Before analysis, samples were removed from the freezer, and body parts were rinsed using a liquid-liquid extraction (100 μl distilled water, 220 μl isopropanol, and 220 μl hexane). Samples were individually rinsed in this solution on a rocker for 3 h at room temperature, and then stored at −20°C for 1 h. This process separated liquid extract into two parts delineated by a meniscus. The top layer of the meniscus was removed, and placed in 1-ml eppendorf tubes. Samples were concentrated to a final volume of 1 μl under a gentle flow of ultra-high purity nitrogen gas, and analyzed using gas chromatography to quantify miticide concentrations.

Instrumentation and Chromatographic Analyses. Analysis of miticide standards and extracts from honey bees was conducted using a Varian 450GC Gas Chromatograph (Varian Canada Inc., Mississauga, Ontario, Canada), equipped with a Flame Ionization Detector (GC-FID). A VF-5 (30 m × 0.25 mm internal diameter × 0.39 mm outer diameter) analytical column was used with helium as a carrier gas. All samples (1.0 μl) were injected manually in split mode at a ratio of 1:20 for 1 min. Helium was used as the carrier gas at a flow of 1.0 ml/min. Temperatures of the injector and detector were 250 and 300°C, respectively. Operating conditions consisted of the following for each miticide:

1. Fluvalinate: For analysis of fluvalinate standards and honey bees treated with fluvalinate, oven temperature was maintained at 50°C for 15 s after injection, then increased by 100°C/min for 1 min to 150°C and then increased by 10°C/min to 250°C and held for 15 min.
2. Amitraz: For analysis of amitraz standards and honey bees treated with amitraz, oven temperature was maintained at 210°C for 1 min, increased by 12°C/min for 2 min to 230°C, then 7°C/min for 4 min to 250°C, then 12°C/min for 4.16 min to 300°C and held for 7.3 min.

Quantities of either fluvalinate or amitraz were measured by comparing heights of peaks in chromatography output relative to a standard curve produced

