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Original article

Selection of VSH-derived "Pol-line" honey bees and evaluation of their *Varroa* -resistance characteristics

Robert G. Danka[1], Jeffery W. Harris[2], Garrett E. Dodds[1]

[1]USDA-ARS Honey Bee Breeding, Genetics and Physiology Laboratory, 1157 Ben Hur Road, Baton Rouge, LA 70820, USA

[2]Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University, Starkville, MS 39762, USA

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Abstract – Honey bees with *Varroa* sensitive hygiene (VSH) have good resistance to *Varroa destructor*. We bred "Pol-line" bees by outcrossing VSH queens to US commercial stocks from 2008 to 2014 and then selecting colonies with low mite infestations. Beginning in 2011, field performance of colonies with outcrossed Pol-line queens was compared to colonies with outcrossed VSH queens. Mite infestations after one season were comparable in colonies of the two bee types. Queens from the most functional colonies of both bee types were added to the Pol-line breeding population each year. Mite resistance was investigated further by exposing mite-infested brood to colonies for 1 week in lab tests. The two bee types did not differ in the percentage of infested brood they removed or in the percentage of non-reproduction among remaining mites. Introgressing the VSH trait into commercial honey bee stock shows promise in creating bees that have useful mite resistance and desirable beekeeping characteristics.

***Apis mellifera* / *Varroa destructor* / mite resistance / *Varroa* sensitive hygiene / breeding**

1. INTRODUCTION

The ectoparasitic mite *Varroa destructor* Anderson and Trueman is widely regarded as a primary threat to the health of honey bees, *Apis mellifera* L., worldwide (Rosenkranz et al. 2010). A desirable approach to mitigating this threat is to select and breed bees for enhanced resistance to the mite. Various breeding efforts have produced some types of bees with demonstrable mite resistance; some show good beekeeping functionality (see reviews by Büchler et al. 2010 and Rinderer et al. 2010). Resistant bees are being used successfully by some beekeepers to reduce or eliminate acaricides when managing mites. A 2005 survey in the USA estimated that up to 25 % of beekeepers were using or had tried bees bred for resistance to *V. destructor* (Kim et al. 2010, J. Westra unpubl. data). However, a large majority of beekeepers are not relying on resistant bees to manage mites. There clearly are expectations by beekeepers, especially by large-scale commercial beekeepers, for more functional mite-resistant bees which are suitable to their established management practices (Dietemann et al. 2012; Danka et al. 2013a).

Here, we describe the selection and evaluation of a population of mite-resistant bees that was developed in conjunction with commercial beekeepers. The bees were derived from bees with mite resistance based on the trait of *Varroa*

Corresponding author: R. Danka,
Bob.Danka@ars.usda.gov
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sensitive hygiene (VSH). High expression of VSH confers high resistance to *V. destructor* (Harbo and Harris 2001) because hygienic activity suppresses mite reproduction (Harbo and Harris 2005). Because the trait is regulated by genes with additive effects (Harbo and Harris 2001). it should be possible to introgress resistance into a variety of desirable bee types. We developed a new population by outcrossing VSH queens to bees managed by commercial beekeeping companies, and then selecting colonies based on good beekeeping functionality (survival, colony population, honey production) and apparent mite resistance (end-of-season infestation). Testing and selection occurred annually from 2008 to 2014. Beginning in 2011, daughters of previously selected queens were added to the testing and selection to determine performance of the selected bees relative to parental VSH bees. The selected population was given the name "Pol-line Hygienic Italian honey bees" when breeding material was first distributed in 2011 by Glenn Apiaries (Fallbrook, California).

The field selection that focused on low end-of-season mite infestations yielded bees with apparent mite resistance (see "Results" section). We investigated this further by bioassaying the selected Pol-line population and the parental VSH population for responses to *V. destructor*. The bioassay provided measures of expression of VSH as hygienic removal of mite-infested brood and as non-reproduction in the mite population that was not removed.

