

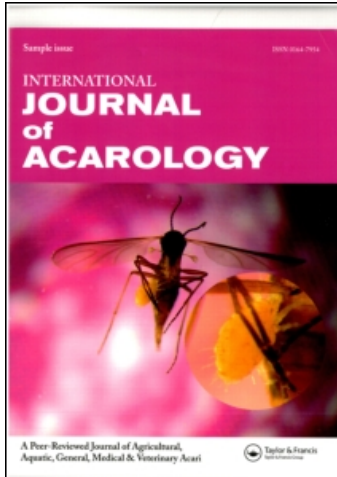
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## International Journal of Acarology

Publication details, including instructions for authors and subscription information:

<http://www.informaworld.com/smpp/title~content=t795426017>

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**To cite this Article** Sammataro, Diana, Untalan, Pia, Guerrero, Felix and Finley, Jennifer(2005) 'The resistance of varroa mites (Acari: Varroidae) to acaricides and the presence of esterase', International Journal of Acarology, 31: 1, 67 – 74

**To link to this Article: DOI:** 10.1080/01647950508684419

**URL:** <http://dx.doi.org/10.1080/01647950508684419>

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## THE RESISTANCE OF VARROA MITES (ACARI: VARROIDAE) TO ACARICIDES AND THE PRESENCE OF ESTERASE

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**ABSTRACT** - Varroa mites (*Varroa destructor* Anderson and Trueman, 2000) are becoming resistant to acaricide treatments via metabolic and/or target site desensitization. Results of a survey of mites from the Carl Hayden AZ lab and from cooperators in five locations (Arizona, California, Florida, Maine, North Dakota) showed that some mites were susceptible to all three acaricides (Amitraz, Coumaphos, Fluvalinate) in the spring of 2003, but by fall most mites were resistant. Mites were resistant to all chemicals, even from beekeepers that do not treat colonies with acaricides. We used esterase native activity gels to test for the presence of specific esterases which might be involved in pesticide resistance in varroa. All mites tested had positive bands for esterase, even those exhibiting susceptibility to some acaricides. Based on the differences between the esterase activity gel profile of the susceptible and cross-resistant *V. destructor*, it is possible that an esterase-mediated resistance mechanism is operative in the population of the mites we analyzed. However, a combination of other resistance mechanisms may be present which make the esterase activity gel method unreliable for use in identifying varroa mites with multiple resistance.

**Keywords** - Varroidae, varroa mites, *Varroa destructor*, acaricide resistance, honey bees, esterase

### INTRODUCTION

During the past decade, chemicals, such as the pyrethroid fluvalinate, and coumaphos, an organophosphate (OP), have been used by beekeepers to control varroa mites (*Varroa jacobsoni*=*V. destructor* Anderson and Trueman) in honey bee colonies. Mite resistance to both fluvalinate and coumaphos has been observed in Europe (Milani, 1995, 1999; Trouiller, 1998; Vedova *et al.*, 1997) and now is being found in the United States (Elzen *et al.*, 1998, 1999a, b, 2000; Pettis *et al.*, 1998 a, b). Fluvalinate-resistant mites first were reported in the U.S. in 1997 (Baxter *et al.*, 1998) and more recently, Varroa mites resistant to coumaphos have been found (Elzen *et al.*, 2000, 2001; Elzen and Westerveldt, 2002). Amitraz, a formamidine, has been used for mite control sporadically since 1992 and is no longer registered for bee mites; nevertheless, resistance to this material has also been found (Elzen *et al.*, 1999c, Mathieu and Faucon 2000).

Reports of resistance to fluvalinate, coumaphos and amitraz throughout the U.S. indicate that resistance is spreading and that cross-resistance might be evident in

some locations (D. Westerfelt and A. M. Jadzack, pers. comm.). Bees are transported across the U.S. for pollination and in the sale of queens and packaged bees. Since many beekeepers have relied upon single-chemical control regimes for about 10 years, resistant mites could exist in every beekeeping operation in the U.S. Currently, the trend is to use multi-chemical rotations so that mites are exposed to widely varying treatment regimes. However, mites could develop cross-resistance.

