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Original article

Field trial of honey bee colonies bred for mechanisms of resistance against *Varroa destructor**

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Abstract – We compared colonies selectively bred for both hygienic behavior and Suppression of Mite Reproduction (HYG/SMR) with colonies bred solely for hygienic behavior (HYG) and unselected control colonies. Colonies were evaluated for strength, brood viability, removal of freeze-killed brood, honey production, mite loads on adult bees and within worker brood, and mite reproductive success on worker brood for two years in two locations. By autumn in both years, the HYG/SMR colonies had significantly fewer mites on adult bees and in worker brood compared to the control colonies, and the HYG colonies had intermediate mite populations. Contrary to expectation, there were no differences among the lines in mite reproductive success. Further studies are required to determine if the genes and neural mechanisms that regulate the SMR trait are the same or different from those regulating hygienic behavior.

***Apis mellifera* / hygienic behavior / suppression of mite reproduction / breeding**

1. INTRODUCTION

This study reports on our progress in breeding honey bees that display mechanisms of resistance to the parasitic mite, *Varroa destructor* Anderson & Trueman. In 1994, we began selecting a line of bees derived from *Apis mellifera ligustica* Spinola for hygienic behavior, a well-known behavioral mechanism of resistance to two brood diseases of honey bees, American foulbrood (Rothenbuhler, 1964; Spivak and Reuter, 2001a) and chalkbrood (Gilliam et al., 1983; Spivak and Reuter, 2001a). The term hygienic behavior was coined by Rothenbuhler (1964) and refers to the genetic ability of bees within a colony to detect and remove diseased worker brood from the nest, thereby limiting disease transmission (reviewed in Spivak and Gilliam, 1998a,b). Hygienic bees most likely use olfactory cues to detect the abnormal brood (Masterman et al., 2001; Spivak et al., 2003).

Hygienic behavior is one of several known mechanisms of resistance against *V. destructor* (Peng et al., 1987; reviewed in Boecking and Spivak, 1999). Bees bred for hygienic behavior detect and remove worker brood infested with the parasitic mite *Varroa destructor* (Spivak, 1996). Other resistance mechanisms against the mites including grooming behavior, in which adult bees remove mites from other adults, damaging the mites in the process (reviewed in Boecking and Spivak, 1999); and an unknown physiological effect of either worker pupae or adult bees in some colonies that reduces mite reproduction (Camazine, 1986; Harbo and Hoopingarner, 1997; Harris and Harbo, 1999). We chose to breed bees for hygienic behavior because it would confer resistance to two diseases and would be one mechanism of defense against *V. destructor*.

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We have been working in collaboration with beekeepers to propagate and test our "Minnesota (MN) Hygienic" line. In our previous studies, we have shown that this line is highly resistant to American foulbrood and chalkbrood (Spivak and Reuter, 2001a). In two studies, this hygienic line had significantly lower mite loads compared to unselected colonies (Spivak and Reuter, 1998a, 2001b); however, to date MN Hygienic colonies are not able to maintain mite loads below a treatment threshold and eventually require some sort of control measure to prevent colony collapse.

In the US in 1997, a new line of bees was bred for the heritable trait, "Suppression of Mite Reproduction" or SMR (Harbo and Hoopingarner, 1997). In colonies of bees bred for SMR, the mites entered worker brood cells to feed and reproduce but mite population growth decreased over time apparently because of the low reproductive success of the mites (Harbo and Harris, 1999). The SMR line showed promise as stock with a good resistance mechanism against *V. destructor*. However, many SMR colonies suffered from poor brood viability and low honey production (M.S., personal observations), and our collaborating beekeepers were unwilling to test the pure SMR line in their colonies.

The goal of this research was to test genetic crosses between the SMR and the MN Hygienic line in apiaries of commercial beekeepers with the aim of maintaining the disease resistance, good brood viability, and high honey production of the hygienic line while increasing the degree of resistance to *V. destructor* through the incorporation of the SMR trait. At the time we initiated the crosses between the lines, the mechanism for the low reproductive success of mites within SMR colonies was not understood. Subsequent studies revealed that in fact SMR colonies selectively remove worker pupae infested with reproductively successful mites (Harbo and Harris, 2005; Ibrahim and Spivak, 2006). This selective removal behavior is a type of hygienic behavior, but this finding was not known to us when we began this study.

We evaluated colonies derived from crosses between the Hygienic and SMR lines (hereafter called HYG/SMR colonies), pure HYG colonies and unselected control colonies, in commercial apiaries in Minnesota and North Dakota. We measured the colonies for colony strength, brood viability, hygienic behavior, honey production, and mite loads on adult bees and within worker brood, and mite reproductive success on worker brood. Although the breeding program and our evaluations in commercial apiaries are ongoing, we report here on the evaluations from 2003 and 2004.

