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ORIGINAL RESEARCH ARTICLE

Brood removal influences fall of *Varroa destructor* in honey bee colonies

Lilia I. de Guzman[a]*, Thomas E. Rinderer[a], Amanda M. Frake[a] and Maria J. Kirrane[b]

[a]*Genetics and Physiology Laboratory, USDA-ARS, Honey Bee Breeding, Baton Rouge, LA, USA;* [b]*Environmental Research Institute (ERI), University College Cork, Cork, Ireland*

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The hygienic removal of *Apis mellifera* brood infested with *Varroa destructor* disrupts the reproduction of the infesting mites, and exposes the foundress mites to potential removal from the colony by grooming. Using brood deliberately infested with marked mites, we investigated the association between the removal of mite-infested brood and the removal of exposed foundress mites in Italian (IHB) and Russian honey bee (RHB) colonies. Our results showed that RHB colonies removed more mite-infested brood in significantly less time (average = 87.9 ± 2.0% for 2.6 ± 0.1 days) than IHB colonies (average = 61.9 ± 7.3% for 3.2 ± 0.1 days or 19.3% per day). For the inoculated brood that was not removed, RHB colonies had lower proportions of brood cells containing: (a) live marked mites regardless of reproductive status (RHB = 4.4 ± 1.3%; IHB = 17.7 ± 5.9%); (b) dead marked mites (RHB = 1.1 ± 0.5%; IHB = 7.1 ± 2.2%); (c) lost introduced marked mites (RHB = 6.6 ± 1.6%; IHB = 13.3 ± 2.8%); and (d) reproductive marked mites (RHB = 8.3 ± 6.3%; IHB = 23.8 ± 6.9%) than IHB colonies did. These observations suggest that RHB colonies indiscriminately remove mite-infested brood regardless of mite status. Regarding trapped mites (i.e., those found below a modified queen excluder), the number of mite-infested brood cells removed positively correlated with the number of mites that were trapped in both honey bee stocks. The majority of the trapped mites fell during the first three days post mite inoculation, which coincided with the highest rates of brood removal. The highest proportions of trapped gravid foundress mites were also recorded during this time, when host bees were early in their development. The comparatively strong and rapid hygienic response of RHB to mite-infested brood and the associated removal of infesting foundresses are probably parts of a suite of factors responsible for suppressing *V. destructor* populations in RHB colonies.

Influencias de retiro cría caída de Varroa destructor en colonias de la abeja de miel

La eliminación higiénica de Apis mellifera crías infestadas de Varroa destructor interrumpe la reproducción de los ácaros que infestan, y expone a los ácaros fundadora a la eliminación potencial de la colonia por la preparación. Usando cría deliberadamente infestada de ácaros marcadas, se investigó la asociación entre la remoción de crías infestadas de ácaros y la eliminación de los ácaros fundadora expuestas en Italiano (IHB) y miel de abeja Rusa (RHB) colonias. Nuestros resultados mostraron que las colonias RHB eliminan más crías infestadas de ácaros en un tiempo significativamente menor (media = 87,9 ± 2,0% para 2,6 ± 0,1 días) que las colonias BHI (promedio = 61,9 ± 7,3% para 3,2 ± 0,1 días o 19,3% por día). Para la cría inoculado que no fue eliminado, colonias RHB tenían proporciones más bajas de celdas de cría que contienen: (a) vivir marcada ácaros independientemente del estado reproductivo (RHB = 4,4 ± 1,3%; IHB = 17,7 ± 5,9%); (b) los ácaros muertos marcadas (RHB = 1,1 ± 0,5%; IHB = 7,1 ± 2,2%); (c) perdió introducido marcó ácaros (RHB = 6,6 ± 1,6%; IHB = 13,3 ± 2,8%); y (d) reproductiva marcado ácaros (RHB = 8,3 ± 6,3%; IHB = 23,8 ± 6,9%) que las colonias IHB hicieron. Estas observaciones sugieren que las colonias RHB eliminan indiscriminadamente crías infestadas de ácaros sin importar el estado de ácaros. En cuanto a los ácaros atrapados (es decir, las que se encuentran por debajo de un excluidor de reina modificado), el número de celdas de cría de ácaros infestadas retirados correlacionó positivamente con el número de ácaros que quedaron atrapados en ambas poblaciones de abejas melíferas. La mayoría de los ácaros atrapados cayó durante los primeros tres días después de la inoculación de ácaros, que coincidió con las mayores tasas de eliminación de cría. También se registraron las proporciones más altas de ácaros fundadora grávidas atrapados durante este tiempo, cuando las abejas anfitrionas fueron temprano en su desarrollo. La respuesta higiénica comparativamente fuerte y rápida de RHB a cavilar-ácaros infestada y la eliminación asociada de infestar fundadoras son probablemente parte de un conjunto de factores responsables de la supresión de las poblaciones destructor V. en colonias RHB.

