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The effect of queen pheromone status on *Varroa* mite removal from honey bee colonies with different grooming ability

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Abstract The objective of this study was to assess the effects of honey bees (*Apis mellifera* L.) with different grooming ability and queen pheromone status on mortality rates of *Varroa* mites (*Varroa destructor* Anderson and Trueman), mite damage, and mortality rates of honey bees. Twenty-four small queenless colonies containing either stock selected for high rates of mite removal (n = 12) or unselected stock (n = 12) were maintained under constant darkness at 5 °C. Colonies were randomly assigned to be treated with one of three queen pheromone status treatments: (1) caged, mated queen, (2) a synthetic queen mandibular pheromone lure (QMP), or (3) queenless with no queen substitute. The results showed overall mite mortality rate was greater in stock selected for grooming than in unselected stock. There was a short term transitory increase in bee mortality rates in selected stock when compared to unselected stock. The presence of queen pheromone from either caged, mated queens or QMP enhanced mite removal from clusters of bees relative to queenless colonies over short periods of time and increased the variation in mite mortality over time relative to colonies without queen pheromone, but did not affect the proportion of damaged mites. The effects of source of bees on mite damage varied with time but damage to mites was not reliably related to mite mortality. In conclusion, this study showed differential mite removal of different stocks was possible under low temperature. Queen status should be considered when designing experiments using bioassays for grooming response.

Keywords *Apis mellifera* · *Varroa destructor* · Queen pheromone · Mite removal · Mite injury

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Introduction

European honey bee (*Apis mellifera* L.) colonies act as a social superorganism in which workers, queens and drones perform different tasks related to colony growth, maintenance and reproduction (Pankiw and Page 2001; Winston 1987). Genetic components strongly affect a variety of honey bee behaviors such as defensive behavior (Guzman-Novoa and Page 1993; Pankiw and Page 2001), foraging and pollen hoarding behavior (Hellmich et al. 1985; Pesante et al. 1987), cleaning behavior (Robinson and Page 1988), swarming (Winston 1980) and mite-resistance behavior (Currie and Tahmasbi 2008; Guzman-Novoa et al. 2012; Harris et al. 2010; Rinderer et al. 2010; Spivak 1996).

Pheromones play a major role in communication and social maintenance within colonies of social insects (Pankiw and Page 2000; Pankiw et al. 1994, 1995; Vander Meer et al. 1998; Winston and Slessor 1998). Honey bee queen pheromones influence various colony functions including queen production (Butler and Fairey 1964; Butler and Simpson 1967; Winston et al. 1991), swarm suppression (Winston et al. 1991), attraction of workers during swarming (Butler and Simpson 1967; Velthuis and Es 1964), drone attraction (Butler and Fairey 1964; Gary 1961a), worker attraction to the queen (Gary 1961b; Zmarlicki and Morse 1964), pollen and nectar foraging (Currie et al. 1992; Higo et al. 1992; Naumann et al. 1994), comb building, brood rearing (Free 1987), orientation at the colony entrance (Ferguson and Free 1981), guarding behavior (Moore et al. 1987), hygienic behavior (Rothenbuhler 1964) and grooming (Post et al. 1987). Interactions between bee genetics and responses to pheromones are known to occur. For example, colony genotype affects pheromone-based retinue responses (Pankiw et al. 1994), mating behaviors (Collins 1979; Pankiw et al. 1995) and ovary activation in worker bees (Barron et al. 2001).

Synthetic queen mandibular pheromone (QMP) in the form of pseudo queen (PQ) (Pseudo Queen, Contech Enterprises, Victoria, BC, Canada) is able to mimic many of the effects of natural queen pheromone in the absence of a queen (Pankiw and Page 2003). For example, synthetic queen pheromone influences defensive behavior (Gervan et al. 2005), queen mating (Pettis et al. 1993), sucrose responsiveness (Pankiw and Page 2003) and comb building (Ledoux et al. 2001). But, it is not known if QMP affects grooming behavior against *Varroa* mites (*Varroa destructor* Anderson and Trueman) in honey bees. Since assays of grooming behavior are typically carried out on individuals or groups of bees in queenless conditions, it is important to know what effects queen pheromone may have on grooming success and if this varies in different stocks of bees.

