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Research

Brood Affects Hygienic Behavior in the Honey Bee (Hymenoptera: Apidae)

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Abstract

Despite receiving much attention, the ectoparasitic mite *Varroa destructor* (Anderson and Trueman) and the pathogens it vectors remain critical threats to the health of the honey bee *Apis mellifera* (Linnaeus) (Hymenoptera: Apidae). One promising intervention approach is the breeding of hygienic honey bees, which have an improved ability to detect and remove unhealthy brood from the colony, and are thus more resistant to *Varroa*. While much hygienic behavior-related research has focused on enhanced adult honey bee olfaction, less attention has been paid to the olfactory signals that originate inside the brood cell, triggering hygienic removal. Here, we hypothesized that selection for hygienic behavior in honey bees has influenced brood signaling, predicting that: 1) in a common social environment, removal rates differ among brood with different selective breeding histories, and 2) the removal rates of brood positively correlate to the hygiene level of the brood's colony of origin. To test these predictions, we cross-fostered brood subjected to control, wound, or *Varroa* treatment in unselected (UNS), Minnesota Hygienic (HYG), and *Varroa*-Sensitive Hygienic (VSH) colonies, and monitored individual brood cells for hygienic removal. Results confirmed both predictions, as brood from hygienic colonies was more likely to be removed than brood from UNS colonies, regardless of where the brood was fostered. These findings suggest that hygiene-related brood signals complement previously identified characteristics of hygienic adults, constituting an important mechanism of social immunity in honey bees. Thus, selective breeding for honey bee hygienic behavior may be improved through the utilization of field assays containing compounds related to larval signaling.

Key words: *Varroa* parasitism, selective breeding, cross-fostering, hygienic behavior, social immunity

Social immunity refers to characteristics associated with the avoidance; reduction; or elimination of pathogens, parasites, and related diseases in social species. Like other eusocial insects, honey bees rely on multiple social immune mechanisms to maintain and improve colony health (reviewed in Simone-Finstrom 2017). One such mechanism is hygienic behavior, defined as the detection and removal of diseased brood from a colony or nest (Spivak and Reuter 2001a, Wilson-Rich et al. 2009). While hygienic behavior is a form of social immunity thought to occur in several social insects (Wilson-Rich et al. 2009, Pull et al. 2018), it has been studied primarily in the honey bee, where it consists of two distinct phases: the uncapping of brood cells and the removal of the diseased brood cell contents (Rothenbuhler 1964). Brood diseases are among the most serious threats to honey bees, contributing to recent, severe losses of managed honey bee colonies (vanEngelsdorp et al. 2012; Spleen et al. 2013; Steinhauer et al. 2014; Seitz et al. 2015; Lee et al. 2015). Although these losses result from multiple, interacting factors (Potts et al. 2010, Nazzi et al. 2012, Goulson et al. 2015, Lee et al. 2015), the parasitic mite *Varroa destructor* is a central threat for honey bee health (Rosenkranz et al. 2010, Annoscia et al. 2012, Nazzi et al. 2012.

The *Varroa* mite is an obligate ectoparasite that reproduces on honey bee brood during the host's metamorphosis inside a capped wax cell (Anderson and Trueman 2000, Rosenkranz et al. 2010). Female foundress mites enter larval cells just before cell capping and initiate feeding and reproduction after cell capping prevents access of adult nurse bees to the cell. Approximately 70 h after cell capping the foundress mite begins to lay eggs. The mite and her offspring feed on the bee host during its larval and pupal stages through an established feeding site (Ifantidis 1988). After emergence, *Varroa* enter a phoretic phase in which they parasitize adult bees, followed by a reproductive phase in which they repeat the reproductive cycle in another brood cell (Rosenkranz et al. 2010). During both phases, *Varroa* act as a physical burden to the bee, reducing body weight and protein levels primarily through the sucking of hemolymph (De Jong et al. 1982, Schatton-Gadelmayer and Engel 1988, Amdam et al.

2004, Garedew et al. 2004). Moreover, *Varroa* transmit honey bee viruses (Bowen-Walker et al. 1999, Kanbar and Engels 2003, Chen et al. 2004) and have been associated with both viral amplification and selection (Yang and Cox-Foster 2005, Martin et al. 2012, Kuster et al. 2014).

Among the ongoing efforts to control *Varroa* (Dietemann et al. 2012), multiple selective breeding programs have been established to increase the frequency of naturally occurring hygienic behavior in order to interrupt the mite reproductive cycle. The Minnesota Hygienic (HYG) honey bees were selected based on a liquid nitrogen-killed brood (LNKB) removal assay, which quantifies a colony's level of hygiene based on the percent of frozen brood that adult bees removed within 24 h (Spivak 1996). *Varroa*-Sensitive Hygienic (VSH) honey bees were originally selected based on apparent suppression of mite reproduction (Harbo and Harris 2001), and were later found to also exhibit a high degree of hygienic behavior (Harbo and Harris 2005, Ibrahim and Spivak 2006). Both HYG and VSH colonies exhibit increased levels of hygiene (Leclercq et al. 2017), and have been shown to be more resistant to *Varroa* mites compared with unselected (UNS) colonies (Spivak and Gilliam 1998, Spivak and Reuter 2001a,b, Harris 2007, Villa et al. 2009, Harris et al. 2012, Danka et al. 2013).