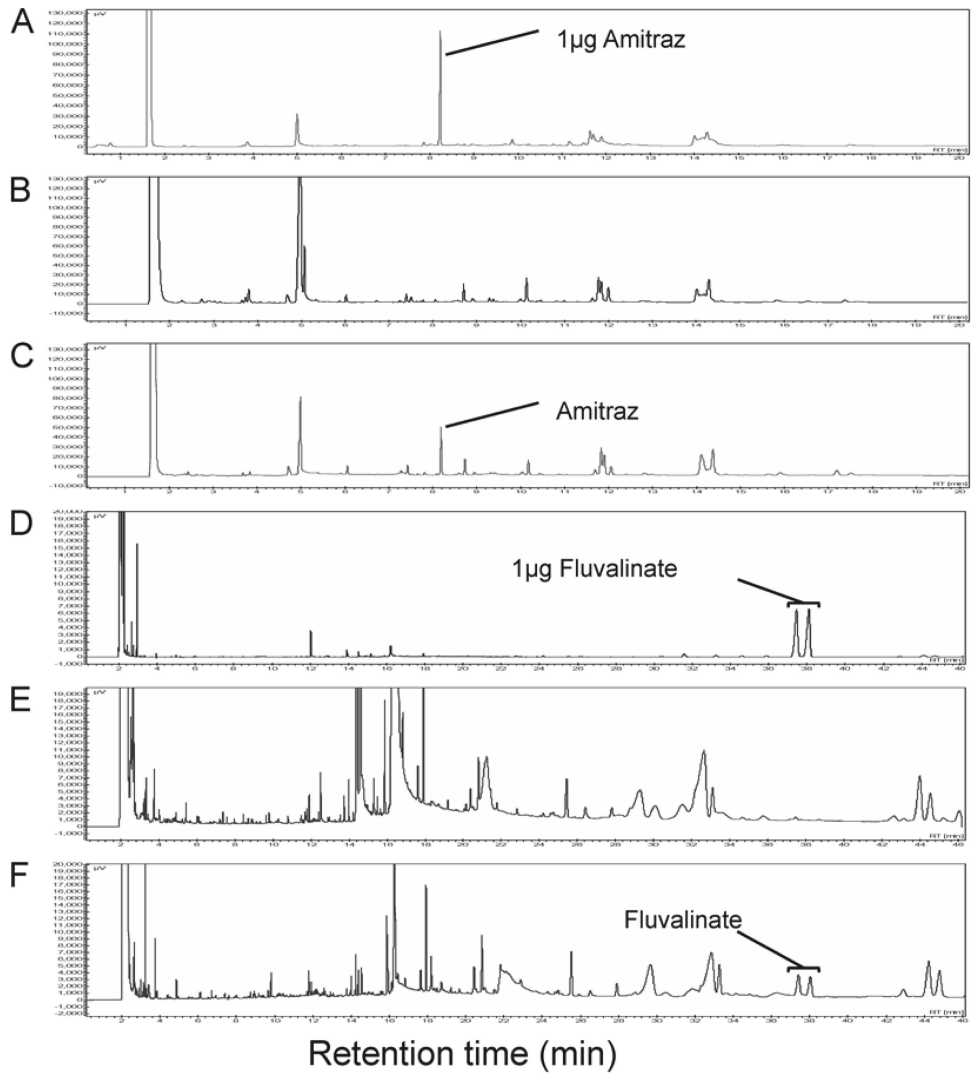

Fig. 1. Exemplar GC traces of 1-μl injections of: (A) 1 μg/μl amitraz standard; (B) an extract of a control honey bee 6 h after dermal application of an acetone blank; (C) an extract from a honey bee 6 h after dermal application of 1.25 μg of amitraz; (D) 1 μg/μl fluvalinate standard; (E) an extract of a control honey bee 6 h after dermal application of an acetone blank; (F) an extract from a honey bee 6 h after dermal application of 1.25 μg of fluvalinate. Data shown are 'absolute measures,' before being standardized by weight.

by running quantified volumes (1 ng to 100 μg) of fluvalinate and amitraz as external standards.

Mortality. Honey bee mortality was quantified at 1, 6, and 24 h posttreatment. When dishes were removed from incubation, dead individuals were removed and not included in residue analysis.

Statistical Analysis. Statistical analyses were conducted in SAS (SAS Institute, Cary, NC). A General Linear model (GLM) was used to test for differences in acaricide residues among body parts of honey bees, among treatments, and among time intervals after exposure. The model initially included all two-way interaction terms; nonsignificant interactions were dropped sequentially to produce final models (Crawley 2005). If interactions were significant, analyses proceeded within each category of class variables. Mortality was assessed using logistic regression with mortality as the response variable, and treatment and time (1, 6, and 24 h) as class explanatory variables.

Results

In total, 349 honey bees were screened for residues (45 in each of the controls, 129 for fluvalinate, and 130 for amitraz), and a total of 1,604 honey bees were observed for mortality.

Residues. Repeatable measures of concentration were obtained from standards of both amitraz and fluvalinate. Furthermore, peaks were easily distin-

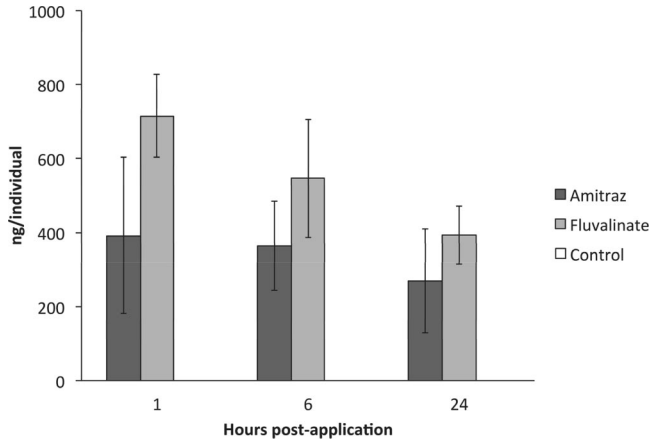

Fig. 2. Total residues (ng) recorded per honey bee at 1-, 6-, and 24-h intervals after 1.25 μg dermal application of amitraz or fluvalinate (n = 15/treatment).

guished from other extraneous material from honey bee preparations (Fig. 1).

Amitraz and fluvalinate were both evident at much higher concentrations in treated versus control honey bees (Fig. 1). Comparison of whole-body miticide content indicated higher overall retention of fluvalinate than amitraz (i.e., 700 vs. 400 ng 1 h posttreatment), but there was a decline in levels of both chemicals with time (Fig. 2).

The interaction between treatment and time was not a significant predictor of miticide residues (GLM, $F = 0.7$; df = 6, 317; $P = 0.68$), and was dropped from the model. Interactions between body part and time ($F = 18.5$; df = 6, 323; $P < 0.0001$) and body part and miticide ($F = 2.7$; df = 4, 323; $P = 0.03$) were significant, so analyses were partitioned accordingly.