Organisms develop resistance via behavioral changes (e.g. avoiding the pesticide), reduced penetration (e.g. cuticle thickening), detoxification of the pesticide by enzymes (i.e. metabolic) or target site desensitization (modifications of action site, e.g. sodium channel mutation) (Scott, 1990; Baars and Driessen, 1984; Hillesheim *et al.*, 1996; Watkins, 1996; Wang *et al.*, 2002). Carrière (2003) suggested that haplodiploid arthropods could develop resistance differently than diploid species. Since varroa are haplodiploid, as well as highly inbred, they may be able to develop pesticide resistance quickly. Resistance mechanisms also could change over time.

In Europe and Israel, the mechanisms of varroa resistance to fluvalinate are reportedly due to high levels of metabolic esterases (Hillesheim *et al.*, 1996; Gerson *et al.*, 1991; Mozes-Koch *et al.*, 2000). Strains of the tick, *Boophilus microplus*, which exhibit cross resistance to both pyrethroids and organophosphates (OPs), possess high levels of metabolic esterase activity (Jamroz *et al.*, 2000). Esterases oxidize and detoxify synthetic pyrethroids and significantly reduce their effect on mites. A second mechanism, target site desensitization, has been described in pyrethroid resistant mites in the U.S. (Wang *et al.*, 2002) and involves mutations in the sodium channel gene sequence. The sodium channel is the target site of pyrethroid binding.

The purpose of this study was to determine the prevalence of resistant mites in beekeeping operations in the U.S. We tested for resistance to fluvalinate, coumaphos and amitraz. We also investigated whether the esterase native activity gel technique used to determine the presence of esterase in cattle ticks and horn flies resistant to pyrethroids and OPs (Guerrero *et al.*, 1997, 1998, 1999, 2001; Pruett *et al.*, 2002; Miller *et al.*, 2001) would be useful for testing resistance in varroa mites.

## MATERIALS AND METHODS

**Sources of mites** - In 2002, mites were collected from untreated colonies at our Laboratory apiary (Carl Hayden Bee Research Center [CHBRC] Tucson, AZ) and from treated colonies in Maine. The mites from Maine were determined to be resistant because they had survived colony treatments with fluvalinate and coumaphos (A. M. Jadcak, pers. comm.). Mites were collected by shaking 200-300 live bees in a quart jar covered with a wire mesh lid. The jar was shaken to dislodge attached mites. The mites were collected into glass vials and stored in a -70°C freezer until ready for esterase activity analysis.

In 2003, mites were collected from colonies located in North Dakota, Florida, Arizona, California, and Maine and from colonies moved between Maine and Florida, as well as colonies from the CHBRC that had annual fluvalinate treatments and one coumaphos treatment. To obtain mites, frames of drone foundation were sent to each cooperator to be placed in their colonies. After the frames were drawn and filled with capped drone brood (and varroa), the frames were returned to CHBRC via overnight mail. Upon arrival, the drone brood frames were stored in an incubator (30°C, 50% RH) until mites could be analyzed for resistance using the vial bioassay.

**Vial bioassay** - Varroa were collected and tested for miticide resistance according to the protocol for varroa described in Elzen *et al.* (1998). Drone cells were uncapped and the brood removed with forceps. Attached live adult female varroa were collected with slender probes and five mites were placed into 20 mL glass scin-

tillation vials for each treatment. The vials were treated with either 0.5 mL acetone (control), 123 µg amitraz, 53 µg coumaphos, or 2.4 µg tau-fluvalinate (Elzen *et al.*, 1998). Acaricide amounts were set to produce approximately 90% mortality in susceptible mite populations (Elzen *et al.*, 1999a, 2000). To ensure mite survival in the low humidity of Arizona (typical ambient humidity in the collection room was 18-25%), the protocol of Elzen (1998) was modified by wetting a 7 mm diameter disc (punched out by a paper punch) of No. 5 Whatman filter paper with 3 to 5 µL of distilled water. One disc was added to each vial during mite collection. A minimum of three replicate vials (5 mites/vial) of each acaricide vs. control was tested for each colony. Depending on the number of mites found in the frame, a minimum of 15 and a maximum of 65 mites were tested. Vials with mites were incubated for 24 hours (Little Giant Still Air Incubator, Miller Mfg., St. Paul, MN) at approximately 30-32°C and approximately 80% RH.