2. METHODS

2.1. Original breeding stock

The MN Hygienic line was derived from a composite of Italian-derived (*A. mellifera ligustica*) colonies that are available commercially in the US. Each year, only the colonies that displayed high honey production, good winter survivorship, gentle temperament, and hygienic behavior (based on a freeze-killed brood assay, see below) were propagated as breeding stock through instrumental insemination. The SMR line was derived from an unknown composite of colonies in the US, mainly from Louisiana, and the breeding stock was also propagated through instrumental insemination.

In 2001 and 2002, we made two crosses between the HYG and SMR lines at the University of Minnesota. In 2001, queens from the HYG line were inseminated with 8 μL semen from drones from the SMR line to produce 50:50 hybrid progeny colonies. In 2002, queens reared from the hybrid colonies were inseminated with semen from SMR drones to produce worker progeny that were, on average, 75% SMR:25% HYG.

2.2. HYG/SMR test crosses

To obtain test crosses for the present study, we gave five queens from the 75% SMR:25% HYG cross to two commercial beekeepers, one from Minnesota (MN) and one from North Dakota (ND) who transport their migratory beekeeping operations to the pine forests in southeastern Texas every year from November to May. In March 2003 in Texas, the beekeepers raised queens from the 75% SMR:25% HYG queens and let the queens mate naturally with drones in the surrounding area. We assumed the drones originated from the HYG line because these beekeepers have been raising

daughter queens annually from that line since 1997. Therefore, the queens' worker progeny would be, on average, approximately 63% HYG and 37% SMR. In March 2004, queens were reared from three of the 63% HYG and 37% SMR colonies and were mated naturally in southeastern Texas, again with drones from the HYG line so that the worker progeny would be approximately 82% HYG:18 %SMR. The colonies containing these HYG/SMR queens were evaluated in summers of 2003 and 2004 in MN and ND after the beekeepers transported them back to their apiaries these northern states.

2.3. HYG and control lines

In 2003 and 2004, we compared the HYG/SMR colonies to two lines: our MN Hygienic line (not crossed with SMR; hereafter called HYG) and with an unselected line of Italian-derived bees that had not been selected for hygienic or SMR traits, hereafter called Control. For the HYG colonies, in 2003 and again in 2004, we gave the two beekeepers queens from the MN Hygienic line that were reared and instrumentally inseminated at the University of Minnesota. Daughters of these queens were allowed to mate naturally with drones in the same beekeepers' apiaries in TX each year. For the Control colonies, naturally mated queens were purchased each year from a different beekeeper in eastern Texas, located about 40 km away from our two collaborators.

2.4. Experimental design

All colonies used in this experiment were initiated from "divides" made from the same stock of parent colonies each year in Texas, and queens from the three lines were introduced into the divides. In this way, initial mite levels were randomized among colonies. All of the colonies owned by the beekeeper from MN had been treated for mites in the fall of 2002 with coumaphos (CheckMite+®) and the test colonies were not treated again until our evaluations were terminated in September 2003. The beekeeper from ND did not treat his colonies for mites in the fall of 2002 or spring of 2003, but used Check Mite+ in the test colonies after our evaluations in September 2003 and did not treat any of his colonies again until after the experiment was terminated in fall of 2004.

The naturally mated queens from the HYG/SMR, HYG and Control lines were marked to indicate their line. In MN 2003, the colonies were situated in four apiaries with 6–8 colonies per line. In ND 2003, the colonies were situated in three apiaries, with 8 colonies per line. In 2004, colonies were first located in one large apiary in ND until mid-June after which they were distributed among 3 apiaries, each with 10–14 colonies per line. In 2003, each colony was maintained in two standard "deep" Langstroth brood boxes, with 9 frames in the top box, and 8 frames and a frame feeder in the bottom box. In 2004, the colonies were maintained in one deep brood box. In all cases, honey supers were added above a queen excluder by the beekeepers as needed.

2.5. Colony evaluation criteria

In late May and early June 2003 in MN and ND, and in June 2004 in ND, colonies with marked queens were evaluated for colony strength (frames of bees and brood), worker brood viability, hygienic behavior, and the number of mites on adult bees. In September, the colonies that still contained marked queens (i.e., those that did not supersede or replace the queens) were evaluated for honey production, number of mites on adult bees, percentage of mites in worker brood, and mite reproductive success.

Combs of bees and brood. Adult worker bee population was visually determined by estimating the number of combs covered by bees in each brood box. The number of combs containing worker brood was calculated by visually estimating the proportion of each side of the frame that contained worker brood.

Brood pattern. In September 2003, brood patterns were measured in each colony by counting, on three different combs, the number of empty cells within a 100 cell area containing sealed worker brood within 1–2 days of emergence. The average number of empty cells in the three 100-cell areas was calculated per colony.