Studies comparing adult workers with different selective breeding histories (henceforth referred to as colony "type") have reported behavioral (Masterman et al. 2000; 2001; Spivak et al. 2003; Harbo and Harris 2009), genetic (Tsuruda et al. 2012, Boutin et al. 2015, Gempe et al. 2016, Jiang et al. 2016), and proteomic (Le Conte et al. 2011, Parker et al. 2012, Guarna et al. 2017) differences between colony types. While many studies regarding hygienic behavior mechanisms (Aumeier and Rosenkranz 2001, Goode et al. 2006, Mondet et al. 2015) have focused on sensitivity and modulation of adult honey bee olfaction, less attention has been paid to the role of the olfactory signals that induce honey bee hygienic behavior. This disregard may be a result of the uncertainty surrounding the signal source. In honey bees, hygienic behavior has been associated with clustered, singly mite-infested cells (Cheruiyot et al. 2018), and with greater numbers of mites (Boecking and Drescher 1992) and mite offspring (Harbo and Harris 2005, Harbo and Harris 2009, Mondet et al. 2016) in individual cells.

Increased hygienic response to *Varroa*-infested brood may be a direct response to the mites themselves, or a consequence of the increased number of mites feeding on the brood. While olfactory signals could originate from the mite (Martin et al. 2002), hygienic behavior is not affected by *Varroa* movement (Aumeier and Rosenkranz 2001) or scent (Aumeier and Rosenkranz 2001, Le Conte et al. 2015). Likely through passive camouflage (Kather et al. 2015), mites are able to mimic host odor profiles with such accuracy that even developmental stage (Martin et al. 2001) and colony-specific differences are reflected (Le Conte et al. 2015). Therefore, signals that elicit hygienic behavior likely originate directly from mite-infested honey bee brood (Salvy et al. 2001, Nazzi et al. 2004, Swanson et al. 2009, Annoscia et al. 2012, Schöning et al. 2012, Jiang et al. 2016, Mondet et al. 2016). Indeed, increased quantities of brood cuticular hydrocarbons (Nazzi et al. 2004) and brood ester pheromones (Mondet et al. 2016) have been associated with hygienic behavior. Furthermore, the inability of *Varroa* to reproduce in worker brood cells of their original host *Apis cerana* may be due to increased susceptibility and related signaling of parasitized worker brood (Page et al. 2016). Thus, signal production and perception by both parties of the hygienic signaling system may co-evolve and contribute to the effective detection of infested cells. Artificial selection for hygienic behavior may have shifted multiple components of that signaling system (Symonds and Elgar 2008) and indirect genetic effects with nonlinear brood–nurse interactions (Linksvayer and Wade 2005) are to be expected. Based on the notion that *Varroa*-parasitized brood play an active role in triggering hygienic behavior, we hypothesized that selection for hygienic behavior in honey bees has influenced brood signaling related to health status. Specifically, we predicted that: 1) in a common social environment, removal rates differ among different types of brood, and 2) the removal rates of brood positively correlate to the hygiene level of the brood's colony of origin. To test these predictions, we measured removal rates of brood cross-fostered within UNS, HYG, and VSH colonies types. Our findings confirmed both predictions, suggesting that hygiene-related brood signaling complements the increased olfactory abilities of hygienic adults, improving the efficiency of the hygienic communication system in honey bees.

Materials and Methods

Overview

Over two consecutive summers, hygienic removal of three different honey bee brood types displaying various levels of hygienic behavior were compared in common colony environments. Experimental frames containing eggs from multiple queens were placed into unrelated colonies for rearing, and were subjected to *Varroa* mite, wound and control treatments. Removal of brood was recorded and compared with respect to treatment, brood type, and colony type.

Materials

Wooden frames, wax foundation, and UNS queens were purchased from Triad Bee Supply in Trinity, NC. HYG queens were provided by Jeff Hull and Amy Weeks in West Monroe, LA. VSH queens were provided by Bob Danka at the United States Department of Agriculture's Agricultural Research Center (USDA-ARC) in Baton Rouge, LA. All queens were open mated and studied for one experimental season only. Sample sizes for colonies of UNS, HYG, and VSH origin were 2, 4, and 3, respectively for 2013, and 2, 2, and 2, respectively for 2014. Before initiating experiments, colonies were established by introducing marked queens to UNS, queenless colonies and waiting a minimum of 7 wk from the time of queen introduction for the worker population to represent the queens' offspring. Following the behavioral experiments each year, LNKB assays were performed to determine the level of hygienic behavior exhibited by each colony (Spivak and Downey 1998). However, due to insufficient brood frames, successful LNKB assay results were only obtained for 11 of the 15 colonies tested.

Methods

All behavioral assays were conducted at the University of North Carolina at Greensboro bee-yard during the summers of 2013 and 2014. Due to the nature of this study, no experimental or mite source colonies used in either study year were treated for *Varroa* mites. Each year, medium wooden frames were sawed vertically into equal thirds and reassembled using metal brackets or staples. Reassembled frames were fitted with new wax foundation. Frames were placed into the top box of UNS colonies above queen excluders, so that the comb could be drawn out without coming into contact with any brood.

Once combs were drawn out, frames were fitted with wire cages that allowed workers to pass but contained the queens. A single queen of UNS, HYG, or VSH origin was placed on each frame. Caged experimental frames were returned to each queen's

respective colony. Once eggs were present on >75% of both sides of a frame, frames were removed from their colonies. A razor blade was used to cut combs into thirds (corresponding to the previous frame cuts) and metal brackets and staples were removed. Sections from different frames were then grafted together using metal brackets and staples such that each new frame contained eggs from at least two queens (depending on availability) of varying origin (VSH, HYG, or UNS). Frames were then redistributed into new colonies, such that no brood was ever placed back into its colony of origin (Fig. 1).

