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ORIGINAL RESEARCH ARTICLE

Selection for resistance to *Varroa destructor* under commercial beekeeping conditions*

John Kefuss[a,b]*, Jacques Vanpoucke[c], Maria Bolt[d] and Cyril Kefuss[a]

[a]*Le Rucher D'Oc, Toulouse, France;* [b]*Pacific Queens, Limache, Chile;* [c]*ufr M.I.G., Université Paul Sabatier Toulouse, Toulouse, France;* [d]*Le Rucher Bolt, Le Pushin, France*

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A survival field test was initiated in 1999 to observe the effects of no treatment against *Varroa destructor* on European honey bee colony survival. After losses of over two-thirds of the 268 original colonies, new colonies were made from the survivors. In 2002, genetic material from these survivors was bred into an independent group of 60 colonies. In 2013, 519 non-treated colonies from both groups were being used for commercial beekeeping, and mite populations were very low. This indicates that under commercial beekeeping conditions, simple methods can be used to select for reduced mite populations.

Selección para la resistencia a *Varroa destructor* bajo condiciones comerciales de apicultura.

En 1999 se inició una prueba de supervivencia en campo para observar los efectos de la ausencia de tratamiento contra *Varroa destructor* en la supervivencia de colonias de abejas europeas. Tras la pérdida de más de dos tercios de las 268 colonias originales, se hicieron nuevas colonias a partir de las supervivientes. En 2002 el material genético de estas supervivientes fue utilizado para crear en un nuevo grupo independiente de 60 colonias. En 2013, 519 colonias no tratadas de ambos grupos estaban siendo utilizadas para la apicultura comercial, y las poblaciones de ácaros eran muy bajas. Esto indica que en condiciones comerciales de apicultura se pueden utilizar métodos simples para la selección de poblaciones reducidas del ácaro.

Keywords: survival tests; resistance; tolerance; hygienic behavior; *Varroa destructor*

Introduction

A major goal of bee breeders is to develop a honey bee resistant to the parasitic mite varroa (*Varroa destructor*). This mite was observed in the USSR on European honey bee (*Apis mellifera* L.) in 1952. From there, it took only 35 years to spread throughout Europe, Asia, South and North America, where it was found in 1987. At the present time, only a few countries such as Australia are free from the mite (Rosenkranz, Aumeier, & Ziegelmann, 2010). In France, the mite caused extensive damage to the beekeeping industry after its arrival in 1982. Measures to limit its progression by restricting hive movements were not effective, and in some cases may have helped spread the mites as beekeepers tried to move their hives from infested to mite free zones. Varroa has been considered as one of the major causes of bee mortality in France (Faucon & Chauzat, 2008) and other parts of the world (Ritter & Dejong, 1984).

While global efforts to develop acaricidal treatments to control varroa were underway, the possibilities of using honey bees resistant to varroa were being considered. Africanized bees in Brazil were discovered to survive infestations without treatment (Moretto, Gonçalves, De Jong, & Bichuette, 1991). Resistance to varroa by European honey bees in Uruguay was reported by Ruttner, Marx, and Marx (1984). When Uruguay bees were tested in Europe in comparisons with *A. mellifera carnica* and a strain of *A. mellifera ligustica* (Starline honey bees developed by Dadant and Sons in the USA), all stocks were equally susceptible to the European mite ecotype (Koeniger, Schmidt, Wilde, Kefuss, & Ducos de Lahitte, 1995). Later it was found that the population dynamics of the bees in Uruguay were different compared to those found in Europe (Rosenkranz, 1999). Additional reports of local resistance to mites in non-Africanized *A. mellifera* subspecies were reported in Europe, the Middle East and tropical South America (Ritter & Dejong, 1984).

In Sedjenane Tunisia, untreated colonies of *A. mellifera intermissa* were able to resist mite infestation and produce honey without chemical treatments for five years (Ritter, 1990). In 1993, queens from these *A. m. intermissa* ecotypes were compared with *A. m. carnica* ecotypes from Germany in Toulouse, France, and it was found that they had fewer mites than colonies of *A. m. carnica*. It is important to note, however, that both ecotypes had colonies that were mite resistant (Kefuss, Vanpoucke, Ducos de Lahitte, & Ritter, 2004). Naturally mated daughters from these queens were the final survivors in a test of 13 European honey bee strains for

*Corresponding author. Email: jkefussbees@wanadoo.fr
*This paper is dedicated to the memory of Steve Taber III.

survival to mites (Büchler et al., 2002). This implies that mite resistance is under genetic control.

Survival of isolated untreated bees to mites has been also observed on Gotland Island in Sweden (Fries, Imdorf, & Rosenkranz, 2006) and in the semi-isolated Arnot Forest in the USA (Seeley, 2007). In those studies, the investigators' main goal was to see if untreated colonies could survive mite infestations, and for that reason, colonies were manipulated as little as possible. Since no attempt was made to select for production, their suitability for commercial use was not determined.

At Le Mans and Avignon France, untreated colonies showing normal development for two or more years were compared with treated colonies for mortality and honey production. No differences were found in mortality but the treated control colonies produced 1.7 times more honey than the untreated (Le Conte et al., 2007).

In the USA, three commercial stocks have been developed with selective breeding that demonstrate different levels of mite resistance. These include: Russian hybrid bees (RHB); Varroa Sensitive Hygiene bees (VSH); and the Minnesota Hygienic line (MNHYG). Each stock has its own specific mechanism(s) of resistance.

Rinderer, Harris, Hunt, and de Guzman (2010) classified resistance mechanisms as either behavioral or physiological. Behavioral mechanisms included hygienic behavior, grooming behavior, and removal of mites from the hive. Physiological mechanisms involved phoresy and brood characteristics such as attractiveness to mites. RHBs exhibit strong grooming traits (Rinderer et al., 2001), high hygienic behavior, reduced brood attractiveness, and decreased reproductive success in combs built by RHB (de Guzman, Rinderer, & Frake, 2008). Hygienic bees quickly remove dead brood from the colonies (Rothenbuhler, 1964). VSH bees hygienically remove mite infested bees (Harbo & Harris, 2005; Ibrahim & Spivak, 2006).

