First data on resistance mechanisms of *Varroa jacobsoni* (OUD.) against tau-fluvalinate

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ABSTRACT

In 1991, the first losses of efficacy of tau-fluvalinate against the honeybee ectoparasite *Varroa jacobsoni* Oud. were recorded in Sicily. Since then, diminished efficacy with available pyrethroid treatments has been encountered in many regions of Italy. The aim of this study was to investigate the type of resistance in *V. jacobsoni* to the pyrethroid tau-fluvalinate by focusing on metabolic resistance mechanisms (detoxication). After developing a suitable application method, two synergists were used: piperonyl butoxide (PBO), as an inhibitor of the microsomal monooxygenases of the cytochrome P450 complex and *S,S,S*-tributylphosphorotrithioate (DEF), which blocks esterases. A significant decrease in the LC_{50} values of the susceptible and of the resistant mite strains after the application of PBO was observed. A slight decrease of the LC_{50} values was also observed after the application of DEF. However, this decrease was not significant. These results indicate that the resistance of *Varroa* mites to tau-fluvalinate can partly be explained by an increased detoxication due to the monooxygenases in the P450 system, which is blocked by PBO. Esterases seems to play a negligible role. Whether glutathione-*S*-transferases are involved, is still unknown, but other mechanisms, such as the modification of the binding sites and/or reduced uptake might be involved as well.

Key words: *Varroa jacobsoni*, resistance, tau-fluvalinate, resistance mechanism, detoxication.

INTRODUCTION

Varroa jacobsoni is a major threat to honeybees in Europe (Beetsma, 1994). Between the *Varroa* mites and their original host, *Apis cerana*, a co-existence without killing the bee colonies have evolved. No such tolerance of the European species *Apis mellifera* to *Varroa* mites is known (Boecking and Ritter, 1994). Therefore, to prevent colony losses, the beekeeper has to reduce the number of *Varroa* mites in the bee colonies. Different types of treatments against these ectoparasitic mites have been developed. Biological mechanical methods include cutting out the drone brood, artificial swarm production, heat treatment of the colony (Engels and Rosenkranz, 1992) or application of organic acids and etheric oils (Imdorf *et al.*, 1995). Apart from these techniques, synthetic acaricides have

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been developed by the industry for beekeeping (for an overview over the different methods see Ritter (1996). One of them is APISTAN®, with the active ingredient tau-fluvalinate, a pyrethroid. The efficacy of APISTAN® against *Varroa* mites is generally between 98 and 100% and the product is registered and used practically world-wide.

However, diminution of its efficacy has been reported in Italy since 1991 (Loglio and Plebani, 1992; Lodesani *et al.*, 1995) and recently resistant mite strains have been reported in the South of France (Vandame *et al.*, 1995) and in the South of Ticino at the border to Italy (Trouiller, 1995).

'Pesticide resistance is a well-known evolutionary phenomenon' (Dobzhansky, 1951). This means that pesticide resistance is a result of selection caused by a changing environment. The development of new insecticides/acaricides has declined during the last decade (Soderlund and Bloomquist, 1990) and selection for *Varroa* mite-tolerant bees is still in its infancy (Ritter *et al.*, 1990; Hoffman *et al.*, 1995). Therefore, resistance management, aiming to prevent, delay or eliminate resistance, is strongly recommended. Knowledge of the resistance mechanism(s) is essential in developing a suitable resistance management strategy. Several different mechanisms of resistance are known in arthropods, such as reduced penetration of the active substance (reduced uptake), a change in the behaviour of the target species, modification of the binding site and metabolism (detoxication processes) (Hassell, 1990, Chapter 9; Bassand, 1993).

Different enzyme complexes – oxygenases, hydrolyses (esterases) and glutathione-*S*-transferases – can be responsible for an increased detoxication rate.