2. MATERIALS AND METHODS

2.1. Selection of Pol-line honey bees

We collaborated with three large (~100,000 colonies total) US beekeeping companies to test colonies with outcrossed VSH queens and to select the best performers to include in the Pol-line population. Company A (Adee Honey Farms; Bruce, South Dakota) pollinates almonds in California, conducts spring management in Mississippi, produces honey in Midwestern states, and overwinters in the Central Valley of California. Company L (Lamb's Honey Farm; Jasper, Texas) produces honey in North Dakota and overwinters and conducts spring management in Texas. Company M (Merrimac Valley Apiaries; Billerica, Massachusetts) pollinates almonds in California, conducts spring management in Louisiana, pollinates apples in New York or highbush blueberries in New Jersey, pollinates lowbush blueberries in Maine, pollinates cranberries in Massachusetts, and overwinters in Louisiana. (We first worked only with company M during 2008–2010 to begin selecting high-functioning colonies with outcrossed VSH queens; Danka et al. 2012.) Queenless colony divisions were produced by each company in spring of 2011, 2012, and 2013. Each new colony was given a queen cell of one of three types: VSH, Pol-line, or Control. VSH and Pol-line queens were produced from USDA-Agricultural Research Service (ARS) populations. Grafting sources for Pol-line queens were either outcrossed VSH queens previously selected from field tests or propagated daughters of such queens. Control queens were produced from the commercial stock (generally Italian) being used by each company. Queens of all three types were grafted from a minimum of five breeder colonies of the appropriate type. Virgin queens were paint-marked before they mated naturally with local drones. Totals of 560, 387 and 425 colonies with mated queens were established in 2011, 2012, and 2013, respectively (Table I). We present data only from colonies that retained their original queens throughout the study period. The infestation by *V. destructor* was determined for each new colony by collecting ca. 300 bees from the brood area, agitating them in soapy water, and counting bees and mites (Rinderer et al. 2004). Infestations generally were similar in colonies of the three bee types within each company each spring; overall starting infestations (mites per 100 bees) across all companies were 0.82±0.06 (SE) in 2011, 1.44±0.07 in 2012, and 1.69±0.10 in 2013).

After spring management, colonies were moved to summer locations and managed as usual by the beekeepers. Four or six colonies of a single bee type were kept together on pallets in each operation for management during the season. Throughout the season, all colonies were moved together and pallets with all three bee types were distributed randomly within apiaries at each location. An exception to normal management was that no treatments against *V. destructor* were applied to any colonies in 2011. In 2012 and 2013, the Control colonies in companies L and M received acaricide treatments that were standard for those companies. Honey production was measured each year by company A and in 2011 and 2013 by company L.

Table I. Numbers of colonies that were started with queens of three bee types (Control, Pol-line, VSH) in each of three beekeeping companies in each of three years

Year	Beekeeping company								
	A			L			M		
	Control	Pol-line	VSH	Control	Pol-line	VSH	Control	Pol-line	VSH
2011	64	60	50	64	80	67	53	60	62
2012	56	53	60	33	21	22	47	46	49
2013	40	43	33	42	61	45	42	60	59

In the autumn of each year, colonies were moved to overwintering locations and were evaluated for the presence of original queens, population size (number of combs at least two thirds covered with adult bees) and infestations of *V. destructor*. In winter, A and M colonies were moved to pollinate almonds. After almond pollination, colonies were moved to spring locations and were evaluated as they had been the prior autumn. At the end of each annual cycle, we usually retrieved original queens from 10 to 15 % of the remaining VSH and Pol-line colonies from each of the three cooperators. We selected colonies that had low mite infestations and large populations of adult bees. The queens from these colonies formed the basis of new maternal queen lines that were added to the Pol-line breeding population.