Standardized masses (by weight of body parts being sampled) show relatively higher residues in the head, and lower residues in the abdomen versus absolute measures in Fig. 1 (Table 1; as would be expected by the relative size of these body parts). Treatment influenced residues in all body parts, but differences among body parts were only distinct in the thorax, where fluvalinate residues were higher than amitraz residues and higher than both controls (Table 2). Residues peaked in the abdomen at 6 h for both treatments; in contrast, residues were higher in the thorax (as might be expected because this is where miticides were applied) 1 h after application, and declined thereafter (Table 2). No detectable residues were noted in control honey bees.

Significant differences were observed between the residual concentration of fluvalinate and amitraz for all body parts. For both fluvalinate and amitraz, significant differences were observed in concentrations among body parts (Figs. 3 and 4; Table 2). Residues of both amitraz and fluvalinate were significantly higher in the thorax than in the head and abdomen (Table 2).

Honey Bee Mortality. Time interval after treatment (Wald $\chi^2_2 = 302.6$; $P < 0.0001$) and treatment (Wald χ^2 (df = 4) = 97.8; $P < 0.0001$) both significantly influenced mortality. Not surprisingly, mortality was significantly higher at 6 h (χ^2 (df = 1) = 10.7; $P = 0.001$) and at 24 h (χ^2 (df = 1) = 294.5; $P < 0.0001$) than at 1 h posttreatment (Fig. 5). Low dose fluvalinate (χ^2(df = 1) = 0.3; $P = 0.59$) and low dose amitraz (χ^2(df = 1) = 2.1; $P = 0.15$) did not cause greater mortality than was observed in controls, but both high dose fluvalinate (χ^2(df = 1) = 80.4; $P < 0.0001$) and high dose amitraz (χ^2(df = 1) = 7.8; $P = 0.005$) treatments were associated with higher mortality than was observed in controls. Mortality in other treatments within time intervals was not significantly different from acetone controls. Finally, high dose fluvalinate caused higher mortality than high dose amitraz at both 6 and 24 h posttreatment (Fig. 5).

Table 1. Mean (±SE) fluvalinate and amitraz residues standardized as ng/mg (=ppm) of body part mass (head, thorax, and abdomen) recorded at three time intervals

Body part mass	Mean (±SE) fluvalinate and amitraz residues (ng/mg)		
	1 h	6 h	24 h
Fluvalinate			
Head	1,360.6 (1236.7)	739.6 (739.0)	758.0 (599.9)
Thorax	21,986.6 (3649.1)	16,714.5 (3287.1)	5,672.3 (1402.8)
Abdomen	0.0	122.0 (121.9)	9 (8.5)
Amitraz			
Head	2,314.1 (712.6)	2,217.6 (536.6)	1,395.2 (544.8)
Thorax	11,319.4 (2235.6)	10,016.5 (1743.2)	7,813.3 (2371.3)
Abdomen	148.2 (57.6)	542.5 (225.6)	123.3 (72.5)

Table 2. Variables that remained in partitioned General Linear Models after sequential removal of nonsignificant terms, and associated statistics

Analysis within	Explanatory variables left in model	*F*	df	*P*	Interpretation[a]
Head	Treatment	3.0	3,107	0.03	Not statistically distinct
Thorax	Treatment	19.8	3,111	<0.0001	Fluvalinate^{A}, amitraz^{B}, Fluvalinate control^{C}, amitraz control^{C}
	Time	2.9	2,111	0.06	1 h^{A}, 6 h^{AB}, 24 h^{B}
Abdomen	Treatment	3.7	3,107	0.01	Not statistically distinct
	Time	3.5	2,107	0.03	6 h^{A}, 1 h^{AB}, 24 h^{B}
Amitraz	Body part	53.4	1,124	<0.0001	Thorax^{A}, head^{B}, abdomen^{B}
Fluvalinate	Body part	68.1	2,121	<0.0001	Thorax^{A}, head^{B}, abdomen^{B}

[a] Items sharing superscripted letters were not statistically different based on Tukey tests.

