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Original article

Effects of synthetic acaricides on honey bee grooming behavior against the parasitic *Varroa destructor* mite

Igor Medici DE MATTOS[1,2], Ademilson E. E. SOARES[1], David R. TARPY[2]

[1]Department of Genetics, Ribeirão Preto Medical School, University of São Paulo, 14.049-900, Ribeirão Preto, SP, Brazil
[2]Department of Entomology, North Carolina State University, Raleigh, NC 27695, USA

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Abstract – *Varroa destructor* is currently one of the main threats for western apiculture. Today, synthetic acaricides (specifically coumaphos, amitraz, and tau-fluvalinate) are the most common methods to control *Varroa* infestations. These compounds, however, are frequently related to a wide range of side effects in the host, as well as a long half-life inside the hive matrices (wax and honey). The western honey bee, *Apis mellifera*, exhibits natural defense mechanisms against the mite such as grooming behavior, which is a sequence of bodily movements where the host scrapes its legs across its body surface to remove the mite. We tested the effects of synthetic acaricides on the performance of grooming behavior by adult honey bee workers. We found that acaricide exposure prior to grooming delayed grooming and reduced the overall duration of grooming behavior. Our data add to a list of other sublethal behavioral consequences of acaricides that may subvert a comprehensive approach to *Varroa* control in managed colonies.

Apis mellifera **/ coumaphos / amitraz / tau-fluvalinate / grooming behavior /** ***Varroa destructor***

1. INTRODUCTION

It is known that honey bee populations are in declining health in Europe and North America (vanEngelsdorp et al. 2008; Mutinelli et al. 2010; Seitz et al. 2016). It is likely that these colony losses result from a combination of multiple factors, including diminished wildflower diversity and habitat, exposure to pesticides, and numerous diseases and parasites (Desneux et al. 2007; Hawthorne and Dively 2011; Dainat et al. 2012; Williamson et al. 2014). Nowadays, the parasitic mite *Varroa destructor* is considered one of the main concerns for apiculture worldwide (De Jong et al. 1982; Le Conte et al. 2010; Rosenkranz et al. 2010). This ectoparasite feeds on the hemolymph of adults and developing bees, vectoring viral pathogens, prompting malformations, undermining colony performance, and eventually resulting in colony death. The original host of *V. destructor* is the Eastern honey bee (*Apis cerana*), but it is believed that the mite host switched to *Apis mellifera* in the first half of the last century in regions where both species of bees were managed (Oldroyd 1999; Rosenkranz et al. 2010).

To this day, the most common practice to control *Varroa* is the use of in-hive acaricides (Ruffinengo et al. 2014; Mullin et al. 2010). Despite the often efficient *Varroa* control promoted by these chemicals, innumerous side effects have been observed. Two acaricides in particular, tau-fluvalinate and coumaphos, were ubiquitously prevalent in colonies and are frequently found at high concentrations (Mullin et al. 2010). Since the half-life of tau-fluvalinate and coumaphos is 5 years in wax (Bogdanov 2004), these pesticides can easily accumulate in colonies to reach unsafe levels (Haarmann et al. 2002; Mullin et al. 2010;

Corresponding author: I. Mattos, igormmattos@usp.br
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Williamson et al. 2014; Zhu et al. 2014). Coumaphos is a neurotoxic organophosphate that inhibits acetylcholinesterase, thus interfering with nerve signaling and function (Boncristiani et al. 2012). Recent studies have shown that coumaphos can alter some immune and detoxification gene expression pathways (Boncristiani et al. 2012; Garrido et al. 2013), affect queen and drone reproductive quality (Pettis et al. 2004; Rangel and Tarpy 2016), and diminish lifespan (Boncristiani et al. 2012). The pyrethroid tau-fluvalinate, an isomer of fluvalinate, targets the sodium channels of mites and insects altering neuronal electrical activity (Dong 2007; Eiri and Nieh 2012; Schmehl 2014). Tau-fluvalinate has already been reported as impacting queen and drone performance and competitiveness (Sokol 1996; Rinderer et al. 1999). Locke et al. (2012) also found direct effects of this pyrethroid on honey bees by increasing susceptibility to deformed wing virus infection. Some antennal olfactory receptor neurons also seem to be strongly sensitive to this pyrethroid (Kadala et al. 2011). Both acaricides are applied by beekeepers through pesticide-impregnated plastic strips and is subsequently distributed throughout a colony by nestmate interaction and trophallaxis (vanBuren et al. 1992; Bevk et al. 2012).