Keywords: Russian honey bees; hygienic behavior; brood removal; mite drop; *Varroa destructor*; resistance

Introduction

Volatiles from the cuticle of honey bee larval hosts trigger oogenesis in *Varroa destructor* (Frey, Odemer, Blum, & Rosenkranz, 2013; Garrido & Rosenkranz, 2003). This facilitates the synchronization of the reproductive cycle of *V. destructor* and the development of the honey bee host which is essential in optimizing foundress fecundity, and the progeny's survival and successful mating (Frey et al., 2013; Kirrane et al., 2011). A disruption in synchronization results in the increase of non-reproductive

*Corresponding author. Email: lilia.deguzman@ars.usda.gov

(NR) foundress mites that produce unmated progeny at the hosts' emergence or no progeny at all (Kirrane et al., 2011).

A high proportion of NR mites is commonly observed in *V. destructor* resistant honey bees such as those with the Varroa Sensitive Hygienic (VSH) trait (Harbo & Harris, 2005, 2009; Ibrahim & Spivak, 2006) and Russian honey bees (RHB) (de Guzman, Rinderer, & Frake, 2007, 2008). Pre- to pink-eyed pupal stages are probably targeted by VSH bees (Harris, 2007). Generally, *V. destructor* mites infesting these early pupal stages of honey bees have begun oviposition of their one male egg. Thus, their removal by workers as a result of hygiene interrupts the reproductive cycle of the infesting foundress mites (Harris, 2007; Kirrane et al., 2011). When such mites infest another larva they produce daughters that will never mate because the males were laid in separate cells.

Consequences of a shift in timing between mite reproduction and bee development have been demonstrated in some "mite transfer" experiments. When mites from pre-pupae were transferred into newly sealed brood (≤24 h), ≥60% of them continued to reproduce, but none of their female offspring were viable or had a chance to mate because of the lack of adult males (Frey et al., 2013; Kirrane et al., 2011). A lower percentage (≤10%) of them produced non-viable progeny when the mites were transferred from white- or pink-eyed pupae to newly sealed brood (Kirrane et al., 2011). Thus, it is possible that volatiles from freshly capped larvae that mites use to activate reproduction may have been insufficient in older brood to stimulate oogenesis or already non-existent at the time of mite re-invasion. This hypothesis is also supported by data of Frey et al. (2013) which show only 40% mite reproduction (production of at least one progeny) despite adding a high amount (5-larvae equivalent) of the compound reported to activate oogenesis to 24 h-old capped larval hosts. It is possible that *V. destructor* are only receptive to the compound once, or there may be an optimum amount of the compound that triggers oogenesis.

RHB colonies are known to have increased removal response toward freeze-killed brood (de Guzman et al., 2002; Kavinseksan, Wongsiri, Rinderer, & De Guzman, 2004; Unger & Guzmán-Novoa, 2009). They are also better at removing mites from their bodies than Italian honey bees (IHB) (Rinderer, De Guzman, & Frake, 2013; Rinderer et al., 2001) and as compared to local bees of Canada (Guzmán-Novoa, Emsen, Unger, Espinosa-Montaño, & Petukhova, 2012). That RHB colonies are also hygienic toward *Varroa*-infested brood is suggested by their high rates of NR (de Guzman et al., 2007) but this has not been observed directly. While there is a correlation between levels of falling mites and the emergence of honey bee brood (Lobb & Martin, 1997), no study has been conducted to determine whether or not removal of mite-infested brood enhances the frequency of mite drop in colonies. The main objectives of this study were: (1) to determine whether or not RHB colonies were also hygienic toward brood deliberately infested with *V. destructor*; and (2) to investigate the relationship between brood removal and the removal of adult mites released by hygiene by counting marked mites that dropped on traps.

Materials and methods

Experimental set up

IHB queens were purchased from a commercial queen breeder in California and RHB queens were naturally mated from our program. Fifty-one colonies (IHB = 25; RHB = 26) were established in April 2010. After two months (when the colonies were comprised of progeny of the introduced queen), 18 colonies (IHB = 9; RHB = 9) having the lowest *V. destructor* infestations (1–5%) were selected for this experiment. At that time, a Cloake board was installed between the two medium (height = 16.8 cm) hive boxes occupied by each of these colonies. A Cloake board, invented by Harry Cloake of New Zealand, consists of a queen excluder mounted to a wooden frame (Cobey, 2005). The wooden frame has grooves which allow a sheet of metal to be slid in and out. The metal sheet also serves as a temporary floor with an upper entrance. One end of the metal sheet is bent which can be used to close the upper entrance. This device allows both a complete separation of upper and lower hive boxes and a separation of them with a queen excluder.

Each colony was set-up as follows. Each queen was confined to the bottom hive box which contained all of the brood frames. The queen excluder (Cloake division board) was placed between the two hive boxes preventing the queen from laying eggs in the top box. The top hive box consisted of four honey and pollen frames, three empty frames and two frames with wax foundation. None of the drawn combs used in the hive box above the Cloake board had ever been used for brood rearing, and were stored in a freezer before being used. These procedures ensured that no entombed or free-roaming mites were present in the top hive box during the experiment.