After allowing 5–7 d for development, the location of uncapped cells containing fifth larval instars were recorded using a permanent marker and transparent plastic sheet secured over each frame with thumb tacks. Cells along wires were avoided since wires are associated with increased brood removal rates (Wagoner and Rueppell 2017). Frames were returned to their colonies for 12–16 h. Cells sealed with a wax cap during this time were then recorded on the transparent plastic sheet. This procedure ensured that treatments were administered to capped cells within 18 h of capping, as is necessary to ensure initiation of mite oogenesis (Frey et al. 2013). Mite, wound, and control treatments were administered to recently capped cells in each section of frame following established protocols (Kuster et al. 2014). To open the caps of experimental cells, one side of the cap was cut with the edge of a razor blade. One side of the cell cap could then be lifted, and resealed after treatment by pressing the cell cap against the cell wall with the edge of the razor. The treatment assigned to each cell was selected at random, and each cell received either one mite, one wound, or no treatment (the control).

Phoretic mites were collected from nonexperimental colonies at the UNCG bee-yard using the sugar shake method (Fakhimzadeh 2001, Dietemann et al. 2013). Mites were shaken on to a damp paper towel, and were gently rinsed with a drop of clean water before being introduced to cells using a fine-tipped paintbrush within approximately 1 h of collection. Mites that could not clutch the paintbrush bristles were considered to be unhealthy, and were not used. Wounds that mimic *Varroa* mite feeding were inflicted with a 50-µm-diameter capillary needles on the dorsal side of the brood between the first abdominal segment and the second thoracic segment according to existing protocols (Herrmann et al. 2005, Dade 2009). This wound was nonlethal, distinct from the pin-killed brood bioassay sometimes used for selection of hygienic behavior (Newton and Ostasiewski 1986). Control cells were opened and resealed just as mite and wound cells, but received neither mite nor wound treatment. In 2013, a total of 1,063 cells were included in the study (361 VSH, 410 HYG, and 292 UNS). UNS brood was placed in one, four, and one UNS, HYG, and VSH colonies, respectively; HYG brood was placed in one, three, and three UNS, HYG, and VSH colonies, respectively; and VSH brood was placed in two, three, and three UNS, HYG, and VSH colonies, respectively. However, UNS brood placed in to UNS colonies in 2013 originated from a single source, and was removed at unexpectedly high rates. This result was indicative of an otherwise undetected issue of brood health, so data from

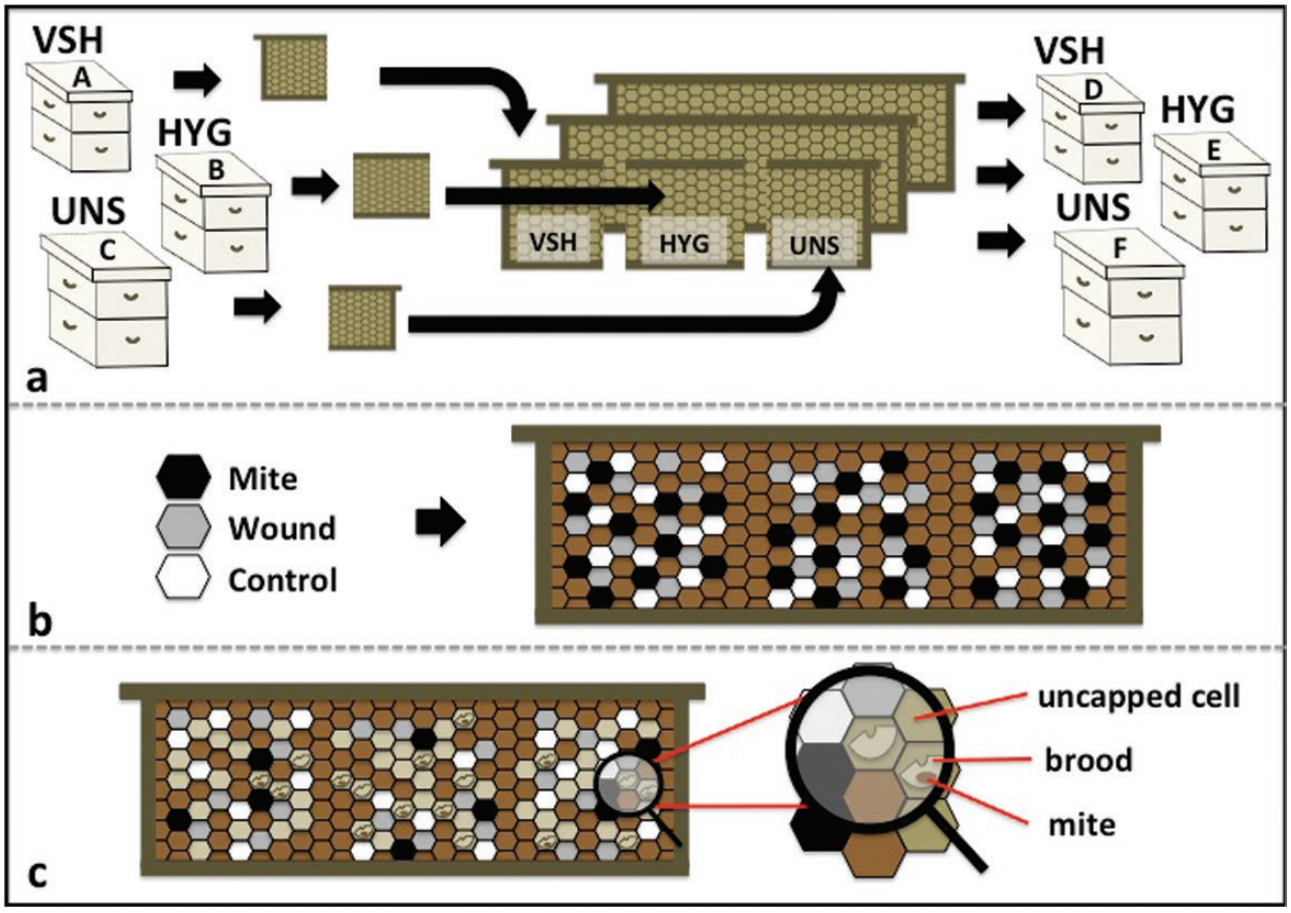

Fig. 1. Experimental methodology. (a) Sections of frame containing brood from VSH, HYG, and UNS colonies were combined and placed into new VSH, HYG, and UNS colonies; (b) mite, wound, and control treatments were introduced to capped brood cells; and (c) treated cells were monitored for evidence of hygienic uncapping and removal.