Grooming behavior is one of several defense mechanisms that honey bees use against the *Varroa* mite (Arechavaleta-Velasco and Guzman-Novoa 2001; Bozic and Valentincic 1995; Currie and Tahmasbi 2008) and the grooming response is affected by external conditions (Currie and Tahmasbi 2008). Grooming can be assessed by an indirect measure of mite fall from clusters of bees (Currie and Tahmasbi 2008), a direct measure of bee behaviors (Andino and Hunt 2011) or by quantifying damage to mites (Andino and Hunt 2011; Guzman-Novoa et al. 2012; Wallner 1994). During grooming worker bees may injure mites with their mandibles (Boecking et al. 1993; Moosbeckhofer and Derakhshifar 1986; Moretto et al. 1993; Morse et al. 1991; Rosenkranz et al. 1997; Ruttner and Hanel 1992; Wallner 1994), but the type of body part injured and amount of damage is highly variable (Rosenkranz et al. 1997; Ruttner and Hanel 1992). Correa-Marques et al. (2000) found no correlation between the percentage of injured mites, resistance behavior and mite infestation level, suggesting that evaluation of the proportion of injured mites on bottom

boards is not a consistently reliable measure of resistance. The use of damage criterion to assess grooming of *Varroa* may be useful in some cases but may not categorize all behavioral components that can result in successful grooming events.

The objective of this study was to assess the effect of queen pheromone status on mite mortality rates, mite injury and bee survival within two groups of honey bee colonies with different grooming ability.

Materials and methods

The experiment was carried out at the University of Manitoba, Winnipeg, Manitoba, Canada (49°54′ N, 97°14′ W). Bees from European honey bee colonies used in this study are referred to as "selected" or "unselected" stocks. "Selected" stock was obtained from the Manitoba Queen Breeders Association from a pool of colonies that had been selected for a combination of criteria related to *Varroa* resistance (ability to reduce mite load overwinter) or tolerance (ability to tolerate high mite infestations with below average bee loss) (Bahreini 2014). Four "unselected" control stock colonies were chosen that were originally headed by New Zealand queens from a single supplier (Arataki Honey, Havelock North, New Zealand). To minimize genetic variation among colonies, one hive from "selected" stock with a high level of grooming response was chosen through a pre-bioassay test at 25 °C and 55–65 RH % (for more details see Currie and Tahmasbi 2008) and bees from the four New Zealand colonies were pooled to form source bees for making the unselected stock colonies. In summer 2007, small broodless colonies were established from the selected stock (n = 12) and unselected stock (n = 12) in nucleus hives (5 frame standard Langstroth hive bodies) with an average of 5,538 ± 153 mixed-age worker bees and then were inoculated with 70 live *Varroa* mites that average mite loads were thus 1.29 mites per 100 bees with a maximum infestation of 1.65 mites per 100 bees. Within each stock the twelve independent queenless colonies were then randomly assigned to one of three "queen pheromone status" treatments: (1) caged, mated queen, (2) synthetic QMP lure, or (3) queenless with no queen or pheromone substitute (n = 4 within each stock). Each mated queen was caged in a JZ/BZ plastic queen cage (QC-800, Mann Lake, Hackensack, MN, USA) and placed between two frames in the center of the colony. The QMP treatment was queenless but contained a PQ Lure as a queen substitute. The lure was placed in the center of the colony. All hives (n = 24) were then randomly assigned to locations in a temperature-controlled environmental chamber (208 × 208 × 273 cm = 11.81 m^3 in the Animal Science/Entomology building, University of Manitoba) and held in constant darkness at 5 °C for 5 days. The temperature (°C) and relative humidity (%) inside the room were monitored using a HOBO C-8® (Onset Computer Corporation, Bourne, MA, USA) data logger.

Collection and inoculation of mites

Mites for this experiment were collected from a separate set of highly infested colonies using a modification of the carbon dioxide (CO_2) method (Ariana et al. 2002). In this method, bees infested with *Varroa* were placed in a box with a screen bottom and put in a Rubbermaid (3.5 L TakeAlongs™, Rubbermaid, Mississauga, ON, Canada) container. The container was then agitated at 400 rpm for 10 min on a Labline® (Fisher, Ottawa, ON, Canada) orbital shaker table while being exposed to CO_2 (5 L/min) (for more details see

Currie and Tahmasbi 2008). Mites falling onto the bottom of the container were collected with the tip of a soft paint brush, placed in Petri dishes lined with a moist paper towel. The mites were then introduced into the small hives by a fine-tipped paint brush through a wire mesh screen (8 squares per inch) that covered the top of the hive and prevented bees from flying out. The mites were placed directly on the bees.

