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Original article

Synthetic and natural acaricides impair hygienic and foraging behaviors of honey bees

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Abstract – Acaricides commonly used to control the honey bee parasitic mite, *Varroa destructor*, may also adversely affect bees. Sublethal LD_{05} doses of synthetic (tau-fluvalinate, amitraz, and coumaphos) and natural (thymol and formic acid) acaricides did not significantly reduce bee survivorship. However, compared to the ethanol solvent control, hygienic behavior critical for pathogen and parasite control was significantly reduced with coumaphos, whereas both pollen and non-pollen foraging behaviors critical for resource acquisition were reduced with tau-fluvalinate, coumaphos, and formic acid. Thymol significantly reduced non-pollen foraging but also reduced the negative effects of ethanol on hygienic behavior. Amitraz did not affect hygienic and foraging behaviors relative to the solvent. Thymol and amitraz appeared to be the safest acaricides based on these tests.

Apis mellifera **/ sublethal / acaricides / hygienic behavior / foraging behavior**

1. INTRODUCTION

The ectoparasitic mite, *Varroa destructor*, is considered to be the greatest threat to honey bee health (Rosenkranz et al. 2010). The mite feeds upon the haemolymph and fat tissue of larvae, pupae, and adult honey bees, shortening their lifespan, which results in weakened colonies, often leading to their death (De Jong 1997; Guzman-Novoa et al. 2010; Ramsey et al. 2019). Honey bee and colony mortality have also been linked to the damage caused by viruses transmitted by the mite, particularly deformed wing virus (Dainat and Neumann 2013; Reyes-Quintana et al. 2019). Beekeepers routinely use commercial products that contain synthetic acaricides, such as tau-fluvalinate, coumaphos or amitraz, to protect their colonies from varroa mites, but there is increasing concern about their use. Both tau-fluvalinate and coumaphos residues have been shown to accumulate and persist in beeswax and honey (Bogdanov 2006; Mullin et al. 2010). While synthetic acaricides were initially very effective at controlling *V. destructor*, mite populations have developed resistance against them reducing their effectiveness (Lodesani et al. 1995). An alternative to synthetic acaricides is commercially available natural acaricides that contain essential oils or organic acids derived from plants. Among these, the most commonly used by beekeepers are thymol and formic acid (Rosenkranz et al. 2010). Natural acaricides do not leave persistent residues in hive products, and there is no evidence that mite populations had developed resistance to them.

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However, there are reports of variable efficacy of natural acaricides due to numerous factors (Imdorf et al. 1999; Underwood and Currie 2003; Emsen et al. 2007).

Acaricides may potentially have detrimental effects on honey bee behaviors. Hygienic behavior of honey bees is a mechanism of disease resistance that involves the workers' recognition (by olfaction) and removal of diseased or *Varroa*-parasitized brood (Rothenbuhler 1964; Spivak and Gilliam 1998; Spivak and Boecking 2001). However, the level of hygienic resistance against varroa mites can be relatively limited (Guzman-Novoa and Morfin 2019) and thus any compromise of it by acaricides would result in colonies having a higher risk of collapsing. Another important behavior is foraging, where worker bees visit flowers to collect pollen and nectar, which are food resources for the colony to reproduce and survive. Hygienic and foraging behaviors thus contribute significantly to the biological success of honey bee colonies.

There are no reports about acaricides being tested for their effects on hygienic or foraging behavior of honey bees, even though there are indications that they may have negative impacts. For example, Teeters et al. (2012) found that bees treated topically with 0.3, 1.5, and 3 μg of tau-fluvalinate/bee flew significantly less distance than control bees. Frost et al. (2013) reported a reduction in memory retention at 24 h after oral exposure to 0.125 and 1.25 μg of tau-fluvalinate/bee, and Williamson et al. (2013) reported impaired memory retention at 24 h following an oral dose of 1.81 ng coumaphos/bee. Effects on memory are important as memory is critical for bees to learn and remember olfactory cues to perform a variety of tasks including foraging and hygienic behaviors (Menzel and Greggers 1992; Masterman et al. 2000; Spivak et al. 2003). Therefore, if memory is impaired, honey bee foraging and hygienic behaviors are most likely negatively affected, which could have detrimental effects at the colony level.