that source colony (n = 51 UNS cells) was removed from the analysis. In 2014, an additional 1,025 cells were included (339 VSH, 256 HYG, and 430 UNS). UNS, and HYG brood were each placed in two UNS, HYG, and VSH colonies, and VSH brood was placed in two, one, and two UNS, HYG, and VSH colonies, respectively.

Each day for 1 wk following treatment administration, experimental frames were removed from their colonies for no more than 30 min/d to allow monitoring of each experimental cell for uncapping and removal. Brood removed on the first day following treatment introductions was excluded from subsequent analyses to avoid experimental artifacts, such as removal triggered by poorly resealed cells. The cross-fostering experimental setup used in this study allowed comparison of removal of different treatment groups 1) between brood types within each colony type, and 2) for each brood type across colony types.

Statistical Analysis

Generalized linear models (GLMs) with binomial distribution and logit links were used to determine 1) the main effect of treatment on removal and 2) the main and interaction effects of colony type and brood type on removal for each treatment by year combination. The effects of treatment on removal were analyzed for each year separately, and again for both years combined. The effects of colony type and brood type on removal of brood in each treatment group were analyzed for the 2 yrs separately, since clear differences in removal responses between the years were apparent (see results). All Wald chi-square and odds ratios data are provided, but only significant effects are reported in the text. Brood and colony types were entered with VSH as the reference category to avoid any potential effects of the missing 2013 data for UNS brood in UNS colonies. To further explore potential effects of this missing data, we excluded all UNS brood and UNS colony data and ran a separate GLM comparing HYG and VSH brood type and colony type effects, and compared these results with results from the full dataset. In order to facilitate comparison of our results with results from previous non cross-fostering studies, we also ran a GLM to determine the effect of colony/brood type on mite-infested brood removal in the subset of data for which colony type and brood type were the same. All GLM results provided in the text are given with parenthetical 95% CIs.

For the 11 colonies with LNKB assay data, Spearman rank correlation coefficients were computed to assess the prediction that the average percent brood removal is positively correlated to the hygiene level (LNKB score) of the brood's colony of origin (overall and for each treatment type: mite, wound, or control). Spearman rank correlation coefficients were also computed to assess the relationship between average percent brood removal and the hygiene level (LNKB score) of the host colony (overall and for each treatment type: mite, wound, or control). One-tailed Spearman rank correlation coefficients were used because of the directionality of our prediction related to hygiene level of the brood's colony of origin, and based on expectations of a positive correlation between brood removal and (host) colony hygiene level (reviewed in Leclercq et al. 2017). All statistical analyses were performed using IBM SPSS Statistics, Version 25.

Results

Effects of Treatment, Colony Type, and Brood Type on Removal Rates

Brood removal status was determined for a total of 2,088 honey bee cells representing three treatment types, three colony types, and three brood types (Table 1). Cell treatment had a significant effect on the likelihood of brood removal in both 2013 (Wald χ^2 = 32.6, df = 2, P < 0.001) and 2014 (Wald χ^2 = 64.1, df = 2, P < 0.001), with mite-infested brood removed most often, followed by wounded brood, and lastly control brood. In 2013, the odds of mite-infested brood removal was 3.8 times (2.4–6.0) higher than that of control brood.

Table 1. Removal data from 2013 and 2014 experiments for each treatment by colony type by brood type combination

		Brood type					
		UNS		HYG		VSH	
Colony type	Treatment	Removed	Not removed	Removed	Not removed	Removed	Not removed
2013							
UNS	Control	8	11	4	34	2	28
	Wound	8	6	10	28	4	25
	Mite	7	11	16	20	4	26
HYG	Control	0	63	1	46	1	48
	Wound	9	49	7	36	3	45
	Mite	8	52	6	41	3	44
VSH	Control	1	19	15	40	4	38
	Wound	1	19	23	28	7	36
	Mite	4	16	29	26	15	28
2014							
UNS	Control	1	54	0	23	4	34
	Wound	3	50	2	23	9	31
	Mite	6	47	5	19	16	24
HYG	Control	4	46	3	31	5	30
	Wound	5	44	1	32	11	23
	Mite	6	44	8	26	19	16
VSH	Control	1	39	0	26	1	36
	Wound	3	36	2	26	3	36
	Mite	9	32	11	18	18	23

Gray text indicates data excluded from analysis.

The odds of wounded brood removal was 2.7 times (1.7–4.4) higher than that of control brood (Supp Table S1 [online only]). In 2014, the odds of mite-infested brood removal was 6.6 times (3.9–11.1) higher than that of control brood. The odds of wounded brood removal was 2.2 times (1.2–3.8) higher than that of control brood (Supp Table S1 [online only]). Since the results were similar for both years, the effects of cell treatment on removal were re-analyzed for 2013 and 2014 combined, and found to be statistically significant (Wald χ^2 = 89.3, df = 2, P < 0.001). After Bonferroni correction, mite-infested brood was significantly more likely to be removed than wounded (X^2 = 25.6, df = 1, P < 0.001) or control (X^2 = 83.9, df = 1, P < 0.001) brood, and wounded brood was significantly more likely to be removed than control (X^2 = 23.5, df = 1, P < 0.001) brood (Fig. 2). In the combined analysis, the odds of mite-infested brood removal was 4.9 times (3.5–6.9) higher than that of control brood. The odds of wounded brood removal was 2.5 times (1.7–3.5) higher than that of control brood (Fig. 2; Supp Table S1 [online only]).