Assessment of mite and bee mortality

To assess bee population size in colonies at the end of the experiment, colonies were scored visually from both top and bottom and the numbers of frame seams completely covered with bees was counted (each frame seam = approximately 2430 worker bees) (Burgett and Burikam 1985; Underwood and Currie 2005). To monitor mite and bee mortality, a piece of white poster board (19 × 61 cm) completely covered with wax paper was placed on the bottom board of each hive so *Varroa* mites and worker bees that fell from the bee cluster could be collected on daily basis. Each hive had a completely open bottom entrance (19 × 2.5 cm). Additional dead bees and mites were collected outside of hive entrance in a dead bee trap (consisting of an Aluminum, three-sided tray, 29 × 19 × 8 cm). The mean abundance of *Varroa* mites [arithmetic mean of the number of *Varroa* mites per bee (Bush et al. 1997; Rozsa et al. 2000)] in each hive was estimated on the final sample date by collecting a sample of adult bees (200–300 worker bees) and using an alcohol wash technique to remove the mites from the bees

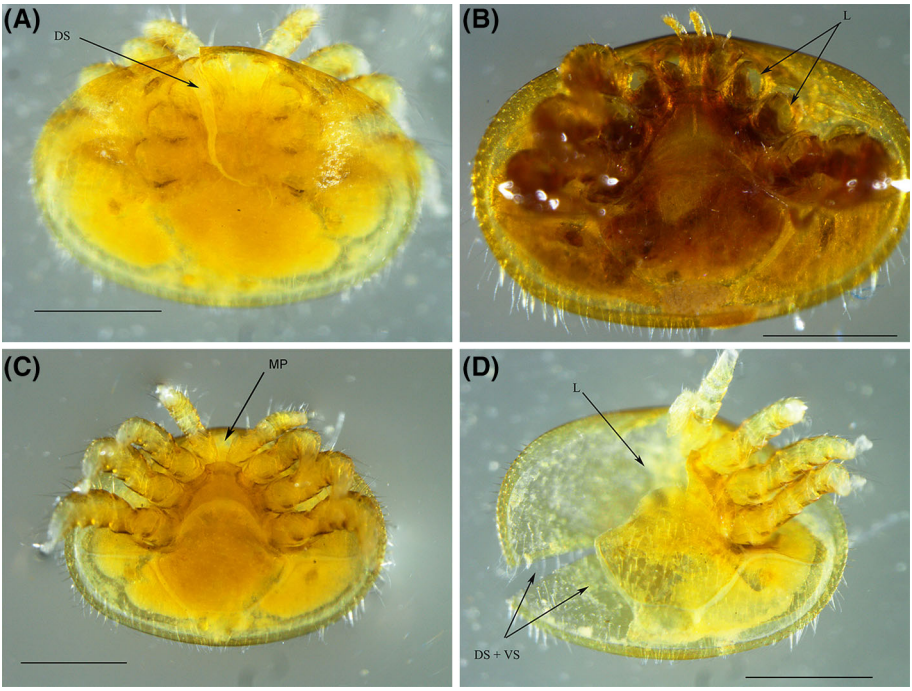

Fig. 1 Injuries in adult *Varroa destructor* collected from the *bottom* of hives. Mites that fell from the bee cluster showed signs of damage to **A** dorsal shield (DS), **B** legs (L), **C** mouth parts (MP), and **D** complex damage to legs, dorsal and ventral (VS) shields. The *scale bars* represent 0.5 mm

(for more details see Gatien and Currie 2003). Mites were removed from dead bees according to Gatien and Currie (2003) by agitating bees in 70 % ethanol for 10 min using a Labline® orbital shaker (Fisher) rotating at 200 rpm. After each sample was shaken, the basket with bees was removed and *Varroa* mites in the alcohol and on the dead bees were counted. In order to confirm that equal numbers of mites were present in each treatment group at the beginning of the experiment, we calculated the initial mean abundance. This was done by adding all mites that dropped during the experiment to those remaining on live bees at the end of the experiment (as measured by multiplying the number of remaining bees by the mean abundance as determined by alcohol wash). Initial mean abundance was determined by dividing the total number of mites by the total number of bees present in the colony on day zero. The rates of daily worker bee and *Varroa* mite mortality were calculated using the following equation (Martin 1998):

$$\text{Daily mortality rate} = 1 - [(1 - a/100)\text{^}1/b]$$

where a denotes percentage of bees or mites lost and b represents length (day) of each sampling period. The "dead mite" values include the number of mites that fell from the bee cluster onto the bottom board, mites found on dead bees on the bottom board and mites found in the dead bee trap. The "dead bee" values consist of the number of bees that fell onto bottom board and into the deasd bee trap.