In this paper, two enzyme complexes, i.e. oxygenases of the cytochrome P450 complex and esterases were investigated, using two selective synergists. As a blocker of the monooxygenases of the cytochrome P450, piperonyl butoxide (PBO) was selected. *S,S,S*-Tributylphosphorotrithioate (DEF) was used as a blocker of esterases (Raffa and Priester, 1985; Scott, 1990). Dose–mortality curves of pyrethroid-resistant and susceptible mites were estimated with and without synergist.

MATERIAL AND METHODS

Mites

The susceptible mites were collected from queenright colonies located in Freiburg i. Br. Germany. These bee colonies had never been treated with pyrethroids.

The resistant mites were descended from colonies from different sites in Northern Italy and from Ticino (South of Switzerland). These colonies were kept under quarantine conditions in a flight room in Freiburg i. Br. Germany.

The mites were collected from sealed bee broods. Only vital dark brown mites were used, which came from just sealed brood (larvae) to bee pupae with pink eyes. According to Stürmer (personal communication), healthy mites weigh

between 0.28 and 0.39 mg. The mites were placed in a Petri dish with bee larvae as a food supply (eight to ten mites per larva). The mites were stored in an incubator at 32.5 ± 0.5°C and 74% RH until the test started.

Development of a test design for applying a synergist

Topical application was used to apply synergists, according to a method developed by Ritter and Roth (1988). Mites were glued dorsally on a microscope slide coated with double face tape (Tesa fix-Doppelband-Fotostrip). Acetone (0.2 µl) (a suitable solvent for the different synergists) was applied ventrally to the mites (oral and contact uptake). The slides with the treated mites were placed for 1 h in a plastic box with an RH of 98%, which was stored in an incubator at 32.5 ± 0.5°C. After 1 h, the mites were gently removed from the microscope slide with a brush and were transferred into the paraffin-coated Petri dishes without active ingredient. They were then treated according to the method of Milani (1995), following the procedure used for the untreated check. After 6 h (no food) the mites were transferred into glass Petri dishes and fed with bee larvae. The status of the mites was recorded after 6, 24 and 48 h. The mites were categorized into three groups: alive (if they moved normally), paralysed (if they moved at least one leg after stimulation with a brush), and dead (if they did not move after repeated stimulations). During the whole test period the mites in the dishes were kept in an incubator at 32.5 ± 0.5°C and 74% RH.

During this procedure mites are submitted to various stress factors: starvation, the effect of gluing and the effect of acetone.

The following tests were designed to investigate the possible undesirable effects of these factors.

(1) Untreated check (Milani's method): collected mites were placed directly into the paraffin-coated Petri dishes for 6 h without food, then transferred into glass Petri dishes and fed with bee larvae (total period of starvation is 6 h).
(2) Effect of prolonged starvation: the mites were kept for 1 h in a glass Petri dish without food. They were allowed to move freely. After 1 h, the mites were gently removed from the glass Petri dish with a brush and confined into the paraffin-coated Petri dishes without active ingredient and were treated according to the method of Milani (total period of starvation is 7 h, (i.e. 1 h glass Petri dish plus 6 h paraffin-coated Petri dish).
(3) Effect of prolonged starvation and gluing: the mites were glued on the microscope slide for 1 h but were not treated with acetone. After 1 h, the mites were gently removed from the microscope slide with a brush and were transferred into the paraffin-coated Petri dishes without active ingredient and were treated according to the method of Milani (total period of starvation is 7 h, (i.e. 1 h glued on the microscope slide plus 6 h in the paraffin-coated Petri dish).
(4) Effect of prolonged starvation, gluing and acetone: mites glued on a microscope slide were topically treated with 0.2 µl of acetone. One hour

later they were transferred into paraffin-coated Petri dishes without active ingredient and were treated according to the method of Milani (total period of starvation is 7 h, i.e. 1 h glued on the microscope slide and treated with acetone plus 6 h in the paraffin-coated Petri dish).

