Environmental Science and Pollution Research (2018) 25:34730–34739
https://doi.org/10.1007/s11356-018-3205-6

RESEARCH ARTICLE

Lethality of synthetic and natural acaricides to worker honey bees (*Apis mellifera*) and their impact on the expression of health and detoxification-related genes

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Received: 12 June 2018 / Accepted: 11 September 2018 / Published online: 15 October 2018

Abstract

In this study, honey bees (*Apis mellifera* L.) were exposed to LD_{05} and LD_{50} doses of five commonly used acaricides for controlling the parasitic mite, *Varroa destructor*. LD_{50} values at 48 h post-treatment showed that tau-fluvalinate was the most toxic, followed by amitraz, coumaphos, thymol, and formic acid. However, the hazard ratios, which estimate the hive risk level based on a ratio of a standard dose of acaricide per hive to the LD_{50} of the acaricide, revealed that tau-fluvalinate was the most hazardous followed by formic acid, coumaphos, amitraz, and thymol. The expression of the honey bee acetylcholinesterase gene increased after treatment with the LD_{05} and LD_{50} acaricide doses and could distinguish three patterns in the timing and level of increased expression between acaricides: one for amitraz, one for tau-fluvalinate and formic acid, and one for coumaphos and thymol. Conversely, changes in cytochrome P450 gene expression could also be detected in response to all five acaricides, but there were no significant differences between them. Changes in vitellogenin gene expression could only detect the effects of tau-fluvalinate, amitraz, or coumaphos treatment, which were not significantly different from each other. Among the acaricides tested, coumaphos, amitraz, and thymol appear to be the safest acaricides based on their hazard ratios, and a good marker to detect differences between the effects of sub-lethal doses of acaricides is monitoring changes in acetylcholinesterase gene expression.

Keywords Acaricides · Acetylcholinesteras · *Apis mellifera* · Cytochrome P450 · Gene expression · *Varroa destructor* · Vitellogenin

Introduction

The varroa mite (*Varroa destructor*) is the most damaging parasite of the honey bee, *A. mellifera* (Ellis and Munn 2005). Current methods used to control this mite primarily depend upon the application of synthetic acaricides in honey bee colonies. Although initially effective, the continuous use of these chemicals has led to the development of mite resistance to the active ingredients of these products (Lodensani et al. 1995; Wang et al. 2002; Li et al. 2005; Sammataro et al. 2005). Synthetic acaricides may also leave chemical residues in honey and wax (Wu et al. 2011). Acaricides like tau-fluvalinate and coumaphos may contaminate 98% of wax combs with up to 204 and 94 ppm, respectively (Mullin et al. 2010). Thus, there will always be residual amounts of some chemicals left over in hive products.

Few studies have been conducted to study the toxicity of synthetic and natural acaricides to bees and most of them were conducted with synthetic compounds. The reported LD_{50} values of topically applied tau-fluvalinate to honey bees range from 0.97 to 200 μg per bee (Atkins et al. 1981; Barnavon 1987; Atkins 1992; Vandame and Belzunces 1998; Santiago et al. 2000; Johnson et al. 2006, 2013; Gashout and Guzman-Novoa 2009). The reported LD_{50} values for topically applied amitraz are similarly variable, ranging between 2.55 and 100 μg per bee (Santiago et al. 2000; USEPA 2011). The

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11356-018-3205-6) contains supplementary material, which is available to authorized users.

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reported LD_{50} values for topically applied coumaphos were less variable, ranging from 14.39 to 31.2 μg per bee (Johnson et al. 2009, 2010; Gregorc et al. 2012). For natural acaricides, even fewer studies have reported on their toxicity to honey bees. Ariana et al. (2002) showed that spraying thyme, savory, or spearmint oil on adult bees caused varroa mortality without apparent harmful effects to the bees. Also, menthol fed to bees in sugar syrup resulted in bee mortality levels that did not significantly differ from controls fed only sugar syrup (Ebert et al. 2007). In other studies, the LD_{50} found for thymol was 210.3 μg per bee (Gashout and Guzman-Novoa 2009) and 55.1 μg per bee (Johnson et al. 2013), whereas that for formic acid was 450 μg per bee (Ebert et al. 2007).

Recent studies have shown that acaricides may have adverse effects on honey bee physiology. For example, flumethrin and coumaphos topically applied to bees affected the transcription levels of several genes encoding antimicrobial peptides (AMP) and immune-related proteins (Garrido et al. 2013). The expression of the AMP, hymenoptaecin, was increased by flumethrin, while coumaphos reduced the expression of hymenoptaecin and abaecin. Boncristiani et al. (2012) found that bees from colonies treated with the recommended hive dose of coumaphos or thymol resulted in down-regulation of vitellogenin (Vg).

So far, no studies have been conducted to compare the most commonly used synthetic acaricides to natural acaricides for their sub-lethal effects on honey bee survivorship and expression of health and detoxification-related genes. This study sought to determine the LD_{05} and LD_{50} for three synthetic (tau-fluvalinate, amitraz, and coumaphos) and two natural (thymol and formic acid) acaricides on adult honey bees. In addition, the pattern of expression of three honey bee genes following exposure to acaricides was determined. The genes selected encoded for the health and lifespan-related protein Vg, which has multiple functions related to development, immunity, health, longevity, and general fitness (Amdam et al. 2003); the detoxification enzyme cytochrome P450 (CYP9Q3), which can metabolize pesticides including the acaricides tau-fluvalinate and coumaphos (Mao et al. 2011); and the enzyme acetylcholinesterase (AChE), which terminates synaptic transmission in invertebrates by hydrolyzing the neurotransmitter acetylcholine (Toutant et al. 1989).