Another known acaricide, amitraz, is a formamidine octopaminergic agonist that can also impact learning and cognition in honey bees (Loucif-Ayad et al. 2008; Boncristiani et al. 2012; Garrido et al. 2013). Amitraz was one of the first acaricides to be registered in the USA, although in 1994 the preparation was withdrawn from the market (Johnson et al. 2013). Amitraz was reported targeting receptors in either the nervous or neuromuscular systems (Evans 1980; Papaefthimiou et al. 2013). Now amitraz is reregistered in some states of the USA and frequently found in beeswax (Mullin et al. 2010; Johnson et al. 2010; Semkiw et al. 2013).

Another negative consequence of the indiscriminate use of acaricides to control *Varroa* infestation is the repeated selection of mites that are resistant to each of these compounds (Milani 1995; Elzen and Westervelt 2002; Maggi et al. 2012). It has also been demonstrated that combined exposure to pesticides may synergize, resulting in the compounds being even more toxic to honey bees than when administered individually (Johnson et al. 2010; Zhu et al. 2014; Johnson 2015). For example, pre-exposure to amitraz can increase the toxicity of other acaricides (Johnson et al. 2013). Evidently, these complex combinations of pesticides may produce synergistic effects on the insect nervous system, especially when they affect the same physiological targets (Johnson et al. 2009; Hawthorne and Dively 2011; Gill et al. 2012).

Controlled breeding programs aimed at *Varroa* resistance have been conducted in some European honey bee populations, in both Europe and North America and, in some cases, has reached satisfactory results (Rosenkranz et al. 2010; Rinderer et al. 2010; Büchler et al. 1992). These programs are based on selecting bees according to various traits and activities that were identified as mechanisms for tolerance to *V. destructor* infestation in *A. mellifera* , most of which are also evident in the original host species. These include hygienic behavior, grooming behavior, and suppressed mite reproduction (Peng et al. 1987; De Jong 1988; Büchler et al. 1992; Rath 1999; Rosenkranz et al. 2010). Grooming behavior is a response to *V. destructor* parasitism that consists of worker bees scraping their legs over their own body in order to remove the ectoparasite (auto-grooming) or by attacking them directly with their mandibles when they are detected on a nestmate's body (allogrooming) (Ruttner and Hänel 1992; Rosenkranz et al. 1997). As a result, the mites can be injured or even killed when successfully groomed (Bienefeld et al. 1999). This behavior appears to be age dependent, and highly specialized workers that frequently employ allogrooming behavior tend to never develop into foragers (Moore et al. 1995). Despite the recent discussion on the effectiveness of traits related to *Varroa* -tolerant *Varroa* -sensitive hygienic (VSH) behavior (Danka et al. 2016), it is undeniable that the grooming behavior towards the ectoparasite promotes lower infestation rates (Rosenkranz et al. 2010; Rinderer et al. 2010).

Although much is known about the side effects of acaricides on bee health and toxicology, very

little is known about the effects of miticides on the behavior of *A. mellifera* , especially the effects of sublethal doses on the innate *Varroa* -resistance activities. Here, we quantify the effects of different acaricides on honey bee workers' grooming behavior in an effort to elucidate the possible side effects of acaricides on the social immunity of this ectoparasite-host relationship.

2. MATERIALS AND METHODS

We obtained European honey bee workers from a single colony to control genotype, kept at the North Carolina State University Lake Wheeler Honey Bee Research Facility (Raleigh, North Carolina, USA; 35.7806° N, 78.6389° W), by collecting them directly from brood frames. Immediately after sampling, we placed 20 sampled worker bees inside individual plastic cages ("holding cages") and fed 50% sucrose solution ad libitum. We maintained the cages in a room with temperatures near broodnest conditions (34 °C and ~50% RH). Within each holding cage, we separately introduced a fraction of one of the three acaricides—coumaphos (CheckMite+®), amitraz (Apivar®), or tau-fluvalinate (Apistan®)—to avoid known synergistic interactions.

The total amount of the acaricide presented in each fragment was calculated through the area (mm^2) of each strip, according to manufacturer specifications of concentration and total amount of acaricide per strip.