The objective of this study was to assess the effects of three synthetic and two natural acaricides commonly used by beekeepers on the lifespan of adult honey bees, as well as on hygienic and foraging behaviors. It is hypothesized that adult bees exposed to sublethal doses of acaricides that are below levels realistically expected within hives might exhibit increased honey bee mortality and impaired hygienic and foraging behaviors.

2. MATERIALS AND METHODS

2.1. Source of bees and chemicals

Experiments were conducted at the University of Guelph's Honey Bee Research Center in Ontario, Canada. Honey bee colonies headed by queens of the Buckfast strain were used as a source of workers. Technical grade (> 98% purity) amitraz, coumaphos, tau-fluvalinate, and formic acid were purchased from Sigma-Aldrich Corporation (St. Louis, Missouri, USA). Thymol was obtained from Fisher Scientific Ltd. (Ottawa, ON, Canada).

2.2. Treatments

To obtain worker bees of the same age, frames with emerging brood collected from source colonies were placed inside wooden emergence cages (50 × 7 × 25 cm) and incubated overnight at 32 ± 2 °C and 60 ± 5% RH. Newly emerged bees were marked with enamel paint of different colors on their thoraces on the day they were obtained from the incubator, to identify cohorts of bees of the same age as well as the different treatments. Marked bees were randomly assigned to different treatments (acaricides, ethanol solvent, or non-treated). Each bee was individually treated with 5 μl of treatment solution, which was applied on the dorsal surface of the thorax using a micropipette (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). For acaricides, bees were treated with the 48 hpt (hours post treatment) LD_{05} of tau-fluvalinate (0.027 μg/bee), amitraz (0.335 μg/bee), coumaphos (0.347 μg/bee), thymol (4.509 μg/bee), or formic acid (8.202 μg/bee) that had been previously determined (Gashout et al. 2018). LD_{05} doses were chosen as they are commonly used to study the effect of sublethal exposure to pesticides on bees (Smirle et al. 1984; Sheila et al. 1991; Ahmadi et al. 2008; Tarek et al. 2018) and are more than 100 times lower than the actual expected exposure in hives from

commercial treatments of these acaricides (Gashout 2017). Bees treated with 95% ethanol (solvent) and non-treated bees were used as controls. After exposure, the bees were introduced into three observation hives until they were 10–14 days old for hygienic behavior assessments (see below), or introduced into three Langstroth hives until they were 19–26 days old for foraging behavior assessments (see below). In total, 850 bees were marked, treated, and introduced into each hive for each treatment group: tau-fluvalinate, amitraz, coumaphos, thymol, formic acid, ethanol (solvent), and non-treated bees.

2.3. Hygienic behavior observations

Three observation hives (47.0 × 4.1 × 96.5 cm) were established to evaluate hygienic behavior. Approximately 500 g of bees from existing colonies that had not been treated against varroa mites for at least 6 months, as well as a Buckfast queen, were introduced into each hive, which contained four standard Langstroth frames, stacked vertically. Upon assembly, the bottom frame of each hive contained a brood comb, the second and third frames contained combs with empty cells and honey, respectively, and the fourth, upper frame had plastic foundation. One side of the observation hive was covered with a glass plate and the other with plexiglass. A small door (10 × 10 cm) was cut into the plexiglass near the bottom of the hive to facilitate manipulation of comb sections containing frozen brood (see below). Each observation hive was placed in the dark in separate windowless rooms of identical dimensions and conditions, maintained at 22–28 °C. Each hive was connected to a ramp, which in turn was connected to an opening leading to the outside, allowing the bees to exit the hive and forage normally (Guzman-Novoa and Gary 1993). The hive entrances were oriented in different directions and painted with different colors (yellow, blue, and orange) to minimize bee drift. Marked bees were introduced into the hives 2 weeks after establishing the colonies.

A census was carried out of all marked bees in each hive 2 days after they were introduced to record the number of accepted bees of each treatment. One additional census was conducted 2 weeks later, during the period when hygienic behavior was evaluated. Censuses were conducted when foraging activities had not started to ensure that no accepted bees were missing due to early foraging. Marked bees on both sides of each frame were captured as digital images using a camera with 100 mm macro lens (Canon EOS 5D Mark II digital camera, Canon Inc. Mississauga, ON, Canada). The pictures were downloaded to a computer and the number of marked bees of each color counted from the images.