Quantification of mite injury

The *Varroa* mites removed from bottom boards and dead bee traps of each hive were classified as to the types of injury found on the idiosoma (dorsal shield), ventral shield, mouthparts, legs or a complex of these parts (Fig. 1). Normal "dimples" that can occur on mites were not categorized as damage (Davis 2009). The proportion of injured mites was assessed by dividing the total number of injured mites by the total number of *Varroa* mites that dropped from the bee cluster onto the bottom board of each hive.

Statistical analysis

The daily mortality rates of worker bees and *Varroa* mites and the proportion of injured mites were analyzed by a repeated measures analysis of variance using a compound symmetry covariance structure with stocks of bees and queen pheromone status as main effects and day as repeated measure (PROC MIXED, SAS Institute 2011). Categorical comparisons among different injury categories in different stocks and treatments were analyzed by the maximum likelihood method (PROC CATMOD, SAS). Proportions were arcsine transformed prior to analyses (Snedecor and Cochran 1980). All data are presented as untransformed means. Where significant interactions were observed, they were partitioned using the SLICE option in LSMEANS statement and differences among treatment means were compared using Tukey's test (PDiff, PROC MIXED, SAS).

Results

Mite mortality

Daily mite mortality rate was higher in the selected stock than the unselected stock ($F = 4.54$; $df = 1, 18$; $P = 0.049$) (Fig. 2), but there was no interaction between source of bees and queen pheromone treatment ($F = 0.37$; $df = 2, 18$; $P = 0.69$) and the three way interaction between source of bees*queen pheromone*time was not significant ($F = 1.18$; $df = 8, 72$; $P = 0.32$). For daily mite mortality rate there was a significant queen pheromone treatment*time interaction ($F = 2.27$; $df = 8, 72$; $P = 0.01$). Colonies with QMP or caged, mated queens had similar mite mortality rates but both were higher than queenless colonies (Fig. 3). LSMEANS slices of the pheromone treatment*time interaction by day showed that significant differences between treatments occurred only on the first day of experiment. Slices by treatment showed mite mortality rate was stable in queenless colonies but fluctuated over time for both caged, mated queen and QMP treatments (Fig. 3) (Table 1).

Bee mortality

The overall daily rate of bee mortality did not differ between different pheromone source treatments (mated queen: $\bar{x} = 0.0041 \pm 0.0014$, QMP: $\bar{x} = 0.0032 \pm 0.0014$ and queenless: $\bar{x} = 0.0038 \pm 0.0014$) ($F = 0.07$; $df = 2, 18$; $P = 0.93$) or with source of bees (selected bees: $\bar{x} = 0.0053 \pm 0.0012$; unselected bees: $\bar{x} = 0.0021 \pm 0.0012$) ($F = 4.12$; $df = 1, 18$; $P = 0.06$). However, the source of bees*time interaction was significant ($F = 2.98$; $df = 4, 72$; $P = 0.03$). Bee mortality in selected bees was higher than in unselected bees only during the first 2 days of the experiment (Fig. 4). The interactions between queen pheromone treatment*source of bees ($F = 0.61$; $df = 2, 18$; $P = 0.55$), queen pheromone treatment*time ($F = 0.26$; $df = 8, 72$; $P = 0.98$), and queen pheromone treatment*source of bees*time ($F = 0.74$; $df = 8, 72$; $P = 0.65$) were not significant.

