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RESEARCH ARTICLE

Effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival

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SUMMARY

Contaminants can affect organisms' behaviour and, as a consequence, survival. Tau-fluvalinate (hereafter fluvalinate) is the active ingredient in a pesticide commonly used in North America to control *Varroa destructor* mites in honey bee (*Apis mellifera*) colonies. Fluvalinate's effects on honey bees are not well known. Honey bee cognitive and neural function can be assessed using the proboscis extension reflex (PER), which applies Pavlovian conditioning techniques. This study used PER to evaluate effects of fluvalinate on honey bee acquisition learning, (long-term) memory recall, responsiveness to sucrose, and mortality. We also evaluated how exclusion criteria for honey bees that did not exhibit PER during training and memory trials affected interpretation of results. Fluvalinate was administered both orally and dermally at high and low doses to mimic routes by which honey bees are exposed. We found negative effects of fluvalinate on honey bee learning, memory, responsiveness to sucrose, and survival, especially in high oral doses. We also found significant consequences to interpretation of results using different exclusion criteria. For example, almost 50% of individuals that failed to show evidence of learning subsequently showed evidence of memory. The latter results have important implications regarding traditional assessment of PER-based learning and memory; the former results suggest that evaluation of honey bee exposure to fluvalinate and attendant consequences warrants further investigation.

Key words: *Apis mellifera*, exclusion criteria, oral *versus* dermal exposure, learning, proboscis extension reflex, pyrethroid, *Varroa destructor*.

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INTRODUCTION

Pesticides are used ubiquitously to control a variety of parasites. Hosts that are being protected by pesticides may suffer collateral damage, particularly when they share targeted biochemical pathways with their parasite. Recent significant losses, including complete colony mortality, of honey bees (*Apis mellifera*) have been closely linked to widespread parasitism by *Varroa destructor* mites (Currie et al., 2010; Guzmán-Novoa et al., 2010; Le Conte et al., 2010). Synthetic chemical pesticides (miticides) remain the primary method of combatting *V. destructor* (e.g. Lipiński and Szubstarski, 2007; Maggi et al., 2009; Calderone, 2010). Because honey bees and mites are both arthropods, collateral damage from miticides is of some concern. Although side effects to honey bees of in-hive miticides and other agrochemicals remain unclear (Chauzat et al., 2009; Mullin et al., 2010), some reduce queen and drone survival (Rinderer et al., 1999; Haarmann et al., 2002; Pettis et al., 2004), decrease sperm viability (Burley et al., 2008), affect memory (Eiri and Nieh, 2012) and increase colony mortality (Johnson et al., 2009; Johnson et al., 2010). Less conspicuous are physiological effects that can alter foraging, flight and homing behaviours (Thompson, 2003; Aliouane et al., 2009), and reduce acquisition learning (hereafter, learning), memory recall and memory extinction (hereafter, memory) (Taylor et al., 1987; Mamood and Waller, 1990; Weick and Thorn, 2002; Abramson et al., 2004; Decourtye et al., 2005; Desneux et al., 2007), and sensitivity to stimuli (Stone et al., 1997; Lambin et al., 2001; Aliouane et al., 2009). These have important implications for honey bees in outdoor settings where, for example, stimuli (e.g. odour, colour, floral patterns) elicit feeding (Menzel and Shmida, 1993; Galizia et al., 2004; Grüter et al., 2006), colony defence (Butler et al., 1969), nestmate recognition (Breed et al., 1995; Breed et al., 1998; Bowden et al., 1998) and other behaviours crucial to a colony's success.

Here, we examine effects of tau-fluvalinate (hereafter, fluvalinate), the active ingredient in Apistan®, one of the commercial treatments frequently used in North America to control *V. destructor*, on honey bee sensitivity to stimuli and on honey bee mortality. Fluvalinate is a pyrethroid, a class of neurotoxins that prolongs membrane depolarization by acting as agonists of voltage-gated sodium channels (Sherby et al., 1986; Ray and Fry, 2006; Davies et al., 2007). Resulting hyperexcitability can lead to musculature changes that culminate in paralysis or death. Honey bees come in direct contact with fluvalinate during in-hive applications. Successive generations may also be exposed through contact with residues in honey and beeswax (Korta et al., 2001; Bogdanov, 2006). Fluvalinate's relatively low toxicity to honey bees has been attributed, in part, to their detoxifying enzymes (cytochrome P450 monooxygenases, particularly CYP9Q1, CYP9Q2 and CYP9Q3) (Johnson et al., 2006; Johnson et al., 2009; Mao et al., 2011; see also Claudianos et al., 2006) that allow honey bees to metabolize the miticide (see also Hillier et al., 2013). In adult honey bees, fluvalinate suppresses excitability of brain neurons and inhibits peak sodium current (Zhou et al., 2011). Few studies have investigated effects of fluvalinate on honey bee cognition (Taylor et al., 1987; Decourtye et al., 2005). Furthermore, we are unaware of published studies that have concomitantly assessed learning (short-term cognitive and neural function), memory (long-term cognitive and neural function) and sucrose responsiveness.

Insect learning, memory and reponsiveness can be quantified using a Pavlovian conditioning method that monitors the proboscis extension reflex (PER) (Bitterman et al., 1983), wherein insects are trained to respond to a sensory cue, typically an odour, paired with a nutritional reward. Conditioning can take place in just one trial if associative learning is unimpeded (Sandoz et al., 1995). More generally, honey bees' responsiveness to sucrose can be used to assess their ability to learn (Scheiner et al., 1999; Scheiner et al., 2001; Scheiner et al., 2003), and their sucrose response threshold indicates gustatory sensitivity (Pankiw and Page, 2003; Kralj et al., 2007). However, propensity for learning and responsiveness to sucrose can vary among individuals within the same colony because of age, season and other variables (Scheiner et al., 2003). To control for this, researchers frequently exclude honey bees based on their initial behaviours, but selection criteria differ widely among studies (Frost et al., 2012). Consistently, individuals that fail to respond to antennal stimulation with sucrose are not retained for conditioning procedures (e.g. Bitterman et al., 1983; Gil and De Marco, 2005; Latshaw and Smith, 2005). However, after conditioning trials are complete, some researchers also discriminate among honey bees based on their PER responses (Smith and Cobey, 1994; Chandra et al., 2000; Latshaw and Smith, 2005; Drezner-Levy et al., 2009). For example, to reduce experimental variation, Scheiner et al. (Scheiner et al., 2003) recommended retaining only honey bees that show the same level of sucrose responsiveness. However, effects of decision rules about excluding individuals that fail to respond during conditioning trials (i.e. degree of stringency in learning requirements) on interpretation of results have not been well studied.

We tested the hypotheses that fluvalinate would reduce honey bee learning, memory, responsiveness to sucrose, and survival. We also evaluated the effects of different exclusion criteria on interpretation of results.

MATERIALS AND METHODS

Honey bee collection and immobilization

Honey bee (*Apis mellifera* Linnaeus) colonies in this study had been treated in spring with oxalic acid to control *Varroa destructor* mites. To minimize variation in age of honey bees in our experiments, we collected only older individuals; these were honey bees found on the outside and at the entrances of colonies. Most honey bees would have been foragers (Higginson and Barnard, 2004) (we did not distinguish between nectar and pollen foragers) but some guards may also have been collected. Approximately 45–50 honey bees (Buckfast strain) were collected for testing on days when experiments were being run. Honey bees were collected at approximately 09:00 h from entrances of colonies in Coldbrook, Nova Scotia, Canada (45°02′N, 64°33′W), in ventilated plastic vials and transported to Acadia University where they were cooled in a –18°C freezer until immobile (~2.5 min) (Frost et al., 2011). Once immobile, they could be safely restrained in plastic 1000 µl pipette tips, leaving only their heads free. Conditioning and extinction experiments described below occurred between 21 June and 8 September 2010; sucrose response threshold experiments were conducted between 17 June and 16 August 2010.