The comb at the bottom of each hive had a 9 × 9-cm section removed and was aligned with the plexiglass door described earlier to allow sections of frozen capped brood to be introduced into this cavity. A 9 × 9-cm section of capped brood was cut from a brood comb of a colony unrelated to the test bees, frozen at – 20 °C for 24 h and subsequently placed into the 9 × 9-cm cavity in the lower comb of the observation hive via the door. The same colony was used as brood donor for the three observation hives as it is known that the colony of origin of freeze-killed brood does not affect the assay results (Spivak and Reuter 1998). One hour later, hygienic events (cell uncapping and brood removal) of the marked bees were observed and recorded continuously for 3 h. The frozen brood squares were always inserted at 09:00 h and observed from 10:00 until 13:00 h. The side of the observation hive being observed was changed every 15 min. This assay was repeated three times for each colony of middle-aged marked bees (at 11, 13, and 14 days old), which is the period when hygienic behavior is typically performed by honey bees (Winston 1987).

2.4. Foraging behavior observations

Three colonies were established in Langstroth hives with marked treated and control workers as described above. Sister Buckfast queens were used for all colonies and each of the colonies contained one frame with brood, one with honey, one with empty drawn comb, and two with foundation. After the new queen and about 500 g of bees were introduced, each colony was not disturbed for 2 weeks to allow for queen acceptance. After that, the marked treated and control bees were introduced to the hives, and the number of

accepted bees of each treatment was recorded in each hive 2 days after they were introduced as described above. This was repeated weekly by opening a hive and retrieving frames until the bees were 38 days old.

The entrance of each hive was connected to a transparent runway (56 × 6 × 35 cm), which allowed for the observation of returning bees. The runway was observed continuously for 3 h (11:30–14:30 h) to record the number of marked bees returning from foraging trips per hour, as well as to record whether or not the returning bees were carrying pollen. The data were collected using a digital voice recorder (Sony ICD-UX533 digital voice recorder, Sony Corp., New York, NY, USA). The counts were used to calculate rates of foraging trips. Observations were repeated four times when the marked bees were 19, 21, 23, and 26 days old. The observations were conducted during a nectar flow period in southern Ontario.

2.5. Statistical analyses

Based on the first census conducted on day 2 after introduction, the number of accepted bees was determined for each treatment and then percentages of surviving bees (from those accepted) were derived from subsequent censuses. The data on the percentage of surviving bees were square-root-arcsine transformed before factorial analysis of variance (ANOVA) to determine the effects of treatment and age on length of life. To analyze hygienic behavior, ratios between counts of hygienic events in 15 min and number of bees that were alive during observation days (based on the censuses of observation hives) were subjected to chi-square tests for comparisons between control untreated bees and the rest of the treatments. To analyze foraging behavior, ratios were obtained between the number of bees foraging per hour of observation and numbers of bees that were alive during observation days. Then, the ratios were log transformed before ANOVA and Fisher LSD analysis to separate means when significance was detected. Transformations were carried out to comply with the assumption of normality required for ANOVA. Statistical analyses were performed using the statistical software R (version 3.3.1) (R Core Team 2012).

3. RESULTS

3.1. Effect of acaricides on adult bee survivorship

There were no significant differences between the treatments for percent survivorship of adult bees ($F_{6,\ 119} = 0.92$, $P = 0.49$; Figure 1). Survivorship declined from 100 to 20% during the course of the experiments, which was significant ($F_{5,\ 120} = 635.35$, $P < 0.0001$). No interaction effects between treatment and age of the bees were detected ($P > 0.05$).

3.2. Effect of acaricides on hygienic behavior

The ethanol control significantly decreased the frequency of hygienic events relative to the non-treated control ($\chi^2 = 178.8$, $P < 0.0001$), which affected the results for all the acaricide treatments as they had ethanol as the solvent (Figure 2). The coumaphos treatment reduced hygienic behavior events by 40% ($\chi^2 = 288.2$, $P < 0.0001$) and the tau-fluvalinate treatment by 34% ($\chi^2 = 190.9$, $P < 0.0001$) relative to the control. However, coumaphos was the only treatment significantly lower than the ethanol control for hygienic behavior events ($\chi^2 = 18.1$, $P < 0.0001$). The thymol treatment resulted in significantly higher hygienic instances than the other acaricide treatments and the ethanol control ($\chi^2 = 51.9$, $P < 0.0001$), even though thymol was dissolved in ethanol. Thus, it appears that thymol reduced the negative effects of ethanol, although bees treated with thymol performed 13% less hygienic behavior instances than the control ($\chi^2 = 30.6$, $P < 0.0001$). The effects of ethanol could not be separated from the effects of the acaricides, but the combination of ethanol and coumaphos was the most detrimental to hygienic behavior, whereas the combination of ethanol and thymol was the least detrimental.