The Pol-line breeding population consisted of queens selected from the most recent field test from 2009 to 2012 and queens of lines that had been propagated for up to four generations after being selected in earlier field tests. The population was propagated annually. Five to ten queens were raised from the most populous colony of each of the 9–31 queen lines that were available each year. Queens were instrumentally inseminated with 8–10 μl of mixed semen collected from drones of all lines. The number of queen lines that was used varied because some lines were lost before the annual propagation and because some were excluded after colonies were found to have relatively high mite infestation or diseased brood, or were too defensive. There was no further selection for improvement in this breeding population. Prior to formally beginning development of the Pol-line population in 2011, a few Pol-line queens were mated to VSH bees during propagation, while some exceptionally resistant Pol-line queens were added to the VSH population.

2.2. Testing Pol-line and VSH breeding populations for parameters related to mite resistance

Pol-line and VSH colonies were tested for responses to *V. destructor* (frequency of successful mite reproduction and removal of mite-infested brood) beginning about 3 months after bees of both types were propagated in 2014. We randomly chose VSH colonies and then chose Pol-line colonies that matched the VSH colonies in size; thus, colonies of the two types had similar populations (as combs of bees) when tested (Pol-line, 6.4±0.6 (SE), $n=15$; VSH, 6.6±1.0, $n=12$; $t=0.17$, df=25, $P=0.869$). Each colony was tested by giving it a comb with sealed brood that was obtained from one of 18 mite-infested donor colonies. The initial mite infestation was measured in 100 sealed brood cells containing larvae, prepupae, or white-eyed pupae of workers. Initial infestation was similar ($F=1.05$; df=1, 36; $P=0.312$) in combs used to test Pol-line colonies (19.6±2.2 %) and VSH colonies (22.7±2.0 %). The comb was inserted into the brood nest of the test colony and allowed to remain for 1 week. The comb was retrieved, and the final infestation was measured in 523±51 cells containing purple-eyed, tan-bodied pupae; these were bees of the same age cohort as those initially measured (Jay 1962). The percentage of removal of infested brood was calculated as ([initial infestation−final infestation]/initial infestation)*100. We also noted whether each cell we opened had evidence of being recapped; recapped cells lack the silk that normally lines the underside of the cap. We recorded the life stage of each mite in a cell and the gender of deutonymphs and adults. For cells that were infested by a single foundress mite, we determined whether the foundress was infertile (i.e., lacked any progeny), was non-reproductive because there were no female progeny old enough to be mature when the worker bee emerged (i.e., were deutonymphs or adults), or was non-reproductive because there were either no female or no male progeny of proper age.

Highly hygienic colonies removed many mite-infested pupae. To accumulate sufficient data for analysis of mite reproduction, two Pol-line colonies were tested twice, five VSH were tested twice, and two VSH were tested three times.

2.3. Statistical analyses

End-of-season infestations of *V. destructor*, adult bee populations (as frames of bees), and honey production in field colonies were compared for VSH, Pol-line, and Control bees within each beekeeping company and within each year with generalized linear mixed models (PROC GLIMMIX in SAS version 9.4; SAS Institute 2013). Models included bee type as a fixed effect and colony within bee type as a random effect. The variables each were modeled using a normal distribution. Mean separation following a significant (P<0.05) effect of bee type was based on differences of least squares means. Percentages of colonies that survived with original queens were compared by chi-square analysis.

Responses to *V. destructor* in bioassays of the propagated populations of Pol-line and VSH were compared with generalized linear mixed models that included bee type as a fixed effect and colony and subsample (i.e., multiple tests on some colonies) as random effects. The variable of percentage change in mite infestation was modeled using a normal distribution. Variables related to mite reproduction were evaluated with models using a binomial distribution that considered the binary responses of reproduction for each mite and that also effectively weighted mite reproduction responses according to the numbers of cells observed. We included responses based on even a few cells because ignoring these data tended to remove colonies that had strong expression of VSH. A logit link function was used to transform mite reproduction variables; however, means and SEs that are reported were back transformed to the original scale (using the ILINK option in LSMEANS statements).