Hygienic behavior. This assay provides an indirect measure of the ability of the colony to uncap and remove diseased and mite-infested brood. All colonies were tested for hygienic behavior by freezing a circular section of sealed worker brood containing 160 cells within the comb using liquid nitrogen (method described by Spivak and Reuter, 1998b). After 24 hours, the number of dead pupae that were in the process of being removed (were uncapped and/or partially removed), and the number completely removed from the cells were recorded. In our selection criteria for breeding stock, only those colonies that uncap and remove > 95% of the

freeze-killed brood within 24 hours are considered hygienic.

Number of Mites on Adult Bees. In June and again in September, approximately 500 adult workers were collected from a central comb containing open brood into a container containing 75% EtOH. In the lab, the mites were shaken off the bees through a strainer (following methods of De Jong et al., 1982). The number of mites per sample was counted and from the weight of bees in each sample and a known weight of 100 wet bees from the same samples, the number of mites per 100 bees was calculated.

Percent infestation in worker brood and mite reproductive success. A section of comb containing 150–200 pupae in the gray wing pad stage (Martin, 1994) was cut out of a comb in each colony, and frozen at –20 ˚C to preserve the integrity of the mites and mite progeny. In the lab, each cell was inspected under a dissecting microscope and the percentage of sealed brood containing mites was calculated. In September each year, two measures of mite reproductive success were recorded. Mite fertility was calculated for each colony by dividing the number of reproductive foundresses (mites that produced at least one male offspring) by the number of pupal cells containing one foundress mite. Number of viable offspring was determined by dividing the total number of adult female daughters by the total number of pupal cells containing one foundress mite (including reproductive and non reproductive foundress). Adult daughters can be recognized by the presence of a live, adult male and the presence of a female with a shed exuvia of the final molt, or if the exuvia could not be found, by light dark brownish color of the mite's body (Martin, 1994; Corrêa-Marques et al., 2003).

Honey production. Honey production was measured by weighing the boxes of honey as they were removed by the beekeepers and subtracting the tare weight of the boxes from the total weight.

2.6. Statistical analysis

When the data were normally distributed, or a suitable transformation was found that would satisfy the assumptions of normality, all measures were analyzed using 2-way ANOVA, with line of bee and apiaries modeled as main effects. Post-hoc comparison of means was done using a Tukey-Kramer HSD test (JMP software, SAS, 1994). Measures of percent mite fertility were analyzed using non-parametric Kruskal-Wallace tests.

3. RESULTS

3.1. Measures of colony strength and honey production

Combs of bees. In June 2003, there were no statistical differences in numbers of combs of bees between the HYG/SMR, HYG and Control colonies in both MN and ND (Tab. I). In June 2004, the Control colonies had significantly more combs of bees compared to the HYG/SMR and HYG colonies.

Combs of worker brood. The HYG/SMR colonies had significantly fewer combs of worker brood compared to both the HYG and Control colonies in June of both years (Tab. I). There was a significant interaction between line and apiary in MN in 2003, when the HYG and Control colonies had less brood in one apiary compared to the other two apiaries.

Brood pattern. In September 2003, there were no significant differences in brood patterns among the lines in either state (Tab. I). The percentage of empty cells on combs containing late stage, sealed worker pupae was not measured in ND in 2004.

Honey production. In MN, the HYG/SMR colonies produced significantly less honey compared to the Control colonies, but this difference was not found in ND in 2003. There were significant apiary effects in 2003 in which all colonies within particular apiaries produced significantly more or less honey compared to colonies in other apiaries, irrespective of their line. There were no significant differences among the lines in honey production in 2004. However, honey production was low overall in ND in 2004 due to unusual weather conditions.

3.2. Measures of hygienic behavior and mite levels

Hygienic behavior. In both states and both years, the HYG/SMR and HYG colonies removed significantly more freeze-killed brood within 24 hours compared to the Control colonies (Tab. II). More specifically, 27 of 42 (64%) of the HYG/SMR colonies tested in 2003 and 32 of 34 (94%) tested in 2004 removed >95% of the freeze-killed brood within

Table I. Measurements of colony strength (frames bees, worker brood, and brood pattern) and honey production for the HYG/SMR, HYG and Control colonies in apiaries in Minnesota (MN) and North Dakota (ND) in 2003 and in ND in 2004. Means for each measure among the three lines of bees, within a state and year, followed by different letters are significantly different at $P < 0.05$ based on Tukey-Kramer HDS tests.