Materials and methods

Source of bees

Experiments were conducted at the Honey Bee Research Center of the University of Guelph in Ontario, Canada. Honey bee colonies containing naturally mated queens of the Buckfast strain were used as a source of workers. To obtain worker bees of the same age, frames with emerging brood were collected from five source hives, placed in wooden emergence cages (50 × 7 × 25 cm) and incubated over night at 32 ± 2 °C and 60 ± 5% RH. The next morning, newly emerged bees were used for experiments.

Chemicals

Technical grade (> 98% purity) amitraz, coumaphos, tau-fluvalinate, and formic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Thymol was obtained from Fisher Scientific (Ottawa, Ontario, CA).

Effect of post-exposure time of acaricides on mortality

A preliminary test using a topical bioassay to measure the acute toxicity of the five selected acaricides on adult honey bees (Edwards et al. 2003) was conducted to select a time point that was later used for estimating LD_{50} and LD_{05} values of each acaricide. For this test, a single concentration of each acaricide was prepared by diluting them in 95% ethanol (2.0 mg/ml) on the day of treatments. Immobile worker bees were scored as dead and removed from the cages. The number of dead bees was recorded at 2, 24, and 48 h post-treatment (hpt). It has been shown that this period of time is sufficient to measure acute toxicity of various pesticides in honey bees (Edwards et al. 2003). The experiment was repeated three times.

Determination of the acaricides' LD_{50} and LD_{05}

To estimate LD_{50} and LD_{05} values, the acaricides were serially diluted in 95% ethanol for final concentrations of 150.0, 50.0, 10.0, 1.0, and 0.1 mg/ml. The doses of each acaricide applied to the bees were 750, 250, 50, 5, and 0.5 μg/bee. The five acaricides and the solvent used were administered individually as a single 10-μg topical dose in 5 μl to the dorsal surface of each worker's thorax (Edwards et al. 2003) using a micropipette (Bio-Rad Laboratories, Mississauga, Ontario, CA). Bees were held between two fingers until the 5 μl of the acaricide was absorbed onto the thorax. For controls, a set of workers were treated with 5 μl of 95% ethanol, while the non-treated control was not exposed to ethanol or the acaricides. Each treated group consisted of 25 bees. After receiving the topical treatment, each group of bees was placed and maintained in a wooden cage (12.7 × 8.5 × 14.5 cm) with a wire-screened wall. The cages were provided with two gravity-top feeders. One contained 50% sugar/distilled water solution, and the other contained dH_2O. The cages were kept in an incubator (32 ± 2 °C, 60 ± 5% RH) and monitored for bee mortality as described above. The number of dead bees was recorded at 48 hpt. The experiment was repeated three times.

Determination of the acaricides' hazard

The hazard ratio was calculated by dividing the hive dose of each acaricide that is recommended by manufacturers, which is the acaricide dose applied to the hive in accordance with the directions on the product's label, by their respective LD_{50} (Table 2) (Smart and Stevenson 1982; Felton et al. 1986).

Sampling of treated bees for gene expression analysis

Bees were treated with the LD_{05} or LD_{50} doses of each of the five acaricides, or 95% ethanol as described previously. The experiment was repeated three times. Samples of live treated and non-treated bees were collected at 2, 24, and 48 hpt and then frozen at – 70 °C until RNA extraction. Approximately 75 bees were collected per sampling time per dose and per treatment.

RNA extraction and cDNA synthesis

RNA was extracted from three pooled sets of 25 bees per sample according to Chen et al. (2000). The concentration and purity of the extracted RNA was measured using a NanoVue spectrophotometer (GE Healthcare, Cambridge, UK). cDNA was prepared using a RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario, CA) following the manufacturer's instructions.

Primers and PCR reactions

Except for AChE (AF213012.1), the primers used to amplify the target and constitutive control gene (glyceraldehyde-3-phosphate dehydrogenase) were obtained from the literature (Table S1). To design primers for AChE (AF213012.1), the sequence of the gene and three related genes were obtained from the NCBI nr database. The sequences were aligned using MUSCLE, and the primers were designed in highly conserved regions using Gene Runner (Version 3.05, Hastings Software, New York, USA). The primers were synthesized by Laboratory Services of the University of Guelph (Guelph, Ontario, CA). Relative RT-PCR reactions were run in an Eppendorf AG 22331 Master Cycler (Eppendorf, Hamburg, DE). For each 15-μl reaction, a 15-μl PCR reaction was composed of 2 μl of cDNA, 1.5 μl of 10× Taq reaction buffer (New England Bio Labs, Pickering, Ontario, CA), 0.5 μl of 10 m M dNTPs (Bio Basics, Markham, Ontario, CA), and 0.25–2 μl of forward and reverse primers for the target gene and the constitutive control gene. Additionally, the reaction contained 0.2 μl of *Taq* DNA polymerase (New England Biolabs, Pickering, Ontario, CA), and 5.3–7.3 μl of dH_2O. Amplification conditions for CYP9Q3, Vg, and AChE were 94 °C for 3 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 60 s, and 72 °C for 60 s, followed by a final extension step at 72 °C for 10 min.