3.3. Effect of acaricides on foraging behavior

For total foraging trips, coumaphos, tau-fluvalinate, and formic acid–treated bees did not differ significantly from each other but performed

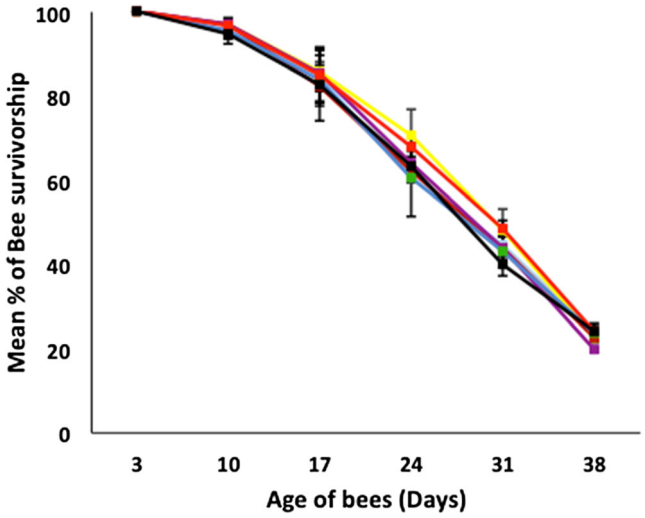

Figure 1. Mean (± SE) percent survivorship of worker honey bees. Bees were topically treated with a LD_{05} of tau-fluvalinate (), amitraz (), coumaphos (), thymol (), or formic acid (); the control treatments consisted of ethanol-treated bees (solvent) () and non-treated bees (control) (). The treated bees were introduced into three Langstroth hives for up to 39 days. A total of 850 bees per treatment were introduced into each colony.

significantly less foraging trips compared to the ethanol and non-treated controls that also did not differ significantly from each other. However, thymol- and amitraz-treated bees did not differ significantly in total foraging trips from the controls ($F_{6, 245} = 7.30$, $P < 0.0001$; Figure 3). Pollen foraging trips comprised approximately one-quarter of the total trips (Figure 4a). Again, coumaphos, tau-fluvalinate, and formic acid–treated bees were not significantly different from each other, but performed significantly less pollen trips than the ethanol and non-treated controls, which were not significantly different from each other ($F_{6, 245} = 5.90$, $P < 0.01$, Figure 4a). Non-pollen foraging trips comprised approximately three-quarters of the total trips, and coumaphos, tau-fluvalinate, formic acid, and thymol-treated bees were not significantly different from each other but were significantly lower than the ethanol and non-treated controls, which were not significantly different from each other (Figure 4b). Thus, the coumaphos, tau-fluvalinate, and formic acid treatments had the greatest negative effects on both total foraging trips and pollen foraging trips, whereas thymol treatment had a negative effect only on non-pollen foraging trips. No interactions between colony and treatment were detected for total, pollen, or non-pollen foraging trips ($F_{12, 239} = 1.38$, $P > 0.05$). The proportion of bees that foraged for pollen did not significantly differ between treatments ($F_{6, 245} = 0.93$, $P > 0.05$).

4. DISCUSSION

Exposure of worker honey bees to LD_{05} doses of either two synthetic or three natural acaricides had mixed effects on hygienic and foraging behaviors. Ethanol, which was used as acaricide solvent, decreased hygienic behavior instances relative to control non-treated bees for all the acaricides tested. The coumaphos treatment was the only one that significantly reduced hygienic behavior events relative to the ethanol control, whereas thymol was the only treatment with significantly more hygienic instances than the ethanol control, indicating a protective effect against the solvent. Unlike hygienic behavior, ethanol did not have an effect on the foraging behavior of the bees. However, tau-fluvalinate, coumaphos, and formic acid had the greatest negative effects on total foraging trips, pollen foraging trips, and non-pollen foraging trips, whereas thymol had a