3. RESULTS

Observations of field colonies suggested that resistance to *V. destructor* was largely retained in selected Pol-line bees relative to VSH bees. Mite infestations after one season in Pol-line colonies were equal to or less than those in colonies having outcrossed VSH queens in seven of nine comparisons (Figure 1). Mite infestations in Pol-line colonies also usually compared favorably to those of Control colonies, i.e., they were similar to treated Controls in four of four comparisons and less than non-treated Controls in two of five comparisons.

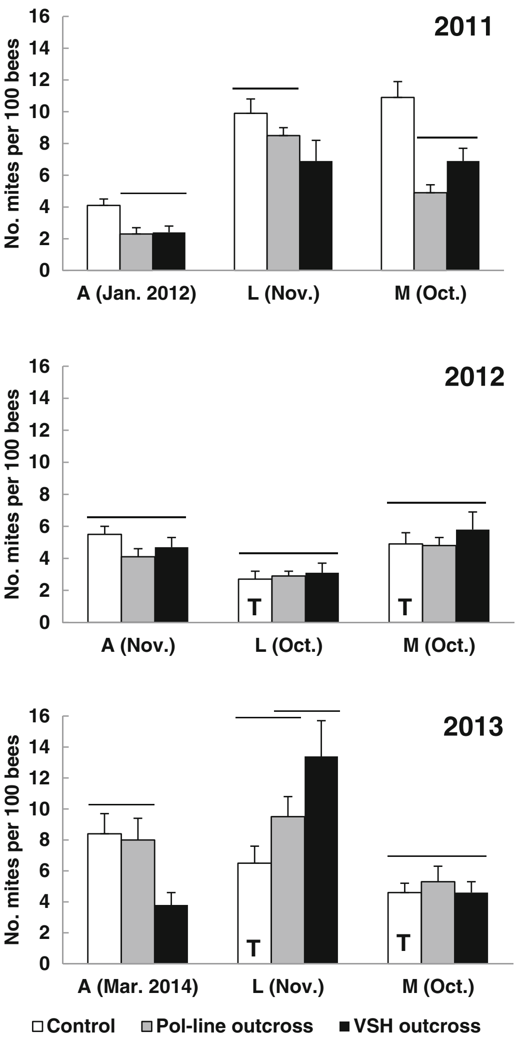

Figure 1. Infestations of *V. destructor* on adult honey bees ($\overline{x} \pm$ SE) in late season each year for three types of bees managed by beekeeping companies A, L, and M. Mite infestation was measured at the end of each year at the times noted for each company. *T* on bars indicates that these Control colonies were treated against *V. destructor*. *Horizontal lines* above bars connect means that did not differ (lsmeans comparison, P>0.05)

Bioassays of propagated VSH and Pol-line bees provided more controlled comparisons of responses to *V. destructor*. Pol-line responses were statistically similar to those of VSH, although VSH colonies had numerically greater responses for each variable (Table II). Pol-line and VSH colonies each removed ~80 % of mite-infested pupae. The difference in recapping frequencies (76 vs 58 % for VSH and Pol-line colonies, respectively) was larger than that of any other variable, and suggests (P=0.133) that VSH bees inspected more pupae that ultimately were not removed. For variables related to mite reproduction, Pol-line and VSH colonies each had mite populations that were ~50 % non-reproductive because they lacked properly aged female progeny; non-reproduction increased by ~10 % for both bee types when lack of male progeny was considered. The difference in frequencies of infertile mites (43 % for VSH vs 29 % for Pol-line colonies) was the largest difference between bee types for any variable of reproduction but was not significant.