Mite inoculation and evaluation of brood removal

Using a push-in wire screen (14 cm 12 cm), each queen was caged overnight in the bottom chamber (about 15 h) on an empty frame not previously used for brood rearing and having a small amount of stored nectar. The section of resulting brood consisted of about 23 rows with 23 cells per row in order to obtain about 500 test brood cells per colony. Queens were released the following day into the bottom hive body and the frames were placed in the upper chamber of their respective

colonies to prevent the queens from laying more eggs on the test combs. On the 8th day or when brood cells were sealed, they were inoculated with mites marked with a small dot of fluid corrector (PRESTO!™) on their idiosoma (Dietemann et al., 2013; Kirrane et al., 2012a). This method ensured that the dropped mites were from the removed inoculated brood. All inoculum mites (foundress, dark) were collected using an insect brush from drone brood (newly sealed larvae to pre-pupae) of two highly infested IHB colonies not included in the experiment. These mite sources were receiving mite-infested brood from other colonies prior to the experiment. At these stages, all foundress mites were gravid and some had initiated oogenesis.

The transfer technique was used in this study (Garrido & Rosenkranz, 2004; Kirrane et al., 2011). Each section was digitally photographed and prints were used to map what treatment type each brood cell received. Marked mites (n = 50 per colony) were introduced randomly among the test brood cells per section. In each section, three groups of brood were established: (a) mite-inoculated through a small opening in the cell capping and then closed; (b) cell cappings opened and closed without mite inoculation (O/C); and (c) undisturbed brood cells as control. Thereafter, each brood frame was returned to their respective colony in the middle of the box above the partially closed Cloake board. To assess brood removal, digital photos of the test brood were also taken after inoculation, and then every day for eight days and compared. On the 8th day, the brood that was not removed was examined for the presence or absence of mites and the status (live, dead, reproductive, NR) of foundress mites used to inoculate the cells. In this study, we classified reproductive mites to be those that produced at least one progeny.

Initial natural infestation of each test brood section was determined by examining 50–100 newly sealed brood cells before mite-inoculation. Final infestation of each brood section was determined by examining O/C and control brood not removed by bees. In addition, brood infestations of the colonies were also monitored in June (one month before the conduct of the experiment in July) and then after the experiment (August and September) by examining 200 brood cells from two frames of capped brood.

Evaluation of mite removal

To compare removal of mites from their own and nest mates' bodies, mite drop was monitored every day for eight days or until the brood were tan-bodied pupae. To collect mites that dropped from the top hive box where the test brood was located, each Cloake board was modified as a mite trap. We placed a white paper insert covered with a screen mesh (8 mesh) (length = 47 cm, width = 37 cm, height = 0.5 cm) to protect it from the bees on top of the metal slide. The metal slide was not pushed all the way to the back leaving about 3 cm for the worker bees to freely move between the top and bottom hive boxes. This method provided the upper hive box a sense of having a queen (to prevent construction of queen cells in the test brood), and also allowed for the creation of an upper entrance for foraging bees. All paper inserts were thickly coated with a mixture of vegetable oil and petroleum jelly (1:1) and were replaced daily. Each paper insert was placed in a labeled plastic bag (~4 L) and frozen until examination. The thick petroleum jelly/vegetable oil coating of the inserts allowed better preservation of the trapped mites while in storage. To determine the location of the dropped mites on the trap, each white paper insert was placed on a plastic tray with three major grids during trap examination. The middle grid encompassed the four middle frames (4–7) including the test frame which was positioned either as the 5th or 6th frame. The two outside grids included the six outer frames (1–3 and 8–10).

Assessment of varroa mite status

Mites were collected using an insect brush and examined under a dissecting microscope as described by Rinderer et al. (2013). In brief, mature and young mites were differentiated based on body coloration. Each mite was also examined for injuries and freshness. Fresh mites were further classified as gravid or non-gravid mites; gravidness also indicated that they were from freshly removed or opened brood cells. Freshness of mites was indicated by the presence of haemolymph and fresh tissues when mites were poked or teased apart with an insect pin (Rinderer et al., 2013). All dropped mites were also examined for the presence of paint. Since bees were able to move between supers, some of the marked mites that were released upon brood removal may have left the top hive box and entered the bottom brood box via phorionts or carrier bees. No traps were installed on the bottom floors of the bottom hive boxes.

Statistical analyses

All data related to the mite-inoculated brood sections (proportion of cells opened, proportion of opened cells that were re-sealed, percentage of the remaining brood that were still infested with inoculum mites, proportion of remaining brood with reproductive mites, and time spent for brood removal) were compared using two-way analysis of variance (ANOVA) with honey bee stock and brood type as main effects (PROC MIXED, SAS v9.2 2008). Data on the natural brood infestation of the test colonies were also analyzed using ANOVA. Where there were interactions, data were further analyzed by honey bee stock and means were compared using *post hoc* t-tests. A paired t-test was used to compare the two honey bee stocks for the cumulative brood removal for each day, differences in initial and

final brood infestations, and proportions of brood with live and dead marked mites and those that did not contain the inoculum mites.

Data on the percentage of brood removed through time were subjected to ANOVA for repeated measures with honey bee stock, brood type and day of observation as fixed effects. Due to an interaction with brood type, data were further separated by inoculation type and a repeated measures ANOVA was performed with honey bee stock and day of observation as fixed effects. There was a two-way interaction in the mite-inoculated group, so a one-way ANOVA was further performed for each honey bee stock to examine the effects of days of observation on brood removal. A paired *t*-test was used to compare the removal of mite-inoculated brood between the two honey stocks.