For reasons beyond our control, instead of using a single colony throughout the experiments, three colonies were used in this study. The first colony was used from 21 June to 28 July; a second (3–17 August) and third (23 August–8 September) colony were used because of queen loss (in Colonies 1 and 2; causes unknown), at which point honey bees were difficult to capture because of reduced foraging departures. Use of multiple colonies has the unintentional benefit of making our results more general than had we used a single colony.

Experimental treatments

Oral applications of fluvalinate were used to mimic consumption of contaminated foods whereas dermal applications were intended to mimic in-hive contact with Apistan® strips. Fluvalinate is a solid that is unsuitable for delivering doses at controlled concentrations over the time frame necessary for experiments, so we had to dissolve fluvalinate in acetone. Fluvalinate was then applied dermally (dorsal surface of the thorax) or orally (proboscis) in loads of 0.125 µg [estimated daily exposure per honey bee in treated colonies (Johnson et al., 2009)] and 1.25 µg using a micropipette. Fluvalinate delivery occurred to immobilized, restrained honey bees in the morning; bees that did not feed were not retained for experiments. After fluvalinate exposure, honey bees were fed ~1 µl of 1.5 mol l^{-1} sucrose solution using a wooden toothpick and left for ~2.5 h in the dark before PER trials.

We had six treatments: control oral and control dermal with acetone only, and low-dose (0.125 µg) dermal, low-dose (0.125 µg) oral, high-dose (1.25 µg) oral, and high-dose (1.25 µg) dermal with fluvalinate. All applications were 1.25 µl in total volume. Individuals from all treatment groups were tested at the same time and their responses were evaluated blind to treatment.

Conditioning and extinction trials

Honey bees were transported to a training arena, where they were exposed to stimulus-free air for 15 s to ensure they were not responding to mechanosensory stimulation. Geraniol (i.e. *trans*-3,7-dimethyl-2,6-octadien-1-ol, Sigma-Aldrich, St Louis, MO, USA) was used as the conditioned stimulus; 3 µl of 'neat' geraniol was put onto small filter paper discs and then placed inside an empty syringe. Airflow was switched from an odour-free constant airflow to the geraniol-laden syringe using a manual valve. The syringe opening was kept ~15 mm from an individual's head, and geraniol-scented air was presented for a total of 6 s. The 1.5 mol l^{-1} sucrose solution was offered 3 s after the onset of geraniol. Sucrose was offered by first touching a honey bee's right antenna for ~1 s with a toothpick dipped in sucrose; sucrose was then offered at the mouthparts for 2 s. Unilateral stimulation was selected to reduce any confounding effect of stimulating right or left antennae during memory trials at differing time intervals (Rogers and Vallortigara, 2008). Residual odours were removed from the training arena using a constant vacuum flow.

Each individual followed this training protocol eight times with an inter-trial interval (ITI) of 8 min. Choice of ITI varies among studies and its importance to results is debated; our choice was influenced by both practicality in processing honey bees and what we judged to be middle ground in the literature (reviewed in Frost et al., 2012). Memory retention was monitored by assessing the PER response 24 h after conditioning trials because healthy honey bees can retain information over that period (Hammer and Menzel, 1995). At this time, odour was presented in the absence of a reward (eight extinction trials; 8 min ITI).

The PER was scored as 0 if there was a lack of response, or extension of the proboscis occurred only after antennal stimulation with sucrose. The PER was scored as 1 when a honey bee opened its mandibles and extended its proboscis following presentation of geraniol but before receiving a sugar reward. For both learning and memory, scores from trials were summed for each honey bee. We did not use exclusion criteria prior to training/extinction because we wanted a more accurate representation of the average responses of individuals within a colony. When obvious issues in motor function (e.g. broken proboscis, stinger used) were noted, individuals were removed from analysis.

After extinction trials and a further 24 h (for assessing mortality), honey bees were freeze-killed, and a new set of honey bees was collected for subsequent trials.

Sucrose threshold trials

Following immobilization, honey bees were fed ~1 µl of 1.5 mol l^{-1} sucrose solution (by stimulating their proboscis directly) and left to recover for 0.75, 1.5 or 3 h. Honey bees then had their PER responses tested by measuring the sucrose threshold response by exposing the antenna to water (0 mol l^{-1} sucrose), followed by ascending sucrose solutions (Pankiw and Page, 2000; Scheiner et al., 2001; Pankiw and Page, 2003; Kralj et al., 2007) of 0.005, 0.01, 0.05, 0.1, 0.5, 1 and 1.5 mol l^{-1} to determine their responsiveness to water and sucrose. Solutions were presented every 3 min to reduce sensitization between successive sucrose stimulations. A positive PER response was assigned when a honey bee extended its proboscis in response to antennal stimulation with sucrose. Scoring was as above, with a maximum score of 8 (because individuals could respond to water). As before, new honey bees were used for subsequent trials.

Mortality

Honey bee mortality was assessed 3 and 24 h post-treatment. Honey bees were classified as dead when there was no evidence of antennation (characteristic constant movement of the antennae) or abdominal movement.

Statistical analyses

Statistical analyses were conducted in SPSS (SPSS, 2008). Learning, memory and sucrose responsiveness of honey bees were compared to test for differences in effects of treatment method (oral/dermal) and dosage (high/low). A general linear model (GLM) was used to test simultaneously for effects of colony and treatment; Tukey's tests were used for *post hoc* pairwise comparisons. Parametric tests were used for analyses because GLMs are typically robust to deviations from normality (Winer et al., 1991). When significant interactions were encountered, main effects were not interpreted (Zar, 1999), data were split by one of the interacting variables, and analyses were run separately for each level of that variable. Mortality was assessed using 2×6 contingency tables for both 3 and 24 h post treatment.

RESULTS

Learning and memory: spontaneous responders and non-responders

Honey bees responding spontaneously (i.e. pre-conditioning) to either geraniol or mechanical stimulation from airflow were removed from statistical analyses [numbers for geraniol precede those for mechanical: Colony 1: 20/322 (6.2%) + 12/322 (3.7%); Colony 2: 16/145 (11.0%) + 1/145 (0.7%); Colony 3: 46/271 (17.0%) + 13/271 (4.8%)]. Two honey bees died in the middle of conditioning, so that we had 628 honey bees in total for these tests. All other individuals were used regardless of response to sugar or odour, and 193/628 (30.8%) did not respond to the odour even once during conditioning trials. Of 358 individuals that responded at least once during conditioning and that survived to extinction trials, only 183 (51.1%) responded at least once during extinction trials. Among 152 individuals that failed to respond (i.e. no PER) to odour stimulation during conditioning trials (suggesting failure to learn) and that survived to extinction trials, 63 (41.5%) recalled the odour successfully ≥1 time 24 h later. Moreover, there was only a weak positive correlation between learning and memory (N=510, r_S=0.07, P=0.05).