4. DISCUSSION

Pol-line stock selected from colonies with outcrossed VSH queens in commercial beekeeping companies retained significant expression of hygiene against *V. destructor*. The average mite resistance of randomly selected colonies with outcrossed queens would be expected to lie midway between the VSH and Control parents, as has been seen in prior studies (Harbo and Harris 2001; Danka et al. 2011). However, mite resistance in Pol-line bees was much closer to that of the VSH parent. The good response to *V. destructor* in the Pol-line population presumably arose because some queens mated with drones from relatively mite-resistant colonies in the local area, and our selection of the best performing colonies identified some of those that derived from these favorable matings. The selection we used (i.e., finding colonies with low end-of-season mite infestations) proved to be useful in lieu of the technically difficult measurements (i.e., measuring rates of hygienic removal of mite-infested brood or percentages of reproducing mites) needed to directly select for high expression of VSH. The technical methods are not well suited for use by commercial bee breeders. Our production of Pol-line honey bee stock using industry-appropriate methods may encourage adoption and further selection of mite-resistant bees with desirable beekeeping characteristics. We note however that the year-to-year and beekeeper-to-beekeeper variation in infestations underscores a need for vigilance when managing

Table II. Responses related to resistance to *V. destructor* of Pol-line and parental VSH bees. Bees were bioassayed by exposing mite-infested brood in test colonies for 1 week. Shown are best estimates of means and SEs, ANOVA results from generalized linear mixed models that account for numbers of colonies (Pol-line, 15; VSH, 12), numbers of total tests with those colonies (Pol-line, 17; VSH, 21) and, for reproduction variables, numbers of cells with single foundress mites per colony (Pol-line, 15.1±2.0 ($\overline{x} \pm SE$), range 2–28; VSH, 15.1±2.0, range 7–31)

Response	Bee type	$\overline{x} \pm SE$	F	df	P
Percentage change in mite infestation	Pol-line	−78±3	1.24	1, 23.7	0.276
	VSH	−84±3			
Percentage of recapped cells	Pol-line	58±8	2.44	1, 22.1	0.133
	VSH	76±9			
Percentage of non-reproductive foundresses (no ♀ progeny of correct age)	Pol-line	47±6	0.61	1, 17.9	0.444
	VSH	53±6			
Percentage of non-reproductive foundresses (either no ♀ or no ♂ progeny of correct age)	Pol-line	57±6	0.64	1, 17.3	0.436
	VSH	64±6			
Percentage of infertile foundresses	Pol-line	29±7	1.85	1, 19.2	0.190
	VSH	43±8			