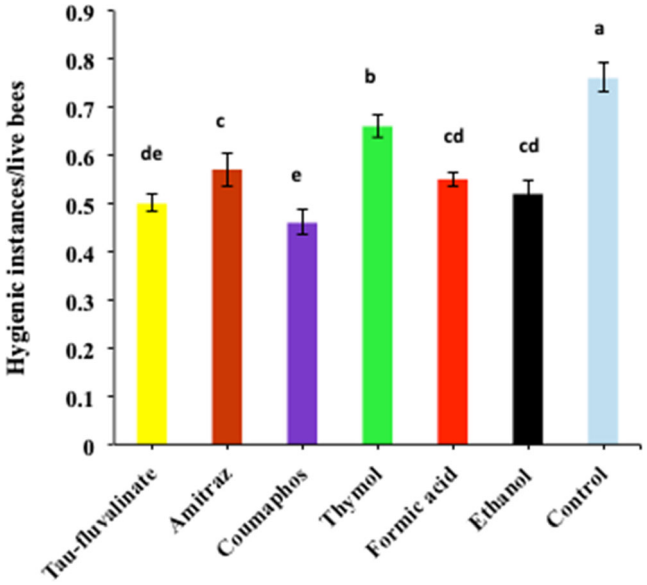

Figure 2. Mean ratio for the number of bees performing hygienic behavior tasks in 15 min to the number of live bees during observation days (± SE). Bees were topically treated with a LD_{05} of tau-fluvalinate (), amitraz (), coumaphos (), thymol (), or formic acid (); the control treatments consisted of () ethanol-treated bees (solvent) and () non-treated bees (control). The treated bees were introduced into three observation hives where hygienic behavior was observed at 2 weeks post-introduction. A total of 850 bees per treatment were introduced into each colony. Different letters above bars indicate significant differences between treatments based on chi square values from contingency table analyses ($P < 0.05$).

negative effect only on non-pollen foraging trips. The detrimental effects of the acaricides on hygienic and foraging behaviors were not due to differences in survivorship. None of the acaricides tested reduced the bees' lifespan in comparison with the control ethanol-treated or non-treated bees.

To the best of our knowledge there are no previous studies of sublethal acaricide effects on the hygienic behavior of honey bees. Hygienic behavior contributes to the biological success of honey bee colonies by reducing the presence of parasites like varroa mites and pathogens like the causative agents of American foulbrood and chalkbrood. Thus, any chemical compound that negatively alters this behavior would presumably increase the susceptibility of colonies to pathogens. Hygienic behavior is mediated by olfactory cues as bees detect the odor of diseased, parasitized, or dead brood under a wax-capped cell, which stimulates them to uncap and remove the cell's contents (Masterman et al. 2000; Spivak et al. 2003). Decreased hygienic behavior of bees can at least in part be due to their impaired ability to detect and react to odor cues as has been shown for ethanol, tau-fluvalinate, amitraz, coumaphos and formic acid (Gashout et al. 2019), tau-fluvalinate (Frost et al. 2013), and coumaphos (Williamson et al. 2013) in laboratory studies using the proboscis extension reflex assay. The effect of ethanol, therefore, may mask the effects of the other acaricides tested. The use of other solvents that do not affect hygienic behavior may have allowed for the detection of the effects of acaricides on hygienic behavior and should be used in further experiments. Despite this, the effects of coumaphos were greater than those of ethanol in reducing hygienic behavior, and thus, it is clearly the most detrimental of the acaricides on hygienic behavior.

The rate of total foraging trips of worker bees was significantly reduced when exposed to LD_{05} doses of all the acaricides, except for the synthetic acaricide amitraz and the natural acaricide thymol.