Rothenbuhler (1964) demonstrated that hygienic behavior is controlled by recessive genes and proposed a two-locus model to explain it. Moritz (1988) re-examined Rothenbuhler's, 1964 paper, and concluded that a three-locus model better fits his data. After testing 500 + hives for hygienic behavior, Kefuss, Taber, Vanpoucke, and Rey (1996) postulated that at least 20 to 30 genetic characters are involved. Seven suggestive QTLs (quantitative trait loci) were found by Lapidge, Oldroyd, and Spivak (2002) each controlling only 9–15% of the observed phenotypic variation in hygienic behavior. They concluded that the genetics of this behavior is complex and probably controlled by many genes. Oxley, Spivak, and Oldroyd (2010) identified six QTLs influencing task thresholds for hygienic behavior. They agreed with Rothenbuhler's (1964) conclusion that independent genetic loci regulate each component of hygienic behavior. Tsuruda, Harris, Bourgeois, Danka, and Hunt (2012) located candidate genes associated with the removal of mite-infested pupae observed in the resistance trait varroa sensitive hygiene (VSH). Studies on the heritability of VSH concluded that selective breeding can probably intensify its expression (Boecking, Bienefeld, & Drescher, 2000). Boutin et al. (2015) were able to correlate hygienic behavior with differential gene expression in 96 genes. Galbraith et al. (2016) found that gene expression in honey bees may have parent-of-origin effects that can change with the individual's physiological state. The possibility of intragenomic conflict between matrigenes and patrigenes should be taken into consideration when studying the genetics of mite resistance. Especially if marker-assisted selection will be used to screen hives for disease resistance.

The above results clearly indicate that there is a genetic basis for mite resistance. The first goal of this field test was to develop simple methods to select colonies for reduced mite populations that could survive without mite treatments under commercial beekeeping conditions. The second goal was to obtain a gene pool expressing low mite phenotypes that could be selected for commercial honey production.

Materials and methods

In this field test, we used only the survival test to select for mite resistance. Exposures to mite-vectored viruses are reduced as non-productive and diseased hives are quickly eliminated from the breeding population. However, most beekeepers and queen breeders will not use this survival test due to the risk of losing large numbers of hives. For them, other tests have been developed for resistance selection where colony losses are reduced (Kefuss, Taber, Vanpoucke, & Rey, 2003; Kefuss, Vanpoucke, Bolt, & Kefuss, 2009 Kefuss et al., 2004).

Genetic material

A collection of commercial colonies ($N = 268$) in standard deep Langstroth hives used for queen rearing and honey production was established in 1999 as test population 1. These colonies were headed by naturally mated queens derived from commercial *A. m. ligustica, A. m. carnica, A. m.caucasica, and A. m. mellifera* breeder queens obtained in 1999 or earlier.

Before 1999, these colonies were systematically treated every year with chemicals to control mites. All chemical treatments were stopped in 1999 and a survival test was initiated (Kefuss et al., 2004). As colonies died out, they were replaced by new colonies with daughter queens made from the best survivors by either splitting survivor hives or requeening non-selected mite infested bees from other beekeepers with open-mated daughters from selected survivor queens.

Test population 2 ($N = 60$) was established in 2002 in Dadant hives 40 km from the location of test population 1. At the origin of this group were six hives purchased from a local beekeeper in 1999 that were split and multiplied to obtain the 60 hives for this test

population. These colonies were requeened using virgin queens from test population 1 naturally mated at location 2. Then, colonies were managed as in group 1 for queen rearing and honey production using the best population 2 virgin queens to naturally mate with population 2 drones. Since 2001, this group has never been treated against mites and colony increase was made only by splitting survivor hives.

Genetic material was exchanged back and forth between these two independent test populations on an irregular basis by requeening with queen cells and virgin queens from the best 1–5 colonies in each group throughout the field test. Low mite levels and general colony performance such as the ability to rear high-quality queens and honey production determined selection of the breeding material. Colonies continued to be used for all aspects of commercial queen rearing and honey production using the same techniques as before 1999.

Usually 20–25 colonies were maintained in 20 + apiaries of the two test populations depending upon the year. Both groups were in contact with non-selected hives of other commercial beekeepers with apiaries of similar or larger sizes sometimes located less than 1 km away from the test groups. Due to this proximity, the non-selected colonies were potential mite sources for mite re-infestation and probably helped to maintain high mite re-infestation pressure on the test hives.

No attempt was made to obtain parallel data from the non-selected chemically treated hives of other beekeepers as an external control. However, this survival test does have a built-in internal control because selection progress of the two test groups are tracked through time in an environment where they are continuously exposed to mite re-infestations from local beekeepers who chemically treat their hives against mites.

Hygienic test (frozen brood insert method)

We tested for hygienic behavior because it has been associated with brood disease resistance (Gilliam, Taber III, & Richardson, 1983) and reduced mite populations (Spivak, 1996). A comb of capped pupae with purple eyes and tan body color was cut into 5 cm squares and frozen for less than 24 h before the start of the test. This brood was furnished by a colony not in the hygienic test headed by a young queen to get maximum brood surface at the correct pupae stage (purple eyes and tan body color). Each square of frozen brood was placed on a comb with brood of the same age. A knife was used to trace the shape of this square on the brood comb and a corresponding brood square was cut out from the comb. The square of frozen brood was then inserted into the hole made where the brood was removed (Figure 1). Dates of brood sampling and measures of hygienic behavior by estimation are given in Table 1.

To estimate hygienic behavior at 24 and 48 h, the inserted piece of frozen brood was examined on both sides and a surface estimation of brood removed was made after taking into account the actual number of empty brood cells in the frozen brood square before insertion. The same person estimated hygienic behavior for all colonies. Colonies that removed 100% of the dead brood at 48 h were considered hygienic. Varroa Sensitive Hygiene was not tested.

The frozen brood insert method was chosen because it is more conservative than the "pin test" method, i.e. fewer colonies with 100% removal (Gramacho, Goncalves, Rosenkranz, & De Jong,1999; Panasiuk, Skowronek, & Bienkowska, 2008; Spivak & Downey, 1998). Also the advantage of this third method over both the pin and the liquid nitrogen methods is that each hive furnishes a brood sample for later mite analysis. Insertion takes less than 2.5 min and thus is less disruptive to the colony (Rey, Kefuss, & Vanpoucke, 2009). Since it makes no difference if brood comes from the same or a different colony (Spivak & Downey, 1998), brood for insertion in our tests was collected and frozen in advance. This reduces time spent in the bee yard.

A trained specialist estimated hygienic behavior in this test. During estimations, the comb is tilted from side to side to observe all relics of brood. Brood photographs may or may not record all these relics and thus may over estimate cell cleaning. Because estimations are fast, they are less disruptive than counting individual cells yet accurate enough to select for hygienic behavior under commercial beekeeping conditions (Table 2; Kefuss et al., 1996; Rey et al., 2009).