Data on the proportions of trapped (total and based on location of mites on the trap), marked, gravid, and injured mites were analyzed with repeated measures ANOVA with honey bee stock and day of observation as the main effects. Effects of location on mite fall were analyzed with a two-way ANOVA with honey bee stock and location as fixed effects. Where there were interactions, data were further separated by honey bee stock and means were compared with *post hoc t*-tests. A correlation analysis was performed to determine if a relationship existed between the number of trapped mites and the number of mite-inoculated brood removed. Prior to analyses all percent variables were transformed with arcsine square-root transformations and all count variables were transformed with square-root transformations to better approximate normality (SAS v9.2, 2008).

Results

Assessment of bees' responses to test brood

We evaluated bees' responses using three brood types: (a) brood cells deliberately inoculated with marked mites (IHB = 450; RHB = 454); (b) brood cells that were opened and O/C (IHB = 459; RHB = 461); and (c) undisturbed brood cells which served as control (IHB = 890; RHB = 1,067). The following parameters were measured.

Percentage of brood removed

ANOVA revealed a significant interaction between honey bee stock and brood type ($F = 7.72$; $p = 0.001$) for the percentage of brood removed (Figure 1). Brood removal differed between IHB and RHB colonies within the three brood types. The highest removal rates were observed in the mite-inoculated group with RHB colonies having higher removal rate than IHB colonies. Both honey bee stocks had similarly low rates of brood removal in the O/C and control groups. Overall, only 13.6 ± 1.9 and 9.7 ± 2.0% of the O/C and control brood cells were removed, respectively. When the cumulative removal of mite-inoculated brood for each day of observation was analyzed, both honey bee stocks showed increased removal during the first three days of observation (Table 1). However, RHB colonies significantly removed more mite-inoculated brood every day than IHB colonies except for day 1. It took only three days for RHB colonies to remove about three quarters of the mite-inoculated brood. In contrast, IHB colonies failed to match or exceed this 3-day removal rate even after eight days of observation.

Time spent for brood removal

For the average time spent in removing brood, no two-way interaction was detected ($F = 1.22$; $p = 0.297$). However, significant honey bee stock ($F = 6.09$; $p = 0.014$) and brood type ($F = 5.58$; $p = 0.004$) effects were observed. Regardless of brood type, RHB (2.5 ± 0.1 days) spent lesser time removing brood than IHB (3.0 ± 0.1 days) colonies. Among brood type, mite-inoculated brood (2.8 ± 0.1 days) = control group (2.8 ± 0.2 days) > O/C group (2.5 ± 0.2 days). We also analyzed the removal time of mite-inoculated brood separately. We found that RHB colonies remove more infested brood cells in a significantly less time (Mean = 2.6 ± 0.1 days). In contrast, IHB spent a long time (Mean = 3.2 ± 0.1 days) removing less brood ($t = 4.74$; $p < 0.0001$).

Proportion of opened brood

The number of opened brood was counted using daily digital photos of the brood sections. For the proportion of opened brood, no interaction between honey bee stock and brood type was detected ($F = 0.04$, $p = 0.966$). However, brood type significantly affected the proportion of brood opened ($F = 12.09$, $p < 0.0001$) with the mite-inoculated group sustaining the highest proportion of opened brood (8.5 ± 1.4%) than the O/C (2.6 ± 0.9%) or control groups (2.2 ± 0.6%), which did not differ from each other. Overall, the proportion of opened brood was low and did not differ significantly ($F = 0.39$; $p = 0.535$) between IHB (4.8 ± 0.9%) and RHB (4.1 ± 1.1%) colonies.

Proportion of re-sealed brood out of the opened brood

ANOVA showed no two-way interaction ($F = 0.47$, $p = 0.629$), honey bee stock ($F = 1.99$, $p = 0.166$) or brood type ($F = 0.12$, $p = 0.891$) effects for the proportion of re-sealed brood. Regardless of brood type, the proportion of re-sealed brood was numerically higher in the IHB (18.1 ± 6.7%) than in the RHB colonies (4.8 ± 2.9%). In the mite-inoculated brood, only seven (5 IHB, 2 RHB) of the opened brood cells were re-sealed. Three (IHB) and one (RHB) cells were later removed.

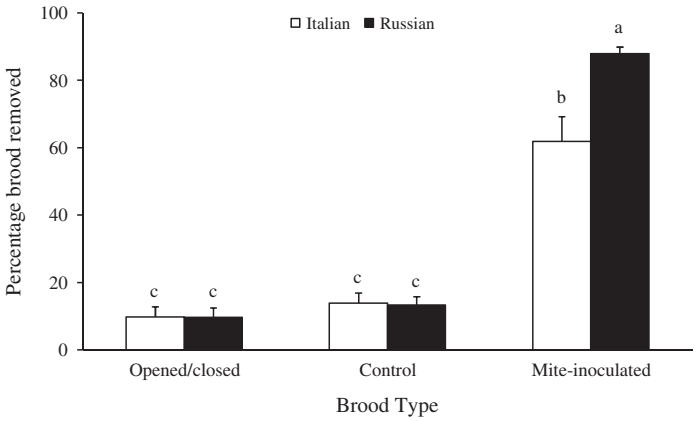

Figure 1. Percentage (mean ± SE) of brood removed in colonies of Italian and Russian honey bees.
Notes: For each brood type, bars with the same letters are not significantly different ($p > 0.05$). A total of 904 brood cells inoculated with marked *V. destructor* mites, 920 brood cells that were opened and closed without mite inoculation (O/C) and 1957 undisturbed brood cells which served as control were examined.