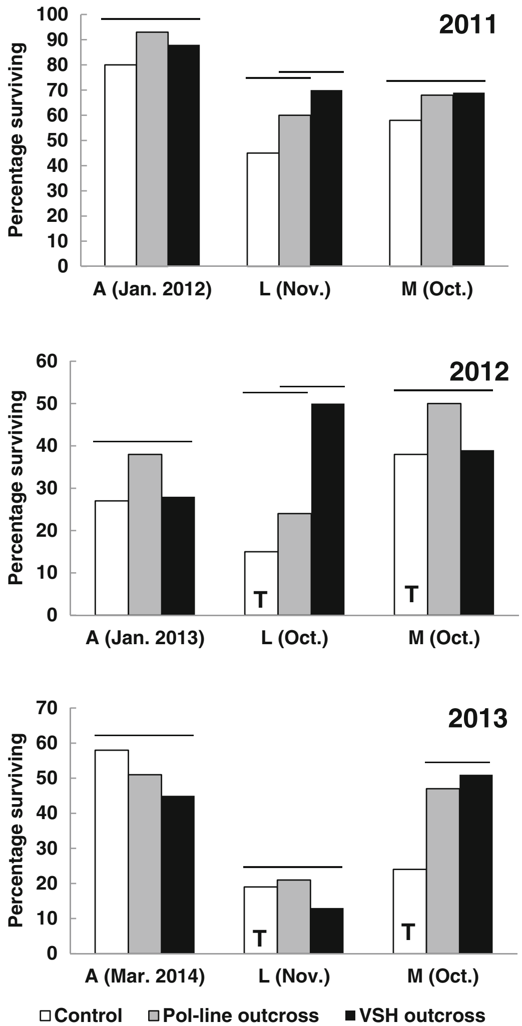

Figure 2. The percentage of colonies of three types of bees that survived with original queens each year in beekeeping companies A, L, and M. Colonies were established in March or April in each year; times of final observations appear with each beekeeper label. *T* on bars indicates that these Control colonies were treated against *V. destructor. Horizontal lines* above bars connect means that did not differ (chi-square comparison, $P>0.05$)

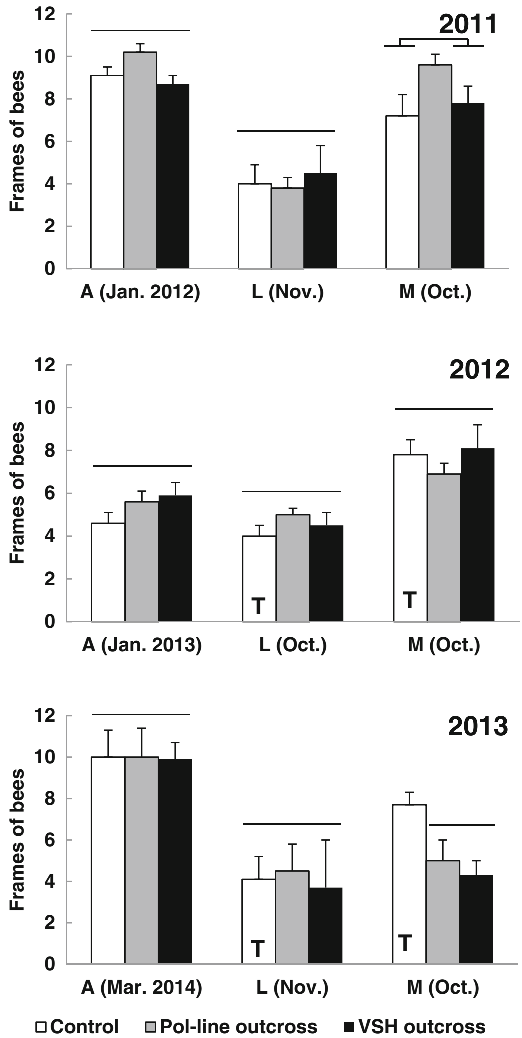

Figure 3. Population sizes of colonies measured as frames of bees ($\overline{x} \pm SE$) in late season each year for three types of bees managed by beekeeping companies A, L, and M. Times of measurements appear with each beekeeper label. *T* on bars indicates that these Control colonies were treated against *V. destructor. Horizontal lines* above bars connect means that did not differ (lsmeans comparison, $P>0.05$)

V. destructor even in bees with good mite resistance. Furthermore, we would expect rapid diminishment of resistance if Pol-line queens supersede and mate with less resistant bees in production settings.

In addition to mite resistance, Pol-line bees showed favorable beekeeping characteristics in the field tests. The percentages of colonies that survived with original queens (Figure 2), populations of adult bees (Figure 3), and honey production (Figure 4) of