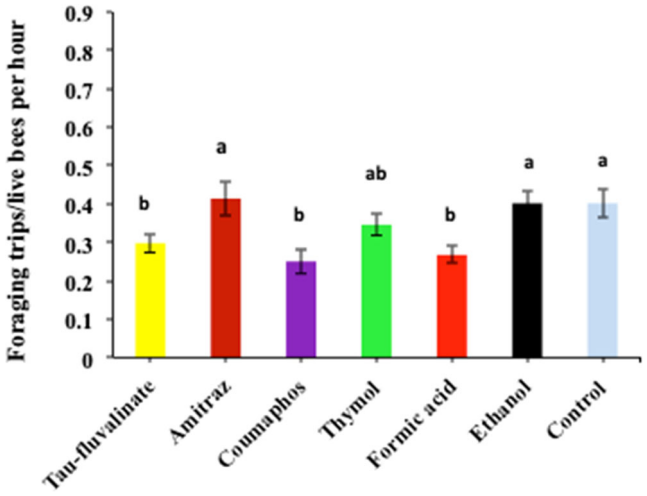

Figure 3. Mean ratio for the number of bees performing total foraging trips per hour of observation to the number of live bees during the observation period (± SE). Bees were topically treated with a LD_{05} of tau-fluvalinate (), amitraz (), coumaphos (), thymol (), or formic acid (); the control treatments consisted of ethanol-treated bees (solvent) () and non-treated bees (control) (). The treated bees were introduced into three Langstroth hives where foraging behavior was observed. A total of 850 bees per treatment were introduced into each colony. Different letters above the bars indicate significant differences based on ANOVA and Fisher's LSD tests performed on log-transformed data ($P < 0.05$). Untransformed data are shown.

Schneider et al. (2009) also found that bees orally fed with 5 μg/bee of coumaphos had reduced foraging activity, which was a much higher dose than the 0.347 μg/bee of coumaphos used in this study. Schneider et al. (2009) also used a different method of exposure than in this study (oral versus topical). A lower frequency of total foraging behavior after exposure to tau-fluvalinate, coumaphos, or formic acid could be due to less foraging trips and/or longer foraging flights. Bees exposed to sublethal doses of other pesticides, such as the neonicotinoids imidacloprid or clothianidin, resulted in a significant reduction of total foraging activity, bee mobility, nursing behavior, and longer foraging flights (Decourtye et al. 2001; Guez et al. 2001; Bortolotti et al. 2003; Medrzycki et al. 2003; Schneider et al. 2012).

The results for total and pollen foraging trips were the same in that only tau-fluvalinate, coumaphos, and formic acid had significant effects. However, the results for non-pollen foraging were different as thymol showed significant negative effects. Nectar foragers (presumably the non-pollen foragers) tend to collect nectar with higher sucrose concentration than pollen foragers (Page Jr. et al., 1998; Riveros and Gronenberg 2010). It might be that thymol increased this behavior, which could have caused the bees to search for more concentrated nectar, thus lengthening foraging trips and reducing the number of nectar foraging trips. Therefore, thymol could have a negative effect on sucrose collection, even though pollen collection was unaffected.

Like hygienic behavior, foraging behavior is also partly mediated by olfactory cues. Bees detect floral odors that guide and help them recognize and find food sources (Burger et al. 2010). Several of the acaricides tested in this study have been reported to decrease the detection of odor cues in honey bees (Frost et al. 2013; Williamson et al. 2013; Gashout et al. 2019). Therefore, acaricides that detrimentally affect the ability of the bees to learn particular scents associated with food rewards should presumably affect their foraging behavior. This could at least partially explain the effects of tau-fluvalinate, amitraz, coumaphos, and formic acid on foraging behavior in this study since all of them have been found to decrease