After 24 hours, the vials were examined under a dissecting microscope. Mites were gently prodded with a probe to encourage movement. Non-moving mites were scored as dead and the mortality rates for each vial were recorded. For each colony tested, total mortality rates from all vials of each type: control, amitraz, coumaphos and fluvalinate were tallied. Vial sets with more than 10% mortality in the control vials were discarded. Samples of susceptible and resistant mites from the vial bioassay were tested for esterase activity.

**Esterase activity analysis** - Live and dead mites from the pesticide treated vials were separated and tested for esterase activity. All mites in the control vials also were tested. Mites were frozen at -70°C in 1.5 ml microcentrifuge tubes and shipped on dry ice for analysis. Ten to 40 mites were used for each esterase activity test.

The mites were pulverized using a disposable pellet pestle (Kontes, Vineland, NJ) and extracted in buffer containing 0.01 M sodium phosphate (pH 6.5), 20% sucrose, 0.001 M EDTA, and 0.5% Triton X-100. Extracts were centrifuged at 4°C, 15,000 RPM for 10 min, and then stored at -80°C. The equivalent of a single mite was loaded onto a lane of a Novex pre-cast 4-12% gradient polyacrylamide Tris-glycine gel (Invitrogen, Carlsbad, CA) and electro-phoresed under native conditions at 4°C. Esterase activity was detected in the gel using the method of Hughes and Raftos (1985) with some modifications, by incubation of the gel in 0.1 M phosphate buffer (pH 6.5) containing 3.2 mM  $\alpha$ - or  $\beta$ -naphthyl acetate and 2.4 mM Fast blue BB salt for 60 min, in the dark, at 37°C. The naphthyl acetate stock solutions were prepared in 1 ml acetone to aid their solubility in the phosphate buffer. Replicate gels were pre-incubated for 15 min in the dark in phosphate buffer with  $1 \times 10^{-3}$ ,  $1 \times 10^{-4}$ , and  $1 \times 10^{-5}$  M eserine sulfate or triphenyl phosphate, which are specific inhibitors of acetylcholinesterases (AChE) and carboxyl-

esterases, respectively, prior to detection of esterase activity with the naphthyl acetate method. To ensure AChE inhibition throughout the 60 min esterase detection step, eserine sulfate was added to the naphthyl acetate-Fast blue BB buffer system. Since this electrophoretic analysis of proteins with esterase hydrolytic activity is performed under native conditions, molecular weights of visualized proteins cannot be determined.

**Statistics** - Mortality from each treatment in the vial bioassay was tested separately using Chi-Square Test for Independence. Survival of mites from each vial per treatment chemical was compared to the expected survival from the control vials. The null hypothesis was that treatments did not differ from control and if the  $\chi^2$  value was greater than the critical values ( $\alpha = 0.05$ ), the hypothesis was rejected. Rejected values were recorded as *miticide-susceptible* (S); samples below the critical values were *miticide-resistant* (R).

## RESULTS

**Vial bioassay** - In April 2003, the three colonies tested from the CHBRC apiary (AZ Lab-1, 2 3) were susceptible to amitraz (Table 1). AZ Lab-1 and 3 also were susceptible to fluvalinate and coumaphos but AZ Lab-2

was resistant. Subsequent testing of AZ Lab-3 was conducted twice in the spring and again in the summer. While the mites were still susceptible to amitraz in the spring, we found resistance to coumaphos and fluvalinate. By the summer, the colony's mites were resistant to all three acaricides. AZ Lab-4 and 5 were tested in the fall and were resistant to all three chemicals.