The estimated mean amount of active ingredient to each tested bee was calculated by the total amount of acaricide per fractionated strip divided by the number of bees presented in each holding cage. Each fraction of acaricide strip used presented 0.5×, 1×, 5×, and 10× the LD_{50} of the tested acaricides (according to the reference LD_{50} described by Dahlgren et al. 2012): amitraz, 2.8 μg/bee (corresponding to 1.3 mm^2 of one strip); coumaphos, 26 μg/bee (4.5 mm^2); and tau-fluvalinate, 20.3 μg/bee (4.2 mm^2). In doing so, we established a total of 13 groups (Table I), repeating each treatment at least three times.

2.1. Behavioral assay

We followed the behavioral bioassay for individual grooming as developed by Aumeier

Table I. All groups tested in this research and their respective acaricide treatments, concentration, and number of tested bees

Group	Acaricide	Amount of acaricide[a]	Number of bees	Number of repetitions	Total
1	Coumaphos	10 times LD_{50}	20	3	60
2		5 times LD_{50}	20	3	60
3		1 time LD_{50}	20	3	60
4		0.5 times LD_{50}	20	3	60
5	Amitraz	10 times LD_{50}	20	3	60
6		5 times LD_{50}	20	3	60
7		1 time LD_{50}	20	3	60
8		0.5 times LD_{50}	20	3	60
9	Tau-fluvalinate	10 times LD_{50}	20	3	60
10		5 times LD_{50}	20	3	60
11		1 time LD_{50}	20	3	60
12		0.5 times LD_{50}	20	3	60
Control	–	–	20	5	100
Total				820	

[a] The area of each one of the acaricides' strips were calculated according to the amount of active compound contained in each one of the tested strips, as well as the LD_{50} tested by Dahlgren et al. (2012)

(2001). Briefly, we placed single bees inside individual transparent-plastic petri dishes (145 mm^2) for at least 20 min to acclimate, after which we placed an adult female mite, collected directly from adult bees (from the same colony used as source of nurse bees), onto each bee's dorsal thorax with a paint brush. We did not use the same Varroa mite more than once. We tested bees after they spent 2, 4, 24, 28, 48, 52, and 72 h inside their respective holding cage. Each bee was tested just once for each time of exposure. No *V. destructor* mite was tested more than once.

We observed and video-recorded the subsequent activities of each bee for 3 min and posteriorly analyzed the frequencies and amount of time of five activities as variables for further statistical analysis: (1) time to react to the presence of a *Varroa* mite, recorded as the time (min) it took for a worker to react to the mite with grooming movements; (2) time spent grooming (min) after we placed the *Varroa* mite onto its thorax; (3) attempts to fly, as many bees initiated flight behavior during the assay; (4) time spent motionless (min) without displaying any movement; and (5) time displaying motor coordination problems (MCP), defined as the time (min) spent by each tested bee upside down or displaying difficulty to remain on its legs (Oliver et al. 2015). These activities were chosen to be recorded in a pilot study since they were the most frequently observed activities. We considered "grooming behavior" (related to defense against the mite infestations) as scraping legs over the bee's body, shaking, and rolling (Aumeier 2001). We did not recorded movements performed to clean body parts that the *Varroa* mite was not present, such as antennae and mouth parts. Data from worker bees that appeared still paralyzed (after 20 min for acclimation), displayed grooming behavior before mite contact, or defecated while tested, were discarded (as in Aumeier 2001).

2.2. Analyses

We compared the behavioral data statistically through two-way ANOVA tests with Dunnet's tests as post hoc analyses. We also compared the mortality rates in each one of the treatment and control groups using survival analyses with non-parametric Kaplan-Meier survival tests. All statistics were analyzed with JMP® Pro v10.0 (SAS, Cary, NC) and are reported as mean ± SEM and with $\alpha = 0.05$, unless otherwise noted.

3. RESULTS

3.1. Behavioral data

Data obtained from 100 bees tested without any acaricide exposure (control group), 215 exposed to different doses of coumaphos, 232 exposed to different levels of amitraz, and 184 exposed to different amounts of tau-fluvalinate were used for statistical analysis. A total of 731 female adult *Varroa* mites were used in this research.