Brood and colony type effects varied by treatment and year (Fig. 3). In the full factorial model no interaction effects were significant (Supp Table S2 [online only]); thus, the model was rerun using main effects only. In 2013, brood type significantly affected removal of the mite-infested (Wald χ^2 = 11.69, df = 2, P = 0.003), wounded (Wald χ^2 = 13.45, df = 2, P = 0.001), and control (Wald χ^2 = 7.12, df = 2, P = 0.029) brood (Table 2). There were also significant colony type effects on removal for the mite-infested (Wald χ^2 = 26.47, df = 2, P < 0.001), wounded (Wald χ^2 = 9.93, df = 2, P = 0.031), and control (Wald χ^2 = 12.81, df = 2, P = 0.002) brood (Table 2). In 2013, significant brood effects existed: the odds of mite-infested, wounded, and control HYG brood removal were 2.6 times (1.5–4.7), 3.3 times (1.7–6.4), and 2.6 times (1.0–6.6) that of mite-infested, wounded, and control VSH brood, respectively (Fig. 3; Table 3). In 2013, the host colony also significantly affected the probability of hygienic removal of brood: the odds of mite-infested, wounded, and control brood removal in VSH colonies were 5.3 times (2.8–10.0), 2.3 times (1.2–4.3), and 12.5 times (2.8–50.0) that of mite-infested, wounded, and control brood in HYG colonies, respectively (Fig. 3; Table 3). Statistical significance of 2013 HYG and VSH brood and colony type effects on removal did not change for any treatment when UNS brood and UNS colonies were added to or removed from the dataset (Supp Table S3 [online only]), and similar effect sizes were observed when comparing HYG and VSH in the models with and without the UNS data (Table 3; Supp Table S3 [online only]). In the subset of 2013 data for which colony type and brood type were the same, significant brood effects existed: the odds of mite-infested VSH brood removal were 3.7 times (1.3–10.6) that of mite-infested HYG brood (Supp Table S4 [online only]).

In 2014, brood type significantly affected removal of the mite-infested (Wald χ^2 = 27.8, df = 2, P < 0.001) and wounded (Wald χ^2 = 12.5, df = 2, P = 0.002) brood (Table 2). There was also a significant colony type effect on removal for the control (Wald χ^2 = 7.3, df = 2, P = 0.026) brood (Table 2). In 2014, significant brood effects existed: the odds of mite-infested and wounded VSH brood removal were 2.3 times (1.2–4.2) and 4.3 times (1.5–12.5) that of mite-infested and wounded HYG brood, respectively (Fig. 3; Table 3). Likewise, the odds of mite-infested and wounded VSH brood removal were 4.8 times (2.7–9.1) and 3.2 times (1.5–7.1) that of mite-infested and wounded UNS brood, respectively (Fig. 3; Table 3). In 2014, the host colony significantly affected the probability of hygienic removal of control brood: the odds of control brood removal in HYG colonies were 6.2 times (1.3–28.9) that of control brood in VSH colonies in 2014 (Fig. 3; Table 3). In the subset of 2014 data for which colony type and brood type were the same, significant brood effects existed: the odds of mite-infested VSH brood removal were 6.1 times (2.2–17.5) that of mite-infested UNS brood (Supp Table S4 [online only]).

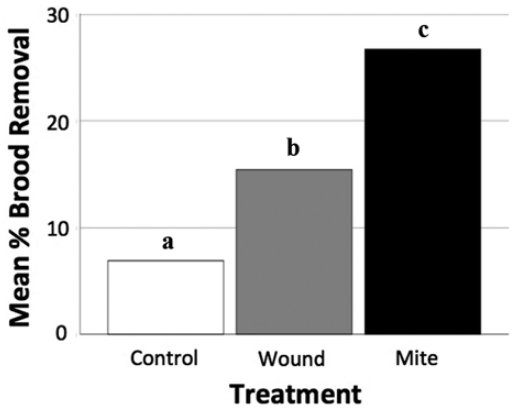

Fig. 2. Treatment effects on mean percent brood removal irrespective of brood or colony type. Different letters indicate significant differences from GLM analyses after Bonferroni correction (P < 0.0167). Overall, mite-infested brood was most likely to be removed and control brood was least likely to be removed.

Effects of Hygiene Level on Brood Removal Rate

LNKB assays revealed variation in hygiene level for individual colonies, and this variation did not strictly correspond to colony type (Table 4). Across both years, the level of colony hygiene (as determined by the LNKB assay) was positively correlated with the likelihood that brood from that colony was removed across all treatments and host colony types (r = 0.75, n = 11, P = 0.008). The relationship between the hygiene level of the colony and the removal rate of its brood (Fig. 4a) was primarily due to significant positive correlations between the LNKB score of the brood's colony of origin, and the percent removal of mite-infested (r = 0.634, n = 11, P = 0.018) and control (r = 0.554, n = 11, P = 0.039) brood. The corresponding correlation for the wounded brood was also positive, but was not statistically significant (r = 0.457, n = 11, P = 0.079). In contrast, the LNKB scores of the host colonies were not significantly correlated with removal of brood fostered in that colony across all treatments and brood types (r = –0.30, n = 11, P = 0.376). The percent of freeze-killed brood removed by the host colony was not significantly positively correlated to the removal rates of co-fostered mite-infested (r = –0.087, n = 11, P = 0.600), wounded (r = –0.691, n = 11, P = 0.991), or control (r = –0.324, n = 11, P = 0.834) brood (Fig. 4b).