Quantification of gene expression

PCR products were separated on a 1% TBE agarose gel with 1% ethidium bromide and visualized using a BioDoc-It ™ Imaging System (UVP, Mississauga, Ontario, CA) under UV light. The gel pictures were quantified using the Scion Image software (Scion Corp., Frederick, Maryland, USA) by measuring the intensity of the amplified bands as per Dean et al. (2002). The ratio of band intensity between the target gene and the control gene was calculated to determine the relative expression units (REUs) of each gene. For each time point, the mean and standard errors were calculated using six measurements from three biological replications with two technical repetitions.

Statistical analyses

Mortality rates were calculated for each treatment and the data were arcsine-square root transformed to normalize means and then subjected to analysis of variance. The LD_{50}, LD_{05} values, inverse 95% confidence limits, slopes, intercepts, and r^2 values were calculated for the different acaricides by Probit analysis using the US Environmental Protection Agency Statistical Program (version 1.5) (Lindberg et al. 2000), which adjusts for the non-treated control mortality (USEPA 1992). The data on relative gene expression were assessed for normality using the Bartlett test and, since they were normally distributed, were subjected to analysis of variance. When significance was detected, means between treatments were compared with Tukey post hoc tests. ANOVA and Tukey tests were performed using the statistical software R, version 3.3.1 (R Core Team 2012).

Results

Lethal dose–response curves and LD_{50} and LD_{05} of acaricides

To choose a post-exposure time for this study, worker bee mortality was determined at 2, 24, and 48 hpt. At 2 hpt, mortality for coumaphos, thymol, or formic acid was not significantly different from that of control bees. However, mortality for tau-fluvalinate or amitraz treated bees was significantly higher than that of the two control treatments ($F_{6,14} = 8.44$, $P = 0.0005$; Fig. S1). At 24 and 48 hpt, mortality in tau-fluvalinate and amitraz treated bees was significantly higher than that in coumaphos treated bees, which was higher than that caused by thymol and formic acid, which were now significantly different than those of both controls ($F_{6,14} = 121.07$, $P < 0.0001$ and $F_{6,14} = 689.33$, $P < 0.0001$, respectively; Fig. S1). Based on these results, 48 hpt was chosen as the post-exposure time to determine lethality. Based on the LD_{50}

values determined by log–dose probit analysis, tau-fluvalinate was the most toxic, followed by amitraz and coumaphos that were not significantly different from each other, and then thymol and formic acid that were also not significantly different from each other (Table 1). The LD_{50} values showed the same ranking of lethality, but each acaricide had significantly different LD_{50} values from each other. The slopes for all the acaricides were similar except for amitraz, which had a higher slope, indicating a stronger dose response in bee mortality. The intercept values, which indicate the sensitivity to the acaricides, corresponded relatively close to the ranking of the LD_{50} values.

Acaricides' hazard

The hazard ratios, calculated from the recommended manufacturer's dose and the LD_{50}, were used to predict the risk of toxicity for bees in a hive. Tau-fluvalinate had the highest hazard, which was six times higher than formic acid, whereas the lowest hazard ratios were for amitraz, coumaphos, and thymol, which were the same and about 55% of that of formic acid (Table 2). This indicates that tau-fluvalinate presented a much greater risk for worker bees at the hive level than the other compounds.

Effect on CYP9Q3 expression

For the LD_{05} dose, expression of CYP9Q3 decreased greatly from 0 to 2 hpt with all treatments (Fig. 1a). At 2 hpt, CYP9Q3 expression with non-treated bees was significantly higher than that of ethanol treated bees, which was significantly higher than in any of the acaricide treated bees, which were not significantly different from each other ($F_{6,34} = 4.72$, $P = 0.0076$). At 24 and 48 hpt, CYP9Q3 expression for ethanol and non-treated bees continued to decline, but expression with non-treated bees was still significantly higher than that with ethanol and all acaricide treated bees at 24 hpt ($F_{6,34} = 21.15$, $P < 0.0001$) and 48 hpt ($F_{6,34} = 8.24$, $P = 0.0004$). However, expression with ethanol was no longer significantly higher than that with the acaricides ($F_{6,34} = 21.15$, $P > 0.5$; $F_{6,34} = 8.24$, $P > 0.5$, respectively). Thus, the effect of the acaricides at the LD_{05} dose could only be distinguished from that of the ethanol solvent at 2 hpt.

The effect of the LD_{50} doses had very similar effects on expression of CYP9Q3 (Fig. 1b). At 2 hpt, CYP9Q3 expression was significantly higher in non-treated bees than in ethanol treated bees, which was significantly higher than that found in tau-fluvalinate, amitraz, coumaphos, thymol, or formic acid treated bees ($F_{6,34} = 9.99$, $P = 0.0002$). At 24 and 48 hpt, CYP9Q3 expression for non-treated bees was significantly higher than that for ethanol or acaricide treated bees, which did not differ from each other ($F_{6,34} = 17.82$, $P < 0.0001$; $F_{6,34} = 5.43$, $P = 0.004$). The highly similar results for CYP9Q3 expression at the LD_{05} and LD_{50} doses indicated that there was no dose response in its expression at those concentrations.