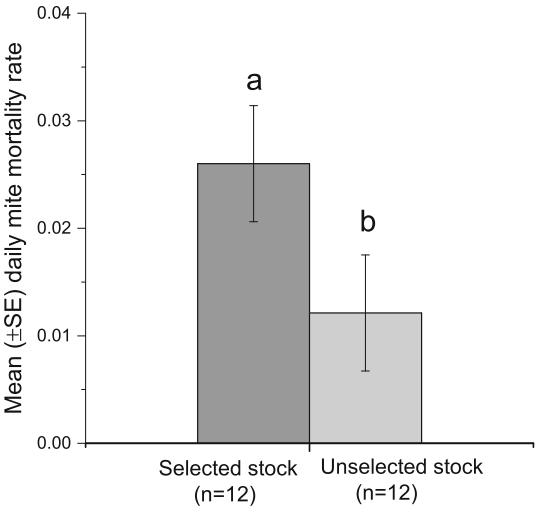

Fig. 2 Mean daily mortality rates of *Varroa* mites from clusters of selected and unselected honey bee stocks. Means capped with different letters are significantly different

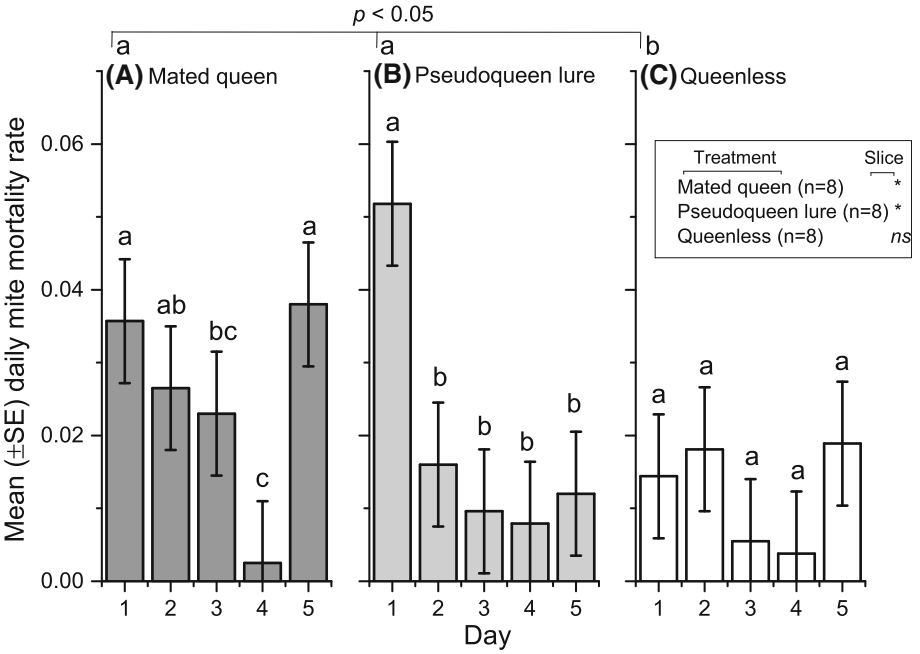

Fig. 3 Effects of queen pheromone treatments (**A** caged, mated queen, **B** pseudoqueen lure, **C** queenless) and day on mean daily mortality rate of *Varroa* mites. *Asterisk* (on legend) indicates a significant difference within queen treatments ($P < 0.05$, slice), *ns* represents a non-significant difference among periods within treatments (see text for results of LSMEANS slice option). $P < 0.05$ indicates significant difference between treatments within days. Means capped with the *same letter* among days within queen treatments and within days among queen treatments (*horizontal line*) are not significantly different

Table 1 Summary of LSMEANS slice option results for the significant treatment*time interaction by day for differences among queen pheromone treatments and by queen pheromone treatment for daily *Varroa* mite mortality rate for differences among days

	F	df	*P*
Slice by day			
1	3.64	2, 72	0.037
2	0.11	2, 72	0.90
3	0.07	2, 72	0.93
4	0.48	2, 72	0.62
5	2.59	2, 72	0.082
Slice by treatment			
Queenless	1.28	4, 72	0.29
Caged queen	8.09	4, 72	<0.0001
QMP	11.00	4, 72	<0.0001

Mite injury

Measurement of the proportion of damaged mites averaged over all treatment combinations showed a significant source of bees*time interaction ($F = 4.46$; $df = 4, 72$; $P = 0.003$). The proportion of injured mites was higher in unselected bees than in selected