Colony measure	State Year	Line of bees	mean ± s.d. (n)		Statistics	
Combs of bees (June)	MN 2003	HYG/SMR	14.5 ± 1.6 (23)	a	Line:	F = 2.91; df = 2,60; $P = 0.062$
		HYG	15.5 ± 1.3 (20)	a	Apiary:	F = 0.27; df = 3,60; $P = 0.845$
		Control	15.5 ± 1.6 (29)	a	L*A:	F = 2.06; df = 6,60; $P = 0.071$
	ND 2003	HYG/SMR	12.2 ± 2.2 (19)	a	Line:	F = 1.48; df = 2,53; $P = 0.234$
		HYG	13.3 ± 3.2 (21)	a	Apiary:	F = 6.97; df = 2,53; $P = 0.002$
		Control	13.0 ± 2.0 (22)	a	L*A:	F = 2.40; df = 4,53; $P = 0.061$
	ND 2004	HYG/SMR	6.1 ± 2.0 (34)	a	Line:	F = 8.24; df = 2,107; $P = 0.0005$
		HYG	6.2 ± 1.8 (32)	a		(colonies located in one apiary in June)
		Control	7.5 ± 1.5 (44)	b		
Combs of worker brood (June)	MN 2003	HYG/SMR	5.7 ± 1.5 (23)	a	Line:	F = 3.31; df = 2,59; $P = 0.043$
		HYG	6.9 ± 1.5 (20)	b	Apiary:	F = 0.64; df = 3,59; $P = 0.590$
		Control	7.1 ± 2.4 (28)	b	L*A:	F = 2.56; df = 3,59; $P = 0.029$
	ND 2003	HYG/SMR	6.1 ± 0.6 (19)	a	Line:	F = 16.97; df = 2,53; $P < 0.0001$
		HYG	7.5 ± 1.4 (21)	b	Apiary:	F = 4.24; df = 2,53; $P = 0.020$
		Control	7.8 ± 0.8 (22)	b	L*A:	F = 1.63; df = 4,53; $P = 0.181$
	ND 2004	HYG/SMR	1.8 ± 0.7 (34)	a	Line:	F = 7.86; df = 2,107; $P = 0.0007$
		HYG	2.6 ± 0.8 (32)	b		(colonies located in one apiary in June)
		Control	2.6 ± 1.1 (44	b		
Brood Pattern % empty cells (Sept.)	MN 2003	HYG/SMR	12.4 ± 5.3 (22)	a	Line:	F = 0.50; df = 2,52; $P = 0.609$
		HYG	12.0 ± 7.0 (17)	a	Apiary:	F = 0.92; df = 3,52; $P = 0.439$
		Control	14.3 ± 7.1 (20)	a	L*A:	F = 0.65; df = 6,52; $P = 0.691$
	ND 2003	HYG/SMR	21.6 ± 8.8 (19)	a	Line:	F = 0.33; df = 2,45; $P = 0.722$
		HYG	20.2 ± 6.4 (13)	a	Apiary:	F = 9.43; df = 2,45; $P = 0.0004$
		Control	19.9 ± 7.5 (22)	a	L*A:	F = 0.85; df = 4,45; $P = 0.498$
Honey kg (Sept.)	MN 2003	HYG/SMR	56.7 ± 20.7 (23)	a	Line:	F = 11.73; df = 2,52; $P < 0.001$
		HYG	71.6 ± 28.0 (19)	ab	Apiary:	F = 2.82; df = 3,52; $P = 0.048$
		Control	92.5 ± 27.4 (22)	b	L*A:	F = 0.76; df = 6,52; $P = 0.601$
	ND 2003	HYG/SMR	68.1 ± 23.2 (19)	a	Line:	F = 2.08; df = 2,46; $P = 0.136$
		HYG	68.4 ± 26.3 (14)	a	Apiary:	F = 3.66; df = 2,46; $P = 0.033$
		Control	80.9 ± 23.7 (22)	a	L*A:	F = 0.78; df = 4,46; $P = 0.547$
	ND 2004	HYG/SMR	28.6 ± 13.0 (18)	a	Line:	F = 2.75; df = 2,44; $P = 0.075$
		HYG	17.8 ± 8.9 (12)	a	Apiary:	F = 0.07; df = 2,44; $P = 0.928$
		Control	31.4 ± 18.6 (23)	a	L*A:	F = 0.45; df = 4,44; $P = 0.773$

24 h and therefore could be considered as potential breeder colonies for this trait. Among the HYG colonies tested in 2003 and 2004, 16 of 34 (47%) and 24 of 32 (75%) respectively, removed >95% within 24 h. In contrast, among the Control colonies, only 6 of 51 (12%) in 2003 and 10 of 44 (23%) in 2004 would be considered hygienic enough for breeding purposes.