Discussion

Hygienic behavior relies on intracolonial communication, and is an important mechanism of social immunity in honey bees. Here, we provide evidence that honey bee brood plays an important role in hygiene-related communication. By cross-fostering honey bees from distinct breeding backgrounds, we were able to compare the relative importance of chemical brood signals and adult detection in triggering this economically important behavior associated with improved colony health. To our knowledge, this is the first behavioral study of honey bee hygiene employing a cross-foster design in order to disentangle the contributions of brood and adult nurses to hygienic behavior. While many previous behavioral studies of honey bee hygiene without a cross-foster design indicate enhanced

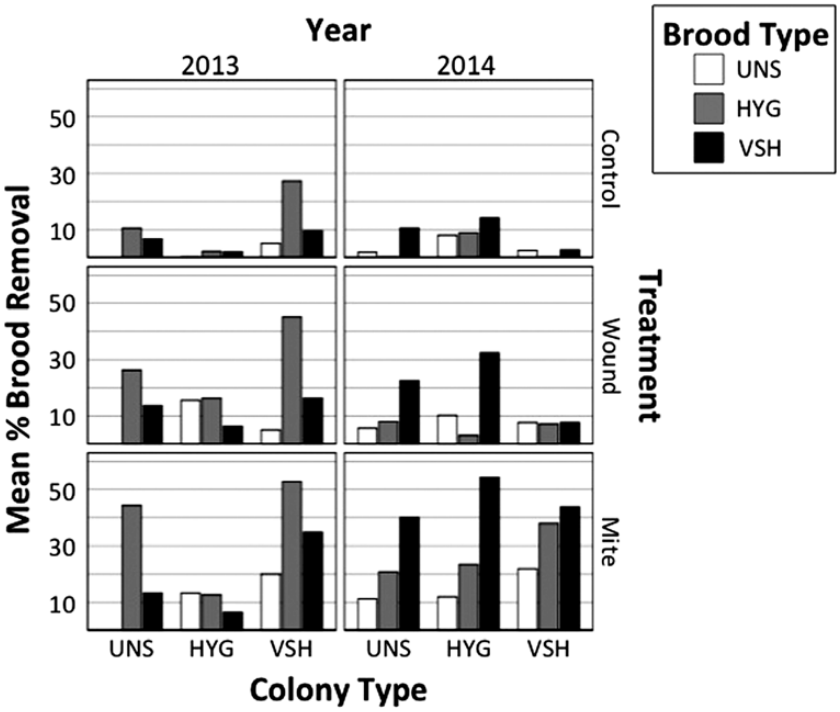

Fig. 3. Brood and colony type effects on mean percent brood removal. White, gray, and black bars represent UNS, HYG, and VSH brood, respectively. Effects of colony type and brood type on brood removal varied by year and by treatment type.

Table 2. Tests of model effects from a GLM of brood type and colony type effects on brood removal for each year by treatment combination

	Wald chi-square	df	Significance
2013			
Mite			
Brood effect	11.69	2	0.003
Colony effect	26.47	2	<0.001
Wound			
Brood effect	13.45	2	0.001
Colony effect	9.93	2	0.031
Control			
Brood effect	7.12	2	0.029
Colony effect	12.81	2	0.002
2014			
Mite			
Brood effect	27.804	2	<0.001
Colony effect	2.736	2	0.255
Wound			
Brood effect	12.498	2	0.002
Colony effect	3.373	2	0.185
Control			
Brood effect	4.416	2	0.110
Colony effect	7.325	2	0.026

Nonsignificant effects are indicated by gray text.

adult olfaction (Spivak 1996, Spivak and Reuter 2001b, Harbo and Harris 2005, Ibrahim and Spivak 2006), our results suggest that hygienic removal is driven by improved brood signaling in addition to improved signal detection by adults.

Our hypothesis that selection for hygienic behavior in honey bees has influenced brood signaling was supported by significant brood type effects on removal in mite-infested and wounded treatments in both 2013 and 2014. These findings indicate that signaling systems may be different in bees with different breeding histories. However, the differences between years also suggest heterogeneity within colony types. In 2014, VSH brood was more likely to be removed than HYG brood despite similarities in VSH and HYG hygiene levels for that year, suggesting that, in addition to hygiene level, a secondary brood characteristic may have been affecting removal. In colonies bred for high and low levels of hygiene, a recent study identified 501 and 342 differently expressed genes (respectively) when comparing hygiene-performing and nonperforming workers (Gempe et al. 2016). However, only 21 of these genes were shared by the two colony types (Gempe et al. 2016), supporting our findings that the effects of selection for enhanced hygienic behavior may vary as a result of breeding history. It is possible that differences in the effects of brood type between years and within years were related to susceptibility of brood to *Varroa*-related damage, virus levels, or other stressors (Page et al. 2016); however, brood susceptibility was not tested.

Evidence of a positive correlation between removal rate and the hygiene level of the removed brood's colony of origin (as determined by LNKB score) also supports our hypothesis that selection of hygienic behavior has influenced brood signaling. Brood from colonies considered highly hygienic based on the freeze-killed brood assay was removed more readily by all colony types (UNS, HYG, and VSH) than brood from less hygienic colonies. Though the positive correlation was only significant for mite-infested and control brood, the trends for all three treatments over both years suggest that the more hygienic the brood's colony of origin, the more likely that brood was to be removed.