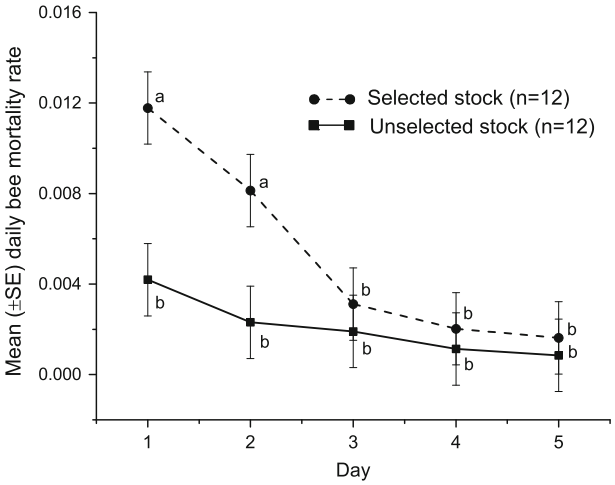

Fig. 4 Mean daily worker bee mortality in different stocks during experiment. Means with the *same letter* among stocks are not significantly different

bees on the first day of the experiment but the reverse trend was seen on day five (Fig. 5). The proportion of injured mites in different pheromone source treatments (mated queen: 0.20 ± 0.08, QMP: 0.25 ± 0.08 and queenless: 0.33 ± 0.08) was similar ($F = 0.42$; $df = 2, 18$; $P = 0.67$). The most frequent category of damage was injury to the idiosoma (dorsal shield) followed by damage to the legs, with damage to the mouthparts being rare. Complexes of either idiosoma, ventral shield and legs or idiosoma, ventral shield, legs and mouthparts occurred in 11 to 20 % of cases (other possible combinations showed no damage) (Table 2). The proportion of damaged mites in different injury categories was similar between different stocks ($\chi^2 = 1.64$; $df = 4$; $P = 0.80$), and among queen

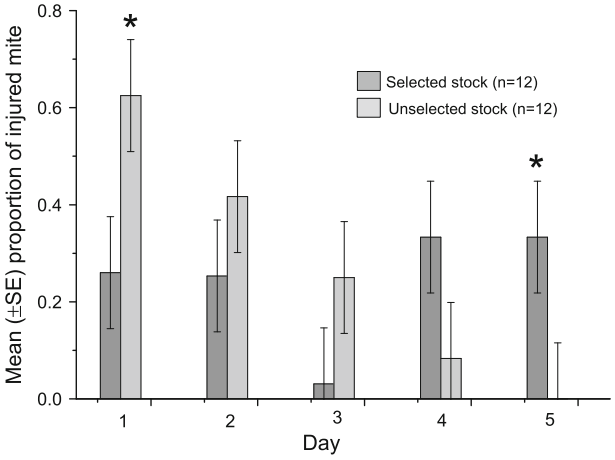

Fig. 5 Mean proportion of injured mites collected from *bottom* boards of selected and unselected stocks during simulated winter storage. *Asterisks* (on bars) indicate significant difference within stocks ($P < 0.05$, slice)

Table 2 Percentage of mites with various combinations of injuries

Stock	Injured mites % (*n*)					
	DS	DS + VS + L	DS + VS + MP + L	L	MP	Total
Selected	64 (18)	14 (4)	11 (3)	11 (3)	0 (0)	100 (28)
Unselected	32 (10)	16 (5)	20 (6)	29 (9)	3 (1)	100 (31)

Injuries were found on the dorsal shield (DS), ventral shield (VS), legs (L) and mouthparts (MP) or combination of the above (+) on *Varroa destructor* collected from bottom boards of selected and unselected stocks during simulated winter storage. (*n*) represents the number of mites examined in each category

pheromone treatments ($\chi^2 = 1.03$; df = 8; $P = 0.99$) and there was no significant source of bees*queen pheromone treatment interaction ($\chi^2 = 3.62$; df = 8; $P = 0.89$).

Discussion

In this study, the effects of queen pheromone status and honey bees with different grooming ability on mite mortality rates of *V. destructor* were assessed in honey bee colonies. As expected, colonies established from colonies selected for increased rates of grooming displayed higher mite mortality rates when compared to colonies established from unselected stock. The difference in mite mortality between stocks could be a result of higher grooming, higher loss of bees infested with mites or other factors that influence mite mortality. When worker bees were treated with different queen pheromone treatments under simulated winter conditions, queen pheromone addition (through the use of PQ lures or caged, mated queens) affected mite mortality rates relative to those in queenless colonies in two different ways. First, pheromone addition caused a short (1 day) transitory increase in mite mortality relative to queenless colonies. Second, both the queen pheromone and caged, mated queen treatments increased the daily variability in mite mortality rates over time relative to the queenless colonies where mortality rates were similar over time.