Effect on Vg expression

After exposure to the LD_{05} doses, Vg expression increased from 0 to 2 hpt for the controls and all the acaricides (Fig. 2a). At 2, 24, and 48 hpt, Vg expression in bees exposed to ethanol and all acaricides was not significantly different from each other or that in non-treated bees ($F_{6,34} = 0.87$, $P = 0.54$; $F_{6,34} = 0.39$, $P < 0.87$; $F_{6,34} = 1.11$, $P = 0.41$, respectively).

The LD_{50} doses also resulted in increased Vg expression from 0 to 2 hpt for the controls and all acaricides (Fig. 2b). At 2 hpt, Vg expression with non-treated bees was significantly lower than that of bees treated with tau-fluvalinate ($F_{6,34} = 16.26$, $P < 0.0001$) but not to any of the other treatments ($F_{6,34} = 16.26$, $P > 0.82$; Fig. 2b). At 24 and 48 hpt, Vg expression with non-treated bees was not significantly different than that of ethanol, thymol, and formic acid treated bees, but

Table 1 LD_{05} and LD_{50} values (μg/bee) and 95% confidence limits estimated for worker honey bees at 48 hpt to five acaricides under laboratory conditions

Acaricide	N[1]	LD_{05}[2, 4] (μg/bee)	95% confidence limits	LD_{50}[3, 4] (μg/bee)	95% confidence limits	Intercept ± SE	Slope ± SE	Chi^2
Tau-fluvalinate	675	0.027 [a]	0.009–0.055	0.448 [a]	0.294–0.643	5.47 ± 0.11	1.34 ± 0.17	9.17
Amitraz	825	0.335 [b]	0.170–0.517	1.986 [b]	1.555–2.461	4.37 ± 0.15	2.13 ± 0.26	2.67
Coumaphos	750	0.347 [b]	0.115–0.710	6.232 [c]	4.100–8.979	3.96 ± 0.19	1.31 ± 0.16	2.76
Thymol	825	4.509 [c]	2.010–7.756	51.250 [d]	37.469–68.775	2.33 ± 0.33	1.56 ± 0.17	1.44
Formic acid	825	6.723 [c]	2.358–13.241	152.452 [e]	105.560–220.640	2.35 ± 0.33	1.21 ± 0.15	4.77

[1] Total number of honey bees used per treatment

[2] Lethal dosage causing 5% mortality after 48 h with 95% confidence limits

[3] Lethal dosage causing 50% mortality after 48 h with 95% confidence limits

[4] Different letters next to the LD_{05} and LD_{50} values indicate significant differences based on ANOVA and Tukey tests ($P < 0.05$). Comparisons are only valid within same dose

Table 2 Determination of hazard ratio of tested acaricides

Formulated acaricide[1]	Active ingredient	Residual time in combs[2]	Dose (g)/hive[3]	Hazard ratio[4]
Apistan®	Tau-fluvalinate	Years	1.6	3.6
Apivar®	Amitraz	Days	1.0	0.5
ChekMite®	Coumaphos	Years	2.8	0.5
Apiguard®	Thymol	Days	25.0	0.5
Mite Away QS®	Formic acid	Days	137.0	0.9

[1] Trade names of acaricides

[2] Residual time of acaricides in combs (Bogdanov 2006)

[3] Dose/hive is the dose of acaricide recommended by manufactures for each colony

[4] Hazard ratios were calculated by dividing the dose of acaricide applied in the hive by their LD_{50s}

was significantly lower than that of bees treated with tau-fluvalinate, amitraz, and coumaphos, which were not significantly different from each other ($F_{6,34} = 15.37$, $P < 0.0001$; $F_{6,34} = 5.43$, $P = 0.004$). The expression of Vg showed a dose response with no response to the acaricides at the LD_{05} doses, but the LD_{50} doses of tau-fluvalinate, amitraz, and coumaphos showed significantly higher expression than both controls at 24 and 48 hpt.

Effect on *AChE expression*

For the LD_{05} doses, AChE expression was not significantly different between the controls or any of the acaricides at 2 and 24 hpt ($F_{6,34} = 0.41$, $P = 0.86$; $F_{6,34} = 0.45$, $P = 0.84$, respectively; Fig. 3a). However, from 24 to 48 hpt, AChE expression increased most with amitraz that had significantly higher expression ($F_{6,34} = 66.07$, $P < 0.0001$) than tau-fluvalinate and formic acid that had significantly higher expression ($F_{6,34} = 66.07$, $P < 0.0001$) than in non-treated and ethanol, coumaphos, and thymol treated bees ($F_{6,34} = 11.17$, $P = 0.0001$).

Exposure to the LD_{50} doses resulted in AChE expression increasing from 0 to 2 hpt with all the acaricides, and AChE expression was significantly higher in bees treated with all the acaricides compared to the two controls ($F_{6,34} = 34.17$, $P < 0.0001$) (Fig. 3b). There was no change at 24 hpt ($F_{6,34} = 76.38$, $P < 0.0001$). However, at 48 hpt, AChE

