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Evaluations of the Removal of *Varroa destructor* in Russian Honey Bee Colonies that Display Different Levels of *Varroa* Sensitive Hygienic Activities

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Abstract The removal of *Varroa destructor* was assessed in Russian honey bee (RHB) colonies with known levels of *Varroa* Sensitive Hygienic (VSH) and brood removal activities. The expression of grooming behaviour using individual bees was also measured using three groups of RHB displaying different VSH levels: low hygiene (RHB-LH, < 35% VSH), medium hygiene (RHB-MH, 35–70%) and high hygiene (RHB-HH, > 70%). Italian colonies (5.43–71.62% VSH) served as control. Our results demonstrated, for the first time, significant relationships between two hygienic responses (VSH activity measured as percent change in infestation and the actual brood removal of *Varroa*-infested donor comb) and two measurements of mite fall (trapped old mites/trapped mites or O/T and trapped young mites/trapped mites or Y/T). However, these relationships were only observed in RHB colonies. In addition, the RHB colonies that displayed the highest levels of hygiene (RHB-HH) also groomed longer in response to the presence of a *V. destructor* mite based on individual bee assays. The positive regressions between the two hygienic measurements and O/T and their negative regressions with Y/T suggest that the removal of infested brood prevented successful mite reproduction, ultimately suppressing *V. destructor* infestations in the RHB colonies. In addition, it is demonstrated that RHB resistance to *V. destructor* rests on both an increased hygienic response and the removal of phoretic

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mites, released by hygienic behaviour, through grooming. Both resistance traits are reflected in the O/T and Y/T ratios found in trapped mites from RHB colonies. None of the measurements involving mite injuries were associated with any measurements of hygiene and colony infestations.

Keywords Russian honey bees · *Varroa destructor* · hygienic behaviour · grooming · mite fall · resistance

Introduction

The mite *Varroa destructor* (Mesostigmata: Varroidae) has become the most important pest of *Apis mellifera* colonies since it first expanded its host range to include this species (Oldroyd 1999). In the early part of the last century, two mitochondrial haplotypes of *Varroa destructor,* shifted host from their native *Apis cerana* to the Western honey bee (Navajas et al. 2010). Grooming and hygienic removal of infested brood are two behaviours of honey bees that help them resist this parasite (Boecking and Spivak 1999). The two behaviours are important means of resistance for the mite's native host, *Apis cerana* (Peng et al. 1987a; Peng et al. 1987b) and are performed to varying degrees by different strains of *A. mellifera* (Bozic and Valentincic 1995; Aumeier et al. 2000; Correa-Marques et al. 2002; Mondragon et al. 2005; Stanimirovic et al. 2010; Balhareth et al. 2012). Together, the two behaviours target the mite during both stages of its life cycle; the phoretic stage when the mite is attached to adult bees and the reproductive stage which occurs within the capped brood cells.

Some honey bee populations resist *V. destructor* mite population growth. Colonies of *A. mellifera* in Sweden (Locke and Fries 2011), France (Buchler et al. 2010), and in forests of the north-eastern US (Seeley 2007) as well as Africanized bees in South America (Aumeier et al. 2000) are all capable of surviving without chemical treatment. Commercially, two stocks of honey bees have been developed that are capable of resisting the mite. These are Russian honey bees (RHB) from the Primorsky region where the host-shift first occurred (Rinderer et al. 2001; Harris and Rinderer 2004; Rinderer et al. 2013) and *Varroa* Sensitive Hygienic (VSH) bees developed at the USDA Honey Bee Breeding, Genetics and Physiology Laboratory in Baton Rouge, LA (Danka et al. 2012). RHB have been bred for low mite populations (Rinderer et al. 2010) while VSH bees were originally bred for the trait of suppression of mite reproduction (Harbo and Harris 1999b; Harbo and Harris 2005).

Natural selection for resistance to *V. destructor* should create a more sustainable host-parasite equilibrium compared with artificially selecting for single characters and forcing further mite adaptations (Le Conte et al. 2007). Honey bees have the highest recombination rate of any metazoan analysed to date (Hunt and Page 1995; Solignac et al. 2007) which should benefit both natural and artificial selection. Recently, Tsuruda et al. (2012) identified a quantitative trait locus (QTL) on chromosome 9 that appears to be related to the VSH trait. We confirmed that this QTL is associated with brood removal in RHB colonies (Kirrane et al. 2015). Arechavaleta-Velasco et al. (2012) identified a putative QTL on chromosome 5 referred to as groom-1 thought to be associated with grooming behaviour. Thus, both behaviours appear to have strong genetic components. A population of honey bees is more likely to be strongly resistant

if it expresses more than one behaviour that affects mite population growth (Rinderer et al. 2013). Africanized honey bees express both hygienic and grooming behaviours (Correa-Marques and De Jong 1998; Guerra Jr et al. 2000; Aumeier 2001; Guzman-Novoa et al. 2012). RHB have been shown to hygienically remove mite infested brood (de Guzman et al. 2016; Kirrane et al. 2015). It is also believed that these bees perform grooming (Rinderer et al. 2001). However, the measurement of grooming behaviour is controversial (Guzman-Novoa et al. 2012; Rinderer et al. 2013).