Our hypothesis that brood signals are important to hygienic behavior was further supported by the lack of a positive correlation between removal rate of brood and the hygiene level (as determined by LNKB score) of the host colony. While these results were unexpected, they were consistent over both years and all three treatments. This lack of effect of host colony hygiene level on removal may be

Table 3. Parameter estimates from a GLM of brood type and colony type effects on brood removal for each year by treatment combination

		Wald chi-square	df	Significance	Odds Ratio with 95% Wald C.I.)
2013					
Mite					
Intercept		9.75	1	0.002	0.42 (0.24–0.72)
UNS	Brood	0.20	1	0.658	1.21 (0.52–2.82)
	Colony	2.46	1	0.116	0.59 (0.31–1.14)
HYG	Brood	10.36	1	0.001	2.63 (1.46–4.74)
	Colony	26.44	1	<0.001	0.19 (0.10–0.36)
Wound					
Intercept		25.70	1	<0.001	0.20 (0.10–0.37)
UNS	Brood	0.39	1	0.535	1.32 (0.55–3.18)
	Colony	1.46	1	0.227	0.63 (0.29–1.34)
HYG	Brood	11.71	1	0.001	3.25 (1.65–6.37)
	Colony	6.84	1	0.009	0.44 (0.23–0.81)
Control					
Intercept		24.45	1	<0.001	0.14 (0.06–0.30)
UNS	Brood	1.14	1	0.285	0.31 (0.04–2.69)
	Colony	3.55	1	0.059	0.39 (0.14–1.04)
HYG	Brood	3.98	1	0.046	2.58 (1.02–6.57)
	Colony	10.78	1	0.001	0.08 (0.02–0.36)
2014					
Mite					
Intercept		0.08	1	0.78	1.08 (0.65–1.77)
UNS	Brood	27.36	1	<0.001	0.21 (0.11–0.37)
	Colony	2.73	1	0.099	0.59 (0.32–1.10)
HYG	Brood	7.07	1	0.008	0.44 (0.24–0.81)
	Colony	0.56	1	0.454	0.80 (0.44–1.44)
Wound					
Intercept		24.67	1	<0.001	0.16 (0.08–0.32)
UNS	Brood	8.49	1	0.004	0.31 (0.14–0.68)
	Colony	1.26	1	0.261	1.73 (0.67–4.49)
HYG	Brood	7.60	1	0.006	0.23 (0.08–0.65)
	Colony	3.36	1	0.067	2.42 (0.94–6.21)
Control					
Intercept		21.59	1	<0.001	0.03 (0.01–0.14)
UNS	Brood	2.87	1	0.090	0.40 (0.14–1.16)
	Colony	0.99	1	0.320	2.35 (0.44–12.62)
HYG	Brood	2.89	1	0.089	0.32 (0.09–1.19)
	Colony	5.46	1	0.020	6.23 (1.34–28.91)

Nonsignificant effects are indicated by gray text.

Table 4. Hygiene levels of individual colonies, based on freeze-killed brood assays

Colony type	Year	% Brood removed (LNKB assay)
UNS	2013	51
UNS	2014	48
HYG	2013	91
HYG	2013	99
HYG	2013	100
HYG	2014	95
HYG	2014	99
VSH	2013	46
VSH	2013	80
VSH	2014	100
VSH	2014	97

an artifact of cross-fostering, which allowed us to differentiate the role of brood signaling from the role of adult signal perception. In a real colony setting this lack of effect would disappear because brood and adults of the same breed would be in the same colony. Indeed, examination of the subset of data in which brood type is the same as colony type is consistent with previous findings that VSH colonies tend to remove more mite-infested brood of their own breed than do HYG colonies (Ibrahim and Spivak 2006, Danka et al. 2013), and both colony types selected for hygienic behavior remove more mite-infested brood than do UNS colonies (Spivak and Reuter 2001b, Harbo and Harris 2005, Danka et al. 2013, Toufailia et al. 2014).

The main conclusions of our cross-fostering experiments are robust despite the small sample size, and are largely consistent with expectations from the literature. The collapse of multiple 2013 UNS colonies before they could be used experimentally was likely the result of our decision not to treat any experimental colonies for *Varroa* mites. These losses prevented repetition of three colony type by brood type combinations during that study year, and led us to repeat the study in 2014. High disease incidence caused by lack of *Varroa* treatment in experimental colonies may also explain the unexpected results for UNS brood in UNS colonies in 2013. The lack of changes to effect significance and the similar effect sizes indicated by the odds ratios in the models with and without this 2013 UNS data indicate minimal influence of the excluded data on the accuracy of the full model. The overall effect of treatment on brood removal