Capped brood mite samples

To study the population dynamics of mite reproduction in the brood, all pieces of brood that were removed to make the hole to insert the 5 cm square of frozen brood for the hygienic test were recovered, frozen, and later examined for the presence of varroa mites. The pupal stage of the recovered capped brood sample (purple eyes and tan body color) permitted mother and daughter mites to be easily distinguished from each other. One hundred cells were opened (under a 300 watt halogen lamp to increase visual acuity) and adult female, daughter, and immature mites were counted. Males were not counted, as we were only interested in the production of potentially reproductive daughters that may or may not have been mated.

Sampling for phoretic mites on bees

At the same time, the brood square was inserted for the hygienic test a bee sample was taken from each hive and frozen for evaluation of phoretic mite infestations on adult bees. Bees (usually between 250–300) in each sample were counted and then washed in a one-liter jar containing about 500 ml of water and one drop of liquid detergent. After shaking 30 times, the jar's content was poured into a double screen honey filter and washed.

Figure 1. Frozen brood insert method used in hygienic test. The frozen brood square is used as a template to trace the hole where it will be inserted.

Table 1. Dates of brood sampling and hygienic behavior testing.

Brood samples	Hygienic test
Population 1	
April 23, 2001	July 9, 1999
August 15, 2002	August 15, 2002
May 21, 2008	May 21, 2008
Population 2	
June 2, 2009	June 2, 2009
June 29, 2010	June 29, 2010

Bees remain on the top screen while mites filtered through to the bottom screen. The bottom screen was then sponged from below to remove water lodged in the screen so that mites could be more easily counted.

Statistical analysis and profile graph

Comparisons are made within each test population and between test populations. To compare mite infestations for different years, Student's two-sample t-tests for comparing two means with unequal variances were used (Kendall & Stuart, 1961; Velleman, 1997). Variance ratios between years indicate whether variability is increasing or decreasing and give additional information on changes in the direction of selection.

We used parallel boxplots analysis to compare hygienic test results within years at 24 and 48 h (Velleman, 1997). These plots show boxplots with 95% confidence intervals around the medians. Significant differences are characterized by non-overlapping confidence intervals.

The profile graph used in the hygienic test is designed to represent ascending scores for a given positive characteristic with a fixed maximum value M (a true non-gaussian variable) of a finite group of individuals. The characteristic spreads from zero to M (in our case, M = maximum hygienic value or 100%) along the y-axis (vertical). Individual hives are located along the x-axis (horizontal) according to their increasing scores at 48 h. This results in a visual ascending effect with platforms for identical scores at 48 h. Each vertical bar represents the scores of one hive for hygienic behavior at both 24 and 48 h.

Results

Mite population growth

In test population 1, mite populations increased in the brood and on the bees between April 2001 and August 2002. There were significantly more daughter mites in the brood for 2002 ($p < .015$) and except for immatures, both means and medians globally increased (Figure 2, Table 3). After 2002, over two-thirds of the colonies were dead in test population 1. At the beginning of 2003, only 164 colonies were alive. When mites were counted again in May 2008 and compared with April 2001 and August 2002, respectively, there were significant reductions for mite infestations in the brood for adults ($p < .0001$, $p < .0001$), daughters ($p < .03$, $p < .004$), immatures ($p < .0001$, $p < .0008$) and on the bees ($p < .0002$, $p < .0048$) (Figures 2, 3,Table 3).

In test population 2 between June 2009 and June 2010, there was a significant mite increase in the brood for adults ($p < .007$) and daughters ($p < .0001$) but not for immatures (NS) nor for mites (NS) on bees (Figures 2, 3 and Table 3). Between test population 1 in August

Table 2. Comparisons of hygienic behavior estimations in France, Chile, and China by two independent observers at different locations and dates in France, Chile, and China; no significant differences between estimations of hygienic behavior by independent observers A and B were found. Significant at $p \leq .05$. NS = not significant. Estimations efficiently discriminate between different levels of hygienic behavior and are less labor intensive than photographic techniques. They are adapted to commercial beekeeping conditions where large numbers of hives need to be screened in a short period of time.

Hygienic estimations							
		April 1999 France			May 2008 France		
		24 h	48 h		24 h	48 h	
	Mean	29.0	47.1		51.9	71.7	
	Median	24	37		40.5	79	
A	*n*	27	27		54	54	
	Variance	**605.4**	**955.8**		**956.3**	**784.3**	
	StdDev	24.6	30.91		30.92	28	
	Mean	29.6	47.4		53.9	64.8	
	Median	25	42		43.5	60	
B	*n*	27	27		54	33	
	Variance	**457.5**	**730.0**		**867.3**	**911.6**	
	StdDev	21.4	27.0		29.5	30.2	
	t-test	**.10**	**.03**		**.49**	**1.06**	
	df	**51**	**51**		**96**	**63**	
	$p \leq$	**.92**	**.98**		**.62**	**.29**	
		NS	**NS**		**NS**	**NS**	
	August 2010 China			February 2013 Chile			
		24 h	24 h	24 h	24 h	48 h	48 h
	Mean	81.0	81.0	81.0	56.8	92.3	92.3
	Median	84	84	84	58.5	99.5	99.5
A	*n*	21	21	21	106	106	106
	Variance	**391.6**	**391.6**	**391.6**	**799.94**	**294.9**	**294.9**
	StdDev	19.78	19.78	19.78	28.28	17.17	17.17
	Mean	78.5	77.6	76.6	55.2	93.3	93.2
	Median	75.5	79.5	75	55	100	100
B	*n*	21	21	21	106	106	89
	Variance	**337.0**	**358.2**	**399.0**	**746.2**	**226.5**	**179.2**
	StdDev	18.4	18.9	19.9	27.3	15.1	13.4
	t-test	**.43**	**.59**	**.72**	**.42**	**.45**	**.41**
	df	**39**	**39**	**39**	**209**	**206**	**191**
	$p \leq$	**.67**	**.56**	**.47**	**.67**	**.65**	**.68**
		NS	**NS**	**NS**	**NS**	**NS**	**NS**

2002 and population 2 in June 2009, mites were significantly lower in 2009 for adults ($p < .0001$), daughters ($p < .002$), immatures ($p < .01$), and on bees ($p < .002$). Also between test population 1 in August 2002 and test population 2 in June 2010, mites were significantly lower in 2010 (excepting daughters) for adults ($p < .0006$), daughters (NS), immatures ($p < .002$), and on bees ($p < .029$) (Figures 2, 3 and Table 4)

There were no significant differences for adults and daughters in the brood and mites on bees between test populations 1 (May 2008) and 2 (June 2009) excepting for an increase in immatures ($p < .018$) in population 2. There was a significant increase in adults ($p < .022$) and daughters ($p < .0001$) when June 2010 is compared to May 2008 but not for immatures or mites on bees. At the start of the test, there were 268 colonies in group 1 and 60 in group 2 (328 colonies). In December 2013, there were 334 colonies in group 1 and 185 colonies in group 2 or a total of 519 colonies not being treated against mites.