The analysis of all treatments showed significant effects of both time of exposure to the miticide ($F = 5.59$; $df = 6, 52$; $P = 0.0002$) and miticide dose ($F = 9.94$; $df = 3, 52$; $P < 0.0001$) on the time spent performing grooming behavior (Figure Fig. 1). The Dunnet's test, used as a post hoc comparison, shows that the mean time spent with grooming behavior by coumaphos-treated bees was significantly lower than in the control group (mean difference = 0.592; 95% CL of difference = 0.262 to 0.922; $P < 0.05$). The amitraz-treated bees, as well as the fluvalinate-treated bees, did not show significant differences from the control group (control × amitraz: mean difference = 0.254; 95% CL of difference = −0.072 to 0.581; $P > 0.05$; control × tau-fluvalinate: mean difference = 0.170; 95% CL of difference = −0.166 to 0.507; $P > 0.05$).

The time to react to the presence of the mite was significantly greater in acaricide-treated bees than in the control group (Figure 2). The two-way ANOVA also demonstrates that both time of exposure to the miticide ($F = 6.76$; $df = 6, 52$; $P < 0.0001$) and miticide dose ($F = 25.71$; $df = 3, 52$; $P < 0.0001$) significantly influenced the time to respond. The post hoc test showed that the reaction time registered for the control group was significantly lower than the ones observed in the group treated with coumaphos (mean difference = −1.845; 95% CL of difference = −2.385 to −1.306; $P < 0.05$), amitraz (mean difference = −1.204; 95% CL of difference = −1.740 to −0.669; $P < 0.05$), and tau-fluvalinate (mean difference = −1.420; 95% CL of difference = −1.971 to −0.869; $P < 0.05$).

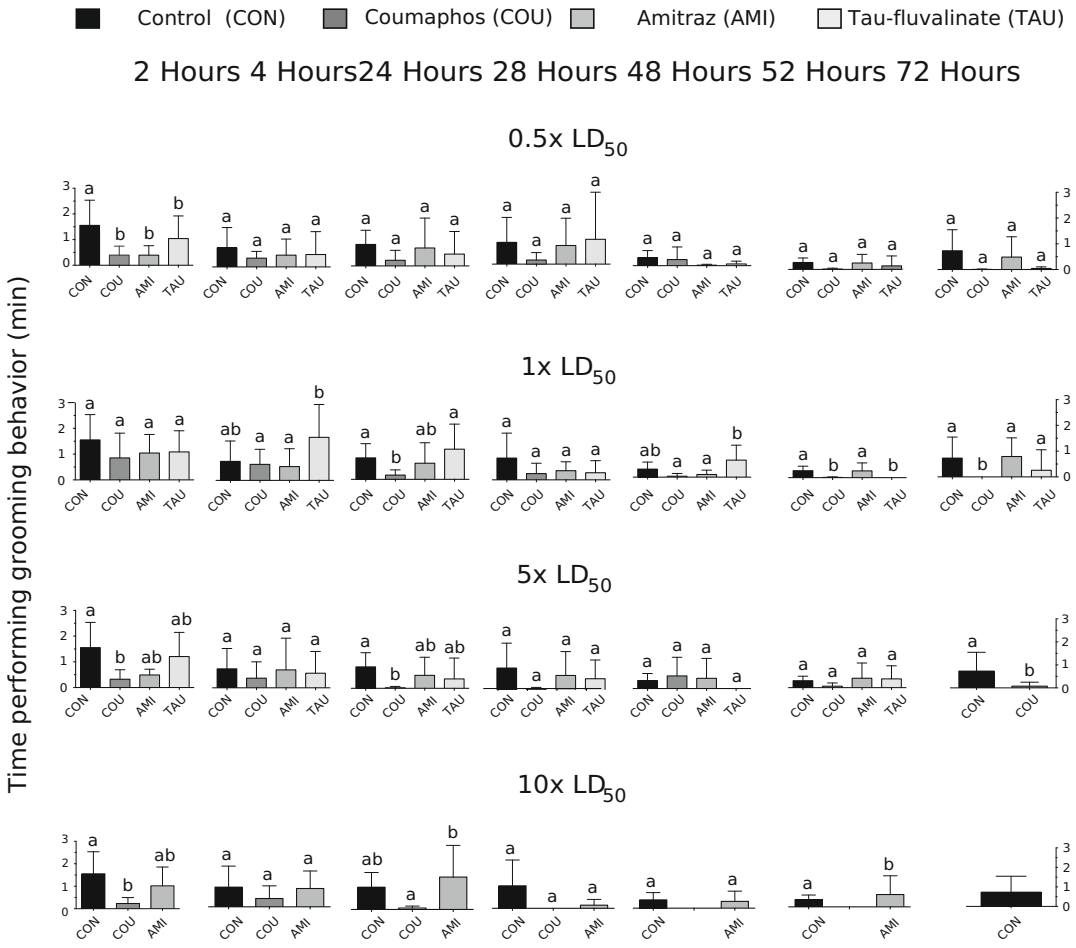

Figure 1. The mean time of performing grooming behavior by the tested honey bees, after being artificially infested with a live *Varroa destructor* mite, in the different treatments. *Different letters* indicate statistically significant difference (Dunnet's test).