General hygiene is measured using either the freeze-killed (Spivak 1996) or pin-killed (Buchler et al. 2010) brood assays while VSH is assessed by measuring the change in infestation of a highly infested donor comb that has been exposed to test bees for one week (Danka et al. 2013). Grooming behaviour has, in different studies, been measured at both the colony (Bienefeld 1999) and the individual levels (Aumeier 2001). The proportion of injured mites trapped in the debris of colonies is the most widely used proxy measurement of grooming behaviour, despite speculation as to its accuracy (Bienefeld 1999; Rosenkranz et al. 1997; Invernizzi et al. 2016). Laboratory assays for grooming expose individual bees in Petri-dishes (Aumeier 2001; Guzman-Novoa et al. 2012), or clusters of bees in cages (Andino and Hunt 2011), to *V. destructor* mites. Both measurements are associated with a certain amount of error. Mite fall is affected by emerging brood (Rosenkranz et al. 1997) and injuries can be caused by other predators (Bienefeld 1999) while laboratory assays may fail to account for the impact of environmental variationon grooming behaviour (Currie and Tahmasbi 2008). However, recent studies have linked the results found in laboratory assays to both injured mites in traps and colony level infestation (Andino and Hunt 2011; Guzman-Novoa et al. 2012; Bahreini et al. 2015).

Rinderer et al. (2013) found that the ratio of older to total trapped mites (O/T) in the debris from RHB and Italian colonies had the strongest negative relationship with colony infestation. This measure is far less time-consuming than examining injuries to mites and can be carried out without the need for a microscope. RHB are known to have a higher relative proportion of phoretic to brood mites (Rinderer et al. 2001). Therefore, high proportions of older mites in traps could be related to high levels of grooming, as bees remove these adult phoretic mites from their bodies (Rinderer et al. 2013). High O/T could also result from reproducing adult mites being removed from brood cells by hygienic behaviour. VSH bees remove mites from cells at 3–5 days post-capping, before a full cohort of offspring have been laid (Harris 2007). In addition, removal due to hygienic behaviour leads to reduced reproduction in the next generation (Kirrane et al. 2011). Combined, this removal of mites and subsequent reduction in reproductive output, could leave a greater proportion of adult mites in the colony which could be reflected by a higher O/T proportion in fallen mites.

The objectives of this study were to determine whether RHB colonies perform both grooming and hygienic behaviours and whether there is a link between the two behaviours. As the measurement of mite fall is controversial in its accuracy for grooming behaviour, we also determined the influence of hygienic removal of cells on mite fall parameters. As O/T has been shown to be strongly related to colony infestation, we determined its association with both brood and phoretic infestation levels as well as with hygienic behaviour and grooming. This study forms part of a larger study aimed at understanding the behavioural resistance of RHB toward *Varroa* parasitism.

Materials and Methods

Experiments were carried out at the USDA-ARS, Honey Bee Breeding, Genetics and Physiology Laboratory in Baton Rouge, Louisiana from July to October of 2013.

Test Colonies and Evaluation of *Varroa* Infestations

Forty six colonies (Italian = 14, Russian = 32) were established in the Spring of 2013. Italian honey bee queens were purchased from a commercial queen producer in California while different RHB queens were obtained from members of the Russian Honeybee Breeders Association (RHBA), based in the USA. All experiments were carried out at least two months after the introduction of queens to ensure that all bees were from test queens. Colonies with supersedure queens were excluded from the experiments. In a companion paper, we described in detail the testing of RHB colonies for the expression of the VSH trait (Kirrane et al. 2015). VSH activity was measured according to the change in mite infestation level after a one week exposure method (Danka et al. 2013). In brief, a test frame of highly infested brood is inserted into a test colony for one week; the infestation of 200 brood cells (of the same cohort) is determined before and after exposure of the test frame (Kirrane et al. 2015). We also counted the actual removal of cells in a small section (~150 cells) of highly infested comb and found this to be significantly related to infestation and reproduction parameters in RHB (Kirrane et al. 2015). This measure is referred to as "brood removal".