Sample variances

From a qualitative point of view in table 3, it is interesting to note that after a global increase of sample variances in test population 1 (excepting for immatures) between April 2001 and August 2002 (adults 1 to 1.4, daughters 1 to 11.7, immatures 1.3 to 1, mites on bees 1 to 1.7), sample variances globally decreased between April 2001 and May 2008 (adults 7.4 to 1, daughters 4.2 to 1, immatures 46.7 to 1, and mites on bees 1.9 to 1). Variances also decreased between August 2002 and May 2008 (adults 10.7 to 1, daughters 48.8 to 1, immatures 36 to 1 and mites on bees 3.1 to 1).

In contrast under very low mite conditions, the variances increased within test population 2 from 2009 to 2010 for adults (1 to 3.8), daughters (1 to 30), mites on

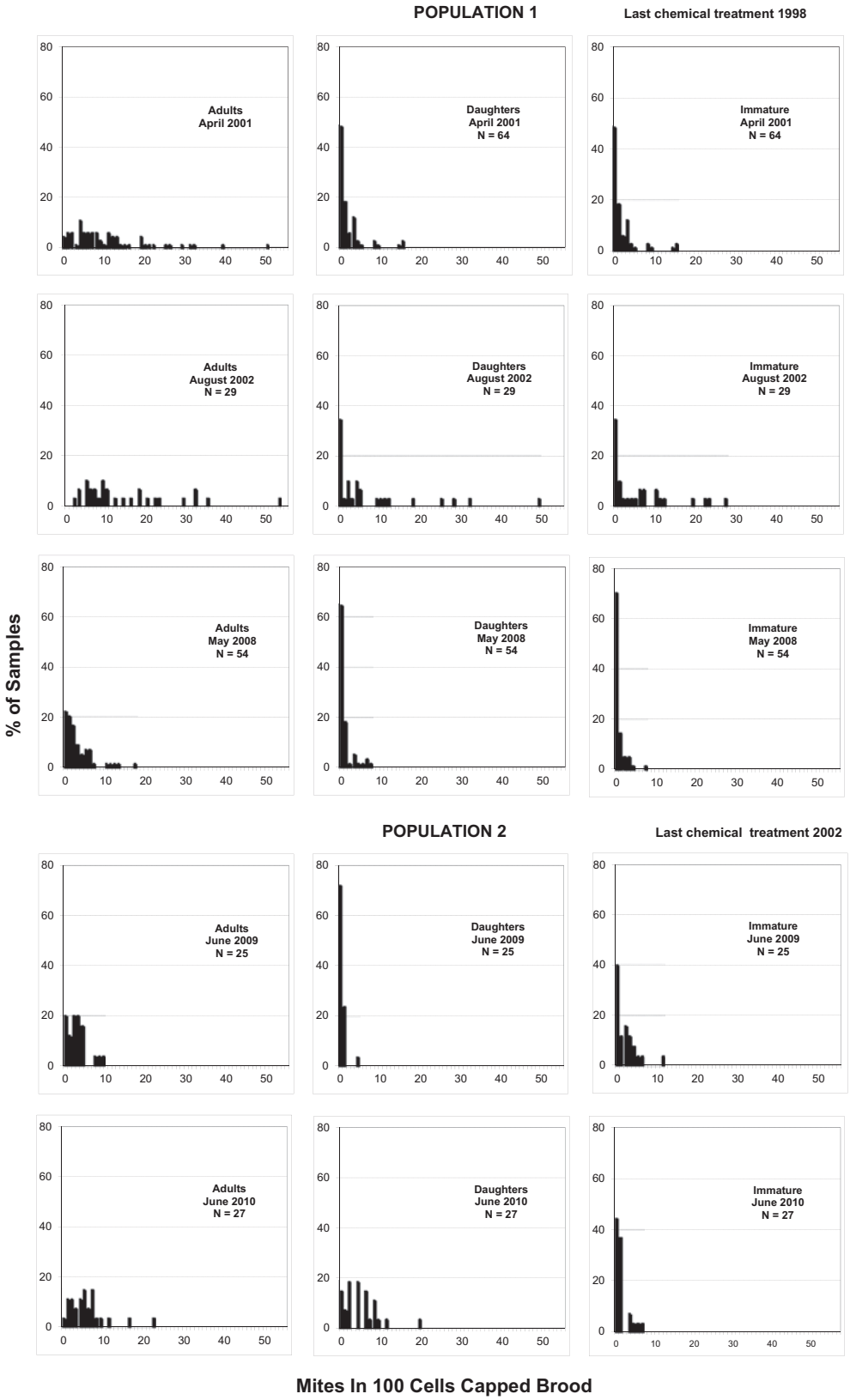

Figure 2. Frequency histograms of adult, daughter, and immature mites in 100 capped brood cells for test populations 1 and 2. The *y*-axis represents % of samples. The *x*-axis represents the amount of mites found in 100 cells. Brood samples were recovered during the hygienic test.

Table 3. Comparisons within populations for mites in brood and on bees. A = adult mite, D = daughter mite, I = immature mite, V.B. = mites on bees, Trend +/− ↘ equals decreasing number of mites. Trend +/− ↗ equals increasing number of mites. Variance ratio is the variance comparison of the first to the second year. Positive ratios indicate decreasing variance and negative ratios indicate increasing variance between years. Significant at $p \leq .05$. NS = not significant.