The mean time displaying motor coordination problems presented by each treatment and control group presented no significant statistical interaction between time of exposure to the miticide. Miticide dose showed significant influence on this behavior ($F = 2.79$; $df = 3, 52$; $P < 0.05$). Pairwise comparison also showed that the mean time displaying motor coordination difficulties presented by the coumaphos-treated group was significantly higher than that presented by bees from the control group (mean difference = −0.242; 95% CL of difference = −0.458 to −0.026; $P > 0.05$). No significant differences were observed when comparing the mean time displaying motor coordination difficulties by the control group and the amitraz-treated, as well as the tau-fluvalinate-treated group.

The number of flight attempts seemed to not be influenced by neither time of exposure to the miticide nor miticide dose ($F = 0.8595$; $df = 18, 51$; $P = 0.625$). Similarly, the mean time spent motionless was not affected by the time of exposure to the miticide or by the miticide dose ($F = 0.91$; $df = 18, 52$; $P = 0.562$).

3.2. Survival analysis

Bee mortality was higher in acaricide-treated groups than in the control group (Figure 3). The Kaplan-Meier survival test showed significant differences among the groups tested with acaricides

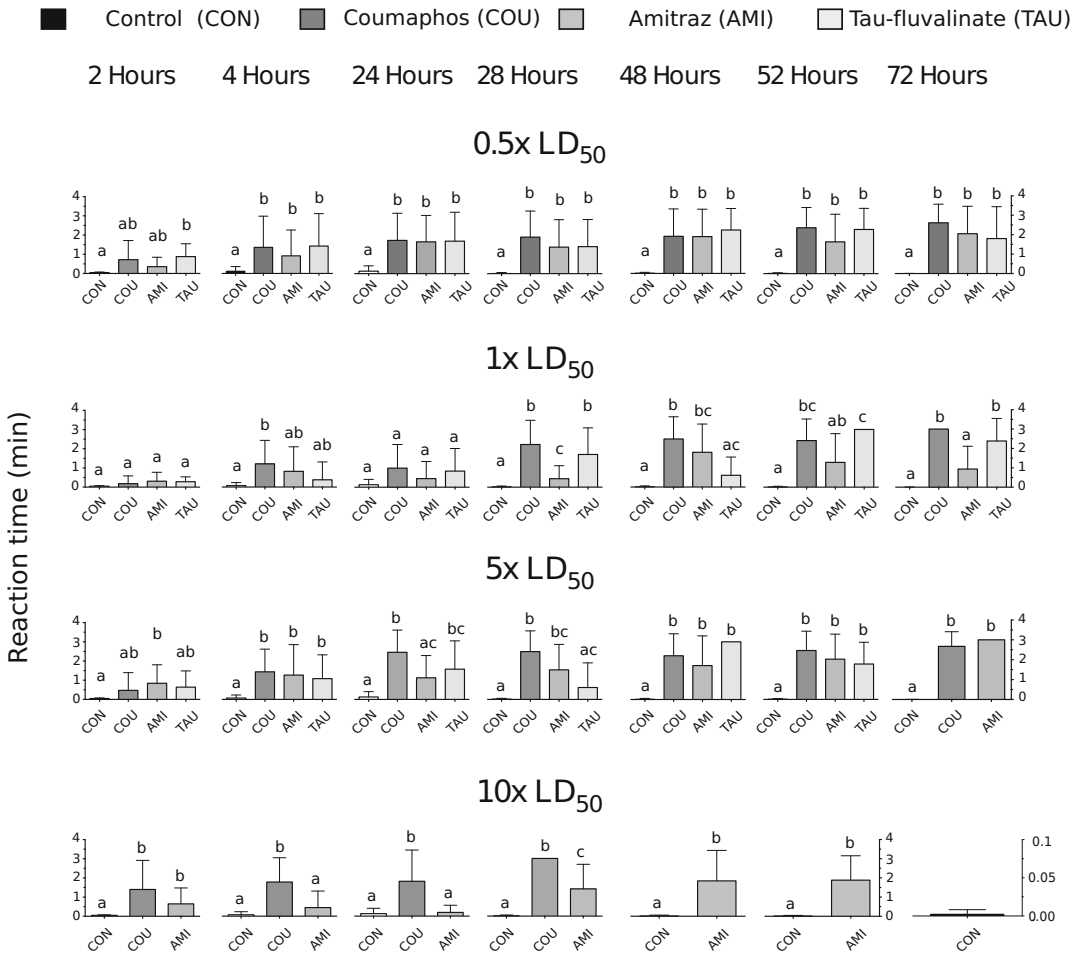

Figure 2. The mean time of reaction of the tested honey bees, after being artificially infested with a live *Varroa destructor* mite, in the different dose and time of exposure of three acaricides. *Different letters* indicate statistically significant difference (Dunnet's test).