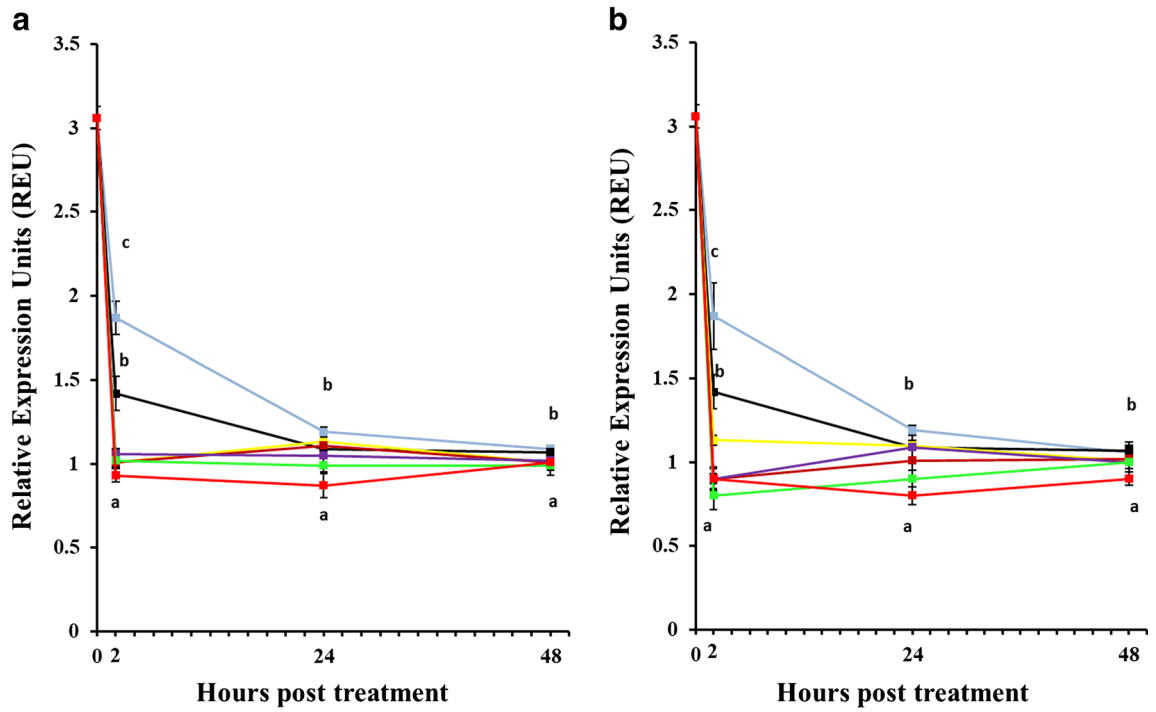

Fig. 1 Relative expression (mean ± SE) of cytochrome P450 (CYP9Q3) for worker honey bees following the treatments with **a** LD_{05} and **b** LD_{50} doses of five acaricides. Bees were topically treated with a LD_{05} dose of (■) tau-fluvalinate, (■) amitraz, (■) coumaphos, (■) thymol, or (■) formic acid. The control treatments consisted of (■) ethanol-treated bees (solvent) and (■) non-treated bees (control). Levels of mRNA were determined relative to RPS5 mRNA levels. Data points represent the mean of six replications. Different letters indicate that the relative expression of cytochrome P450 in treated bees was significantly different from those of non-treated bees based on ANOVA and Tukey tests ($P < 0.05$)

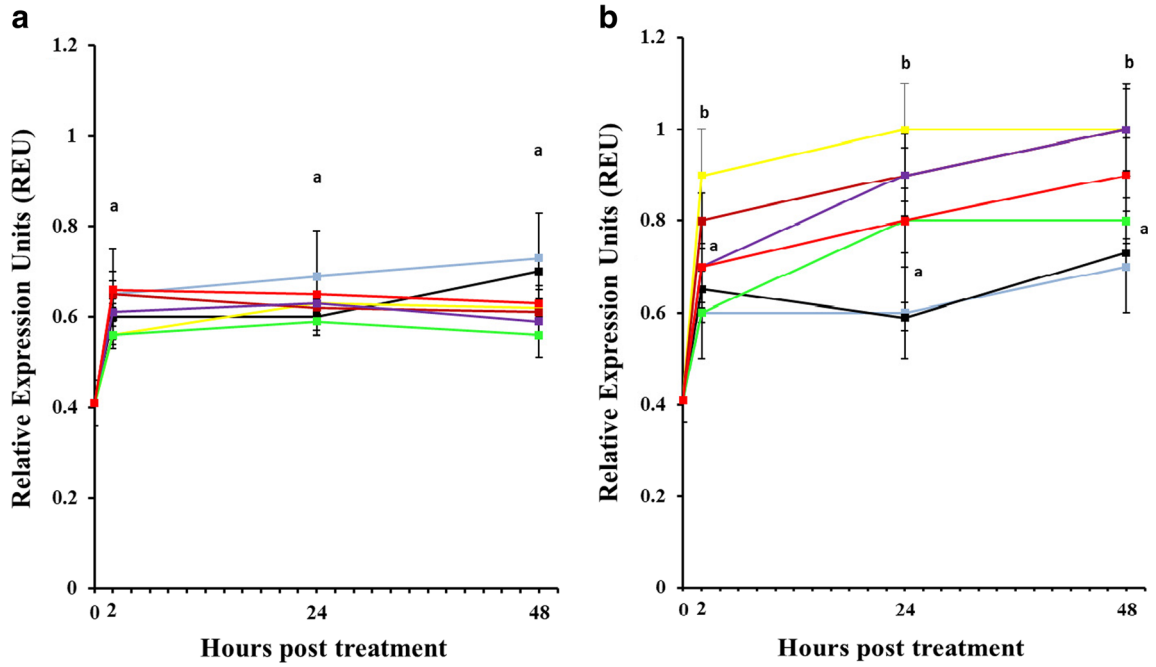

Fig. 2 Relative expression (mean ± SE) of vitellogenin (Vg) for worker honey bees following treatments with **a** LD_{05} and **b** LD_{50} doses of five acaricides. Bees were topically treated with a LD_{05} dose of (■) tau-fluvalinate, (■) amitraz, (■) coumaphos, (■) thymol, or (■) formic acid. The control treatments consisted of (■) ethanol-treated bees (solvent) and (■) non-treated bees (control). Levels of mRNA were determined relative to RPS5 mRNA levels. Data points represent the mean of six replications. Different letters indicate that the relative expression of vitellogenin in treated bees was significantly different from those of non-treated bees based on ANOVA and Tukey tests ($P < 0.05$)