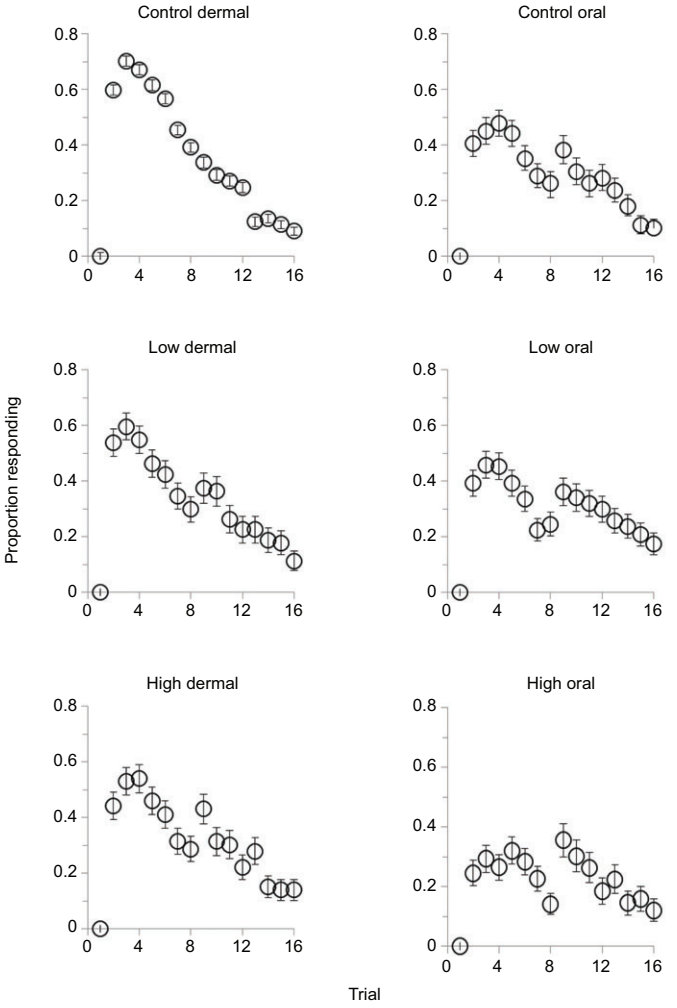

Fig. 1. Proportion of positive proboscis extension responses by honey bees after excluding spontaneous responders at trial 1 (±1 s.e.m.). Trials 2–8 are learning trials and trials 9–16 are memory trials.

Learning: conditioning trials

Evidence of learning was highest in early trials (Fig. 1). Responses began to wane after the fourth trial in all treatments (Fig. 1), suggesting satiation, antifeedant effects or just poor health, although this same protocol did not previously produce this result (Frost et al., 2011). In any case, the more important result is consistency among treatments. There was a significant effect of both colony ($F_{2,608}$=8.1, P<0.0001) and treatment ($F_{5,608}$=9.2, P<0.0001) on learning and memory scores (*post hoc* pairwise comparisons are indicated in Fig. 2). Because the colony by treatment interaction approached significance ($F_{10,608}$=1.7, P=0.09), and because colony was confounded with time of year, data were reanalyzed within each colony. Treatment remained a significant predictor of learning and memory for all colonies (all F≥4.9, all P≤0.007). In general, oral treatments interfered more than dermal treatments with honey bees' ability to learn an odour–reward association. Consistently, the most significant differences occurred between control dermal and high oral fluvalinate treatments.

To test for effects of exclusion criteria, data were re-analyzed by selecting only individuals that recalled the odour at least once during conditioning trials [this form of exclusion is not uncommon (e.g. Sandoz et al., 2000; Drezner-Levy et al., 2009)]. Although treatment

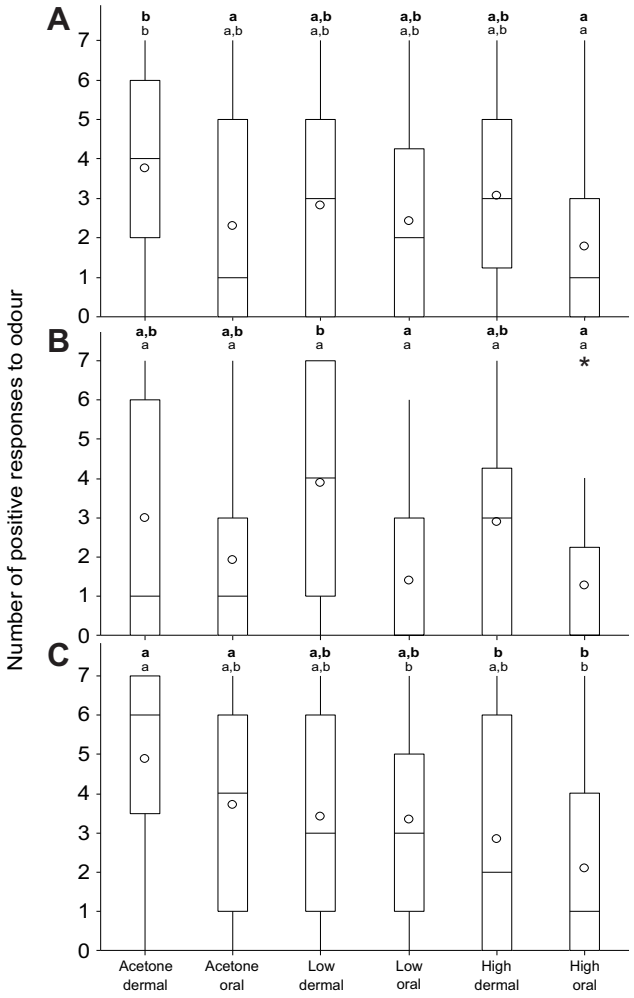

Fig. 2. Effect of treatment on learning in (A) Colony 1, (B) Colony 2 and (C) Colony 3. Plots show all data. Treatments not sharing letters (pooling data from the three colonies) differed significantly (P<0.05, Tukey's pairwise comparisons); bold letters (top row) represent results of *post hoc* comparisons for pooled data, Roman letters (lower row) represent comparisons when only individuals responding ≥1 time to odour were retained. Asterisks indicate outliers (values that are more than 1.5 times the interquartile range from the mean), horizontal lines represent medians, top and bottom of box indicate upper and lower quartiles, vertical lines depict range, and open circles represent means.

was significant in this model ($F_{5,415}$=4.5, P<0.0001), colony ($F_{2,415}$=2.0, P=0.13) and the colony by treatment interaction ($F_{10,415}$=0.9, P=0.57) were not. To allow comparison with the original analysis, data were reanalyzed by colony. Treatment significantly affected learning for Colonies 1 and 3 ($F_{5,189}$=2.3, P=0.05; $F_{5,159}$=2.8, P=0.02, respectively), but not for Colony 2 ($F_{5,67}$=1.9, P=0.11). In pairwise comparisons for Colony 1, the only significant difference was between control dermal and high oral treatments (P=0.05), with the latter treatment having a significantly lower mean response score; for Colony 3, individuals treated with both high and low oral treatments had significantly lower response scores than did control dermal treatments (P=0.04 and P=0.03, respectively).

To illustrate potential effects of selecting individuals for analyses based on their conditioning scores, significance values were calculated using more stringent learning criteria (Table 1). For conditioning experiments, removal of non-responders affected significance of all three factors on honey bee learning.