Initial brood and adult bee infestations of each colony were determined by measuring the infestation of 200 brood cells (from two to three frames) and mite washes from 300 to 500 bees (Rinderer et al. 2001; de Guzman et al. 2007).

Colony Level Grooming (Mite Fall)

All 46 colonies were used to determine grooming at the colony level, which was evaluated by measuring daily mite drop over four weeks in September 2013. Cafeteria trays were covered with grease-proof paper coated with a 1:1 mixture of vegetable oil and petroleum jelly and placed between two bottom boards (wire screen on top of a solid board providing an entrance for bees and a rear facing access for the trap) of each hive (Rinderer et al. 2013). Papers were collected from trays and replaced with new ones every day, for the 28 day period. Immediately after collection, samples were placed individually in plastic bags and frozen for later analysis. On analysis, mites were collected from the papers using an insect brush and examined under a dissecting microscope at 40X magnification. The mites were categorized based on age, injuries and recency of death. The latter was determined by assessing how "fresh" the mite was on prodding the carapace with an insect pin and noting the presence of haemolymph or tissues exuding from the wound (Rinderer et al. 2013). The presence of the vegetable oil and petroleum jelly on the papers ensured that freshness was preserved, in spite of freezing the samples. Mites lighter than ochre brown were classified as "young" (Rinderer et al. 2013). Damaged or missing legs and damaged idiosoma and/or gnathosoma were considered injuries; dents in the dorsal shield were not (Davis 2009).

Rinderer et al. (2013) demonstrated several measurements of mite fall that related to a decrease in colony mite numbers (Table 1). Measurements that do not require an estimation of colony infestation are ideal criteria for selection.

Auto-Grooming

Individual grooming behaviour was measured in the laboratory by observing individual bees in Petri-dishes, modified from the methods of Guzman-Novoa et al. (2012) and Khongphinitbunjong et al. (2012). Twenty six colonies were selected based on their VSH measures (Kirrane et al. 2015), 20 RHB colonies displaying low (RHB-LH, < 35% change in infestation, $n = 6$ colonies), medium (RHB-MH, 35–70% change in infestation, n = 6 colonies) and high (RHB-HH, > 70% change in infestation, $n = 8$ colonies) levels of VSH and six Italian colonies (VSH activity = 5.43–71.62%), which were randomly selected from the existing 14 colonies. The Italian colonies served as control. Phoretic mites for inoculation were collected from heavily infested Italian colonies different from the ones being tested. To obtain inoculum mites, infested bees were allowed to emerge in an incubator overnight and then the mites were removed from the young bees with an insect brush. Only adult mother (darker than ochre brown) mites were chosen. Mites were collected in this manner, because the powdered sugar method of obtaining mites from adult bees could result in damage to the mites. The mites were maintained on moist filter paper in the lab. Bees from test colonies were scooped from frames of open brood within the brood-nest. This ensured that the bees were of uniform age, and reduced the risk that any bees collected might have drifted from a nearby colony. In the laboratory, the bees were placed individually into Petri-dishes (diameter = 60 mm) and allowed to acclimatize to the conditions for about 3 min before being tested. Bees were visually inspected for the presence of mites and those that already bore a mite were not used. In addition, any bees that defecated while in the Petri-dish were excluded, as this might affect their propensity to groom. Thirty bees were subjected to the treatment of having a mite inoculated onto their thorax with an insect brush, while 30 bees touched with insect brush only (no mite inoculation) served as control.

The behaviours of the individual bees were recorded for three min using a video recorder (Panasonic HDC-HS250). The test and control bees were placed in separate Petri-dishes, both of which were within the same field of view, and recorded simultaneously. A bee was said to groom if it used sweeping motions of its legs over the head, thorax and abdomen

Table 1 Mitefall measurements estimated in study

Criteria	Name
Injured mites/total trapped mites	I/T
Injured mites/total fresh mites	IF/F
Old mites/total trapped mites	O/T
Young mites/total trapped mites	Y/T
Fresh mites/total trapped mites	F/T
Dry mites/total trapped mites	D/T
Injured dry mites/total dry mites	ID/D

(Peng et al. 1987a). In the event that the mite was successfully removed from the bee, recording stopped. On analysis, total time spent grooming (duration) and latency to onset of first grooming event were recorded. After the test, mites were removed from the test bee with an insect brush and examined for injuries under a dissecting microscope.