Mites on adult bees. In June of 2003, there were no significant differences among the lines in the number of mites per 100 adult bees

Table II. Measurements of hygienic behavior, mite levels on adult bees and in worker brood, and mite reproductive success for the HYG/SMR, HYG and Control colonies in apiaries in Minnesota (MN) and North Dakota (ND) in 2003, and in ND in 2004. Means for each measure among the three lines of bees, within a state and year, followed by different letters are significantly different at $P < 0.05$ based on Tukey-Kramer HDS tests.

Colony measure	State Year	Line of bees	mean ± s.d. (n)		Statistics	
Hygienic Behavior	MN	HYG/SMR	91.0 ± 14.1 (23)	a	Line:	F = 15.61; df = 2,60; $P < 0.0001$
% removed 24 h	2003	HYG	86.7 ± 13.9 (20)	a	Apiary:	F = 0.08; df = 3,60; $P = 0973$
(June)		Control	67.5 ± 20.5 (29)	b	L*A:	F = 1.30; df = 6,60; $P = 0.271$
	ND	HYG/SMR	91.2 ± 9.2 (19)	a	Line:	F = 17.85; df = 2,46; $P < 0.0001$
	2003	HYG	94.8 ± 5.9 (14)	a	Apiary:	F = 2.25; df = 2,46; $P = 0.117$
		Control	72.4 ± 15.6 (22)	b	L*A:	F = 1.37; df = 4,46; $P = 0.258$
	ND	HYG/SMR	99.2 ± 2.3 (34)	a	Line:	F = 43.5; df = 2,107; $P < 0.0001$
	2004	HYG	94.8 ± 7.9 (32)	a		
		Control	82.2 ± 15.1 (44)	b		
Mites/100 adult bees	MN	HYG/SMR	0.02 ± 0.11 (22)	a	Line:	F = 0.49; df = 2,50; $P = 0.616$
(June)	2003	HYG	0.07 ± 0.22 (20)	a	Apiary:	F = 1.31; df = 3,50; $P = 0.282$
		Control	0.06 ± 0.11 (20)	a	L*A:	F = 1.19; df = 6,50; $P = 0.326$
	ND	HYG/SMR	1.5 ± 2.2 (19)	a	Line:	F = 0.43; df = 2,53; $P = 0$ .651
	2003	HYG	1.2 ± 1.0 (21)	a	Apiary:	F = 0.01; df = 2,53; $P = 0.990$
		Control	1.2 ± 1.1 (22)	a	L*A:	F = 3.80; df = 4,53; $P < 0.009$
	ND	HYG/SMR	0.2 ± 0.2 (33)	a	Line:	F = 0.12; df = 2,105; $P = 0.888$
	2004	HYG	0.2 ± 0.3 (31)	a		
		Control	0.2 ± 0.3 (44)	a		
Mites/100 adult bees	MN	HYG/SMR	0.9 ± 1.0 (23)	a	Line:	F = 7.74; df = 2,51; $P = 0.001$
(Sept)	2003	HYG	2.7 ± 3.7 (19)	b	Apiary:	F = 6.02; df = 3,51; $P = 0.001$
		Control	3.9 ± 5.0 (20)	b	L*A:	F = 0.67; df = 6,51; $P = 0.675$
	ND	HYG/SMR	7.8 ± 6.3 (19)	a	Line:	F = 5.42; df = 2,46; P =0.008
	2003	HYG	8.3 ± 4.6 (14)	a	Apiary:	F = 0.38; df = 2,46; $P = 0.689$
		Control	14.4 ± 9.4 (22)	b	L*A:	F = 2.36; df = 4,46; $P = 0.067$
	ND	HYG/SMR	0.6 ± 0.5 (20)	a	Line:	F = 6.03; df = 2,53; P =0.004
	2004	HYG	2.1 ± 2.2 (16)	b	Apiary:	F = 0.48; df = 2,53; $P = 0.621$
		Control	2.1 ± 2.8 (26)	b	L*A:	F = 1.55; df = 4,53; $P = 0.202$
% Mites in Brood	MN	HYG/SMR	0.9 ± 1.1 (22)	a	Line:	F = 10.6; df = 2,45; $P = 0.0002$
(Sept)	2003	HYG	2.5 ± 2.1 (16)	a	Apiary:	F = 4.41; df = 3,45; $P = 0.008$
		Control	6.9 ± 7.7 (19)	b	L*A:	F = 3.69; df = 6,45; $P = 0.005$
	ND	HYG/SMR	24.6 ± 17.1 (18)	a	Line:	F = 14.0; df = 2,39; $P < 0.0001$
	2003	HYG	27.1 ± 16.1 (11)	a	Apiary:	F = 0.28; df = 2,39; $P = 0.757$
		Control	53.9 ± 18.7 (19)	b	L*A:	F = 0.77; df = 4,39; $P = 0.550$
	ND	HYG/SMR	2.3 ± 3.3 (21)	a	Line:	F = 4.72; df = 2,53; $P = 0.013$
	2004	HYG	5.5 ± 5.2 (16)	ab	Apiary:	F = 1.40; df = 2,53; $P = 0.256$
		Control	6.4 ± 5.3 (25)	b	L*A:	F = 0.76; df = 4,53; $P = 0.556$
Mite Fertility (% foun-	MN	HYG/SMR	91.6 ± 26.8 (14)	a	Line:	Chi-sq = 1.55, df = 2, $P = 0.460$
dress with at least one	2003	HYG	93.8 ± 11.1 (14)	a		
offspring)		Control	96.0 ± 6.9 (14)	a		
(Sept.)						
	ND	HYG/SMR	92.7 ± 7.6 (18)	a	Line:	Chi-sq = 1.47, df = 2, $P = 0.479$
	2003	HYG	91.8 ± 7.5 (11)	a		
		Control	90.0 ± 8.8 (19)	a		
	ND	HYG/SMR	96.3 ± 8.6 (18)	a	Line:	Chi-sq = 0.97, df = 2, $P = 0615$
	2004	HYG	97.1 ± 10.7 (14)	a		
		Control	97.1 ± 7.6 (23)	a		
Number of Viable						
female mite	MN	HYG/SMR	1.07 ± 0.51 (14)	a	Line:	F = 0.05; df = 2,30; $P = 0.955$
Offspring (NVO)	2003	HYG	1.11 ± 0.33 (14)	a	Apiary:	F = 0.08; df = 3,30; $P = 0.971$
(Sept.)		Control	1.05 ± 0.10 (14)	a	L*A	F = 0.57; df = 6,30; $P = 0.752$
	ND	HYG/SMR	0.94 ± 0.37 (18)	a	Line:	F = 0.99; df = 2,39; $P = 0.382$
	2003	HYG	1.10 ± 0.28 (11)	a	Apiary:	F = 0.16; df = 2,39; $P = 0.849$
		Control	1.12 ± 0.38 (19)	a	L*A	F = 1.13; df = 4,39; $P = 0.354$
	ND	HYG/SMR	0.93 ± 0.36 (18)	a	Line:	F = 0.30; df = 2,46; $P = 0.742$
	2004	HYG	0.95 ± 0.08 (14)	a	Apiary:	F = 0.12; df = 2,46; $P = 0.889$
		Control	0.98 ± 0.21 (23)	a	L*A	F = 0.63; df = 4,46; $P = 0.647$