(0.5×, 1×, 5×, and 10× LD_{50}) and the control group (respectively: log-rank (Mantel-Cox): Chi square = 194.00, df = 3.0, $P < 0.0001$; Chi square = 73.15, df = 3.0, $P < 0.0001$; Chi square = 49.00, df = 3.0, $P < 0.0001$; and Chi square = 153.021, df = 3, $P < 0.001$).

Tau-fluvalinate resulted in the highest mortality among all the acaricides tested; in the highest dose (10× LD_{50}), the pyrethroid induced mortality within only 2 h of topical contact. Both coumaphos and tau-fluvalinate killed 100% of the tested bees within 72 h (10× LD_{50}).

The pairwise comparison among the survival curves demonstrates that the control group had a higher survival rate than all the treatment groups (Table II). Pairwise comparisons also showed that the mortality rates observed in the amitraz treatment (5×, 1× and 0.5× LD_{50}) were lower than those in the coumaphos and tau-fluvalinate treatments (Table II). No significant differences were observed when comparing the mortality rates between coumaphos and tau-fluvalinate (Table II).

4. DISCUSSION

The chemical control of *Varroa* has been important for the maintenance of beekeeping production as well as the pollination services performed by *A. mellifera*. The status quo of unsustainable colony losses, however, has been of great concern

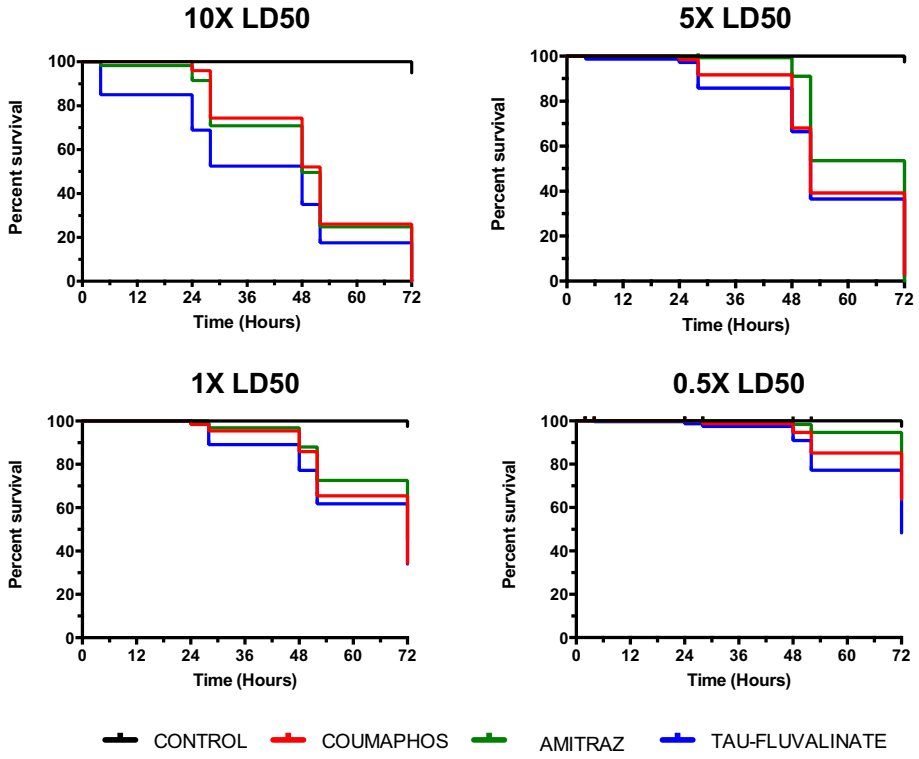

Figure 3. Survival curves observed for honey bees confined with three different acaricide treatments and their negative control group.