Statistical Analysis

Variables were tested for normality and percentage data were subjected to arcsin square root transformation to approximate normality. Untransformed means are presented. Individual grooming behaviour was analysed using a two factorial Analysis of Variance (ANOVA) with bee group (RHB-LH, RHB-MH, RHB-HH and Italian colonies) and mite treatment (mite or no mite inoculation) as independent variables. No interaction was determined thus no post hoc test was required. Repeated measures ANOVA was carried out on mite fall measurements with stock and week and fixed effects and colony as the repeated subject. No significant effect of time was detected. Thus, mites collected daily from the *Varroa* traps for 28 days were added and then subjected to one-way ANOVA. Simple linear regression was used to determine the effect of hygiene (VSH level and brood removal) on mite fall measurements and on the proportion of phoretic and brood mites in the colonies. Regressions were carried out on the entire data set as well as for both stocks individually. All statistics were carried out using the software package R (R Core Team 2013) except for the repeated measures ANOVA which was carried out using SAS (SAS Institute 2008).

Results

In July 2013, when test bees populated all colonies, brood infestations were 2.1 ± 0.6% and 3.2 ± 0.9% for RHB and Italian colonies, respectively. The adult infestations were also low: RHB = 2.7 ± 1.1%; Italians 2.2 ± 0.4%. Because of the introduction of an infested donor comb to each of the test colonies to determine VSH activity, infestations increased at the end of the experiment (October) for both honey bee stocks. However, the average brood infestation of RHB colonies was 7.24 ± 0.9% while the Italian colonies supported a higher average rate of 16.41 ± 1.9% in late fall. A similar trend was observed for the adult bee infestations: RHB = 5.68 ± 1.1%, Italians = 14.4 ± 3.4%. The average infestation rates of the colonies used in the individual grooming assays were: Italian = 14.31 ± 1.8% (brood) and 16.06 ± 2.5 (adult bees); RHB-LH = 5.91 ± 4.0% (brood) and 7.72 ± 1.8 (adult bees); RHB-MH = 5.74 ± 4.0% (brood) and 5.6 ± 1.6 (adult bees) and RHB-HH = 4.51 ± 1.7% (brood) and 4.75 ± 0.8 (adult bees).

Colony Level Grooming (Mite Fall)

From the 46 test colonies, a total of 22,828 trapped *V. destructor* mites were analysed for the presence of injuries, age, and recency of death. Significantly greater O/T and F/T were recorded in RHB compared with the Italian honey bee colonies (Table 2). Conversely, the Italian honey bee colonies had higher Y/T and D/T than the RHB colonies. I/T was higher in the Italian than in the RHB colonies. IF/F and ID/D did not differ between honey bee stocks. A similar trend was observed when the 26 colonies

Table 2 Measurements of mite fall (Means ± SE) and the results of the analyses of variance (ANOVA) for the Russian and Italian honey bee colonies

Parameters	Honey bee stock		*P*-value
	Italian	Russian	
Old/trapped mites (O/T)	55.62 ± 4.46^{b}	66.54 ± 2.46^{a}	0.0054
Young/trapped mites (Y/T)	42.15 ± 4.1^{a}	32.67 ± 2.41^{b}	0.0112
Injured/trapped mites (I/T)	24.32 ± 2.54^{a}	18.87 ± 1.47^{b}	0.0152
Fresh/trapped mites (F/T)	54.58 ± 3.25^{b}	65.39 ± 1.54^{a}	0.0004
Dry/trapped mites (D/T)	43.18 ± 3.07^{a}	33.83 ± 1.92^{b}	0.0013
Injured fresh mites/fresh mites (IF/F)	3.06 ± 0.47	3.14 ± 0.52	0.661^{ns}
Injured dry mites/dry mites (ID/D)	51.69 ± 2.74	48.35 ± 2.79	0.368^{ns}

Different letters denote significance, *ns* indicates non-significant. Degree of significance presented in *P*-value column

n = 46 colonies

used for the individual grooming assay were considered. We found that the RHB-HH and RHB-MH colonies had more old (O/T) than young mites (Y/T) on their traps compared to the RHB-LH and Italian honey bee colonies (Table 3). In addition, higher proportions of fresh (F/T) compared with dry mites (D/T) were recorded from these two Russian honey bee groups. In contrast, traps from the Italian colonies had the lowest F/T and thus, the highest D/T. The RHB-LH colonies were intermediate in both of these variables. The proportions of injured mites (I/T), injured fresh mites to fresh mites (IF/F) and injured dry mites to dry mites (ID/D) did not vary among the honey bee groups.

Relationship between Mite Fall and Hygienic Behaviour

O/T was positively correlated with both VSH activity and brood removal measurements in the RHB colonies only (Table 4). Both VSH activity and brood removal also led to lower Y/T particularly in RHB colonies. Overall, O/T showed a significant negative correlation with brood infestation but did not show this correlation when stocks were analysed separately. Adult bee infestation was also negatively correlated with O/T but more pronounced in the Italian than RHB colonies. VSH activity and brood removal were negatively correlated with Y/T, but this was only observed in the RHB colonies. A positive correlation between Y/T and brood infestation was observed in the RHB colonies, while a positive correlation with adult bee infestation was only observed in the Italian colonies. Adult bee infestations were negatively correlated with F/T but positively correlated with D/T. No mite injury measurements (I/T, IF/F and ID/D) correlated with any measurements of hygienic behaviour or *V. destructor* infestation.