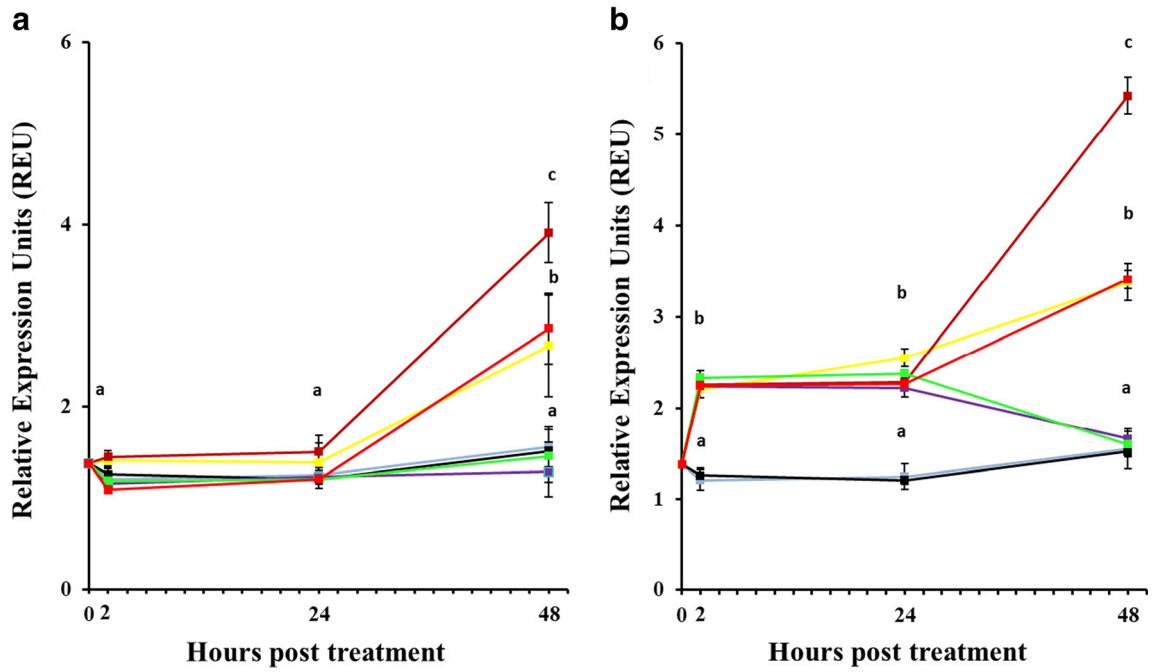

Fig. 3 Relative expression (mean ± SE) of acetylcholinesterase (AChE) for worker honey bees following treatments with **a** LD_{05} and **b** LD_{50} doses of five acaricides. Bees were topically treated with a LD_{05} dose of (■) tau-fluvalinate, (■) amitraz, (■) coumaphos, (■) thymol, or (■) formic acid. The control treatments consisted of (■) ethanol-treated bees (solvent) and (■) non-treated bees (control). Levels of mRNA were determined relative to RPS5 mRNA levels. Data points represent the mean of six replications. Different letters indicate that the relative expression of acetylcholinesterase in treated bees was significantly different from those of non-treated bees based on ANOVA and Tukey tests ($P < 0.05$)

expression in only non-treated and ethanol treated bees remained unchanged. Expression declined for coumaphos and thymol treated bees, which were no longer significantly different from the two controls ($F_{6,34} = 66.07$, $P > 0.99$). AChE expression in bees treated with tau-fluvalinate and formic acid increased moderately and was significantly higher than that of the controls ($F_{6,34} = 66.07$, $P < 0.0001$) but not significantly different from each other. The greatest increase in expression was with amitraz, which was significantly higher than that for the other treatments ($F_{6,34} = 66.07$, $P < 0.0001$). The results for AChE expression showed a dose response with the LD_{50} dose having greater effects of expression than that of the LD_{05} dose, but the ranking of the impacts of the acaricides at 48 hpt was the same with the LD_{50} and LD_{05} doses.

Discussion

Based on the estimated LD_{50} values in this study, the three synthetic acaricides, tau-fluvalinate, amitraz, and coumaphos, in that order, were the most toxic acaricides tested on adult honey bees. These differences are likely due to differences in their acaricidal modes of action. Tau-fluvalinate acts on the voltage-gated sodium ion channels in nerves (Eto 1974), amitraz binds to octopamine receptors (Hollingworth and Lund 1982), and coumaphos inhibits acetylcholinesterase in the nervous system (Davies et al. 2007). The natural acaricides were significantly less toxic to the bees than the synthetic acaricides, with thymol having intermediate LD_{50} values and formic acid having the lowest LD_{50} values. This could also be related to their acaricidal modes of action. Thymol binds to octopamine (Enan 2001) or gamma-aminobutyric acid (GABA) receptors (Priestley et al. 2003), and formic acid inhibits mitochondrial respiration (Liesivuori and Savolainen 1991).