For each set-up 2 × 25 mites were used. Data were recorded after 48 h and analysed statistically with a Brandt–Snedecor χ^2 test (Sachs, 1984 pp. 357–361); the paralysed and mobile mites were pooled and tested against dead mites.

Bioassay with the synergists

The dose–mortality relationships of the synergists applied alone were measured for each strain with the methods described above, using design number 4. PBO was selected to block the oxygenases, and DEF to block the esterases.

To investigate the effect of the synergists on the toxic effect of tau-fluvalinate, a single concentration of an acetonic synergist solution (500 μl l^{-1}, was applied to the mites .Thus, each mite received 0.1 nl of synergist in a droplet of 0.2 μl. The control group was treated with 0.2 μl of pure acetone. Dose–mortality curves were estimated using a series of four to 12 different concentrations of tau-fluvalinate, depending on the mite strain, susceptible or resistant. For each concentration, 20–30 mites were treated. Generally, 60 mites were used for the control group. Following the method of Milani (1995), the data after 48 h were used to calculate the LC_{50} values. Paralysed and mobile mites were pooled and tested against dead mites. LC_{50} values were calculated using logit analysis, according to Berkson (1953). Analyses of regression were performed according to Linder and Berchtold (1976, Chapter 3).

RESULTS

Development of application method

The results of the four different designs are given in Table 1. The mortality among the four designs is significantly different after 48 h ($\chi^2 = 34.2$; df = 3; $p \ll 0.01$). This is due to an increased mortality in the group 2, which could be explained by a higher energy consumption caused by the searching behaviour of the mites for food.

There is no significant difference between the mortalities in the three other groups (1, 3 and 4) ($\chi^2 = 2.4$; df = 2; $p = 0.30$). It can be concluded that the modified design (4) has no influence on the mortality of the mites. Therefore, design 4 was used for applying different synergists.

PBO

The dose–mortality relationships of PBO used alone showed the same shape for the resistant and for the susceptible strains (Fig.1). The dose of 0.1 nl per mite was selected for both strains, because the mortality induced by this amount of

TABLE 1

Results of the four different designs for the development of the test method

Design	Replicate	After 48 h					
		Mobile	Paralysed	Dead	Total	% pooled mortality	
1	1	20	0	5	25		
	2	24	0	1	25	12.0	a
2	1	15	1	9	25		
	2	10	0	15	25	48.0	b
3	1	19	0	5	24		
	2	21	0	3	24	16.7	a
4	1	22	0	2	24		
	2	25	0	0	25	4.1	a

(1) Untreated check (Milani's method), (2) effect of prolonged starvation (the mites were allowed to move), (3) effect of prolonged starvation and gluing, (4) effect of prolonged starvation, gluing and acetone. (For a detailed description of the designs see Material and Methods.)
Designs with the same letter did not differ significantly at the 5% level (Brandt-Snedecor χ^2 test).

PBO was not significantly different from that of the untreated check (susceptible, $\chi^2 = 0.54$, df $= 1$, $p > 0.05$; resistant, $\chi^2 = 0.30$, df $= 1$, $p > 0.05$). Moreover, evidence obtained elsewhere (Milani, personal communication), indicates that a mortality of up to 25% in the untreated check is not uncommon under the test conditions, provided a clear dose–mortality relationship is observed. The dose–mortality relationship starts from 0.1 nl per mite for resistant strains and 0.2 nl per mite for susceptible strains.