Memory: extinction trials

Treatment significantly affected honey bee memory ($F_{5,504}$=3.2, P=0.007), but there was no significant effect of colony ($F_{2,504}$=0.1, P=0.87), nor was there a significant interaction between colony and treatment ($F_{10,504}$=0.7, P=0.75). After re-running the model without colony as a variable, treatment remained significant ($F_{5,504}$=4.5, P<0.0001). In general, individuals treated with high oral doses of fluvalinate had the lowest memory scores whereas the low dermal application had the least effect on honey bee memory (Fig. 3).

When only individuals that recalled the odour at least once were retained for analysis, treatment went from being non-significant ($F_{5,240}$=1.7, P=0.13) to being significant ($F_{5,240}$=2.4, P=0.04) after colony ($F_{10,240}$=1.0, P=0.36) was removed from the model (Table 1; for pairwise comparisons see Fig. 3). *Post hoc* tests were not used for selection criteria more stringent than ≥1 response because sample sizes were too small to continue analyzing the data separately by colony. However, none of the factors had a significant effect on memory when non-responders were eliminated (Table 1).

Sucrose responsiveness

In only honey bees treated with a high oral dose of fluvalinate, ~30% of individuals (19/63) were repeatedly observed with a perpetually extended proboscis (prior to sucrose stimulation) that drooped distally.

There was a significant effect of both colony ($F_{1,340}$=10.4, P=0.001) and treatment ($F_{5,340}$=11.4, P<0.0001) on honey bee PER; the interaction between colony and treatment was not significant ($F_{5,340}$=1.6, P=0.17). There was no significant effect of recovery time (1.5 h *vs* 3 h; P=0.86). Data were reanalyzed by colony (only Colonies 1 and 2 were used for sucrose responsiveness experiments) and for both colonies treatment significantly affected sucrose responsiveness (P<0.0001 in both cases; Fig. 4). *Post hoc* testing (Table 2) suggested that control dermal treatments had the least effect on response scores, whereas high oral treatments of fluvalinate had the most detrimental effects on overall responsiveness to sucrose.

Table 1. Effects of using different selection criteria (number of times honey bees responded to odour) on statistical results from conditioning and extinction trials

	Conditioning			Extinction		
Criterion	Colony	Treatment	Colony × treatment	Colony	Treatment	Colony × treatment
≥0	<0.0001	<0.0001	0.09	0.87	0.007	0.75
≥1	0.13	<0.0001	0.27	0.36	0.13	0.99
≥2	0.009	<0.0001	0.12	0.57	0.86	0.83
≥3	0.38	0.009	0.06	0.11	0.90	0.54
≥4	0.97	0.29	0.18	–	–	–

Data are P-values from general linear models. –, insufficient data.

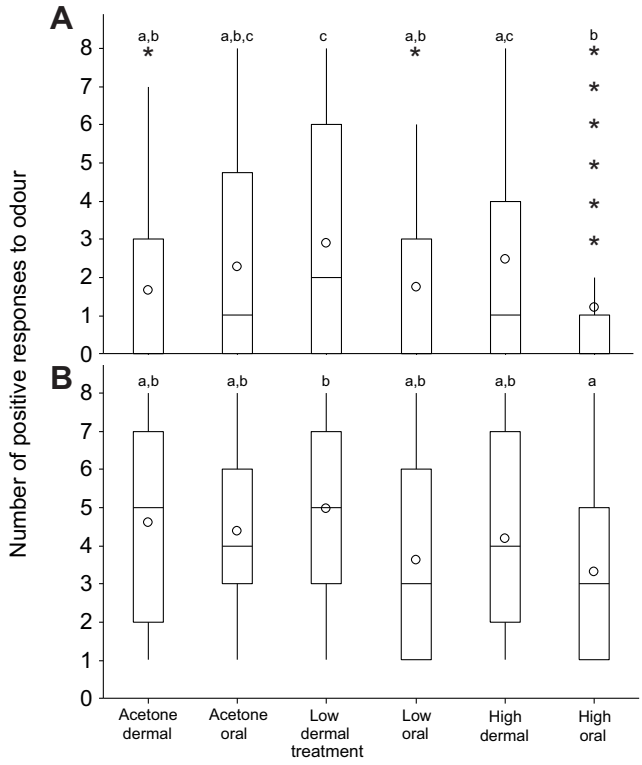

Fig. 3. Effect of treatment on memory recall of a learned odour when (A) all individuals were included in analyses and (B) only individuals responding ≥1 time to odour were retained. Treatments not sharing letters differed significantly (P<0.05, Tukey's pairwise comparisons). Asterisks indicate outliers (values that are more than 1.5 times the interquartile range from the mean), horizontal lines represent medians, top and bottom of box indicate upper and lower quartiles, vertical lines depict range, and open circles represent means.

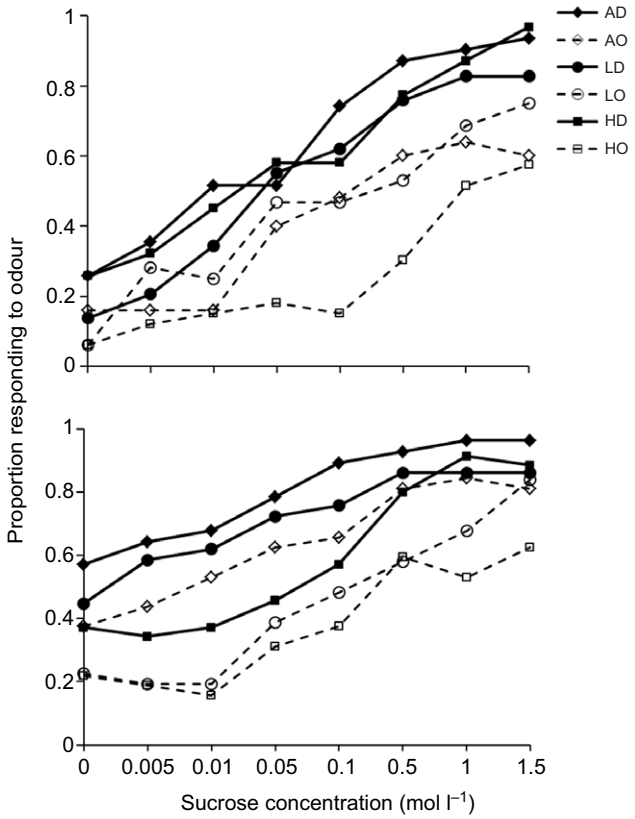

Fig. 4. Effect of treatment on honey bee responsiveness to different concentrations of sucrose from Colony 1 (top) and Colony 2 (bottom). Letter codes denote treatment: A, acetone-only control; L, low dose (0.125 µg fluvalinate); H, high dose (1.25 µg fluvalinate); D, dermal application; O, oral application. Statistical analyses are presented in Table 2.

Mortality

Treatment did not significantly affect honey bee mortality 3 h post-treatment for Colony 1 (χ^2_5=2.9, N=383, P=0.70) or Colony 2 (χ^2_5=4.6, N=216, P=0.50), but did in Colony 3 (χ^2_5=12.9, N=368, P=0.02), where honey bees were most susceptible to high oral, high dermal and low dermal treatments. Mortality 24 h post-treatment was significantly affected by treatment in Colony 1 (χ^2_5=11.9, N=383, P=0.04) and Colony 3 (χ^2_5=24.8, N=368, P<0.0001), and approached significance in Colony 2 (χ^2_5=12.9, N=216, P=0.06). We describe only general patterns here (Fig. 5) to avoid excessive (15) *post hoc* comparisons. For 24 h cumulative mortality in Colony 1, control dermal (control) and low oral treatments had the lowest lethal toxicity rates whereas acetone oral and high oral treatments were highest. In both Colony 2 and 3, honey bees were most susceptible to high oral treatments and mortality was lowest among control dermal honey bees.