Auto-Grooming

Analysis of the duration of time spent grooming by individual bees during the 3-min observation showed no significant interaction between mite treatment and bee group

Table 3 Measurements (Means ± SE) of behavioural responses, mite fall and the results of the analyses of variance (ANOVA) for the Russian honey bees displaying different VSH levels as compared to the Italian honey bees

Measurements	Bee group				
	Italian (5.43–71.62% VSH level)	Russian (Low, >35% VSH level)	Russian (Medium, 35–70% VSH level)	Russian (High, >70% VSH level)	*P*-value
A. Behavioral					
Latency (s)	21.84 ± 1.43	18.89 ± 1.99	21.17 ± 3.12	19.17 ± 2.19	0.976^{ns}
Duration (% total time)	7.74 ± 0.84^{a}	9.4 ± 1.23^{ab}	8.28 ± 1.49^{a}	10.46 ± 0.8^{b}	0.0025
B. Mite fall					
Old/trapped mites (O/T)	60.29 ± 6.58^{b}	58.11 ± 3.45^{b}	74.14 ± 6.12^{a}	73.96 ± 5.28^{a}	< 0.0001
Young/trapped mites (Y/T)	37.26 ± 5.85^{a}	41.52 ± 3.62^{a}	25.02 ± 5.76^{b}	25.10 ± 5.32^{b}	< 0.0001
Injured/trapped mites (I/T)	24.29 ± 3.48	17.88 ± 3.32	16.08 ± 4.27	19.81 ± 4.13	0.0582^{ns}
Fresh/trapped mites (F/T)	55.66 ± 4.62^{b}	60.91 ± 4.85^{ab}	70.34 ± 4.93^{a}	66.16 ± 4.07^{a}	0.0009
Dry/trapped mites (D/T)	41.88 ± 4.15^{a}	34.69 ± 4.97^{ab}	28.81 ± 4.85^{b}	32.91 ± 4.23^{b}	0.0047
Injured fresh mites/fresh mites (IF/F)	2.37 ± 1.09	2.96 ± 1.18	3.63 ± 2.75	3.76 ± 2.15	0.936^{ns}
Injured dry mites/dry mites (ID/D)	54.09 ± 4.41	45.41 ± 4.99	45.99 ± 9.29	50.19 ± 9.4	0.538^{ns}

VSH was calculated as the change in infestation of *Varroa*-infested donor combs

Different letters denote significance, *ns* indicates non-significant. Degree of significance presented in *P*-value column

$n = 46$ colonies

(F = 0.689, d.f. 1=, $P = 0.559$). However, significant effects of mite treatment (F = 54.779, d.f. = 1, $P < 0.0001$) and bee group (F = 4.803, d.f. = 3, $P < 0.05$) were detected (Table 3). Overall, test bees groomed significantly longer when challenged with *Varroa* mites (11.15 ± 0.74% total time spent grooming) than those bees touched by an insect brush only (= no mite inoculation) (7.02 ± 0.52% total time). RHB-HH displayed the longest grooming duration as compared to RHB-MH and Italian honey bees. RHB-LH did not differ from RHB-HH, RHB-MH or Italian. For the latency (time from mite introduction to the onset of first grooming activity), no two-way interactions and no bee group (F = 0.084, d.f = 3, $P = 0.976$) effect were observed. However, a significant effect of mite treatment was identified (F = 14.867, d.f. = 1, $P < 0.001$). Mite-inoculated bees (18.24 ± 1.3 s) responded to the presence of *Varroa* mites more quickly than those bees touched by an insect brush only (no mite inoculation) (22.19 ± 1.5 s) (Table 3). All bee groups responded similarly to the presence of mites (Table 3).