Table 1. Cumulative proportion (mean ± SE) of *V. destructor*-inoculated brood removed through time for Italian and Russian honey bees. For each day, means with the same letters are not significantly different (*t*-test, $p > 0.05$).

Days of observation	Italian	Russian	Analysis
1	11.9 ± 4.8[a]	22.9 ± 5.6[a]	$t = -1.70$; $p = 0.108$
2	25.9 ± 6.5[b]	56.6 ± 6.5[a]	$t = -3.19$; $p = 0.006$
3	41.1 ± 9.0[b]	70.0 ± 7.1[a]	$t = -2.51$; $p = 0.023$
4	48.8 ± 8.3[b]	76.6 ± 6.1[a]	$t = -2.69$; $p = 0.016$
5	53.5 ± 8.3[b]	80.4 ± 5.2[a]	$t = -2.78$; $p = 0.013$
6	57.7 ± 7.8[b]	85.3 ± 2.1[a]	$t = -3.40$; $p = 0.006$
7	61.0 ± 7.5[b]	87.1 ± 2.1[a]	$t = -3.43$; $p = 0.006$
8	61.9 ± 7.3[b]	87.9 ± 2.0[a]	$t = -3.53$; $p = 0.005$

Of the three re-sealed brood cells left, 1 (RHB) and 1 of 2 IHB brood cells still had live mites at the end of the experiment.

Infestation of test brood section

The test sections for the IHB colonies had higher natural mite infestations than in the RHB colonies both before mite inoculation (IHB = 4.7 ± 1.4%, RHB = 1.2 ± 0.5%; $t = 2.36$, $p = 0.031$) and after the experiment (IHB = 6.1 ± 1.6%, RHB = 1.4 ± 0.7%; $t = 3.63$, $p = 0.002$). Natural infestations at the end of the experiment included both the control and O/C groups.

For the mite-inoculated brood cells, a total of 227 cells (IHB = 172; RHB = 55) were not removed by the honey bees at the end of the experiment. However, not all of them contained the introduced marked mites. The IHB colonies had higher proportions of brood containing live marked mites, dead marked foundress, and escaped introduced mites than RHB colonies (Table 2). Four brood cells (all IHB) in the mite-inoculated brood cells were also naturally infested; none in the RHB colonies. Regardless of honey bee stock, low levels of infestation were observed in the control (3.8 ± 1.0%; 62 out of 1759 brood cells remaining) and O/C (3.5 ± 1.1%; 27 out of 794 brood cells remaining) groups at the end of the experiment.

For the proportion of brood with reproductive mites (foundress produced at least one progeny), no significant interaction between honey bee stock and brood type ($F = 0.26$, $p = 0.775$) and no honey bee stock ($F = 0.87$, $p = 0.357$) effects were detected. However, a significant influence of brood type was recorded ($F = 10.38$, $p = 0.0003$). Of the foundress mites that were examined at the end of the experiment, higher reproduction was recorded in mites that were naturally infesting the control (74.2 ± 9.3%) and O/C (59.3 ± 12.6%) brood groups than those mites that were deliberately introduced (16.5 ± 4.9%) into the newly sealed brood. When the mite-inoculated brood was analyzed separately, IHB (23.8 ± 6.9%) supported numerically higher reproductive success of mites than RHB colonies (8.3 ± 6.3%) but no difference was detected ($t = 1.96$, $p = 0.069$). This lack of differences between the two stocks was probably due to low sample size; the majority of the inoculated brood were removed by RHB.

Table 2. Parameters related to the removal and infestation of test brood sections for Italian and Russian honey bee colonies. Some of the marked mites were presumed to have lost their paint.

					Infested brood, not removed (%)		Overall mite distribution (%)		
Col. #	Stock	# Brood inoc.	Inoc. brood removed (%)	Inoc. brood not removed, no mites (%)	Dead mites	Live mites	Trapped	Brood	Missing
901	I	50	88.0	6.0	6.0	0.0	72.0	6.0	22.0
903	I	50	78.0	12.0	0.0	10.0	50.0	16.0	34.0
905	I	50	32.0	6.0	6.0	56.0	34.0	64.0	2.0
907	I	51	27.5	27.5	21.6	23.5	31.4	45.1	23.5
908	I	50	56.0	6.0	4.0	34.0	36.0	42.0	22.0
911	I	50	66.0	12.0	8.0	14.0	44.0	22.0	34.0
912	I	50	58.0	22.0	10.0	10.0	38.0	20.0	42.0
918	I	50	62.0	22.0	8.0	8.0	58.0	16.0	26.0
919	I	49	89.8	6.1	0.0	4.1	44.9	4.1	51.0
Mean		50	61.9	13.3	7.1	17.7	45.4	26.1	28.5
939	R	50	98.0	0.0	2.0	0.0	62.0	2.0	36.0
943	R	51	78.4	11.7	0.0	9.8	43.1	9.8	47.1
954	R	51	86.3	7.8	3.9	2.0	45.1	5.8	49.0
956	R	50	86.0	14.0	0.0	0.0	72.0	0.0	28.0
957	R	50	82.0	6.0	2.0	10.0	52.0	12.0	36.0
958	R	50	94.0	4.0	0.0	2.0	40.0	2.0	58.0
959	R	50	88.0	6.0	0.0	6.0	64.0	6.0	30.0
960	R	52	86.5	9.6	0.0	3.9	44.2	7.7	48.1
964	R	50	92.0	0.0	2.0	6.0	72.0	8.0	20.0
Mean		50.4	87.9	6.6	1.1	4.4	54.9	5.8	39.1