DISCUSSION

Spontaneous and delayed responses

Percent spontaneous response to odour (PER on the first odour presentation) varied among the three colonies, increasing by ~6% with each successive colony used. Although it is impossible to distinguish between colony-level and temporal effects because individuals were not collected from the three colonies concurrently, if the increase in spontaneous responses is temporal (intraseasonal) it is consistent with other studies (Erber et al., 1980; Harris and Woodring, 1992). This variation may be a result of a plethora of things including increased exposure during foraging to volatile geraniol produced by flowers (Gerber et al., 1996; Arenas et al., 2007), or fluctuations in octopamine levels (Harris and Woodring, 1992). Each of these may also explain apparent satiation observed here but not in Frost et al. (Frost et al., 2011).

Approximately 30% of individuals did not respond to odour even once during conditioning trials (these are traditionally classified as non-learners). This seems to indicate a failure to learn the odour–reward association, and there was a weak positive relationship

Table 2. Number of positive responses to sucrose (out of a possible maximum of eight) for honey bees treated with different concentrations and exposure routes of fluvalinate

	Colony 1			Colony 2		
Treatment	Median	Mean	N	Median	Mean	N
Control dermal	7.0	6.4^{a}	28	5.0	5.1^{a}	31
Control oral	5.5	5.0^{a,b}	28	3.0	3.2^{b,c}	25
Low dermal	7.0	5.7^{a}	29	5.0	4.3^{a,b}	29
Low oral	3.0	3.6^{b,c}	31	4.0	3.5^{a,b,c}	32
High dermal	5.0	4.7^{a,b}	35	5.0	4.8^{a,c}	31
High oral	3.0	3.0^{c}	32	2.0	2.1^{c}	33

Treatments not sharing letters differed significantly (all P<0.05, Tukey's pairwise comparisons).

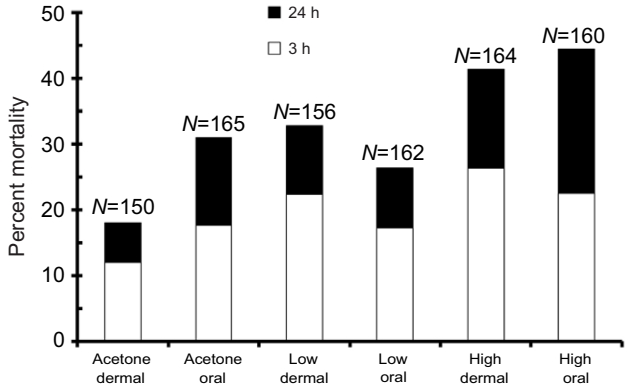

Fig. 5. Cumulative honey bee mortality 3 and 24 h post-treatment. For simplicity, data are pooled for all colonies. Numbers above bars are the total number of honey bees tested for each treatment.

between successful learning and memory (see Results). Intuitively, this suggests that honey bees that did not learn an association would be unable to accurately recall this association at a later time. However, almost 50% of individuals classified as non-learners during conditioning trials recalled the odour successfully during extinction trials 24 h post-conditioning. There are several important implications of this result. First, a higher proportion of non-learners received oral treatment applications; the highest proportion occurred within the high fluvalinate oral treatment. This may suggest an antifeedant response caused by ingestion of sub-lethal doses of fluvalinate that may be compounded by acetone [see Nauen et al. (Nauen et al., 2001), Schmuck et al. (Schmuck et al., 2003) and Ramirez-Romero et al. (Ramirez-Romero et al., 2008) for similar antifeedant effects of imidacloprid, dihydroxy imidacloprid and Cry1Ab protein, respectively].

Perhaps more importantly, odour recall by non-learners during extinction trials suggests that an odour–reward association was learned despite a lack of PER. This highlights the importance of testing both learning and memory (Abramson et al., 1999; Hussaini et al., 2009) when assessing honey bees' learning, memory and sensitivity to stimuli, something previous studies on effects of fluvalinate have not done (Taylor et al., 1987; Decourtye et al., 2005). Furthermore, individuals that fail to respond to odour during conditioning trials should be retained for extinction trials and included in statistical analyses (Smith and Cobey, 1994).

Effects of fluvalinate on honey bee learning, memory, sucrose responsiveness and mortality

Oral treatments negatively affected honey bees' learning abilities more than did dermal treatments. Heightened susceptibility to oral applications of a pesticide has been noted elsewhere [e.g. imidacloprid (Suchail et al., 2000; Nauen et al., 2001)], and may be related to the rapid movement of fluvalinate into the digestive system, whereas dermal treatments rely on passive absorption. This may be further complicated by the fact that oral administration may be less accurate than dermal, due to variability in the standard amount that any individual may ingest (Suchail et al., 2000). The most pronounced differences occurred between the control dermal control and high oral fluvalinate treatments. In contrast, other pesticides, such as chlorpyrofos and bifenthrin, are five to seven times more toxic when applied dermally than orally (Nauen et al., 2001).

Individuals treated with high oral doses of fluvalinate performed poorest during memory recall tests; low dermal applications had the least effect on honey bee memory, and may indicate a time-sensitive detoxification of fluvalinate. Cytochrome P450 monooxygenases are effective at metabolizing fluvalinate over time (Johnson et al., 2006; Johnson et al., 2009; Mao et al., 2011). In the present study, memory recall was assessed 24 h after fluvalinate application, which may be a sufficient period for honey bees to detoxify some fluvalinate (Yu et al., 1984; Hillier et al., 2013), eliminating potentially negative effects on long-term retention of learned associations.

In addition, high oral treatments of fluvalinate had the most detrimental effects on sucrose responsiveness, whereas control dermal treatments had the least effect. More generally, oral treatments appeared to reduce responsiveness, and this may be related to some antifeedant effect that is more pronounced when honey bees ingest compounds directly. This is similar to reduced trophallaxis observed in honey bees exposed to dosages of imidicloprid exceeding 200 ng per honey bee (Suchail et al., 2000).

Only oral treatments of fluvalinate induced unique proboscis extension behaviours. If this was simply a result of noxious qualities of acetone, a similar response should have been noted among oral acetone control and low-dose fluvalinate treatments. Thus, it suggests that the high dose of fluvalinate (a 10-fold increase over the low-oral dose) has distinctive behavioural effects (e.g. hyperexcitability of muscles) unrelated to oral contact with acetone. Physiologically, disruption in normal feeding may be the result of pharmacological action. High spontaneous PER responses may result from exposure to organophosphate compounds that inhibit acetylcholinesterase (Decourtye et al., 2005). Fluvalinate, like other pyrethroids, results in initial hyperexcitability of cells *via* static opening of sodium channels, followed by tetanic paralyses of muscles (Johnson et al., 2009). The effects of such disruption on neurophysiological function, particularly in reference to memory acquisition and consolidation in the antennal lobes, remain unclear.