to beekeepers and the general public. The growing number of studies that highlight the side effects of acaricides (even in sublethal doses) and the long half-life presented by those chemicals (specially in wax) increases the priority for alternative *Varroa* control methods that are less harmful for bees (Mullin et al. 2010; Boncristiani et al. 2012; Locke et al. 2012; Williamson et al. 2014; Zhu et al. 2014). Mullin et al. (2010) found alarming concentration of acaricides contaminating colony wax, reaching up to 3.82 μg/g of amitraz, 91.9 μg/g of coumaphos, and 188 μg/g of tau-fluvalinate. The combined exposure produced by *Varroa* control treatment and contaminated wax can produce doses higher than LD_{50} (such as the studied by Dahlgren et al. 2012). Our results show that all the tested synthetic acaricides have a significant effect on the survival of worker honey bees. In the highest dose tested (10× LD_{50}), 100% of the worker bees died within 72 h. When subletal doses were applied (0.5 LD_{50}), the acaricide treatments resulted in death rates of 20% but still significantly higher than those in the control group. Compared with Dahlgren et al. (2012), the same dose of acaricide produced lower mortality rates (within 48 h) in our tests. The differences observed are most likely a consequence of our different methods; we used fractions of acaricide strips inside cages instead of individual topical application of the active ingredient. Our approach may be subjected more to individual heterogeneity of the contacted dose, but it represents a more realistic facsimile of the in-hive exposure to the chemicals.

The side effects of these acaricides seem not to be restricted to mortality or contamination of beekeeping products, as important activities related to natural defense against *Varroa* were also significantly affected. Worker honey bees exposed to acaricide presented significantly less time spent performing grooming behavior after a *Varroa* mite was introduced onto its thorax.

Both the dose of the acaricides applied and the time of exposure presented significant effects on behavioral performance. Groups treated with

Table II. Log-rank (Mantel-Cox) pairwise comparison of survival rates observed among all treatments

Concentration	Acaricide	Amitraz		Control		Coumaphos		Tau-Fluvalinate	
		Chi square	*P*	Chi square	*P*	Chi square	*P*	Chi square	*P*
5 times LD_{50}	AMI	–	–	120.390	<0.001	11.275	0.001	18.060	<0.001
	CON	120.390	<0.001	–	–	150.464	<0.001	162.289	<0.001
	COU	11.275	0.001	150.464	<0.001	–	–	0.802	0.371
	TAU	18.060	<0.001	162.289	<0.001	0.802	0.371	–	–
1 time LD_{50}	AMI	–	–	120.390	<0.001	8.454	0.004	2.789	0.095
	CON	120.390	<0.001	–	–	63.199	<0.001	73.111	<0.001
	COU	8.454	0.004	63.199	<0.001	–	–	1.030	0.310
	TAU	2.789	0.095	73.111	<0.001	1.030	0.310	–	–
0.5 times LD_{50}	AMI	–	–	9.8820	0.002	5.171	0.023	17.640	<0.001
	CON	9.882	0.002	–	–	23.836	<0.001	41.235	<0.001
	COU	5.171	0.023	23.836	<0.001	–	–	4.142	0.042
	TAU	17.640	<0.001	41.235	<0.001	4.142	0.042	–	–

coumaphos presented the steepest reduction on the total time performing grooming behavior after the mite was introduced. Even at subletal doses, this organophosphate was able to significantly reduce grooming behavior. It is known that coumaphos has been reported to impact foraging behavior (Schneider et al. 2012), trophallaxis (Bevk et al. 2012), and motor activity (Williamson et al. 2013). Nonetheless, very little is known about the effects of this acaricide on natural Varroosis resistance, such as grooming. Williamson et al. (2013) observed a notable increase on grooming activity when coumaphos was fed to bees in sublethal doses. The authors observed the behavior of coumaphos-treated workers without the presence of the mite, as well as counted activities that are not directly related to attempts to remove the mite, such as grooming antennae. Considering the antagonistic results obtained by this research, as well as the differences in the experimental design, we may hypothesize that the presence of *Varroa* plays an important role in the triggering of grooming behavior (attempts to remove the mite) by the host.