Mites from the apiary of the Arizona cooperator who reported using no chemical treatments had only one sample of fluvalinate-susceptible mites in May (Table 2). All other samples were resistant to all three acaricides. Samples from cooperators in other states also were resistant to all chemicals, with the exception of mites from Florida; they were all susceptible. The mite samples from a cooperator in Maine (ME) and one that moves colonies between Maine to Florida (Migratory ME/FL) all tested resistant. Migratory ME/FL provided mites from untreated and treated colonies, but all were resistant to the three acaricides. Mites obtained in early July samples from North Dakota were susceptible to amitraz and fluvalinate, but were resistant to coumaphos. In late July, mites from the same cooperator were resistant to all three chemicals.

**Esterase activity analysis** - There was an intense band of esterase activity present in the OP-resistant mites

Fig. 1. Native Esterase Activity Gel. Test run of esterase gel of varroa vs. *B. microplus* larvae (from TX lab) with various toxicological profiles. These were assayed for general esterase activity by extraction in phosphate buffer containing Triton X-100, fractionation by native polyacrylamide gel electrophoresis, and incubation with  $\alpha$ -naphthyl acetate and Fast blue BB. Lanes represent one mite equivalent. Lane 1: *B. microplus* Gonzalez strain susceptible to both Pyrethroid and OP; Lane 2: OP-resistant Tuxpan strain of *B. microplus*; Lane 3: Coatzoacoalcos pyrethroid-resistant strain of *B. microplus*; Lane 4 and 5: Verbal report of Pyrethroid- and OP- resistant varroa from Maine/Florida mites, 2002; Lane 6 and 7: varroa susceptible to both pyrethroid and OP (Lab mites, Tucson AZ 2002). The pesticide resistant ticks possess both qualitative and quantitative differences in esterase activity.

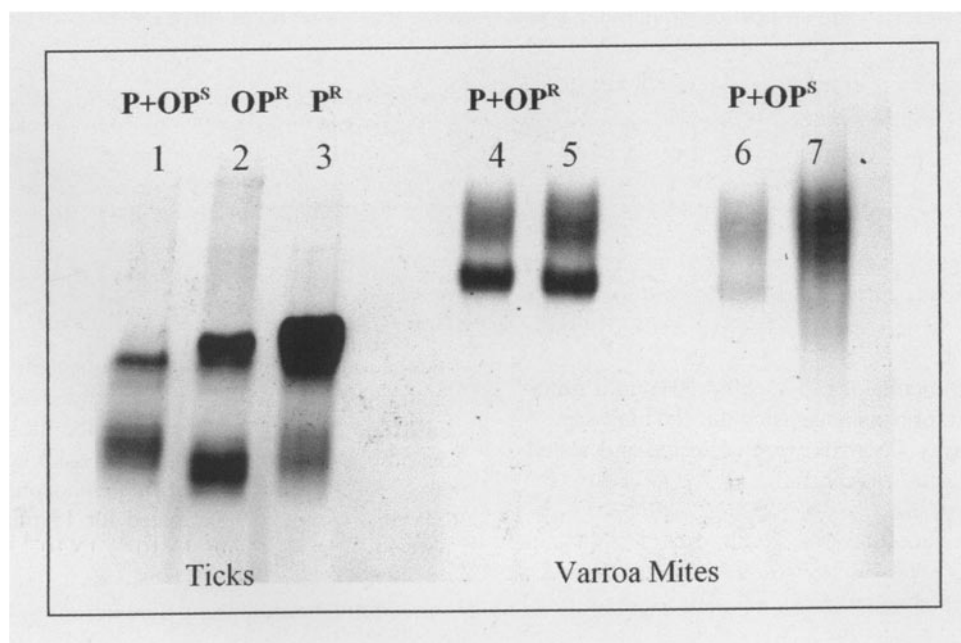


Table 1. Vial bioassay results from varroa mites from Carl Hayden Honey Bee Research Center (CHRC), Tucson AZ. The null hypothesis was that treatments did not differ from control and if the  $\chi^2$  value was greater than the critical values ( $\alpha = .05$ ), the hypothesis was rejected. Rejected values were recorded as *miticide-susceptible* (S); samples below the critical values were *miticide-resistant* (R). The *Time of sampling* included Early Spring (April/May), Spring (June) and Summer/Fall (July-Sept). The number of samples taken at each time is represented by the letters in each column. For example, in Lab-3 colony, two samples were taken in the Spring and the Summer. The numbers under the Chi Square column correspond to the sampling times, and indicate degrees of freedom and Chi Square value at  $\alpha = .05$ . Mite samples from Lab 2 and 3 colonies were used in the esterase activity gel in Figure 2.