Assessment of mites that dropped below the test brood sections

Of the total mites that dropped, the proportions of marked mites were 50.8 ± 5.5% from IHB and 63.8 ± 4.1% from RHB colonies. It is possible that the paint may have come off some mites. Also, all test colonies were naturally infested with low levels of mites (although significantly higher in IHB than in RHB colonies). Thus, it was impossible to ascertain whether the unmarked mites were in fact the inoculum mites or naturally infesting mites. Also, some marked mites may have moved to the lower hive boxes where the brood was located via adult bees. However, no marked mites were observed in any of the 7200 brood cells examined one-two months after the experiment. Since there was no other brood except the test brood section and all combs used above each Cloake board had never been used for brood rearing, we presumed all trapped mites (marked and unmarked) were from the inoculated brood. Only half of the inoculated mites were recovered from the traps while more than one quarter (RHB = 39%, IHB = 29%) were not accounted for (Table 2). The following parameters associated with mite drop were measured.

Proportion of mites that dropped

For the proportion of mites that dropped out of the inoculum mites, ANOVA revealed a significant interaction between honey bee stock and day of observation ($F = 2.49$, $p = 0.020$) and day of observation effect ($F = 22.59$, $p < 0.0001$) (Figure 2). No significant effect of honey bee stock was detected ($F = 1.05$, $p = 0.308$). In the IHB colonies, the highest proportion of trapped mites was observed on the 1st day, which was similar to that observed on the 2nd and 3rd day. There was a steady decline after the 1st day with another small peak on the 7th day. For RHB, the highest proportions of trapped mites were recorded during the first two days of observation. There was a sharp decline in trapped mites thereafter with a small peak again on the 6th day. For both honey bee stocks, the number of mites trapped was significantly correlated with the number of mite-inoculated brood removed by the bees (IHB, $r = 0.706$, $p < 0.0001$; RHB, $r = 0.843$, $p < 0.0001$) (Figure 3).

Proportion of gravid mites

Analysis of the proportion of gravid mites out of the total number of trapped mites showed no significant interaction between honey bee stock and day of observation ($F = 0.85$, $p = 0.574$) and no honey bee stock effect ($F = 2.32$, $p = 0.130$). However, a significant day effect was observed ($F = 3.21$, $p = 0.004$) (Figure 4). The highest proportions (more than 50%) of trapped gravid mites were recorded during the first three days of observation (when test brood was L6 to pre-pupal stages) with the lowest proportion observed during the last day of the experiment when test brood were already tan-bodied pupae.

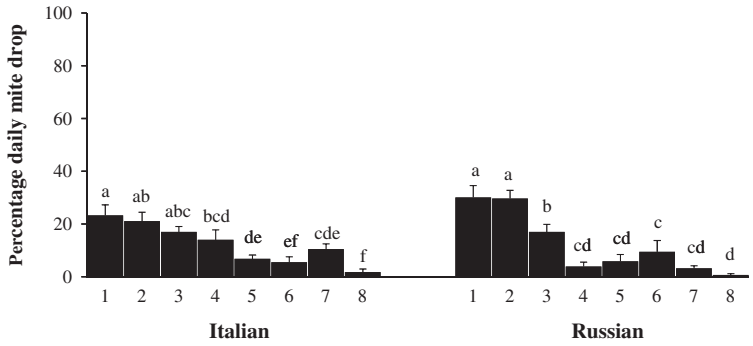

Figure 2. Percentage (mean ± SE) of *V. destructor* mites that dropped on bottom board traps of Italian and Russian honey bees.
Note: For each honey bee stock, bars with the same letters are not significantly different ($p > 0.05$).

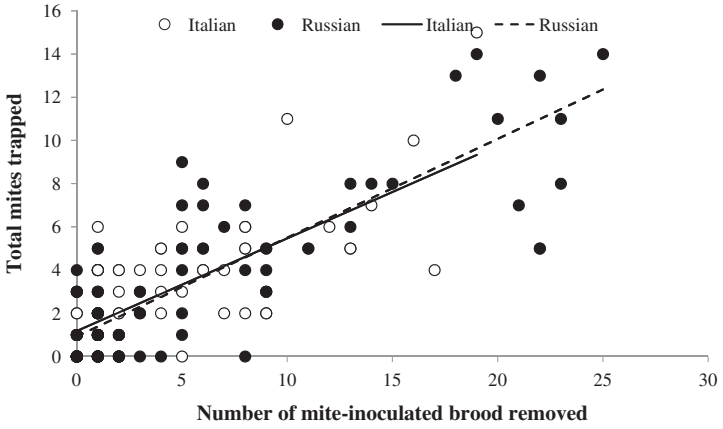

Figure 3. Correlation between the number of *V. destructor* mites trapped and number of mite-inoculated brood removed by Italian and Russian honey bees.
Note: IHB, $r = 0.706$, $p < 0.0001$; RHB, $r = 0.843$, $p < 0.0001$.