in MN or ND (Tab. II). The mite levels in all colonies in MN in 2003 were very low, and most colonies did not have a detectable number of mites. In contrast, mite levels were higher in ND colonies in 2003 because that beekeeper had not treated his colonies the previous fall. By September 2003, the HYG/SMR colonies had significantly fewer mites on adult bees than the HYG and the Control colonies in MN (Tab. II). In ND in September 2003, both the HYG/SMR and HYG colonies had significantly fewer mites on adult bees compared to the Control colonies.

There were no significant differences in the number of mites per 100 adult bees among the lines in June 2004 (Tab. II). However, by September 2004, the HYG/SMR colonies had significantly fewer mites on adult bees than the other two lines, although the mite levels in all colonies were still relatively low.

Mites in Brood. In 2003 in both states, the HYG/SMR and HYG colonies had significantly fewer mites in worker brood compared to the Control colonies (Tab. II). In September 2004, the HYG/SMR colonies had significantly fewer mites in brood compared to the Control colonies.

Mite reproductive success. No significant differences were found among the lines in September 2003 and 2004 for either measure of mite reproductive success. The average percent fertility of the foundress mites and the number of viable female offspring (NVO) produced by each foundress were equivalent among the lines in both states and both years (Tab. II).

3.3. Relationship between hygienic behavior and mite levels

The percentage of mites in brood and the number of mites on adult bees were plotted as a function of hygienic behavior (% removal of freeze-killed brood) for each colony in ND in the fall of 2003 (Fig. 1). Both revealed significant slopes (mites on adults: $F = 12.64$; df = 1,53; $P < 0.001$; mites in brood: $F = 25.30$; df = 1, 42; $P < 0.0001$). The R-squared value for mites in brood as a function of hygienic behavior ($r^2 = 0.376$) was higher than for mites on adult bees ($r^2 = 0.193$).