Comparisons within populations									
		2001 vs 2002				2001 vs 2008			
		April		August		April		May	
Year		A	D	I	V.B.	A	D	I	V.B.
1	Mean	10.8	2.0	6.8	4.9	10.8	2.0	6.8	4.9
	Median	8	1	3.5	3.89	8	1	3.5	3.89
	n	64	64	64	65	64	64	64	65
	Variance	**99.9**	**12.1**	**79.4**	**12.3**	**99.9**	**12.1**	**79.4**	**12.3**
	StdDev	10	3.48	8.91	3.51	10	3.48	8.91	3.51
2	Mean	14.7	7.8	6.1	5.5	3.2	.9	**.6**	2.8
	Median	10	3	3	4.49	2	0	0	1.98
	n	29	29	29	29	54	54	54	54
	Variance	**144.2**	**141.6**	**61.2**	**20.3**	**13.5**	**2.9**	**1.7**	**6.5**
	Std Dev	12.0	11.9	7.8	4.5	3.7	1.7	1.3	2.6
	t-test	**1.54**	**2.58**	**.37**	**.59**	**5.66**	**2.16**	**5.45**	**3.86**
	df	**46**	**30**	**61**	**43**	**82**	**94**	**66**	**115**
	$p \leq$	**NS**	**.015**	**NS**	**NS**	**.0001**	**.03**	**.0001**	**.0002**
	Trend +/−	↗	↗	↘	↗	↘	↘	↘	↘
	Variance ratio (Year 1:2)	**.69**	**.09**	**1.30**	**.61**	**7.42**	**4.14**	**46.46**	**1.89**

Year		2002 vs 2008				2009 vs 2010			
		August		May		June		June	
		A	D	I	V.B.	A	D	I	V.B.
1	Mean	14.7	7.8	6.1	5.5	2.7	.4	2.0	2.5
	Median	10	3	3	4.49	2	0	1	2.1
	n	29	29	29	29	25	25	25	25
	Variance	**144.2**	**141.6**	**61.2**	**20.3**	**5.9**	**.6**	**6.7**	**2.5**
	StdDev	12.0	11.9	7.8	4.5	2.41	.79	2.58	1.57
2	Mean	3.2	.9	**.6**	2.8	5.7	4.7	1.1	3.3
	Median	2	0	0	1.98	5	4	1	3.02
	n	54	54	54	54	27	27	27	26
	Variance	**13.5**	**2.9**	**1.7**	**6.5**	**22.6**	**17.8**	**2.7**	**7.3**
	StdDev	3.7	1.7	1.3	2.6	4.76	4.22	1.63	2.7
	t-test	**5.06**	**3.10**	**3.74**	**2.99**	**2.84**	**5.18**	**1.41**	**1.26**
	df	**30**	**28**	**28**	**37**	**39**	**27**	**40**	**40**
	$p \leq$	**.0001**	**.004**	**.0008**	**.0048**	**.007**	**.0001**	**NS**	**NS**
	Trend +/−	↘	↘	↘	↘	↗	↗	↘	↗
	Variance ratio (Year 1:2)	**11**	**48**	**36**	**3**	**.26**	**.04**	**2.50**	**.34**

bees (1 to 2.9) but not for immatures (2.5 to 1) (Table 3). When the group 2 sample variances of June 2010 are compared with those of group 1 in August 2002, variances are still globally lower in Group 2 (adults 6.4 to 1, daughters 8 to 1, immatures 22.7 to 1, mites on bees 2.8 to 1) (Table 4).

Distribution of mites on bees

In both test populations, the number of mites found per 100 bees decreased compared to that found on the original population 1 of 1999 where 75% of the colonies had more than 5 mites per 100 bees. In 2001, 66.2%, 2002 65.5%, 2008 87%, 2009 92%, and 2010 80.8% of the colonies had less than 5 mites/100 bees (Figure 3).

Hygienic behavior

No correlations were found between hygienic behavior and mite infestations either on bees or in the brood. Within groups, significant differences were always found between hygienic behavior at 24 and 48 h excepting for group 2 in 2009 where no significance

Table 4. Comparisons between populations for mites in brood and on bees. A = adult mite, D = daughter mite, I = immature mite, V.B. = mites on bees, Trend +/− ↘ equals decreasing number of mites. Trend +/− ↗ equals increasing number of mites. Variance ratio is the variance comparison of the first to the second year. Positive ratios indicate decreasing variance and negative ratios indicate increasing variance between years. Significant at $p \leq .05$. NS = not significant.

Comparisons between populations									
		2001 vs 2009				2001 vs 2010			
		April		June		April		June	
Year		A	D	I	V.B.	A	D	I	V.B.
1	Mean	10.8	2	6.8	4.9	10.8	2	6.8	4.9
	Median	8	1	3.5	3.89	8	1	3.5	3.89
	n	64	64	64	65	64	64	64	65
	Variance	**99.9**	**12.1**	**79.4**	**12.3**	**99.9**	**12.1**	**79.4**	**12.3**
	StdDev	10	3.48	8.9	3.5	10	3.48	8.9	3.5
2	Mean	2.7	.4	2	2.5	**5.7**	**4.7**	**1.2**	**3.3**
	Median	2	0	1	2.1	**5**	**4**	**1**	**3.02**
	n	25	25	25	25	**27**	**27**	**27**	**26**
	Variance	**5.9**	**.6**	**6.7**	**2.5**	**22.6**	**17.8**	**2.7**	**7.3**
	StdDev	2.41	.79	2.58	1.57	4.8	4.2	1.63	2.7
	t-test	**6.02**	**3.42**	**3.89**	**4.55**	**3.31**	**2.93**	**4.87**	**2.44**
	df	**78**	**77**	**82**	**85**	**87**	**41**	**72**	**59**
	$p \leq$	**.0001**	**.001**	**.0002**	**.0001**	**.0013**	**.0055**	**.0001**	**.018**
	Trend +/−	↘	↘	↘	↘	↘	↗	↘	↘
	Variance Ratio (Year 1:2)	**16.93**	**20.21**	**11.85**	**4.94**	**4.42**	**.68**	**29.41**	**1.69**
		August		June		August		June	
		2002 vs 2009				2002 vs 2010			
Year		A	D	I	V.B.	A	D	I	V.B.
1	Mean	14.7	7.8	6.1	5.5	14.7	7.8	6.1	5.5
	Median	10	3	3	4.49	10	3	3	4.49
	n	29	29	29	29	29	29	29	29
	Variance	**144.2**	**141.6**	**61.2**	**20.3**	**144.2**	**141.6**	**61.2**	**20.3**
	StdDev	12	11.9	7.8	4.5	12	11.9	7.8	4.5
2	Mean	2.7	.4	**2**	2.5	5.7	4.7	**1.1**	3.3
	Median	2	0	1	2.1	5	4	1	3.02
	n	25	25	25	25	27	27	27	26
	Variance	**5.9**	**.6**	**6.7**	**2.5**	**22.6**	**17.8**	**2.7**	**7.3**
	StdDev	2.41	.79	2.58	1.57	4.8	4.2	1.63	2.7
	t-test	**5.27**	**3.34**	**2.66**	**3.37**	**3.77**	**1.32**	**3.33**	**2.26**
	df	**30**	**28**	**34**	**35**	**37**	**35**	**30**	**46**
	$p \leq$	**.0001**	**.002**	**.01**	**.002**	**.0006**	**NS**	**.0023**	**.029**
	Trend +/−	↘	↘	↘	↘	↘	↘	↘	↘
	Variance ratio (Year 1:2)	**24.6**	**225.2**	**9.2**	**8.2**	**6.4**	**8**	**22.9**	**2.8**
		2008 vs 2009				2008 vs 2010			
		May		June		May		June	
Year		A	D	I	V.B.	A	D	I	V.B.
1	Mean	3.2	.9	.6	2.8	3.2	.9	.6	2.8
	Median	2	0	0	1.98	2	0	0	1.98
	n	54	54	54	54	54	54	54	54
	Variance	**13.5**	**2.9**	**1.7**	**6.5**	**13.5**	**2.9**	**1.7**	**6.5**
	StdDev	3.6	1.71	1.3	2.6	3.6	1.71	1.3	2.6
2	Mean	2.7	.4	**2**	2.5	5.7	4.7	**1.1**	3.3
	Median	2	0	1	2.1	5	4	1	3.02
	n	25	25	25	25	27	27	27	26
	Variance	**5.9**	**.6**	**6.7**	**2.5**	**22.6**	**17.8**	**2.7**	**7.3**
	StdDev	2.41	.79	2.58	1.57	4.8	4.2	1.63	2.7
	t-test	**.65**	**1.84**	**2.51**	**.63**	**2.382**	**4.46**	**1.44**	**.75**
	df	**67**	**76**	**29**	**70**	**41**	**30**	**43**	**47**
	$p \leq$	**NS**	**NS**	**.018**	**NS**	**.022**	**.0001**	**NS**	**NS**
	Trend +/−	↘	↘	↗	↘	↗	↗	↗	↗
	Variance ratio (Year 1:2)	**2.3**	**4.65**	**.26**	**2.63**	**.6**	**.16**	**.63**	**.89**