The time it took for a worker bee to react with grooming movements to the presence of the mite on its body was also significantly affected by both the dose of the acaricide applied as well as the time each tested bee was exposed to the miticide. Groups treated with coumaphos and tau-fluvalinate exhibited significantly longer reaction times. Even at subletal doses, all acaricides dramatically increased the reaction time of the host.

Oliver et al. (2015) observed that bees exposed to tau-fluvalinate spent more time upside down and fanning their wings, although the authors did not find significant effects of tau-fluvalinate on grooming behavior. That experiment, however, was conducted in the absence of mites and is thus, once again, a notable difference from the experimental design adopted by the current study.

The behavioral side effects observed in acaricide-treated worker bees is concerning, considering that these activities are highly correlated to efficacy in grooming behavior against *Varroa*. Aumeier (2001) asserts that honey bee strains described as "efficient groomers" (e.g., African-derived honey bees) display grooming movements for longer periods in the presence of the ectoparasite compared with susceptible strains (e.g., Carniolan European honey bees). The author also shows that efficient grooming performers react faster to the presence of the mite. de Mattos et al. (in review) similarly observed a significant positive statistical correlation between the total time performing grooming behavior and the resistance against *Varroa* in African-derived honey bees (as measured by total infestation rate). The same study also detected a highly significant negative correlation relating the time it took for a worker bee to initiate grooming movements.

It has been shown that the neurologic effects of acaricides may affect olfactory ability of antennae, cognition, learning, and memory in honey bees (Williamson et al. 2013). The neural circuits that drive olfactory learning and memory are all mediated by cholinergic neurotransmission (Gauthier 2010; Williamson et al. 2013). Substances able to disrupt acetylcholinesterase (such as coumaphos and amitraz) in the brain have been shown to produce significant impairments on those circuits (Loucif-Ayad et al. 2008; Williamson et al. 2013; Palmer et al. 2013). Frost et al. (2013) found that fluvalinate can also produce negative effects on honey bee olfactory learning and memory in their responsiveness to sucrose. Thus, it is possible that impairment of antennae olfactory sensitivity to cause delay on the process of triggering a fast grooming response. Rosenkranz et al. (2010) hypothesize that a specific scent of the mites could be detected by the bees then eliciting grooming movements. On the other hand, Kather et al. (2015) and Le Conte et al. (2015) highlight the ability of *V. destructor* to chemically mimicry host cues and avoid hygienic bees that would detect and remove the mites. Effects of acaricides on tactical sensitiveness of bees, as well as the role those tactical skills play on *Varroa* resistance, still need to be tested.

New studies are also required to better understand how acaricides impair the triggering and performance of grooming behavior, as well as the role played by olfactory sensitiveness, learning, and memory on the success of grooming defense against *V. destructor*.

The current study is not designed to determine which acaricide is the safest for *Varroa* control by beekeepers, although we illuminated a possible side effect of acaricide treatments against *Varroa*.

It is also possible that our results indicate that chemical treatment can produce a scenario of dependence; when used, these acaricides can undermine the performance of naturally evolved mechanisms of defense against the mite (such as grooming behavior). The recurrent use of these acaricides can result in higher in-hive concentrations and consequently a possible sharp impairment of the performance of grooming against *Varroa*. According to this scenario, faster growth rates of mite populations, increasing the incidence of viruses, precocious weakening of colonies, and, consequently, higher rates of mortality can also be related to acaricide exposure.

We suggest that the discussion concerning the health status of the honey bee must address more narrowly the behavioral side effects of chemical treatments to mitigate Varroosis. Our data also imply that studies regarding natural defenses of the host against *Varroa*, as well as the ones addressing breeding honey bees for *Varroa* resistance, are important future directions for the development of sustainable beekeeping.

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Contributions All authors equally contributed to this research accomplishment.

Effets d'acaricides de synthèse sur le comportement de toilettage de l'abeille contre l'acarien parasite *Varroa destructor*

***Apis mellifera* / coumaphos / amitraze / tau-fluvalinate / toilettage / Acari**

Eindluss von synthetischen Akariziden auf das Grooming-Verhalten von Honigbienen gegenüber der parasitischen Varroamilbe

Apis mellifera* / Coumaphos / Amitraz / tau-Fluvalinat / Grooming-Verhalten / *Varroa destructor

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