Discussion

GC-FID reliably quantified fluvalinate and amitraz from honey bee body part samples. Amitraz and fluvalinate were not found in detectable concentrations within control honey bees, suggesting that the colonies selected for study had low initial exposure to both miticides (only oxalic acid had been used for treatment of these hives). Fluvalinate is generally present at higher concentrations than amitraz within the body after similar dermal application. This may indicate that fluvalinate is more readily transported across the cuticle or is more soluble in acetone. The majority of all fluvalinate and amitraz was localized in the thorax. When standardized by body part mass, concentrations were still greatest in the thorax, followed by the head and abdomen. Such concentrations are high in comparison with previous research. For example, Smodiš Škerl et al. (2010) found residues ranging between 0.11–0.26 ng/mg in honey bee heads and as high as 1.00 ng/mg in larvae. This, of course, may be attributed to the relatively higher dosage treatment tested for residue analysis in our study.

Residues of fluvalinate generally declined over time (the small concentration within the head being the exception, but this may be related to the limits of detection for this assay). Concentrations of fluvalinate were consistently higher in the thorax even when the mass of each body part was used to standardize concentrations recorded. Over the course of 24 h, honey bees eliminated almost 50% of the fluvalinate in the thorax, indicating rapid removal or degradation of fluvalinate. Despite having a lower concentration at 1 h, amitraz concentrations decreased more slowly, from 10.8 ng/mg at 1 h to 7.2 ng/mg at 24 h. Given that honey bees were able to reduce concentrations of both compounds over 24 h, this also provides clarity regarding interpretation of residue studies. Mechanisms for eliminating these compounds within honey bees include direct metabolism, advection, deposition, and detoxifying P450 enzymes (Tremolada et al. 2004, Johnson et al. 2009). Concentrations of miticides in bees may occur from recent or recurrent exposure, rather than a consistent bioaccumulation that might occur over a long time period (i.e., overwintering). This is, of course, assuming that reductions in concentration remain linear and do not become asymptotic over time frames greater than those tested here.

It is also possible that the fate of these miticides is affected by the age of individual honey bees. Johansen (1977) discusses differential sensitivity to insecticides, with newly emerged honey bees being most sensitive to DDT, dieldrin, and carbaryl, whereas older bees were more susceptible to malathion and methyl-para-

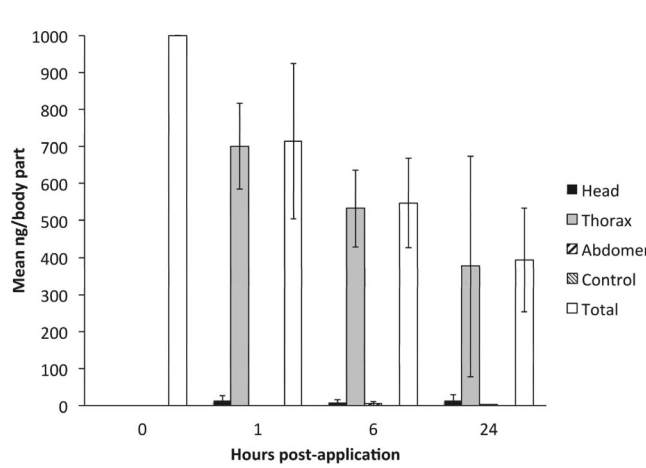

Fig. 3. Mean residues (ng) in body parts recorded per honey bee at 1-, 6-, and 24-h intervals after 1.25 μg dermal application of amitraz (n = 15/treatment). Highest concentrations were consistently noted within the thorax.

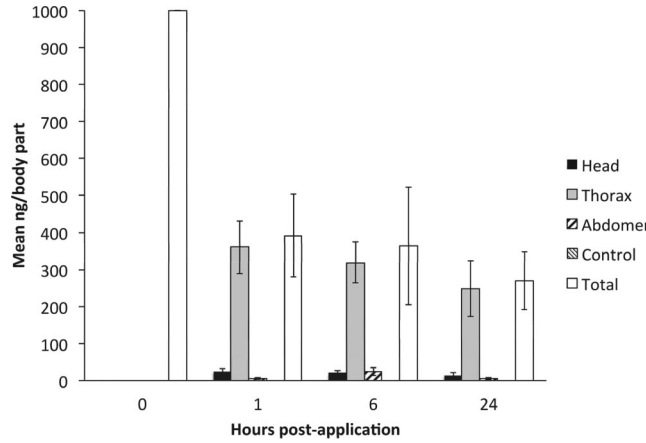

Fig. 4. Mean residues (ng) in body parts recorded from honey bee body parts at 1-, 6-, and 24-h intervals after 1.25 μg dermal application of fluvalinate (n = 15/treatment). As with amitraz, highest concentrations were consistently noted within the thorax.

thion. This may be confounded under hive conditions by the exposure of foragers to other contaminants in the field. For our study, forager honey bees were selected; however, further experiments are required to determine if similar trends are present for miticides within other ages/castes of honey bees.