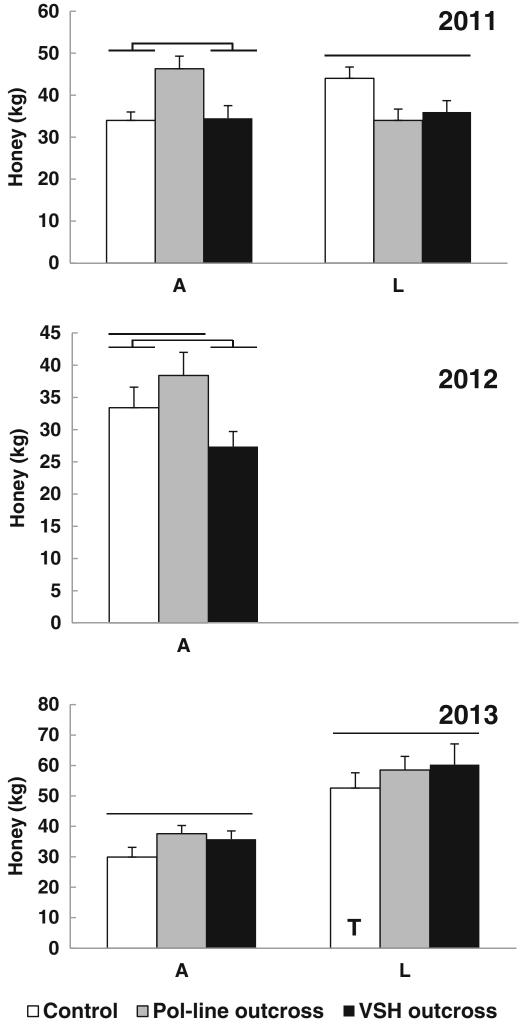

Figure 4. Surplus honey ($\bar{x} \pm SE$) that was harvested per colony each year from three types of bees managed by companies A and L. Queen status was not noted at the time of honey production, so the data are from colonies whose queens were either original or superseded. *T* on bars indicates that these Control colonies were treated against *V. destructor*

Pol-line colonies were adequate relative to the performances of the two other bee types.

Responses to *V. destructor* determined by the bioassays generally were similar to those found in prior work on VSH bees but trended toward greater hygiene and toward lower responses of non-reproduction (especially infertility). Removal of pupae from naturally mite-infested cells by VSH and Pol-line bees here (84 and 78 %, respectively) is greater than that found for VSH bees in most reports (67–76 % in Harbo and Harris 2009; Villa et al. 2009; Danka et al. 2011; Harris et al. 2012 and Danka et al. 2013b; 91 % in Danka et al. 2010). Non-reproduction by mites in VSH and Pol-line colonies here (53 and 47 %, respectively) is much less than for VSH bees in two reports (80–100 %; Harbo and Harris 2001; Harbo and Harris 2005) but greater than in another (~40 %; Harris and Harbo 1999). Infertility of mites in VSH and Pol-line colonies here (43 and 29 %, respectively) is similar to that in two reports (Villa et al. 2009; Danka et al. 2013b). less than in two others (55, 76 %; Harbo and Harris 2005; Danka et al. 2011), and greater than in another (~10–30 %; Harris and Harbo 1999). Recent selection of VHS bees at our laboratory has been based largely on removal of mite-infested pupae rather than on the original criterion of the percentage of non-reproducing mites (Harbo and Harris 1999). Harbo and Harris (2009) point out that selection for hygiene alone potentially misses other mechanisms that suppress mite reproduction; the current data suggest that this may be occurring in the current VSH population. The significant responses to *V. destructor* by Pol-line bees are noteworthy because of the repeated outcrossing (up to four generations in a queen line) and the relatively low percentage of original VSH parentage ($\bar{x} = 46\%$; range=33–54 %) in the pedigrees of the 15 Pol-line colonies that we bioassayed. This suggests that the VSH trait, once infused into a population, subsequently can be maintained for at least several generations by relatively simple selection.

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Sélection d'abeilles de la lignée 'Pol-line' issue de celles au comportement VSH et évaluation de leurs caractéristiques concernant leur résistance au Varroa

***Apis mellifera* / *Varroa destructor* / résistance aux acariens / comportement hygiénique sensible au varroa / élevage**

Selektion von Honigbienen der VSH-abgeleiteten "Pol-line" und Beurteilung ihrer Merkmale der Varroa-Resistenz

***Apis mellifera* / *Varroa destructor* / Milbenresistenz / Varroa-sensitive Hygiene / Zucht**

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