CHRC Bee Lab			Time of sampling		Esterase	df, Chi Square $\alpha = .05$		
Source	Colony	Treatments	E.Spring	Spring	Summer/Fall	E.Spring	Spring	Summer/Fall
AZ lab	Lab-1	Amitraz	S			3, 10.25		
		Coumaphos	S			3, 8.0		
		Fluvalinate	S			3, 7.25		
	Lab-2	Amitraz	S			1, 5.56		
		Coumaphos	R			1, 2.93		
		Fluvalinate	R			1, 0.04		
	Lab-3	Amitraz	S	S S	R R	2, 5.89	2, 9.98; 4, 19.72	2, 1.2; 4, 3.4
		Coumaphos	S	R S	R R	2, 4.11	2, 4.96; 4, 13.18	2, 1.0; 4, 0.2
		Fluvalinate	S	R S	R R	2, 5.44	2, 3.54; 4, 10.92	2, 1.0; 4, 0.2
	Lab-4	Amitraz			R	2, 0.84		
		Coumaphos			R	2, 0.25		
		Fluvalinate			R	2, 0.25		
	Lab-5	Amitraz			S			2, 5.99
		Coumaphos			R			2, 1.46
		Fluvalinate			R			2, 1.46

Fig. 2. Native gel profiles of varroa from 2003 multi-state vial assay survey. Lane 1: AZ lab-1 susceptible; Lane 2: FL mites (susceptible); Lane 3: AZ lab-2 (mixed resistance); Lane 4: AZ cooperator reported no treatments (mites mostly resistant); Lane 5: Migratory ME/FL (treated), mites all resistant; Lane 6: AZ lab-3 (mixed resistance); Lane 7: ND #1 (mixed resistance); Lane 8: *Boophilus microplus* Coatzoacoalcos. Table II represents the colonies that were tested in this gel run. The mites in Lane 2 (FL) tested out susceptible in the vial assay; however they came up positive for esterase activity in the gel profile.

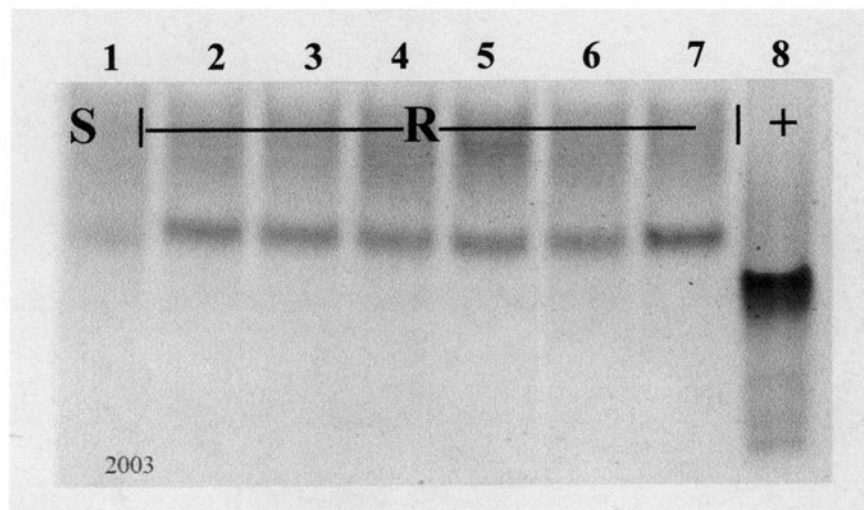


Table 2. Vial Bioassay results of varroa mites from the cooperators. Miteicide-susceptible (S); and miteicide-resistant (R) varroa are indicated in the columns. The *Time of sampling* included Spring (May), Summer (June/July) and Fall (Aug-Sept). The number of samples taken at each time is represented by the letters in each column; for example in AZ, there were three spring samples and 2 summer samples taken. The *Esterase* column (shaded) indicates whether the mite sample was used for the activity gel test (Y= Yes). The numbers under the Chi Square column correspond to the sampling times, and indicate degrees of freedom and the Chi square critical value, at  $\alpha=.05$ .