Published mortality rates from fluvalinate vary [LD_{50} of 0.97 μg per honey bee in Santiago et al. (Santiago et al., 2000); 0.56 μg per honey bee in Elzen et al. (Elzen et al., 2000); 6.75 μg per honey bee in Johnson et al. (Johnson et al., 2009)] and can be affected by various physiological and environmental factors (Johnson et al., 2009). In general, we found mortality rates from fluvalinate increased with dosage and that fluvalinate was more lethal with oral than dermal application.

Effects of data exclusion

Within a colony, there is variation among individuals in learning, memory and sensitivity to stimuli; although some researchers may favour selecting uniform responders (e.g. Scheiner et al., 2003), these selection rules will affect results obtained and consequently interpretation of treatment. For example, spontaneous responders are almost always eliminated from further observation (Erber et al., 1980; Bitterman et al., 1983; Pelz et al., 1997; Morgan et al., 1998; Dacher et al., 2005) because it is difficult to isolate learning capabilities if individuals are transferring previously learned associations within a new context (Gil and De Marco, 2005; Farina et al., 2007). Furthermore, individuals that fail to respond to sucrose prior to or during conditioning are often removed from further testing (Bitterman et al., 1983; Gil and De Marco, 2005; Latshaw and Smith, 2005). However, in this study, many honey bees with relatively low gustatory response and no PER to odour [non-responders are common (Smith, 1991)] during conditioning trials did learn the odour–reward association, indicated by positive responses during extinction trials. Thus, in studies that assess both learning and memory, researchers should err on the side of caution when

determining selection criteria, and substantiate their application of selection criteria.

When learning and memory recall responses in this study were tested using different selection criteria, statistical significance of various factors changed depending on the exclusion rule. There may be no standardized way of dealing with abnormal honey bee responses, and appropriate selection criteria undoubtedly will vary based on the specific question(s) being posed. However, it is imperative that authors justify their choice of data and, ideally, provide a comparison of results using different selection criteria (Smith and Cobey, 1994). Data exclusion is commonly practised by researchers using PER paradigms. However, this makes it difficult to compare results among studies that apply different selection criteria. Furthermore, if researchers eliminate individuals based on a lack of response to odour during learning trials, important data regarding long-term effects of a treatment on honey bee memory could be lost. In our study, almost 50% of individuals that failed to respond once during conditioning trials responded to the odour during extinction trials. Therefore, researchers need to justify removal of test subjects and discuss implications for statistical analyses.

Overall patterns

There are two important findings that emerge from this study. The first is methodological: researchers need to think carefully about their exclusion criteria when using PER. We present an objective way of analyzing results with a plurality of exclusion criteria; if results are consistent regardless of the criterion, greater confidence in results can be conveyed. Second, we found a slight negative effect of fluvalinate on learning, memory responsiveness to sucrose, and survival that was positively related to dose. It is unlikely that honey bees would be exposed all at once to oral doses as high as in our single exposures because residues in honey and beeswax are not that concentrated. However, it is important to note that these were one-time applications, so inferences can only be drawn regarding immediate, rather than cumulative, effects of fluvalinate. Little research has been carried out to record the build-up of fluvalinate in honey bee tissues over time, although Haarmann et al. (Haarmann et al., 2002) found that honey bees from colonies treated the previous year with Apistan® contained up to 0.1 µg fluvalinate per honey bee. Data suggest partial detoxification of fluvalinate over 24 h (Hillier et al., 2013), but honey bees in the colony are exposed to fluvalinate daily for up to 8 weeks during treatment with Apistan®, and long-term ability of cytochrome P450s to detoxify fluvalinate is unknown (Johnson et al., 2009). Future research should investigate effects of repeated low-dose treatments of fluvalinate to more closely mimic conditions in treated colonies.

LIST OF ABBREVIATIONS

CYP	cytochrome P450
GLM	general linear model
ITI	inter-trial interval
PER	proboscis extension reflex

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AUTHOR CONTRIBUTIONS

All authors contributed to developing the questions and experimental design and setup. E.H.F. conducted the experiments, did the majority of the analyses, and wrote paper as part of her Master's thesis. D.S. provided funding, ran some follow up statistical analyses and edited the paper for submission. N.K.H. provided laboratory facilities, funding and expertise on pesticides.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES

Abramson, C. I., Aquino, I. S., Ramalho, F. S. and Price, J. M. (1999). The effect of insecticides on learning in the Africanized honey bee (*Apis mellifera* L.). *Arch. Environ. Contam. Toxicol.* **37**, 529-535.

Abramson, C. I., Squire, J., Sheridan, A. and Mulder, P. G., Jr (2004). The effect of insecticides considered harmless to honey bees (*Apis mellifera* L.): Proboscis conditioning studies using the insect growth regulators. *Environ. Entomol.* **33**, 378-388.

Aliouane, Y., El Hassani, A. K., Gary, V., Armengaud, C., Lambin, M. and Gauthier, M. (2009). Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behavior. *Environ. Toxicol. Chem.* **28**, 113-122.

Arenas, A., Fernández, V. M. and Farina, W. M. (2007). Floral odor learning within the hive affects honeybees' foraging decisions. *Naturwissenschaften* **94**, 218-222.

Bitterman, M. E., Menzel, R., Fietz, A. and Schäfer, S. (1983). Classical conditioning of proboscis extension in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **97**, 107-119.

Bogdanov, S. (2006). Contaminants of bee products. *Apidologie (Celle)* **37**, 1-18.

Bowden, R. M., Williamson, S. and Breed, M. D. (1998). Floral oils: their effect on nestmate recognition in the honey bee, *Apis mellifera*. *Insectes Soc.* **45**, 209-214.

Breed, M. D., Garry, M. F., Pearce, A. N., Hibbard, B. E., Bjostad, L. B. and Page, R. E. (1995). The role of wax comb in honey bee nestmate recognition. *Anim. Behav.* **50**, 489-496.

Breed, M. D., Leger, E. A., Pearce, A. N. and Wang, Y. J. (1998). Comb wax effects on the ontogeny of honey bee nestmate recognition. *Anim. Behav.* **55**, 13-20.

Burley, L. M., Fell, R. D. and Saacke, R. G. (2008). Survival of honey bee (Hymenoptera: Apidae) spermatozoa incubated at room temperature from drones exposed to miticides. *J. Econ. Entomol.* **101**, 1081-1087.

Butler, C. G., Fletcher, D. J. C. and Watler, D. (1969). Nest-entrance marking with pheromones by the honeybee *Apis mellifera* L., and by a wasp, *Vespula vulgaris* L. *Anim. Behav.* **17**, 142-147.

Calderone, N. W. (2010). Evaluation of Mite-Away-II for fall control of *Varroa destructor* (Acari: Varroidae) in colonies of the honey bee *Apis mellifera* (Hymenoptera: Apidae) in the northeastern USA. *Exp. Appl. Acarol.* **50**, 123-132.

Chandra, S. B. C., Hosler, J. S. and Smith, B. H. (2000). Heritable variation for latent inhibition and its correlation with reversal learning in honeybees (*Apis mellifera*). *J. Comp. Psychol.* **114**, 86-97.

Chauzat, M.-P., Carpentier, P., Martel, A.-C., Bougeard, S., Cougoule, N., Porta, P., Lachaize, J., Madec, F., Aubert, M. and Faucon, J.-P. (2009). Influence of pesticide residues on honey bee (Hymenoptera: Apidae) colony health in France. *Environ. Entomol.* **38**, 514-523.