Although this is the first study to compare the toxicity of synthetic acaricides to natural acaricides for adult honey bees, other studies have analyzed the acute toxicity of individual acaricides to honey bees. Compared to previously reported values, the LD_{50} values for the acaricides in this study were 2–2.2 times lower for tau-fluvalinate (Santiago et al. 2000; Johnson et al. 2006), 2.3–5 times lower for coumaphos (Klochko et al. 1994: Johnson et al. 2010), 1.3 times lower for amitraz (Santiago et al. 2000), 3 times lower for formic acid (Ebert et al. 2007), and 4 times lower for thymol (Gashout and Guzman-Novoa 2009). Thus, the LD_{50} values were consistently lower in this study. Reasons as to why such lower LD_{50} values were found in this study are the age of the bees and the time post-treatment when toxicity was determined. All the acaricide toxicity studies cited above used older adult bees while this study used newly emerged (< 24 h old) bees, and it is known that older adult bees have harder cuticles than newly emerged bees (Elias-Neto et al. 2010). Bees with softer cuticles could have facilitated a rapid and more abundant penetration of the acaricides, causing higher mortality with lower doses of the chemicals. Also another possible reason for the unusually low LD_{50} values in this study was that it used the Buckfast genotype, which could be more susceptible to the toxic effects of acaricides compared to other genotypes. It is known that different bee genotypes vary in their susceptibility to insecticides (Elzen et al. 2003). A more significant factor may be that all the acaricide toxicity studies cited above calculated LD_{50} values at 24 hpt, whereas this study calculated them at 48 hpt. The toxicity of the 10 μg/bee dose of all the acaricides tested was considerably greater at 48 than at 24 hpt, and thus greater toxicity would be expected with the same dose at a longer time after exposure compared to the shorter times in the previous reports.

A comparison of the hazard ratios, which are estimates of hive level toxicity, to the LD_{50} values, showed that some acaricides, like formic acid, had the lowest toxicity but the second highest hazard ratio. This is due to the relatively high doses of formic acid applied per hive. Another example is amitraz that had approximately 25 times lower LD_{50} values than thymol, but the same hazard ratios as thymol, because thymol is applied at doses that are 25 times higher per hive than amitraz. However, it should be recognized that there is inherent uncertainty in hazard ratio calculations as bioavailability is difficult to predict. Only a relatively small proportion of tau-fluvalinate in Apistan® and coumaphos in Checkmite® strips is actually released into the hive environment (Tremolada et al. 2004; Bonzini et al. 2011). Residual time in combs is also an important consideration, which is not included in the hazard ratio calculation. Residues of tau-fluvalinate and coumaphos could remain in the wax combs for years, potentially accumulating (Bogdanov et al. 1998a; Wallner 1999), whereas amitraz, thymol, and formic acid break down much faster (Korta et al. 2001; Bogdanov et al. 1998b).

The expression of three honey bee genes, i.e., CYP9Q3, Vg, and AChE, was examined at the same time period after treatment as the assessment of mortality in order to determine how their expression was affected by the LD_{05} and LD_{50} doses. CYP9Q3 is a member of the midgut cytochrome P450 family of honey bees (Claudianos et al. 2006). Cytochrome P450 monooxygenases are detoxification enzymes metabolizing naturally occurring xenobiotics and pesticides (Guengerich 2005; Mao et al. 2011). CYP9Q3 can metabolize pesticides including the acaricides tau-fluvalinate and coumaphos and enhance tolerance of honey bees to other pesticides such as cypermethrin and bifenthrin (Mao et al. 2011). Vg is a yolk protein synthesized in the abdominal fat body of insects that is released to the hemolymph and acts as an antioxidant (Corona et al. 2007). In honey bees, Vg has multiple functions related to development, immunity, health, longevity, and general fitness (Amdam et al. 2003). AChE encodes a serine hydrolase that terminates synaptic

transmission in invertebrates by hydrolyzing the neurotransmitter acetylcholine (Toutant et al. 1989). Organophosphorus and carbamate insecticides bind to the serine site of acetylcholinesterase, which blocks the cleavage of the transmitter acetylcholine resulting in accumulation of acetylcholine in the synaptic area, thereby causing excitation, paralysis, and death.

The significant change in CYP9Q3 expression was lower expression than both controls at the LD_{05} and LD_{50} doses by all tested acaricides at 2 hpt. It did not show any differences between the acaricides or any effect of the doses of the acaricides. Mao et al. (2011) observed that CYP9Q3 expression increased 1.5-fold with tau-fluvalinate and decreased 1-fold with bifenthrin, a pyrethroid insecticide that interferes with an insect's nervous system (Wolansky et al. 2007) after oral exposure to honey bees. Thus, our results are opposite of what was expected for CYP9Q3 expression in response to tau-fluvalinate, but more like that of the response to a pyrethroid. One difference in the studies was that Mao et al. (2011) dissected the guts of honey bees to extract their RNA, whereas in our case, whole bees were used. High levels of CYP9Q3 expression have been reported in the midgut compared to the hemolymph of honey bees (Claudianos et al. 2006). Another difference is that Mao et al. (2011) applied much higher amounts (15 μg/bee tau-fluvalinate) compared to the 0.45 μg/bee of tau-fluvalinate used in this study. For CYP9Q3 expression, the results indicate that this gene is not a good marker for distinguishing between the effects or the doses of the five acaricides tested, unlike the LD_{05} and LD_{50} values.