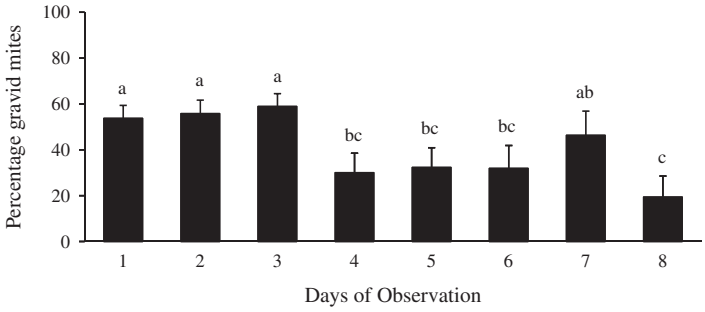

Figure 4. Percentage (mean ± SE) of gravid *V. destructor* mites that dropped on bottom board traps through time.
Note: Bars with the same letters are not significantly different ($p > 0.05$).

Proportion of trapped mites based on where they dropped on the traps

When the proportion of trapped mites were analyzed based on the location where they dropped, no significant interaction between honey bee stock and location ($F = 1.96$, $p = 0.171$) and no honey bee stock effect was detected ($F = 0.00$, $p = 0.999$). However, a significant effect of location ($F = 295.01$, $p = 0.0001$) was observed. Out of the total mites that dropped, 87.8 ± 2.4% were collected in the middle of the traps while only 12.2 ± 2.4% were found along the edges of the traps. When we examined the proportions of marked mites, a similar trend was observed. No two-way interactions ($F = 0.84$, $p = 0.366$) and no honey bee stock ($F = 2.87$, $p = 0.100$) effect were observed. However, a significant location ($F = 7.78$, $p = 0.009$) effect was detected. Of the mites that dropped in the middle of the traps, 60.0 ± 3.6% of them were marked. For those mites that dropped along

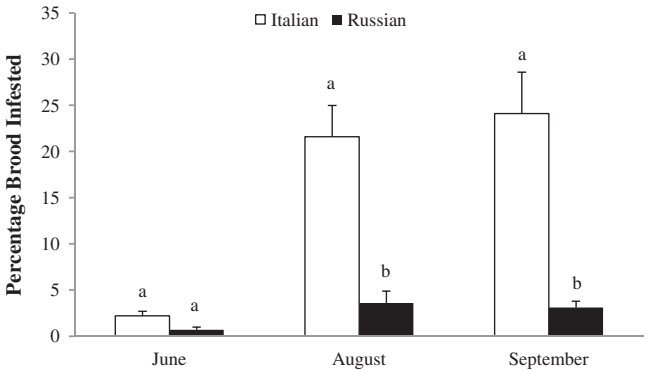

Figure 5. Percentage (mean ± SE) of worker brood infested in colonies of Italian and Russian honey bees.
Note: Bars with the same letters are not significantly different ($p > 0.05$). A total of 5400 brood cells were examined for each honey bee stock.

the edges of the traps, only 30.6 ± 9.0% of them were marked.

Proportion of injured mites

For the proportion of injured mites, no two-way interactions ($F = 0.86$, $p = 0.537$) and no honey bee stock ($F = 0.22$, $p = 0.641$) or day of observation ($F = 1.94$, $p = 0.069$) effects were detected. The average proportion of injured mites in the IHB was 22.3 ± 3.7 and 20.6 ± 2.9% in the RHB colonies. Also, the proportion of injured mites out of the mites that dropped was higher in those mites that were collected from the middle (24.1 ± 2.8%) than from the edge (6.4 ± 3.0%) of the traps ($t = -5.66$, $p < 0.0001$).

Prevalence of V. destructor in test colonies

The prevalence of *V. destructor* in the test colonies was monitored one month before and two months after the test. A significant interaction of between honey bee stocks and month of observation was detected ($F = 9.43$, $p = 0.0007$) for the percentage of brood infested. Except in June, when the populations of test colonies were changing to the progeny of the introduced queens, RHB colonies had significantly lower infestation rates than IHB both in August and September (Figure 5). No marked mites were observed in any of the brood cells examined in August and September.

Discussion

This study demonstrated that RHB colonies have strong and rapid response to brood deliberately infested with *V. destructor*. Overall, 88% of the brood inoculated with mites was removed by RHB after eight days, 70% of which was removed within three days post mite-inoculation. This 3-day cumulative removal was even higher than the 62% cumulative removal rate displayed by IHB colonies by the end of the experiment (after eight days). This observation agrees with the findings of Harris (2007) showing peak removal of infested brood during 3–5 days postcapping by VSH bees. In general, mites have laid eggs during this first three days of postcapping development. Thus, the presence of progeny within the capped brood cells may have intensified brood removal during this time. Since all inoculum mites were obtained similarly from the same colony sources, similar reproductive success was expected in both honey bee stocks. Yet, removal of brood during the first three days post mite introduction was more pronounced in RHB than in IHB colonies. Thus, it is likely that RHBs were removing infested brood regardless of the reproductive status of the mites, similar to the observation made by Harris, Danka, and Villa (2009, 2010) on VSH bees.