We observed that honey bees exposed to amitraz defecated large volumes of feces during our experiments. It is unclear if this effect is detrimental or beneficial to honey bees, because it may cause physiological stress and dehydration, or may be a mechanism for rapid elimination of toxins from the body. Future work should include fecal analyses to determine if there are high levels of amitraz and metabolites within feces of treated honey bees.

Hazard assessments of synthetic pesticides to honey bees and other nontarget insects are most commonly based upon a 50% lethal dosage (=LD_{50}; Decourtye et al. 2005). However, a growing body of evidence indicates that sublethal effects of immediate pesticide exposure may have other harmful consequences (Johnson et al. 2009). This includes impaired physiological function, learning, memory, and behavior that ultimately affect colony maintenance and development (Abramson et al. 2004, Decourtye et al. 2005, Desneux et al. 2007, Han et al. 2010, Frost 2011). Therefore, it is important to evaluate carefully effects of such pesticides and potential for residual activity within honey bee bodies. This is particularly important in the case of miticides applied directly to colonies to manage *V. destructor* mites.

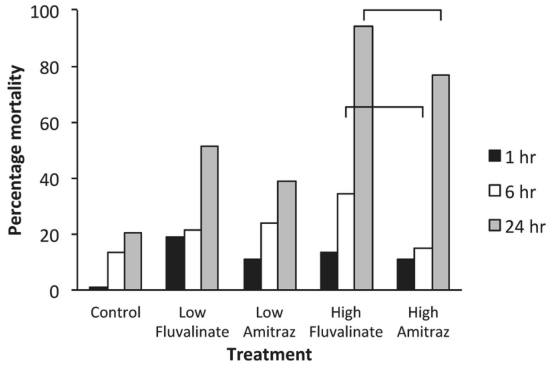

Fig. 5. Percentage mortality during three intervals after dermal application of low and high concentrations of amitraz and fluvalinate (n = 15/treatment). Horizontal parentheses indicate where significantly higher mortality occurred from fluvalinate than from amitraz (6 h after application $\chi^2_1 = 11.9$, $P = 0.0006$; 24 h after application $\chi^2_1 = 15.8$, $P < 0.0001$; χ^2_1 and Ps from remaining comparisons $\leq$2.4 and $\geq$0.12).

Honey bees exposed to low concentrations of fluvalinate and amitraz had relatively low mortality (not significantly different from controls), whereas high dosages did cause significant mortality. This was a somewhat delayed effect, most evident 24 h posttreatment and not significant at 1 or 6 h. Although our results indicate that high concentration dermal applications of amitraz and fluvalinate have a significant effect on honey bee mortality, these concentrations exceed those to which honey bees would normally be exposed. For honey bees that survive treatment, we found relatively rapid detoxification of high concentrations of amitraz and fluvalinate during the time course of this experiment (i.e., a reduction from a 1.25 μg application rate to under 400 ng in honey bee bodies). Though the concentrations of these applications are high, it is also worth noting that these are single-dose acute applications, whereas honey bees in the hive can be exposed to lower dosages of both fluvalinate and amitraz for months. Furthermore, this study examined exposure to miticides using a single dermal application. Given standard treatment methods for these miticides, and the complexity of hive environments, it is important to consider other routes of exposure. This includes airborne exposure, along with ingestion and trophallaxis of contaminated materials in hives (honey, pollen, etc.). Therefore, one must consider the rate of uptake through multiple

routes versus the rate of detoxification. Further studies are required to examine cumulative effects of these exposure pathways, and their interactions under normal hive conditions.

This study indicated that fluvalinate caused greater mortality from acute dermal exposure than did amitraz, and that residues broke down more slowly over the time scale examined (24 h). Such a result might suggest that amitraz would therefore provide greater safety for honey bee usage. However, this must be considered in terms of the lethal dosages of these compounds and their likelihood to bioaccumulate over time. Increased honey bee mortality may occur in instances where multiple, lipophilic, synergistic miticides build up in comb and wax (Johnson et al. 2009). Under these conditions, highly lipophilic miticide residues such as fluvalinate may be released from comb and wax long after application, and increase honey bee mortality should a second miticide be applied at a sublethal threshold at a later date (i.e., the combination of two sublethal compounds may increase toxicity). Cumulative effects of miticide exposure and synergism between in-hive compounds are poorly understood, and will require further investigation; this study provides an important starting point by examining removal rates from honey bee body parts.

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