COOPERATORS	Treatments	Time of sampling			Esterase Y=tested	df, $\chi^2$ ; $\alpha=0.05$	Spring	Summer	Fall
		Spring	Summer	Fall					
AZ	Amitraz	R R R	R R			2, 4.25; 1, 0.66; 2, 1.0	4, 0.76; 3, 0.22		
not treated	Coumaphos	R R R	R R		Y	2, 0.6; 1, 0.66; 2, 2.3	4, 2.26; 3, 0.22		
	Fluvalinate	S R R	R R			2, 1.53; 1, 1.93; 2, 0.77	4, 0.14; 3, 0.22		
FL	Amitraz	S			Y	1, 2.43			
	Coumaphos	S				1, 0.41			
	Fluvalinate	S				1, 1.29			
CA	Amitraz		R		*		4, 13.9		
	Coumaphos		R				4, 11.0		
	Fluvalinate		R				4, 13.2		
ME	Amitraz		R		*		7, 12.18		
	Coumaphos		R				7, 2.33		
	Fluvalinate		R				7, 6.88		
ND #1	Amitraz		S S		Y		4, 20.1; 4, 11.0		
early July	Coumaphos		R R				4, 7.3; 4, 3.6		
	Fluvalinate		S R				4, 12.4; 4, 9.23		
ND #2	Amitraz		R R		*		4, 1.31; 4, 2.55		
Late July	Coumaphos		R R				4, 0.4; 4, 1.7		
	Fluvalinate		R R				4, 0.83; 4, 1.7		
Migratory ME/FL	Amitraz			R				3, 3.86	
not treated	Coumaphos			R	*			3, 0.266	
	Fluvalinate			R				3, 0.157	
Migratory ME/FL	Amitraz			R	Y			2, 1.32	
treated	Coumaphos			R				2, 0.021	
	Fluvalinate			R				2, 0.062	

that was missing in the susceptible varroa from the 2002 samples (Fig. 1). The pesticide-resistant tick strains (Lanes 2 and 3) possess both qualitative and quantitative differences in esterase activity compared with the pesticide-susceptible strain (Lane 1). The esterase profile for both the susceptible and resistant mites was not affected by eserine sulfate or triphenyl phosphate (data not shown), indicating the esterases were probably not acetylcholinesterases or carboxylesterases.

The column labeled *Esterase* in Table II identifies those samples that were tested by the gel method, shown in Fig. 2. The "Y" in the column identifies those mites in the sample that were tested for esterase activity. Lane 1 was the original susceptible colony in our research yard from 2002 which subsequently died. This colony had no esterase band. The mites from the Florida cooperator (Lane 2 in Fig. 2) had a strong esterase band although in the bioassay they were susceptible to all chemicals. All other mites run in the gel had esterase activity, regardless of their resistance history; the results in the vial bioassay showed strong resistance to all acaricides.

## DISCUSSION

Mites from our lab generally were susceptible to the acaricides used for controlling varroa. However, almost all the mites we received from our cooperators, regardless of the treatment regimes used, had resistance to all acaricides. The only exception was the mites from Florida which were susceptible despite being in a region that has been heavily treated with acaricides. In colonies where we were able to test mites more than one time, we found mites susceptible in the spring but by late summer the mites became resistant to all three acaricides, despite not being treated with amitraz. We were unable to test the susceptible Florida mites again in the fall to determine if this trend continued. Esterase activity was not a reliable indicator of resistance in our samples. Mites resistant to some acaricides had an esterase band, as did the susceptible mites.