Expression of Vg by acaricides was only observed with the LD_{50} doses where it increased at 48 hpt after exposure to tau-fluvalinate, coumaphos, amitraz, and formic acid relative to the controls. Thus, it was unable to detect the effects caused by the LD_{05} doses, and even the changes in expression at the LD_{50} doses could not detect effects due to thymol or formic acid. While it did detect an effect of tau-fluvalinate, coumaphos, and amitraz, it could not distinguish differences between them. Boncristiani et al. (2012) showed that coumaphos and thymol treatments downregulated Vg expression between 25 and 33%. The reason for the reverse results in this study could be due to the application of single LD_{50} doses topically to individual bees, compared to application to the whole colonies at an estimated 300-times-higher dose per bee by Boncristiani et al. (2012) based on an approximate spring hive population of 10,000 bees. Based on the changes in Vg expression, it appears that it is also not a good marker for differentiating between the effects of the acaricides, although it could distinguish between the effects of the less toxic natural acaricides (thymol and formic acid) from the synthetic acaricides (tau-fluvalinate, amitraz, and coumaphos).

Significant changes in AChE expression due to certain acaricides were observed at 48 hpt for the LD_{05} doses and 2, 24, and 48 hpt for the LD_{50} doses. This indicated a dose response with a faster response in expression at the higher doses. Also, AChE gene expression showed a greater differential response to the different acaricides, particularly at 48 hpt with the LD_{05} and LD_{50} doses. There have been no studies on acaricides on AChE expression in bees, but Boily et al. (2013) showed that bees exposed to the neonicotinoid insecticides, i.e., imidacloprid and clothianidin, increased AChE enzyme activity. However, Weick and Thorn (2002) found that a topical application of 0.1, 1, or 100 ng/bee of coumaphos did not affect AChE enzyme activity in bee brains. For AChE gene expression, expression in greenbugs increased 1.5-fold in a resistant strain compared to a susceptible strain in response to organophosphorus insecticides (Gao and Zhu 2002). Thus, it is not surprising that expression increased with acaricide exposure.

Compared to the other genes tested in this study, AChE was the only marker to distinguish effects between both natural and synthetic acaricides, as well as distinguish between the effects of different synthetic acaricides. While the degree of change in expression compared to the control was greatest for treatments with amitraz followed by tau-fluvalinate and formic acid and then coumaphos and thymol, this did not correlate well with the toxicity as indicated by the LD_{50} values. The modes of action of coumaphos (inhibiting acetylcholinesterase in the nervous system) and thymol (binding to octopamine or GABA receptors) are quite different, and so it is unclear why they would have almost identical effects on AChE expression. It is possible that these acaricides affected AChE expression due to secondary effects, after affecting other aspects of the nervous system or bee metabolism, increasing the need for the cycling of acetylcholine. That would not necessarily be related to their modes of action. However, the mode of action of coumaphos, inhibition of AChE activity, may also explain why AChE expression increased temporarily after exposure to coumaphos. Feedback inhibition may have been reduced due to lower AChE activity, resulting in greater expression of the gene to return AChE activity levels to pre-exposure levels.

In conclusion, the relative toxicity to honey bees of the acaricides tested in this study was very different. While the natural acaricides have lower toxicity, they do not necessarily present lower hazards to bees in the hives, although they degrade faster than some of the synthetic acaricides. Among the acaricides tested, amitraz, coumaphos, and thymol appear to be the safest acaricides based on their hazard ratios. Among the genes tested, only expression of AChE showed both a dose response between the LD_{05} and LD_{50} doses and the ability to distinguish between the effects of several of the five acaricides tested. Thus, it appears to be good general marker for acaricide effects on bees, rather than being related to a specific mode of action of an acaricide. It also shows that the natural acaricides tested either do not affect or only transiently affect its expression depending upon the dose. Thus, changes in AChE

expression may be a good way to assess non-target impacts of acaricides on honey bees and to distinguish between the intensities of those non-target effects. AChE plays a critical role in the functioning of the central nervous system of bees and changes in its expression could be related to negative impacts on the bee (Galizia et al. 2011). For example, sub-lethal neonicotinoid doses increased expression of AChE in adult bees possibly explaining the negative effects of neonicotinoids on memory, learning, and foraging behavior (Blacquière et al. 2012; Boily et al. 2013). Thus, it would be interesting for future studies to relate changes in AChE expression following xenobiotic exposure to changes in bee behaviors related to mental functioning, like the proboscis extension reflex, which measures learning and memory (Galizia et al. 2011).

Acknowledgments The following people contributed in different ways in the experiments conducted: Paul G. Kelly, Mollah Md. Hamiduzzaman, Abril Soria-Martínez, Nancy Bradbury, Mariana Guzman, Masha Burelo, Brooke Wallace, David Stotesbury, and Angela Gradish. This study was partially funded by a grant from the Ontario Ministry of Agriculture, Food and Rural Affairs to EG.

Compliance with ethical standards

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

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