Claudianos, C., Ranson, H., Johnson, R. M., Biswas, S., Schuler, M. A., Berenbaum, M. R., Feyereisen, R. and Oakeshott, J. G. (2006). A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Mol. Biol.* **15**, 615-636.

Currie, R. W., Pernal, S. F. and Guzmán-Novoa, E. (2010). Honey bee colony losses in Canada. *J. Apic. Res.* **49**, 104-106.

Dacher, M., Lagarrigue, A. and Gauthier, M. (2005). Antennal tactile learning in the honeybee: effect of nicotinic antagonists on memory dynamics. *Neuroscience* **130**, 37-50.

Davies, T. G. E., Field, L. M., Usherwood, P. N. R. and Williamson, M. S. (2007). DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life* **59**, 151-162.

Decourtye, A., Devillers, J., Genecque, E., Le Menach, K., Budzinski, H., Cluzeau, S. and Pham-Delègue, M. H. (2005). Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. *Arch. Environ. Contam. Toxicol.* **48**, 242-250.

Desneux, N., Decourtye, A. and Delpuech, J. M. (2007). The sublethal effects of pesticides on beneficial arthropods. *Annu. Rev. Entomol.* **52**, 81-106.

Drezner-Levy, T., Smith, B. H. and Shafir, S. (2009). The effect of foraging specialization on various learning tasks in the honey bee (*Apis mellifera*). *Behav. Ecol. Sociobiol.* **64**, 135-148.

Eiri, D. M. and Nieh, J. C. (2012). A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. *J. Exp. Biol.* **215**, 2022-2029.

Elzen, P. J., Baxter, J. R., Spivak, M. and Wilson, W. T. (2000). Control of *Varroa jacobsoni* Oud. resistant to fluvalinate and amitraz using coumaphos. *Apidologie (Celle)* **31**, 437-441.

Erber, J., Masuhr, T. H. and Menzel, R. (1980). Localization of short-term memory in the brain of the bee, *Apis mellifera*. *Physiol. Entomol.* **5**, 343-358.

Farina, W. M., Grüter, C., Acosta, L. and Mc Cabe, S. (2007). Honeybees learn floral odors while receiving nectar from foragers within the hive. *Naturwissenschaften* **94**, 55-60.

Frost, E. H., Shutler, D. and Hillier, N. K. (2011). Effects of cold immobilization and recovery period on honeybee learning, memory, and responsiveness to sucrose. *J. Insect Physiol.* **57**, 1385-1390.