Only six out of the 600 mites inoculated onto RHB bees were successfully removed by grooming. These were evenly distributed across groups with two mite-removals

Table 4 Associations among mite fall parameters, measurements of hygiene (VSH activity and brood removal), and *Varroa* infestations in Russian and Italian honey bee colonies ($n = 45$ colonies)

	Overall			Russian			Italian		
	r	r^2	*P*-value	r	r^2	*P*-value	r	r^2	*P*-value
Old mites (O/T)									
VSH activity	0.404	0.163	0.004	0.526	0.277	0.001	0.126	0.016	0.297
Brood removal	0.561	0.314	< 0.0001	0.454	0.206	0.008	0.484	0.234	0.054
Brood infestation	−0.319	−0.102	0.032	−0.315	0.156	0.079	−0.177	−0.031	0.563
Adult bee infestation	−0.629	−0.396	< 0.0001	−0.454	−0.206	0.009	−0.726	−0.527	0.008
Young mites (Y/T)									
VSH activity	−0.425	−0.181	0.002	−0.555	−0.309	0.001	−0.044	−0.002	0.344
Brood removal	−0.549	−0.301	< 0.0001	−0.466	−0.217	0.006	−0.411	−0.169	0.090
Brood infestation	0.300	0.090	0.026	0.310	0.096	0.047	−0.024	−0.057	0.563
Adult bee infestation	0.594	0.353	< 0.0001	0.104	0.011	0.171	0.687	0.472	0.008
Injured mites in trap (I/T)									
VSH activity	−0.095	−0.009	0.236	−0.114	−0.013	0.443	−0.167	−0.028	0.431
Brood removal	−0.104	−0.011	0.457	−0.138	−0.019	0.497	−0.307	−0.094	0.162
Brood infestation	0.120	0.014	0.432	−0.038	0.001	0.835	−0.118	0.014	0.702
Adult bee infestation	0.259	0.067	0.088	0.024	0.000	0.897	0.086	0.007	0.789
Fresh mites (F/T)									
VSH activity	0.138	0.019	0.178	−0.507	−0.258	0.643	−0.063	−0.004	0.350
Brood removal	0.207	0.043	0.101	0.044	0.002	0.342	−0.232	−0.054	0.547
Brood infestation	−0.228	−0.052	0.132	0.016	0.000	0.933	0.033	0.001	0.916
Adult bee infestation	−0.398	−0.158	.007	−0.155	−0.024	0.396	−0.086	−0.007	0.790
Dry mites (D/T)									
VSH activity	−0.151	−0.023	0.161	−0.144	−0.021	0.554	−0.187	−0.035	0.458
Brood removal	−0.151	−0.023	0.170	−0.044	−0.002	0.336	−0.179	−0.032	0.263
Brood infestation	0.236	0.056	0.119	0.029	0.000	0.871	−0.005	0.000	0.985
Adult bee infestation	0.346	0.119	0.022	0.128	0.016	0.484	−0.006	0.000	0.984
Injured fresh (IF/F)									
VSH activity	−0.158	−0.025	0.772	−0.164	−0.027	0.634	0.173	0.030	0.301
Brood removal	−0.055	−0.003	0.353	0.182	0.033	0.174	0.401	0.161	0.097
Brood infestation	−0.100	−0.010	0.457	−0.077	−0.006	0.373	−0.279	−0.078	0.722
Adult bee infestation	−0.155	−0.024	0.997	−0.179	−0.032	0.819	−0.263	−0.069	0.600
Injured dry (ID/D)									
VSH activity	−0.158	−0.025	0.795	−0.187	−0.035	0.930	−0.148	−0.022	0.394
Brood removal	−0.118	−0.014	0.506	−0.158	−0.025	0.583	0.226	0.051	0.226
Brood infestation	−0.138	−0.019	0.686	−0.114	−0.013	0.446	−0.167	−0.028	0.430
Adult bee infestation	−0.126	−0.016	0.566	−0.167	−0.028	0.702	−0.100	−0.010	0.368

occurring in each of the RHB-HH, RHB-MH, RHB-LH groups. Three out of the 180 mites inoculated onto Italian bees were removed. However, none of the mites that had been successfully removed by a test bee were damaged.

Discussion

Our earlier experiment showed a positive correlation between the removal of brood infested with paint-marked *V. destructor* and the number of mites that were trapped, in both RHB and Italian colonies (de Guzman et al. 2016). This study confirmed these earlier findings. Our results demonstrated, for the first time, significant relationships between two measurments of hygienic responses (VSH activity measured as percent change in infestation, and actual brood removal of *Varroa*-infested donor comb) and two measurements of mite fall (O/T and Y/T) in RHB colonies only. While O/T was found to be positively correlated with VSH activity and brood removal, Y/T was negatively associated with these two parameters. In addition, the RHB-HH colonies, that displayed the highest levels of hygiene, also groomed longer in response to the presence of a mite in our individual assays. The RHB colonies that displayed the highest levels of hygienic behaviour also had high O/T and low Y/T in traps. Although Y/T showed a positive association with brood mite infestation in RHB colonies, it was weak. Daughter (young) mites are known to be more prone to grooming compared with older mites (Kirrane et al. 2012) though in the case of RHB colonies, bees may be aggressive to both young and old mites. Combined, these observations suggest that RHB removed young infested brood, a trend also observed in VSH bees (Harris 2007). This behaviour also allowed the removal of old mites before they have a chance to reproduce. Consequently, RHB colonies had significantly lower mite infestations compared with the Italian controls within the cells. These findings are indicative of a persistent response to *V. destructor* mites released by hygienic brood removal in RHB colonies.