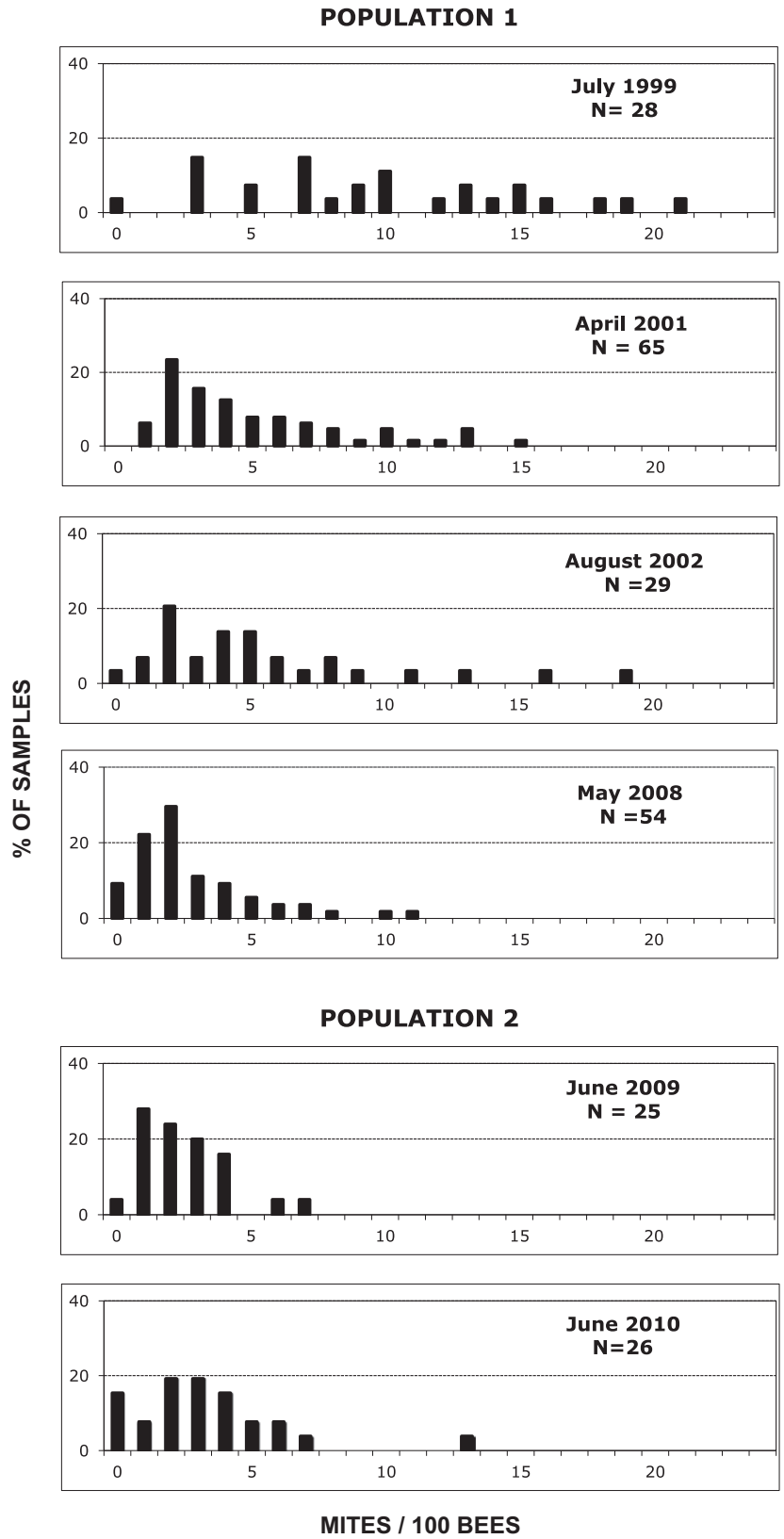

Figure 3. Mites on 100 bees for test populations 1 and 2. The *y*-axis represents % of samples. The *x*-axis represents mites on 100 bees. Bee samples were recovered during the hygienic test.

difference occurred due to the high hygienic behavior already present at 24 h (Figure 4). In group 1, colonies had high hygienic behavior in 1999 at the start of the survival test (Figure 4). No significant differences were found for hygienic behavior at 24 h between the years 1999, 2002, and 2008. However, a significant decrease in hygienic behavior at 48 h occurred between the years 1999 and 2008 ($p < .0003$) and 2002 and 2008 ($p < .0068$).