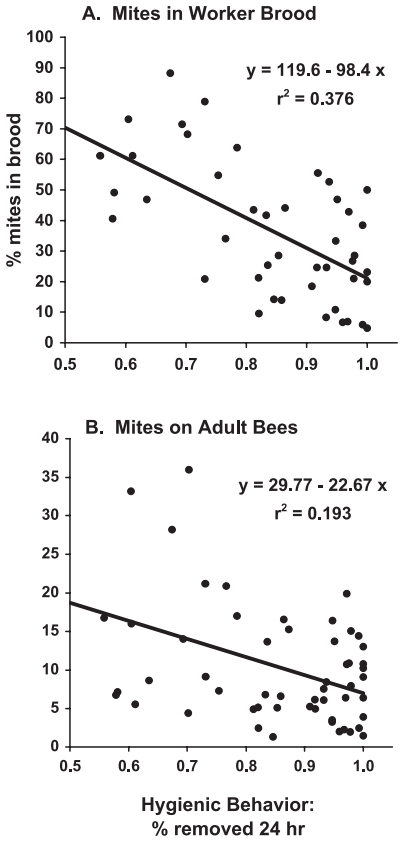

Figure 1. The percentage of mites in brood (1a) and the number of mites per 100 adult bees (1b) plotted as linear functions of hygienic behavior for all colonies in North Dakota in September 2003 when mite levels were relatively high in all colonies.

4. DISCUSSION

The main aim of this study was to determine if the incorporation of the SMR trait into the "MN Hygienic" line of bees would decrease the mite level in the HYG/SMR colonies relative to the MN Hygienic (HYG) and Control colonies, while maintaining high levels of hygienic behavior (correlated with disease resistance), good brood viability and high honey production. In September in both years and both states, the HYG/SMR colonies had significantly fewer mites on adult bees and in worker brood compared to the Control colonies. Overall, the HYG colonies had intermediate levels of mites on both adult bees

and in brood compared to the HYG/SMR and Control colonies, which shows that the addition of the SMR trait to the HYG line did help lower mite levels. Concomitantly, the proportion of colonies within each line that expressed hygienic behavior, (i.e., removed >95% of the freeze-killed brood within 24 h) was higher in the HYG/SMR colonies (64% and 94% in 2003 and 2004, respectively), and HYG colonies (47% and 75%) compared to the Control colonies (12% and 23%). Although we did not test disease resistance directly in this study, based on previous studies (reviewed in Spivak and Gilliam, 1998a, b; Spivak and Reuter, 2001a) the results of the freeze-killed brood assay suggest that majority of HYG/SMR colonies would not show clinical symptoms of chalkbrood (*Ascosphaera apis*) or American foulbrood (*Paenibacillus larvae*). Although we did notice problems in brood viability in our initial HYG/SMR crosses in 2001 and 2002 (unpublished data), brood viability was the same among the lines in the tested crosses in 2003. The HYG/SMR colonies produced significantly less honey than the Control colonies in MN in 2003, but there was no significant difference among the three lines in ND in 2003 and 2004. Therefore, the addition of the SMR trait into the MN Hygienic line tended to decrease mite levels on adult bees and in worker brood and to maintain or increase the level of hygienic behavior, but did not affect brood viability or honey production relative to the HYG line.

Based on previous studies (Harbo and Hoopingarner, 1997; Harris and Harbo, 1999), and those run concurrently with the present study (Harbo and Harris, 2005; Ibrahim and Spivak, 2006), we expected to see significantly lower mite reproductive success within the HYG/SMR colonies relative to the HYG and particularly the Control colonies. However, measures of fertility and number of viable female offspring were the same among the three lines in both years. Harbo and Harris (2005) concluded that the SMR trait could be explained by the hygienic behavior of adult SMR bees, which selectively remove only pupae infested with mites that have laid eggs. If SMR bees do not remove pupae infested with mites that lay no eggs, the relative frequency of infertile mites within the colony should increase over time, which could explain the mechanism for the low reproductive success we observed in the SMR line (Ibrahim and Spivak, 2006). In the present study, the majority of HYG/SMR colonies expressed a high degree of hygienic behavior based on the freeze-killed brood assay, suggesting they are highly sensitive to cues that trigger the removal of abnormal brood. But they did not selectively remove pupae with fertile mites leaving pupae with infertile mites, and the mites on worker pupae that were not removed produced the same number of viable offspring as the other lines. Further studies are required to determine if the selective removal of pupae with fertile mites is observed only in pure SMR colonies. It also remains to be determined if the genes and neural mechanisms for the SMR trait are the same or different as those for hygienic behavior (Rothenbuhler, 1964; Lapidge et al., 2002; Spivak et al., 2003).