Our finding that O/T is associated with hygienic removal of infested brood, though new, is not surprising. Rosenkranz et al. (1997) found increased numbers of mites falling onto traps in colonies with hatching brood, compared to those without hatching brood. These authors suggested that foundress mites having just reproduced are in a "stressed" condition which causes them to fall to the bottom of the hive on host emergence (Rosenkranz et al. 1997). We now show that hygienic removal of infested brood by adult bees also influences mite fall measurements. While mites removed by hygienic behaviour appear to be more susceptible to grooming, the higher O/T in RHB could also reflect a higher proportion of mites on adult bees in these colonies than in the brood as shown by Rinderer et al. (2001). VSH activity suppresses mite reproduction (Ibrahim and Spivak 2006; Harbo and Harris 2005) and this has been shown to occur in RHB (Kirrane et al. 2015). High proportions of non-reproductive mites have been reported in RHB colonies (de Guzman et al. 2007; 2008). This lower reproductive success of mites in RHB colonies may lead to an overall greater proportion of older mites in the hive, reflected in mite fall. O/T may therefore be a good measurement of the ability of a colony to suppress mite reproduction and could be used as a selection tool for *V. destructor* resistance.

Overall, O/T showed a negative relationship with brood infestation regardless of stock. RHB colonies supported the highest O/T and also maintained lower brood infestations as compared to Italian colonies (7.24 vs. 16.41%). Further, a negative correlation between O/T and adult bee infestation was detected, but this was larger in

Italian compared with RHB colonies. Yet, the adult infestation of Italian (14.4%) colonies was over double that of RHB colonies (5.68%). That O/T had negative associations with both brood and phoretic mite infestations is in accordance with the results of Rinderer et al. (2013).

The large negative regression between Y/T and hygienic measurements suggests that the removal of infested brood prevented successful mite reproduction, suppressing *Varroa* infestation in the RHB colonies. Since hygienic measurements did not influence Y/T in the Italian colonies, the large positive regression between Y/T and adult infestation may suggest that young mites emerging with the bees were removed. Nonetheless, this removal of young phoretic mites did not significantly suppress varroa infestations in the Italian colonies. Indeed since these young mites are more susceptible to grooming behaviour (Kirrane et al. 2012), their removal is probably not indicative of a strong defensive response. In fact, the Italian colony that displayed the highes level of hygienic behaviour, in this study, supported a relatively low brood infestation but a high proportion of phoretic mites. This indicates that although this colony was removing infested brood, they were not successful at removig significant amounts of phoretic mites from the hive. Indeed the grooming duration of this colony in the indivisual assays was below average for that group.

Injuries to mites may be more common in colonies with higher rates of infestation (Rinderer et al. 2013). In this study, higher I/T was observed in the Italian colonies, which supported higher rates of infestations and D/T. However, I/T was not associated with any of the hygiene or infestation measurements. These observations may suggest that most mite injuries in the Italian colonies may be associated with the removal of dead and dried mites during house cleaning and not from the hygienic removal of infested brood. Indeed, Italian colonies supported a higher proportion of injured dry mites than RHB colonies. This observation supports the conclusions of Harbo and Harris (1999a), Lodesani et al. (2002), Correa-Marques et al. (2002), and Rinderer et al. (2013) that the percentage of damaged mites is not a good measure of resistance. Guzman-Novoa et al. (2012) linked the proportion of injured mites on the hive floor to colony level infestation in a number of susceptible and resistant stocks in Canada. However, when looking at individual stocks, they did not find an association between these measurements in their RHB colonies. They suggested that this lack of correlation may be a result of low sample size, having only tested five colonies of RHB. The results of the present study, as well as those of Rinderer et al. (2013) do not support the use of injured mites as a measure of grooming behaviour, or resistance, in either RHB or Italian colonies.

Despite the call for greater standardization and accuracy in measurements of grooming, many recent studies continue to use varying methods for collecting mite fall. Guzman-Novoa et al. (2012) collected mites every three days and stored them in 96% ethanol. Andino and Hunt (2011) collected mites from full-sized colonies after 72 h and froze them. Locke and Fries (2011) placed traps in the colonies for seven days. The latter also included dimples in the dorsal shield as damage, despite the fact that these are known to be related to ontogenetic effects (Davis 2009). We carried out our mite fall measurements in the same location and using the same methods of Rinderer et al. (2013). Both of these studies failed to find significant relationships between injured mites in the traps and infestation levels. Both did however find a relationship between O/T and infestation parameters. This supports the use of O/T as an estimate of

resistance, at least in RHB colonies. The fact that it is strongly associated with VSH and brood removal indicates that it would also be a useful measure in other honey bee stocks that display that trait.