In group 2, there were no significant differences between 2009 and 2010 for hygienic behavior at 24 and 48 h. Between groups, no significant differences for hygienic behavior at 24 h and 48 h were found when the years 1999 and 2002 (Population 1) were compared to 2009 and 2010 (population 2). A significant difference did exist when 2008 (less hygienic) was compared to 2009 at 24 h ($p < .0049$) and to 2009 ($p < .005$) and 2010 ($p < .0005$) at 48 h.

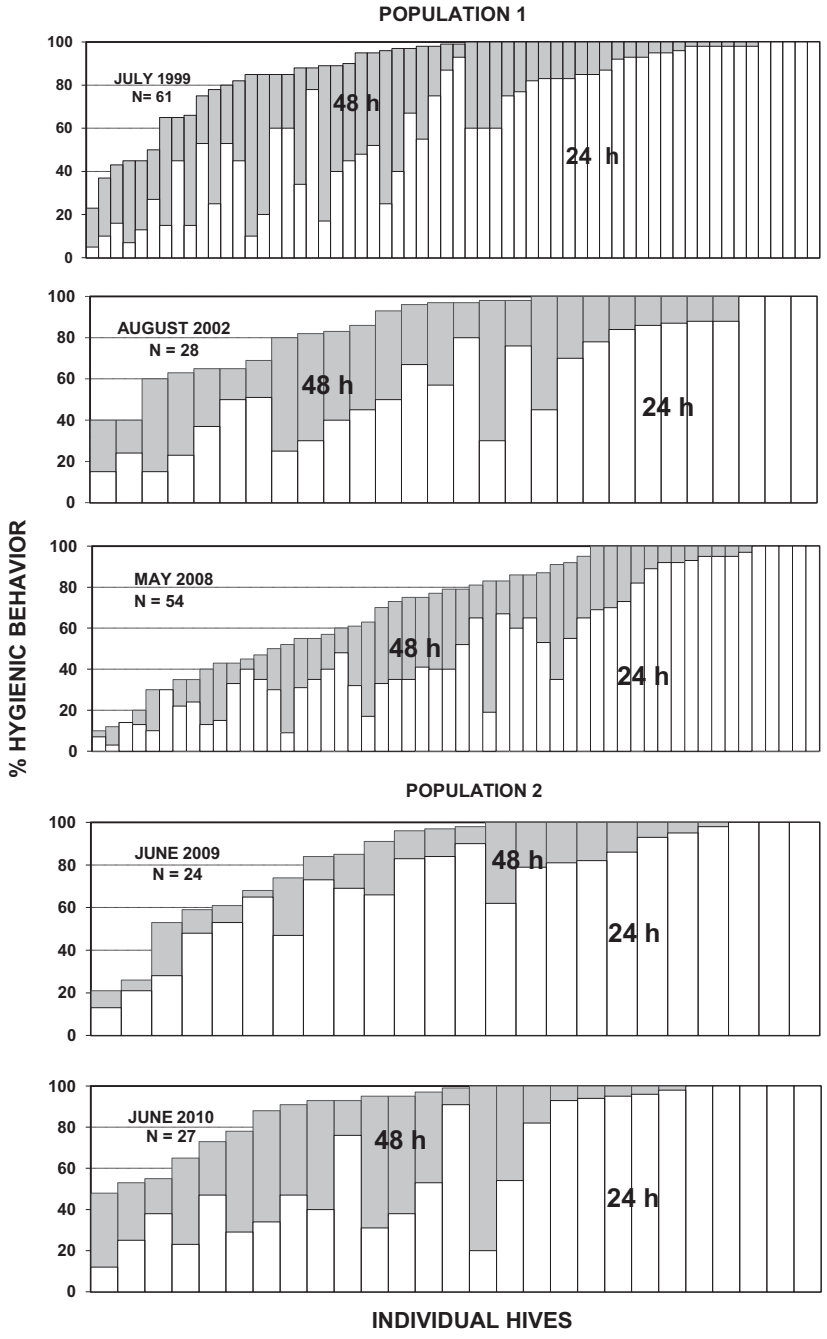

Figure 4. Hygienic behavior in test populations 1 and 2. Global group and detailed individual performances are shown. The *y*-axis represents % hygienic behavior. Columns on the *x*-axis are results for individual colonies. This graph includes a black bar profile graph of ascending removal scores at 48 h. The removal score of each individual colony at 24 h is shown in white over its score at 48 h. For each 48 h plateau, ascending scores at 24 h make a local white sub-profile for these colonies.

Discussion

Precise definitions make it easier to focus on a problem. At the present time, semantic confusion exists among bee scientists whenever the terms resistance and tolerance are utilized. According to Schneider and Ayres (2008), resistance and tolerance are two separate major pathways for survival to infestations. Raberg, Graham, and Read (2009) define resistance as "the ability of a host to limit parasite burden" and tolerance as the ability of a host "to limit the damage caused by a given parasite burden". They state that the advantage of their definitions is that resistance and tolerance can be considered both independently and in parallel. Accepting their definitions for resistance and tolerance, mite populations should decrease as bees become more resistant and would be expected to remain at the same level or even increase if tolerant. Hence, according to these definitions what many bee researchers are describing as tolerance should actually be considered as resistance.

Techniques developed to study resistance and tolerance in other animals may have direct applications to the honey bee and *vice versa* (Bishop, Doeschl-Wilson, & Woolliams, 2012). Doeschl-Wilson et al. (2012a, 2012b) pointed out the problems of making accurate phenotype measures for tolerance and indicated the type of measurements that would have to be made when selecting for tolerance. Tolerance mechanisms that prevent or repair damage may offer individuals wider generic (group) protection when they are exposed to a variety of diseases and may be good candidates for genetic improvement in the immune system (Doeschl-Wilson & Kyriazakis, 2012).

In honey bees, Danka, Rinderer, Spivak, and Kefuss (2013) defined resistance as the ability of a hive to "keep *V. destructor* at a relatively low level". Efforts to document resistance to varroa focus on the maintenance of colony fitness being associated with reduced numbers of infesting mites. Fitness in honey bees can be measured in a number of ways such as amount of brood, colony size, survival, queen, and honey production.

Although tolerance to the haplotype of varroa found in Europe has not been demonstrated (according to the above definitions), efforts to do so would have to focus on the maintenance of colony fitness with elevated numbers of infesting mites.