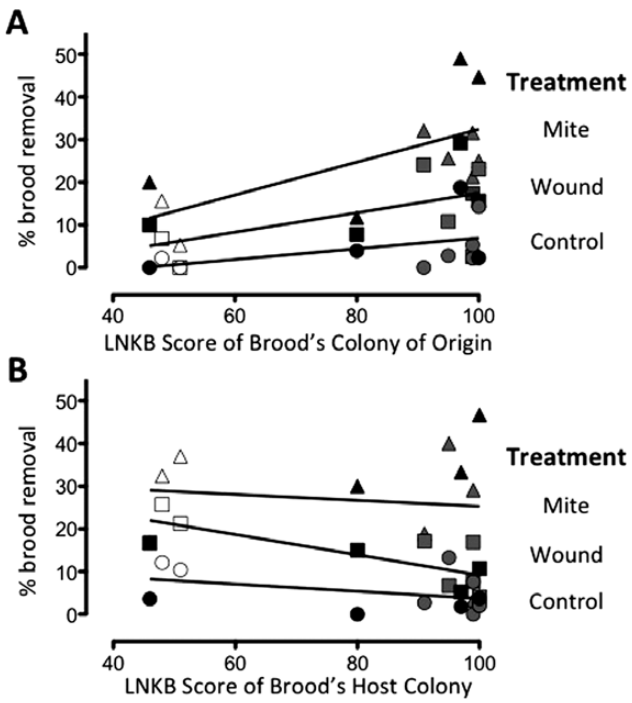

Fig. 4. Percent brood removal plotted against (a) the LNKB score of the brood's colony of origin and (b) the LNKB score of the brood's host colony. Triangular, square, and circular markers represent mite, wound, and control treatments, respectively, and correspond to the lines of best fit drawn beside respective treatment labels. White, gray and black symbols represent UNS, HYG, and VSH colonies, respectively. Colonies without LNKB data (n = 4) are not included. For mite-infested and control brood, there were significant positive correlations between brood removal and the LNKB score of the brood's colony of origin. There were no significant positive correlations between brood removal and the LNKB score of the brood's host colony.

in our study was similar to findings from previous studies (Spivak 1996, Harris et al. 2010), both in terms of absolute and relative brood removal rates of mite-infested and control brood. As stated previously, removal of mite-infested brood in the subset of data in which brood type was the same as colony type was consistent with previous research indicating the highest removal rates in VSH colonies, followed by HYG and then UNS colonies (Spivak and Gilliam 1998, Spivak and Reuter 2001a, Ibrahim and Spivak 2006, Harris 2007, Harris et al. 2012, Danka et al. 2013). However, it should be noted that performance of VSH colonies in 2013 LNKB assays was unexpectedly low.

Collectively, our findings suggest that honey bee brood might be as, or more important than honey bee adults in determining the frequency of hygienic tasks. These findings may explain why the hypothesis of increased olfactory sensitivity in hygienic honey bees was not supported by functional annotation of candidate genes previously associated with hygienic behavior (Le Conte et al. 2011). However, a number of studies have associated adult traits with stocks of bees selected for hygienic behavior (Masterman et al. 2001, Martin et al. 2002, Spivak et al. 2003). Thus, enhanced brood signaling and better adult detection of brood signals likely complement each other, co-evolving such that the more pronounced one ability, the weaker the other. This signal-detection coevolution would explain the inverse relationship between brood and colony type effects in our results. Interestingly, our 2013 data suggest that while HYG colonies removed less brood than VSH or UNS colonies, HYG brood was removed with greater frequency than both other brood types. Likewise in 2014, adult bees in VSH colonies removed less brood than adult bees in HYG colonies, but VSH brood was removed with greater frequency than HYG brood. This apparent inverse relationship between signal intensity and receiver sensitivity is consistent with response threshold models, which predict that increasing stimulus strength leads to recruitment of a larger number of task performers, and thus to greater genotypic variety among individuals involved in stimulus response (Fewell and Page 1993, Beshers and Fewell 2001). Consequently, colonies capable of producing strong brood signals in response to mite infestation would need relatively fewer adults with enhanced olfaction capabilities to complete hygienic tasks, although further research is necessary to test this idea. In a recent study, Gempe et al. (2016) reported that colonies bred for improved hygiene had three times the number of workers performing hygienic tasks than colonies bred for low hygienic performance. While the authors suggest that this may be a result of a lower threshold for stimulus perception in hygienic adults, our results suggest that it may also be a result of improved brood signaling. Increased numbers of workers performing hygienic tasks in certain colonies may also be related to increased brood susceptibility (Page et al. 2016), and/or effects of hygienic task performance on olfactory threshold sensitivity (Theraulaz et al. 1998). Regardless of the exact nature of the relationship between signal intensity and olfactory sensitivity, our findings demonstrate the likely importance of brood signaling to pest and pathogen resistance in Apidae, and potentially other social insects.

If left untreated, non-hygienic colonies of Western honey bees parasitized by *Varroa* typically die within 1–3 yr (Korpela et al. 1992, Fries et al. 2006), pointing to the urgency with which novel control methods are needed. This vulnerability to *Varroa* at the colony level is not seen in *Varroa*'s original host *A. cerana*, whose resistance is due at least in part to the evolution of social apoptosis (Page et al. 2016), and possibly to related brood signals responsible for triggering hygienic removal. Since *Varroa*-resistant bees do not require the time and expense of new compound development and approval, and do not lead to development of resistance or pesticide exposure and accumulation in bees, humans or ecosystems (Rinderer et al. 2010, Dietemann et al. 2012, reviewed in Plettner et al. 2017), one sustainable and environmentally responsible intervention approach is to further develop biological strategies for selective breeding of pest- and disease-resistant Western honey bees. The evidence for colony-type specific brood signals presented here not only highlights a previously neglected aspect of social immunity, but also represents a critical step toward the improvement of breeding strategies for *Varroa*-resistant honey bees. For example, improved signaling capabilities and lower thresholds of disease and/or *Varroa* detection may be achieved by basing honey bee selection on an olfactory trigger released by hygienic brood, rather than the less biologically relevant olfactory indicators released by LNKB. Selection may also be performed to improve brood signaling efficiency by increasing signals that unambiguously distinguish healthy and compromised brood. Our results predict differences between UNS, HYG, and VSH brood signaling abilities, encourage further studies to identify the chemical nature of honey bee brood signals, and have important implications for honey bee hygiene breeding strategies that could lead to improved colony health and survival. Augmented honey bee health resulting from improved *Varroa*-resistance through enhanced hygienic performance and the related decrease in exposure to chemical *Varroa* treatments may result in increased crop and honey bee product yields, and could prevent the spread of disease between honey bees and other more ecologically important native pollinators. Furthermore, these findings may lead to important insights regarding

communication within and between other social insects, including both beneficial and pest species.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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