Removal of a mite from the bee's body was rare in our laboratory assay. While a number of bees in the individual grooming assay responded to the presence of a mite, others were noted to behave as normal without responding in any way. A similar phenomenon was noted by Ruttner and Hanel (1992). There is considerable variation in the degree to which a mite will move when on the bee's body in laboratory assays (Guzman-Novoa et al. 2012; Arechavaleta-Velasco et al. 2012). Unfortunately, this cannot be controlled. During the course of these assays, some mites were observed to move straight to the abdominal sternites, and remain there for the duration of the test period while others consistently moved around the bee's body. It has been suggested, therefore, that an assay that measures a bee's response to abiotic stimuli might provide a more reliable measure (Guzman-Novoa et al. 2012; Arechavaleta-Velasco et al. 2012). We measured the response of bees to both a mite and an abiotic stimulus (touching with a brush). Despite a considerable degree of variation, bees groomed for significantly longer when challenged with a mite than touched with a brush only and the trend across the four honey bee groups was similar.

We selected test bees from within the brood-nest of the hive in order to ensure that they were of a similar age and from the test colony. It is possible that bees perform grooming behaviour at a certain age (Kolmes 1989). By sampling open brood we ensured the collection of nurse bees, for which mites show a preference (Boot et al. 1994). If auto-grooming is an important component of RHB resistance these bees would be expected to express this behaviour. There is also a genetic component to task specialization in bees (Frumhoff and Baker 1988) which could be missed when sampling a small number of bees over a short time period. Determining the age at which bees groom should be a priority for future research in order to ensure greater standardization in laboratory assays.

It appears therefore that a population of bees that has been selectively bred for low infestations, or those that have been subjected to natural selection, can develop more than one mechanism of resistance. Geographical location (Guzman-Novoa et al. 2012) and environment (Currie and Tahmasbi 2008) could dictate the costs and benefits of different resistance traits (Vandame et al. 2002). Conditions that allow for a longer brood rearing season, for example, could select for increased hygienic behaviour at the cost of brood removal. Lower humidity conditions on the other hand have been shown to improve grooming success in the first two days (Currie and Tahmasbi 2008). This hypothesis would further support the breeding of bees for low mite populations as opposed to a single particular resistance trait (Le Conte et al. 2007). RHBs are known to have higher proportions of mites on adult bees than in brood cells (Rinderer et al. 2001). Nevertheless, they display removal behaviour toward infested brood as well as phoretic mites, and have been shown to be resistant to *V. destructor* across a range of geographical locations (Rinderer et al. 2000; Guzman-Novoa et al. 2012).

We have shown that RHB colonies that display high levels of VSH spend a considerably greater amount of time grooming in response to mite pressure, in comparison to control colonies. These colonies also had higher O/T and F/T, indicative of high removal of infested brood. Indeed, O/T was positively correlated with VSH activity and brood removal while Y/T was negatively correlated in RHB colonies only,

all of which are indicative of increased hygienic behaviour toward *Varroa*-infested brood. Thus, it appears that while VSH and brood removal are responsible for a significant amount of RHB resistance, removal of phoretic mites or mites "released by hygiene" by adult bees, also plays a role. The Minnesota hygienic bees, which are bred for high removal of freeze-killed brood (FKB) are only moderately resistant to *V. destructor* (Danka et al. 2012; Spivak and Reuter 2001). Thus, our results may indicate that highly resistant stocks of *A. mellifera* resist *V. destructor* through a combination of traits. Investigating the expression of both behaviours across a larger number of colonies could provide more concrete evidence of this observation.

In conclusion, the increased hygienic response of RHB to brood infested with *V. destructor* as well as removal of phoretic mites released by hygiene are probably major contributors in the ability of these bees to resist mite parasitism. Further, the impact of hygienic removal of *Varroa*- infested brood on mite fall in colonies displaying this behaviour needs to be taken into account when using mite fall as a tool for measuring grooming behaviour. This might explain why Harbo and Harris (1999a) reported low heritability for grooming behaviour, measured as percentage of damaged mites, in their colonies that displayed high levels of hygienic behaviour. Nevertheless, a good understanding of a colony's resistance mechanisms can be gained from examining the appropriate mite fall parameters.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflicts of interest.

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