Frost, E. H., Shutler, D. and Hillier, N. K. (2012). The proboscis extension reflex to evaluate learning and memory in honeybees (*Apis mellifera*): some caveats. *Naturwissenschaften* **99**, 677-686.
Galizia, C. G., Kunze, J., Gumbert, A., Borg-Karlson, A. K., Sachse, S., Markl, C. and Menzel, R. (2004). Relationship of visual and olfactory signal parameters in a food-deceptive flower mimicry system. *Behav. Ecol.* **16**, 159-168.
Gerber, B., Geberzahn, N., Hellstern, F., Klein, J., Kowalksy, O., Wüstenberg, D. and Menzel, R. (1996). Honey bees transfer olfactory memories established during flower visits to a proboscis extension paradigm in the laboratory. *Anim. Behav.* **52**, 1079-1085.
Gil, M. and De Marco, R. J. (2005). Olfactory learning by means of trophallaxis in *Apis mellifera*. *J. Exp. Biol.* **208**, 671-680.
Grüter, C., Acosta, L. E. and Farina, W. M. (2006). Propagation of olfactory information within the honeybee hive. *Behav. Ecol. Sociobiol.* **60**, 707-715.
Guzmán-Novoa, E., Eccles, L., Calvete, Y., McGowan, J., Kelly, P. G. and Correa-Benítez, A. (2010). Varroa destructor is the main culprit for the death and reduced populations of overwintered honey bee (*Apis mellifera*) colonies in Ontario, Canada. *Apidologie (Celle)* **41**, 443-450.
Haarmann, T., Spivak, M., Weaver, D., Weaver, B. and Glenn, T. (2002). Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. *J. Econ. Entomol.* **95**, 28-35.
Hammer, M. and Menzel, R. (1995). Learning and memory in the honeybee. *J. Neurosci.* **15**, 1617-1630.
Harris, J. W. and Woodring, J. (1992). Effects of stress, age, season, and source colony on levels of octopamine, dopamine and serotonin in the honey bee (*Apis mellifera* L.) brain. *J. Insect Physiol.* **38**, 29-35.
Higginson, A. D. and Barnard, C. J. (2004). Accumulating wing damage affects foraging decisions in honeybees (*Apis mellifera* L.). *Ecol. Entomol.* **29**, 52-59.
Hillier, K., Frost, E. and Shutler, D. (2013). Fate of dermally applied miticides fluvalinate and amitraz within honey bee bodies. *J. Econ. Entomol.* **106**, 558-565.
Hussaini, S. A., Bogusch, L., Landgraf, T. and Menzel, R. (2009). Sleep deprivation affects extinction but not acquisition memory in honeybees. *Learn. Mem.* **16**, 698-705.
Johnson, R. M., Wen, Z., Schuler, M. A. and Berenbaum, M. R. (2006). Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. *J. Econ. Entomol.* **99**, 1046-1050.
Johnson, R. M., Pollock, H. S. and Berenbaum, M. R. (2009). Synergistic interactions between in-hive miticides in *Apis mellifera*. *J. Econ. Entomol.* **102**, 474-479.
Johnson, R. M., Ellis, M. D., Mullin, C. A. and Frazier, M. (2010). Pesticides and honey bee toxicity – USA. *Apidologie (Celle)* **41**, 312-331.
Korta, E., Bakkali, A., Berrueta, L. A., Gallo, B., Vicente, F., Kilchenmann, V. and Bogdanov, S. (2001). Study of acaricide stability in honey. Characterization of amitraz degradation products in honey and beeswax. *J. Agric. Food Chem.* **49**, 5835-5842.
Kralj, J., Brockmann, A., Fuchs, S. and Tautz, J. (2007). The parasitic mite *Varroa destructor* affects non-associative learning in honey bee foragers, *Apis mellifera* L. *J. Comp. Physiol. A* **193**, 363-370.
Lambin, M., Armengaud, C., Raymond, S. and Gauthier, M. (2001). Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Arch. Insect Biochem. Physiol.* **48**, 129-134.
Latshaw, J. S. and Smith, B. H. (2005). Heritable variation in learning performance affects foraging preferences in the honey bee (*Apis mellifera*). *Behav. Ecol. Sociobiol.* **58**, 200-207.
Le Conte, Y., Ellis, M. and Ritter, W. (2010). Varroa mites and honey bee health: can *Varroa* explain part of the colony losses? *Apidologie (Celle)* **41**, 353-363.
Lipiński, Z. and Szubstarski, J. (2007). Resistance of Varroa destructor to most commonly used synthetic acaricides. *Pol. J. Vet. Sci.* **10**, 289-294.
Maggi, M. D., Ruffinengo, S. R., Damiani, N., Sardella, N. H. and Eguaras, M. J. (2009). First detection of *Varroa destructor* resistance to coumaphos in Argentina. *Exp. Appl. Acarol.* **47**, 317-320.
Mamood, A. N. and Waller, G. D. (1990). Recovery of learning responses by honeybees following a sublethal exposure to permethrin. *Physiol. Entomol.* **15**, 55-60.
Mao, W., Schuler, M. A. and Berenbaum, M. R. (2011). CYP9Q-mediated detoxification of acaricides in the honey bee (*Apis mellifera*). *Proc. Natl. Acad. Sci. USA* **108**, 12657-12662.
Menzel, R. and Shmida, A. (1993). The ecology of flower colours and the natural colour vision of insect pollinators: the Israeli flora as a study case. *Biol. Rev. Camb. Philos. Soc.* **68**, 81-120.
Morgan, S. M., Butz Huryn, V. M., Downes, S. R. and Mercer, A. R. (1998). The effects of queenlessness on the maturation of the honey bee olfactory system. *Behav. Brain Res.* **91**, 115-126.
Mullin, C. A., Frazier, M., Frazier, J. L., Ashcraft, S., Simonds, R., Vanengelsdorp, D. and Pettis, J. S. (2010). High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE* **5**, e9754.
Nauen, R., Ebbinghaus-Kintscher, U. and Schmuck, R. (2001). Toxicity and nicotinic acetylcholine receptor interaction of imidacloprid and its metabolites in *Apis mellifera* (Hymenoptera: Apidae). *Pest Manag. Sci.* **57**, 577-586.
Pankiw, T. and Page, R. E. (2000). Response thresholds to sucrose predict foraging division of labor in honeybees. *Behav. Ecol. Sociobiol.* **47**, 265-267.
Pankiw, T. and Page, R. E., Jr (2003). Effect of pheromones, hormones, and handling on sucrose response thresholds of honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **189**, 675-684.
Pelz, C., Gerber, B. and Menzel, R. (1997). Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. *J. Exp. Biol.* **200**, 837-847.
Pettis, J. S., Collins, A. M., Wilbanks, R. and Feldlaufer, M. F. (2004). Effects of coumaphos on queen rearing in the honey bee, Apis mellifera. *Apidologie (Celle)* **35**, 605-610.
Ramirez-Romero, R., Desneux, N., Decourtye, A., Chaffiol, A. and Pham-Delègue, M. H. (2008). Does Cry1Ab protein affect learning performances of the honey bee *Apis mellifera* L. (Hymenoptera, Apidae)? *Ecotoxicol. Environ. Saf.* **70**, 327-333.
Ray, D. E. and Fry, J. R. (2006). A reassessment of the neurotoxicity of pyrethroid insecticides. *Pharmacol. Ther.* **111**, 174-193.
Rinderer, T. E., De Guzman, L. I., Lancaster, V. A., Delatte, G. T. and Stelzer, J. A. (1999). Varroa in the mating yard: I. The effects of *Varroa jacobsoni* and Apistan® on drone honey bees. *Am. Bee J.* **139**, 134-139.
Rogers, L. J. and Vallortigara, G. (2008). From antenna to antenna: lateral shift of olfactory memory recall by honeybees. *PLoS ONE* **3**, e2340.
Sandoz, J. C., Roger, B. and Pham-Delègue, M. H. (1995). Olfactory learning and memory in the honeybee: comparison of different classical conditioning procedures of the proboscis extension response. *C. R. Acad. Sci. III, Sci. Vie* **318**, 749-755.
Sandoz, J. C., Laloi, D., Odoux, J.-F. and Pham-Delègue, M. H. (2000). Olfactory information transfer in the honeybee: compared efficiency of classical conditioning and early exposure. *Anim. Behav.* **59**, 1025-1034.
Santiago, G. P., Otero-Colina, G., Sánchez, D. M., Guzmán, M. E. R. and Vandame, R. (2000). Comparing effects of three acaricides on *Varroa jacobsoni* (Acari: Varroidae) and *Apis mellifera* (Hymenoptera: Apidae) using two application techniques. *Fla. Entomol.* **83**, 468-476.
Scheiner, R., Erber, J. and Page, R. E., Jr (1999). Tactile learning and the individual evaluation of the reward in honey bees (*Apis mellifera* L.). *J. Comp. Physiol. A* **185**, 1-10.
Scheiner, R., Page, R. E., Jr and Erber, J. (2001). Responsiveness to sucrose affects tactile and olfactory learning in preforaging honey bees of two genetic strains. *Behav. Brain Res.* **120**, 67-73.
Scheiner, R., Barnert, M. and Erber, J. (2003). Variation in water and sucrose responsiveness during the foraging season affects proboscis extension learning in honey bees. *Apidologie (Celle)* **34**, 67-72.
Schmuck, R., Nauen, R. and Ebbinghaus-Kintscher, U. (2003). Effects of imidacloprid and common plant metabolites of imidacloprid in the honeybee: toxicological and biochemical considerations. *Bull. Insectology* **56**, 27-34.
Sherby, S. M., Eldefrawi, A. T., Deshpande, S. S., Albuquerque, E. X. and Eldefrawi, M. E. (1986). Effects of pyrethroids on nicotinic acetylcholine receptor binding and function. *Pestic. Biochem. Physiol.* **26**, 107-115.
Smith, B. H. (1991). The olfactory memory of the honeybee *Apis mellifera*. I. Odorant modulation of short- and intermediate-term memory after single-trial conditioning. *J. Exp. Biol.* **161**, 367-382.
Smith, B. H. and Cobey, S. (1994). The olfactory memory of the honeybee *Apis mellifera*. II. Blocking between odorants in binary mixtures. *J. Exp. Biol.* **195**, 91-108.
SPSS (2008). *SPSS Statistics for Windows*, Version 17.0. Chicago, IL: SPSS Inc.
Stone, J. C., Abramson, C. I. and Price, J. M. (1997). Task-dependent effects of dicofol (kelthane) on learning in the honey bee (*Apis* mellifera). *Bull. Environ. Contam. Toxicol.* **58**, 177-183.
Suchail, S., Guez, D. and Belzunces, L. P. (2000). Characteristics of imidacloprid toxicity in two *Apis mellifera* subspecies. *Environ. Toxicol. Chem.* **19**, 1901-1905.
Taylor, K. S., Waller, G. D. and Crowder, L. A. (1987). Impairment of a classical conditioned response of the honey bee (*Apis mellifera* L.) by sublethal doses of synthetic pyrethroid insecticides. *Apidologie (Celle)* **18**, 243-252.
Thompson, H. M. (2003). Behavioural effects of pesticides in bees – their potential for use in risk assessment. *Ecotoxicology* **12**, 317-330.
Weick, J. and Thorn, R. S. (2002). Effects of acute sublethal exposure to coumaphos or diazinon on acquisition and discrimination of odor stimuli in the honey bee (Hymenoptera: Apidae). *J. Econ. Entomol.* **95**, 227-236.
Winer, B. J., Brown, D. R. and Michels, K. M. (1991). *Statistical Principles in Experimental Design*, 3rd edn. Boston, MA: McGraw-Hill.
Yu, S. J., Robinson, F. A. and Nation, J. L. (1984). Detoxication capacity in the honey bee, *Apis mellifera* L. *Pestic. Biochem. Physiol.* **22**, 360-368.
Zar, J. H. (1999). *Biostatistical Analysis*, 4th edn. Upper Saddle River, NJ: Prentice Hall.
Zhou, T., Zhou, W., Wang, Q., Dai, P. L., Liu, F., Zhang, Y. L. and Sun, J. H. (2011). Effects of pyrethroids on neuronal excitability of adult honeybees *Apis mellifera*. *Pestic. Biochem. Physiol.* **100**, 35-40.