Hygienic behavior is associated with reduced mite populations (Harbo & Harris, 2005; Ibrahim & Spivak, 2004, 2006; Spivak,1996; Spivak & Reuter, 1998). At present, two types of hygienic behaviors are known. General hygienic behavior that is associated with removal of diseased larvae, pupae, and mites can easily be selected for using the frozen brood technique. The second, Varroa Sensitive Hygiene is characterized by a higher removal rate of mites and manipulation of cell contents but is more difficult to select.

Comparisons between four different lines of honey bees in the USA indicated that lines selected only for general hygienic behavior and those selected for varroa sensitive hygiene removed freeze killed brood at about the same percent in 48 h. However, mites were removed to a lesser degree in the lines selected for general hygienic behavior (14%) than in the group selected for Varroa Sensitive Hygiene (66%)(Danka, Harris, Villa, & Dodds, 2013). This suggests that beekeepers should select for both behaviors to get maximum disease and varroa resistance.

Hygienic tests were performed to see if this trait would be influenced during the survival test. That we globally (excepting for comparisons with 2008) did not find significant changes in hygienic behavior between years is not surprising. Population 1 colonies had high hygienic behavior in 1999 at the start of the test. This might partially explain the high colony survival after 2002 when almost one-third of the colonies survived. After 2002, no more selection for hygienic behavior was made until 2008 when a significant reduction in hygienic behavior at 48 h was observed (Figure 4).

This indicates that when colonies within a breeding population have been selected for high general hygienic behavior that is controlled by recessive genes (Rothenbuhler, 1964) and selection is stopped, the attained level of hygienic behavior can remain stable over a long period of time.

This might explain the results of Locke and Fries (2011) and our field test where no correlations between general hygienic behavior and mite infestations were found. Groups containing colonies with both high and low general hygienic behaviors such as those in Danka et al. (2013) would have to be tested to see if they differ in mite infestations.

It is clear that mechanisms of resistance and tolerance (whatever they are) may require years to be expressed before they can be utilized for selection. Chemical mite control masks and destroys natural selection for these mechanisms. Short-term experiments lasting only a few months can lead to erroneous conclusions. This is well illustrated in the 1993–2004 *A. m. intermissa* survival experiment where clear differences were observed only after 12 months of testing (Kefuss et al., 2004). Similar results were found by Fries and Bommarco (2007) and Locke and Fries (2011).

That over 33% of the population 1 colonies would survive after two years was not expected at the start of the tests. At present, parasite burden is low in both test populations indicating that colonies are resistant but probably not tolerant to mite infestations. This is clear when we consider the low number of mites found for the test populations in 2008, 2009, 2010 (Tables 3, 4, Figures 2, 3). We wish to stress that within test population 1 from April 2001 to May 2008, mite populations on the bees and in the brood significantly decreased indicating a clear progression toward higher resistance

(Figures 2, 3, Table 3). Extreme values of variation diminished between 2001 and 2008 suggesting again that a selection toward resistance had occurred (Table 3). Some of the significant differences observed in the 2002–2008 comparison might have been due to a seasonal effect as mite populations are usually but not always lower in the spring when compared to those of late summer (see Kefuss et al., 2004 for an example of high Spring and low Fall mite populations). In an open mating population, fluctuations in mite numbers and variation are to be expected. This might explain the significant increase of adult and daughter mites between 2009 and 2010 in test population 2. However, mite populations for both years in population 2 were still significantly lower than that of population 1 in 2002 (Table 4).

The population of adult mites on bees and in the brood of the two test populations was significantly limited after 2002. This might have been due to reproductive failure of either female or male mites as many adults were found without daughters (Figure 2 and Table 3). Varroa Sensitive Hygienic behavior (VSH) where bees selectively remove mites might also have played a role (Harbo & Harris, 2005) but was not tested for in this survival test. We are in agreement with Locke and Fries (2011) and Locke, Le Conte, Crauser, and Fries (2012) who concluded that factors such as reduced mite reproduction opportunities (delayed mite egg laying) and suppression of mite reproduction success (high mite infertility) probably play a major role in limiting mite populations. This does not however exclude other unknown factors that might be less, equally, or even more important for colony survival.

Since genetic material was exchanged between the two test populations over time, the populations, though independent, are probably very closely related to each other genetically despite differences in location and mite sources. This situation corresponds to that of a beekeeper who buys queens from a queen breeder to change the genetic composition of his colonies. It also implies that beekeepers should be able to incorporate selected mite resistance material from outside sources into their own populations with little difficulty using queen cells for example. Harbo and Harris (2001) found similar results when they exchanged resistant and non-resistant queens between hives. Resistant colonies became non-resistant and non-resistant became resistant.

If a new lethal mite-vectored virus occurs in a mite tolerant bee, high colony mortality might result until resistance or tolerance to that virus is found. Given the problems of virus transmission by mites (Chen, Pettis, & Feldlaufer, 2005; Locke et al., 2012; Mirinda, Gauthier, Ribierre, & Chen, 2012) we suggest that beekeepers should first select their colonies for mite resistance to reduce colony mite populations. Then, select for tolerance to the damage caused by the mites and the diseases they vector. For example, recent studies indicate that tolerance to the deformed wing virus may be under genetic control (Khongphinitbunjongaet et al., 2015; Locke, Forsgren, & de Miranda, 2014). Al Toufailia Amiri, Scandian, Kryger, and Ratnieks (2014) found that worker bees from colonies that were more than 95% hygienic had significantly fewer mites and lower levels of RNA copies of DWV. The ideal situation for beekeepers would be a mite-resistant bee that is both tolerant to the damage caused by the mites and the diseases they vector.

There are clear reasons why beekeepers should select for mite resistance and why chemicals should not be used in mite control. Chemicals used to treat against mites have been clearly found to impact colony health, immunity, and potentiate the effects of insecticides; Haarmann, Spivak, Weaver, Weaver, and Glenn (2002), Collins, Pettis, Wilbanks, and Feldlaufer (2004), Mullin et al. (2010), Locke et al. (2012) and Johnson, Dahlgren, Siegfried, and Ellis (2013).

Breeding projects in different parts of the world have demonstrated that it is possible to select bees with increased levels of resistance to *V. destructor* (see reviews of Büchler, Berg, & Le Conte, 2010; Rinderer et al., 2010) and that this is a commercially viable situation (Danka et al., 2012). Our results demonstrate that it is possible to select bees that lower mite populations using simple methods adapted to commercial beekeeping conditions and to breed this genetic material into other honey bee gene pools even when the underlying resistance mechanism is not understood (blind selection). We believe that it is the responsibility of everyone who breeds bees to try to select for mite resistance to reduce chemicals in hives. We owe this effort to the general public and to future generations of beekeepers.

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