The effects of QMP on grooming against *Varroa* have not been studied. However, Naumann (1991) has shown bees initiate a range of grooming behaviors after coming in contact with queen pheromone. These same grooming behaviors may be related to the enhanced rates of *Varroa* removal from the colony that we observed when bees were exposed to the two queen pheromone treatments, but we were not able to observe bees directly in our experiment.

The short-duration effects of queen pheromone on mite mortality rates relative to queenless colonies may be related to changes in release rates from both PQ lures and caged, mated queens. PQ is a synthetic pheromone containing 10 queen equivalents per dispenser (Ledoux et al. 2001). One queen equivalent is the average amount of pheromone found in a pair of queen mandibular glands: 200 μg 9-keto-2-(E)-decenoic acid, 100 μg 9-hydroxy-2-(E)- decenoic acid [88 % R-(−) and 12 % S-(+)], 20 μg methyl p-hydroxybenzoateand 2 μg 4-hydroxy-3-methoxyphenylethanol (Melathopoulos et al. 1996). Mean daily release rates of pheromone average 0.3–1.4 queen equivalents of QMP over a five-day period, but the amount released decreases over the first 2 days (Gervan et al. 2005). The amount of QMP released from PQ was not measured in our study, but the diminished response after 1 day suggests a dose-related reduction may have been at least partly responsible for lower mite mortality rates found after day one. Similar effects may

have occurred with the caged, mated queens. Queens were caged in this experiment to prevent confounding effects that would result from brood production by the queen and to prevent queen movement so that it would make pheromone dispersion similar to the stationary lure. Worker bees directly or indirectly (through queen-to-bee and bee-to-bee contacts) receive and disperse pheromone from the queen. Thus, a queen "running free" through the colony is better able to disperse pheromone (Gervan et al. 2005; Naumann et al. 1991). Higher variability in mite mortality rates that we observed in both the queen and pheromone treatments relative to queenless colonies may have been related to uneven fluctuation dispersion of pheromone from the lure or caged, mated queens. However, we did not measure daily release rates or have a free-running queen treatment in our experiment so this could not be assessed. Varroa mites may also be more susceptible to being removed from their hosts during the first 2 days of introduction to a cage environment when it is possible that they are seeking suitable feeding sites and not as protected as when they finally position themselves under the sternites of their host.

Our experiment showed the synthetic QMP (in the form of the PQ Lure) had differential effects on mite mortality in colonies established from two different sources of bees, although further testing on colonies with a broad range of genetic diversity is required. If QMP influences grooming across a wide array of genetic sources of bees it may have a role as an alternative or a supportive tool to improve management of *Varroa* mites in commercial operations, but this needs testing in commercial operations on longer time scales.

Currie and Tahmasbi (2008) showed mite removal from caged bees is affected by interactions between environmental conditions and the genotype of bees. Under conditions that simulated winter temperatures of 5 °C, grooming was less effective and there appeared to be a transient cost associated with grooming at low temperatures (Currie and Tahmasbi 2008). In the larger colonies utilized in this study at low temperature (5 °C), we found greater mite removal in colonies established from the selected stock than in the colonies established from unselected stock. There was also a short transitory cost in terms of increased bee mortality rates in the selected stock when compared to the unselected stock in the first 2 days of the experiment. However, the overall bee mortality rates did not differ between these two treatments.

The increased rates of bee mortality in selected stock relative to unselected stock may have contributed to increased mite mortality if those bees that died were also mite infested. In the overwintering period, mites may die, be groomed from a host and drop from the winter cluster of bees, or leave the colony attached to a dead host (De Jong 1990; Fries et al. 1991). However, this latter occurrence was not likely to have been responsible for increased mite mortality in the queen pheromone treatments as queen treatment did not affect bee mortality rates. Longer term studies replicated on multiple source colonies are required to get a true measure of the potential costs of resistance.