The presence of resistant mites in most colonies, especially those sampled in the fall, may be explained by the over-use or misuse of registered varroa acaricides. Surprisingly, mites also showed resistance to amitraz, which is not a registered acaricide. These results suggest either cross-resistance between amitraz and other registered chemicals, or mite exposure to amitraz. Resistance could increase quickly in colonies because mites not killed by acaricides reproduce, thus reinforcing resistant genes. It was surprising to find resistant mites from cooperators who did not treat with acaricides (e.g. the Arizona and Maine cooperators). The presence of resistant mites in their operations may be due to: 1) bees robbing honey from a weak or dying hive (with resistant mites) within the flight range of the apiary and in the process acquiring those mites, 2) introduction of package bees and queens

from other states that have resistant mites, or 3) drifting bees, a common phenomenon in large apiaries where phoretic mites can be swiftly distributed throughout the whole apiary in a short time.

Our findings indicated that as the summer progresses, the population of bees and (resistant) mites increased. Perhaps the resistant mites were able to out-compete the susceptible mites. This may explain the switch from susceptible to resistant mites from spring to fall. Why the resistant mites appear to overwhelm colonies may be expressed in genetic terms. Resistance is rarely totally dominant (Carrière, 2003) but could be expressed at some level in heterozygotes especially if the resistance confers a gain of function, e.g. detoxification of chemicals by enzymes, reduced penetration and enhanced elimination of toxins. Females that have two copies of a resistant allele (RR) would produce offspring that also are homozygotes. Heterozygote foundress mites would produce 0.5 heterozygote and 0.5 homozygote susceptible offspring if the male parent had the susceptible allele, and 0.5 homozygous resistant and 0.5 heterozygous offspring if their male parent carried the resistant allele. Therefore, each heterozygote has a 0.5 probability of producing all resistant individuals and a 0.5 probability that half of their offspring will be resistant depending on the genotype of the male parent. The homozygous resistant state would not change due to brother-sister mating and its frequency would increase with each generation. Under these conditions, it is not surprising to find increased frequency of resistant individuals over time, especially if pressure from acaricides is removing homozygous susceptible individuals from the population. Varroa resistance could also be sex-linked, but since varroa males do not come in direct contact with the acaricides (other than through the accumulation in the wax) and their resistance has never been tested, this is only a speculation. Of course, unless we are able to rear varroa off host and in an artificial environment, we can only hypothesize such events.

Based on the esterase activity gel profile of the susceptible and resistant *V. destructor* from 2002, it is possible that an esterase-mediated resistance mechanism is operative in the population of mites we analyzed. However, the reliability of this method was not apparent in the 2003 tests, as all mites had the esterase band. Since other resistance mechanisms are operating on varroa mites, including esterase detoxification (Gerson *et al.*, 1991; Thompson *et al.*, 2002), monooxygenases in the P450 system (Hillesheim *et al.*, 1996), and sodium channel mutations (Wang *et al.*, 2000), testing varroa for any one method may not be the most reliable way to determine mite resistance.

Once the operating systems of varroa resistance are determined, it may be possible to develop a successful management program to counteract resistant varroa. According to Milani and Vedova (2002), resistant mites left untreated for 4-6 years will lose their resistance to

fluvalinate. Why this is so and what mechanisms are being used for such a switch, need to be determined. Identifying resistance mechanisms in varroa will be challenging though, because it will require rearing esterase-free and susceptible mites in an isolated area and subjecting them to known chemical regimes. This will require strains of mites (and colonies) that are not contaminated from outside sources or mites of known resistance reared in the laboratory.

#### ACKNOWLEDGEMENTS

This paper is dedicated to Dr. Patti Elzen for her help and support in sharing her technique and teaching us the vial assay. James Waddell pulled a lot of mites and tested many vials, for which we are grateful. Thanks also to Drs. Dave Gilley, Gloria DeGrandi-Hoffman, Judith Hooper and Gerry Loper for their helpful suggestions on the manuscript. And thanks to all the cooperators for sending us drone frames (R. Abel, B. and B. Woodworth, F. Terry, T. Glenn) and to the able help of bee inspectors from many states, especially Anthony M. Jadcak (Maine) and Dave Westerfelt (Florida) for sending us the extra bees and mites. Anonymous reviewers were also very helpful.

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