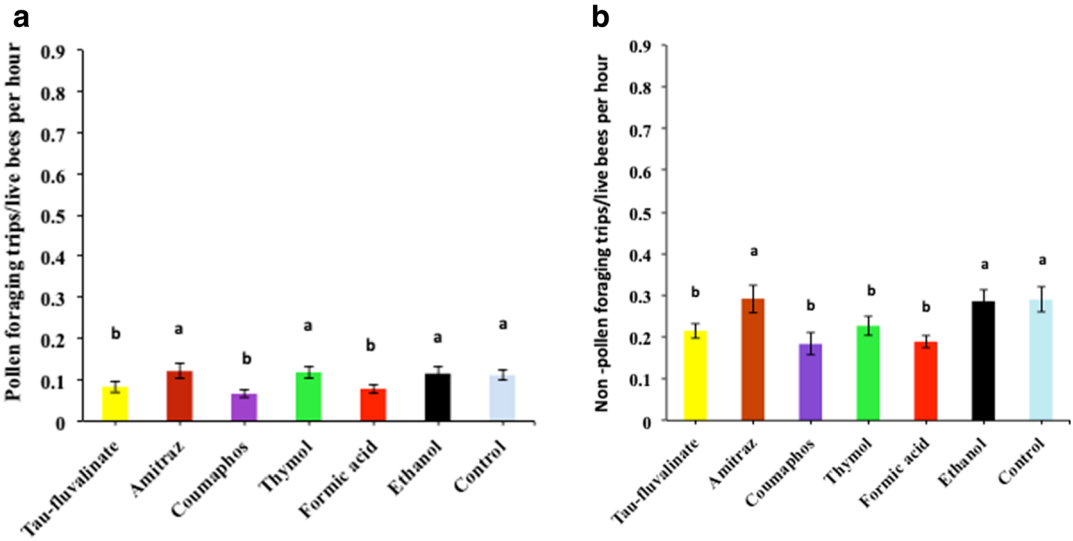

Figure 4. Mean ratio for the number of bees performing pollen foraging trips (**a**) and non-pollen foraging trips (**b**) per hour of observation to the number of live bees during the observation period (± SE). Bees were topically treated with a LD_{05} of tau-fluvalinate ( ), amitraz ( ), coumaphos ( ), thymol ( ), or formic acid ( ); the control treatments consisted of ethanol-treated bees (solvent) ( ) and non-treated bees (control) ( ). The treated bees were introduced into three Langstroth hives where foraging behavior was observed. A total of 850 bees per treatment were introduced into each colony. Different letters above the bars indicate significant differences based on ANOVA and Fisher's LSD tests performed on log-transformed data ($P < 0.05$). Untransformed data are shown.

responses to odor cues (Gashout et al. 2019). Decreased foraging behavior from acaricide treatments could compromise a colony's fitness by reducing pollen and nectar collection. A recent study demonstrated that neonicotinoid insecticides, which also affect neural processes, resulted in impairment of hygienic and foraging behaviors of honey bees (Morfin et al. 2019).

The only acaricide that negatively affected both hygienic and foraging behaviors in this study was coumaphos. Coumaphos may have had a greater impact than other acaricides as it inhibits the enzyme acetylcholinesterase, which is important to hydrolyze acetylcholine, the excitatory neurotransmitter in the nervous system of insects (Roulston et al. 1966). Thus, acetylcholinesterase inhibition may interfere with the normal integration of chemical cues within the brain. As a result, there could be a loss of olfactory capacity that may interfere with many important behaviors in bees, including hygienic and foraging behaviors. In contrast, the only acaricide that did not affect hygienic and foraging behaviors in this study was amitraz. Amitraz is an octopaminergic agonist in arthropods causing over-excitation of the central nervous system and paralysis (Hollingworth and Lund 1982). In a previous study, exposure to the LD_{05} dose of amitraz significantly increased honey bee acetylcholinesterase gene expression, unlike coumaphos and thymol. However, it decreased honey bee long-term memory similar to coumaphos (Gashout et al. 2018, 2019). It appears that those changes are not predictive of the differences in the effects of the LD_{05} doses of amitraz, coumaphos, and thymol on hygienic and foraging behaviors. While the LD_{05} of amitraz caused no significant effects on those behaviors, its excitatory mode of action likely means that effects would be observed at higher doses.

In summary, this study showed that LD_{05} doses of synthetic and natural acaricides do not cause acute mortality, but have the potential to affect hygienic and foraging behaviors in honey bees.

Only amitraz never negatively affected foraging or hygienic behaviors, and thymol only had a negative effect on non-pollen foraging but reduced the adverse impact of ethanol for hygienic behavior. Thus, those two acaricides appear to be the safest to use. While both foraging and hygienic behaviors are important, they appear to differ in their sensitivity to acaricides with non-pollen foraging being most sensitive (affected by four acaricides), followed by pollen foraging (affected by three acaricides) and hygienic behavior being the least sensitive (affected by one acaricide and the solvent). This implies that measuring non-pollen foraging is the best behavior to screen for potential negative impacts of acaricides and other xenobiotics. While foraging and hygienic behaviors are important, future research should test acaricides in field studies where brood production, adult population, honey storage, treatment efficacies, and winter colony survival could also be measured.

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AUTHORS' CONTRIBUTIONS

HG and EG designed experiments, performed experiments, data collections, artwork, and data analysis; HG, EG, and PG wrote the paper. All authors read and approved the final manuscript.Funding information

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest The authors declare that they have no conflict of interest.

Les acaricides synthétiques et naturels altèrent les comportements hygiéniques et de butinage des abeilles.

***Apis mellifera* / sublétal / acaricides / comportement hygiénique / comportement de butinage.**

Synthetische und natürliche Akarizide verhindern das Hygiene- und Sammelverhalten bei Honigbienen.

***Apis mellifera* / sublethal / Akarizide / Hygieneverhalten / Sammelverhalten.**

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