The use of mite damaged as an indicator to select and to breed resistant stocks with enhanced grooming is still debatable (Boecking and Spivak 1999; Correa-Marques et al. 2000; Liebig 1997). In numerous studies, higher percentages of injured mites found on the bottom board of the hives are suggested as criterion for selection for grooming responses that provide resistance to *Varroa* (Arechavaleta-Velasco and Guzman-Novoa 2001; Correa-Marques et al. 2000; Moosbeckhofer 1992; Peng et al. 1987; Ruttner et al. 1984). Guzman-Novoa et al. (2012) showed significant correlations between the proportion of injured mites and mite removal rate at the colony level. They also found mite-resistant strains of bees show higher mite damage than unselected genotypes. Correa-Marques et al. (2002) suggest mite damage is not sufficient to explain resistance of Africanized bees (*Apis mellifera scutellata* Lepeletier) against *Varroa* mite. Our results were mixed as the

proportion of injured mites was greater in unselected colonies than in selected colonies early in the experiment but the reverse was true later in the experiment. From the results of their study and ours it appears that successful grooming can occur without visible damage to mites. Other mechanisms that increase mite fall from clusters without resulting in visible mite damage may be present. In our study the reason why the proportion of mites damaged fluctuated with time in selected colonies relative to unselected colonies could be related to temperature. Our study was conducted at 5 °C and mites that fell to the bottom board as a result of "grooming" or some other mechanisms would have difficulty relocating a host. The cold temperature would interfere with host seeking and questing behaviors by the mite and reduce the number of potential host bees on the bottom board (as they would remain in the winter cluster at this temperature). Thus, mites that are removed by bees in these conditions may be less likely to be damaged after removal from individual bees (when they are on comb) or removal from the colony (on the bottom board of the hive). Other studies that showed positive relationship between mite damage and resistance to mites were conducted at warmer temperatures where undamaged mites may have been more likely to relocate potential host bees and be removed multiple times or be damaged by bees after removed from the bee's body.

Correa-Marques et al. (2000) classified mite damage into six categories and show injured legs are more frequent than other types of damages. Phoretic *Varroa* mites use the front legs to attach to the body of host, and these legs are therefore most likely to be subjected to breakage during attempts to remove the mites by the worker bees (Zaitoun et al. 2001). Several studies confirmed that damage to legs is the most frequent category of injury [19.3 % (Boecking and Ritter 1993), 22.8 % (Zaitoun et al. 2001), 23 % (Bahreini 2001), 25 % (Rosenkranz et al. 1997), 46–47.4 % (Correa-Marques et al. 2002), 30–50 % (Ruttner and Hanel 1992) and 54–72 % (Correa-Marques et al. 2000)] and damage to legs, alone or in conjunction with other damage categories, in our study was also frequent (11–51 %). It has been suggested that injured mite legs are indicative of an active defense against *Varroa* (Ruttner and Hanel 1992) but our results do not agree as injury categories did not differ with stock. The type and degree of mite damage also did not vary with queen pheromone treatment. The most frequent category of "unique" injury occurred on the dorsal shield (47 %). In a study on Africanized and European honey bee colonies, approximately 16 % and 37–47 % of the mites had injured dorsal shields, respectively (Correa-Marques et al. 2000). In addition to worker–worker grooming, damage to the body of *Varroa* mites can also result from hygienic brood removal behaviors (usually distinguished from auto- and allo-grooming of adults) or other commensal animals or scavengers in the hive (Davis 2012; Guzman-Novoa and Page 1999; Harbo and Harris 1999; Rosenkranz et al. 1997). In our experiment, hives were broodless and maintained under controlled conditions, that excluded external sources of damage from scavengers. Therefore, damage that was observed would be due to grooming responses of bees during the process of removing mites or damaging them after they were removed. Although small numbers of damaged mites could have been removed by hygienic bees from empty combs we controlled for this by randomly assigning queen treatments to each colony within stocks.

In summary, this study showed the presence of queen pheromone or caged, mated queen caused transient increases in mite mortality relative to queenless colonies that lasted for about a day, and queen pheromone treatments increased the variability in mite mortality rates over a period of 5 days relative to queenless treatments. Our findings revealed that the colonies from selected stock removed more *Varroa* mites than the colonies from unselected stock under low temperature. Injury signs on the mites' bodies commonly associated

with grooming behavior were not reliably linked to grooming success as measured by mite mortality rates. Our results suggest assessment of mite damage may not be reliable as a selective criterion for breeding programs under low temperatures and that studies examining grooming behavior need to consider the effects of queen pheromones on their results. Resistance behaviors against mites increased mite mortality in overwintering hives, and this may be a useful tool against *Varroa* in some regions. Further studies are needed to define the range of environmental conditions where it might be useful.

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

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