Aside from the presence of *V. destructor*, other stimuli may have triggered the removal of the mite-inoculated brood. It is possible that the worker bees may have responded to the odor of the correction fluid used to mark the inoculum mites. However, it is unclear whether or not RHB are more sensitive to the smell of the paint than IHB since they removed more brood than IHB did. Also, the natural mite infestations in the IHB colonies were significantly higher than in the RHB colonies. In spite of this, the presence of more naturally infesting mites in the IHB colonies did not accelerate brood removal in this stock. At the end of the experiment, four of the mite-inoculated brood cells that were not removed by IHB also contained naturally infesting mites. About 12% (55 out 454) and 38% (172 out 450) of the brood inoculated with mites were not removed by the bees at the end of the experiment for RHB and IHB, respectively. However, 36% (20 out of 55) (RHB) and 46% (80 out of 172) (IHB) of the remaining mite-inoculated brood were still infested with live mites. An examination of the remaining mite-inoculated brood showed that the RHB (8%) supported a lower proportion of brood containing foundress mites with progeny than the IHB (24%) colonies. This observation agrees with our earlier study showing reduced reproduction of

mites that are naturally infesting RHB (de Guzman et al., 2007, 2008).

It is also interesting to note that 13% (60 out of 450) (IHB) and 7% (30 out of 454) (RHB) of the mite-inoculated brood were not removed and yet did not contain the introduced mites at the end of the experiment. It is possible that some of the introduced mites were able to escape from the small holes created in the cell cappings for mite introduction. However, the bees may have facilitated the escape of the inoculum mites by widening the holes before re-sealing the cell cappings. Also, RHB may have removed previously inoculated brood because of the presence of mite feces or volatiles from wounds inflicted by the introduced mites. Only half of the marked mites were recovered from the traps while more than 1/4 (RHB = 39%, IHB = 29%) were not accounted for. These unrecovered freed mites were either phoretic on adult bees, had died and fallen to the floor of the bottom box or were carried away from the hive by worker bees, as described by Morse, Miksa, and Masenheimer (1991). Neither adult bee infestation nor mite drop in the bottom box was determined during the experiment. The mites freed from the bees' hygienic activities may have also invaded new hosts below the Cloake board. However, no marked mites were observed in the 7200 brood cells we examined 1–2 months after the experiment. Assuming that the 39% missing mites were able to invade new hosts below the Cloake board of RHB colonies, they certainly did not dramatically increase brood infestation in this stock. It is possible that the infested brood were again removed by the bees or the majority of them were unable to produce viable progeny (=NR) if not removed (Kirrane et al., 2011). A high proportion of NR mites is one of several factors that contribute to the slow growth of mite populations in RHB colonies (de Guzman et al., 2007). Hence, increased removal of mite-infested brood appears to be one of the contributors to the low levels of brood infestations of *V. destructor* in RHB colonies observed in this study. Also, the low proportions of brood with live and dead foundress mites and those that were not removed but did not contain the introduced marked mites in RHB colonies suggest that RHB remove infested brood indiscriminately regardless of mite status.

The number of mite-infested brood removed was also associated with the number of mites recovered from the traps. However, no difference between the two honey bee stocks for the correlation between the number of brood removed and trapped mites was detected. It also appeared that the mites exposed by brood removal were quickly pursued by both IHB and RHB bees since about 88% of the trapped mites were recovered from the middle of the traps (60% still having the paint), just beneath the test brood or its surrounding area. This quick and successful removal response by both RHB and IHB bees was likely facilitated by the absence of suitable brood for invasion above the Cloake board forcing the exposed mites to become phoretic. About 96% of trapped mites were fresh, another indication that the mites were from the removed brood. Also, only about half of the trapped mites were gravid (swollen opisthosoma) because swollenness generally diminishes when oviposition stops as the hosts pupae get older or due to oosorption as mites become phoretic on adult bees (Kirrane et al., 2012b).

Several factors have been reported to act together to substantially reduce *V. destructor* populations in RHB colonies (de Guzman et al., 2007). Increased grooming as measured by the proportions of damaged mites (Guzmán-Novoa et al., 2012; Rinderer et al., 2001) or total mite drop (Rinderer et al., 2013), and hygienic response towards frozen brood (de Guzman et al., 2002; Kavinseksan et al., 2004; Unger & Guzmán-Novoa, 2009) have been demonstrated in RHB colonies. Since RHB colonies showed strong and rapid hygienic responses to mite-infested brood and grooming responses to the mites released by hygienic activities in this study, these attributes probably also are important components of this suite of factors responsible in suppressing mite population in RHB colonies. Further, two out of 10 IHB colonies (Table 2) showed high rates of brood and mite removal. These important traits, now documented to be involved in *V. destructor* resistance in RHB, can probably be improved through selective breeding in IHB.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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