One important finding is that we are now able to document is that it is possible to maintain the hygienic trait through natural matings of queens bees. Hygienic behavior is a recessive trait (Rothenbuhler, 1964). Honey bee queens are highly polyandrous and mate with 10–20 males (Estoup et al., 1994). Previous studies have shown that for a colony to express hygienic behavior, at least 50% of the workers in the colony must express the trait (Trump et al., 1967; Arathi et al., 2001). Therefore, at least 50% of the drones the queen mates with must have the genes for hygienic behavior. We have maintained the MN Hygienic line by instrumentally inseminating queen bees from hygienic colonies with semen collected from drones from other hygienic colonies. The beekeepers from MN and ND have been raising daughters queens from these breeders and have been introducing the daughters into their colonies in TX for over 6 years. Our findings of high levels of hygienic behavior (Tab. II) indicate that there are sufficient drones from hygienic colonies in the areas surrounding their apiaries in southeastern Texas so that worker progeny from naturally mated queens express the trait. This finding indicates that similar results could be obtained in any location if sufficient beekeepers use hygienic stock.

Another important point we reinforce in this study is that selecting colonies for hygienic behavior does in fact reduce mite levels compared to unselected colonies (see also Spivak and Reuter, 1998a, 2001b). But the regression of mites on adult bees and in worker brood as a function of hygienic behavior (Fig. 1) indicates that expressing hygienic behavior based on our criteria for the freeze-killed brood assay is not sufficient for mite resistance. In addition to selecting colonies that are hygienic and that contain mites in worker brood with low reproductive success, it may be worthwhile to also select for another trait, such as grooming behavior, which would limit the number of mites on adult bees (Ruttner and Hänel, 1992; Thakur et al., 1997; Arechavaleta-Velasco and Guzmán-Novoa, 2001; Mondragon et al., 2005).

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Test sur le terrain de colonies d'abeilles domestiques sélectionnées pour des mécanismes de résistance contre l'acarien *Varroa destructor*.

***Apis mellifera* / comportement hygiénique / sélection / *Varroa destructor* / Acari / mécanisme de résistance / suppression de la reproduction**

Zusammenfassung – Feldversuch mit Honigbienenvölkern die auf Resistenz gegenüber *Varroa destructor* gezüchtet wurden. Wir stellen einen Feldversuch mit Bienenvölkern vor, die in den USA auf Resistenz gegenüber der parasitischen Milbe *Varroa destructor* gezüchtet wurden. Wir verglichen Bienenvölker, die entweder hinsichtlich Hygieneverhalten (HYG) oder Unterdrückung der Milbenreproduktion (SMR) selektiert wurden mit Bienenvölkern, die auf beide Resistenzparameter hin selektiert wurden (HYG/SMR). Als Kontrolle dienten unselektierte Bienenvölker. Die Versuche wurden in Minnesota und Nord-Dakota in den Jahren 2003/2004 durchgeführt. Bei den Versuchsvölkern wurden die Volksstärke, die Überlebensfähigkeit der Brut, das Ausräumen gefrorener Brut, der Honigertrag, der Bienen- und Brutbefall sowie der Reproduktionserfolg der Milben in der Arbeiterinnenbrut (Fertilität und lebensfähige weibliche Nachkommen) erfasst. In beiden Jahren hatten im Frühsommer die HYG/SMR-Völker signifikant weniger Brutwaben als die HYG- und die Kontrollvölker, obwohl die Überlebensfähigkeit der Brut in allen drei Versuchsgruppen identisch war. Auch die Honigproduktion war in allen drei Versuchsgruppen gleich außer im Jahr 2003, als die HYG/SMR-Völker in Minnesota signifikant weniger Honig als die Kontrollvölker sammelten. Beim Hygieneverhalten entfernten die HYG/SMR- und die HYG-Völker innerhalb von 24 h signifikant mehr gefrorene Brut als die Kontrollvölker an allen Bienenständen und in beiden Jahren. Die HYG/SMR-Völker hatten im September beider Untersuchungsjahre einen signifikant geringeren Milbenbefall der Bienen und der Brut im Vergleich zu den Kontrollen. Insgesamt lag der Milbenbefall der Bienen und der Brut bei den HYG-Völker zwischen dem Befall der HYG/SMR-Völker und dem der Kontrollvölker. Dies zeigt, dass durch den zusätzlichen Resistenzparameter „SMR" bei den HYG-Linien der Milbenbefall reduziert wurde. Entgegen unseren Erwartungen gab es zwischen den Versuchsgruppen keine Unterschiede im Reproduktionserfolg der Milben. Diese Ergebnisse bestätigen unsere früheren Ergebnisse, nach denen Bienenvölker, die über das Ausräumen gefrorener Brut auf Hygieneverhalten selektiert wurden, einen geringeren Milbenbefall aufweisen als unselektierte Völker. Allerdings muss in weiteren Versuchen überprüft werden, ob die genetischen Regulationsmechanismen für die SMR-Eigenschaft dieselben sind wie die für das Hygieneverhalten.

***Apis mellifera* / Hygieneverhalten / Unterdrückung der Milbenreproduktion / Zucht**

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