Figure 2 shows the dose–mortality lines of a susceptible strain with and without PBO together with the dose–mortality lines of a resistant strain with and without PBO. The LC_{50} values of susceptible and resistant mite strains with and without PBO together with their confidence intervals are given in Table 2. The LC_{50} values of the mites treated with and without PBO are in all cases significantly different from each other (the confidence limits of the LC_{50}s in trials 2, 3 and 4 did not overlap. Overlapping of the confidence limits was observed in trial 1. Consequently, a *t*-test of the LC_{50}s was carried out and indicated a significant difference ($t = 3.03$, df $= 6$, $p_{\text{two-tailed}} = 0.02$; Linder and Berchtold, 1976). Large differences between the LC_{50} values of the two trials with the resistant mites (3 and 4) occurred. Nevertheless, a clear level of resistance was observed for both trials. The resistance ratio (RR; LC_{50} of the resistant strain divided by the LC_{50} of the susceptible strain) and the synergistic factor (SF, LC_{50} of one strain without synergist divided by the LC_{50} of the same strain with synergist), together with their 95% confidence limits (Robertson and Preisler, 1992), were calculated (Tables 2 and 3). The RRs were calculated between each measured LC_{50} value of the susceptible strain and each LC_{50} value of the resistant strain (Table 3). The RRs after the application of PBO strongly

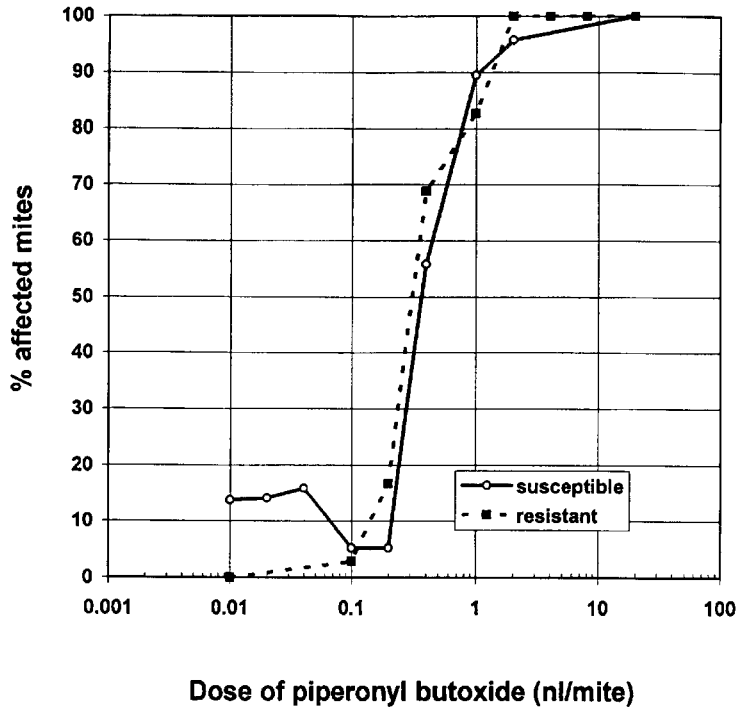

Fig. 1. Percentage of affected susceptible and resistant *Varroa* mites (dead and paralysed mites) after 48 h treatment with the synergist PBO. Susceptible mites, $n = 418$; resistant mites, $n = 270$.

decreased, but did not reach 1, which would indicate the potential for complete reversion of the resistance by applying PBO. As a consequence, the large SFs obtained on the resistant strains reflect the effect of PBO, increasing the sensitivity of resistant mites against tau-fluvalinate.

The slopes obtained from the susceptible mites (with and without PBO) are much steeper than the slopes of the resistant mites (Table 2). The difference between these slopes was compared with a parallelity test according to Linder and Berchtold (1976). The common slopes of the two different mite strains were significantly different from each other ($\chi^2 = 5.24$; df $= 1, p \ll 0.01$).

DEF

The dose–mortality relationships of DEF used alone showed the same shape for the resistant and for the susceptible strains (Fig. 3). The dose of 0.1 nl per mite was selected for both strains, because the mortality induced by this amount of DEF was not significantly different from that of the untreated check (susceptible, $\chi^2 = 2.27$, df $= 1, p > 0.05$; resistant, $\chi^2 = 0.49$, df $= 1, p > 0.05$).

The LC_{50} values with their confidence intervals are given in Table 4. A significant difference between the LC_{50} values of the susceptible and the resistant

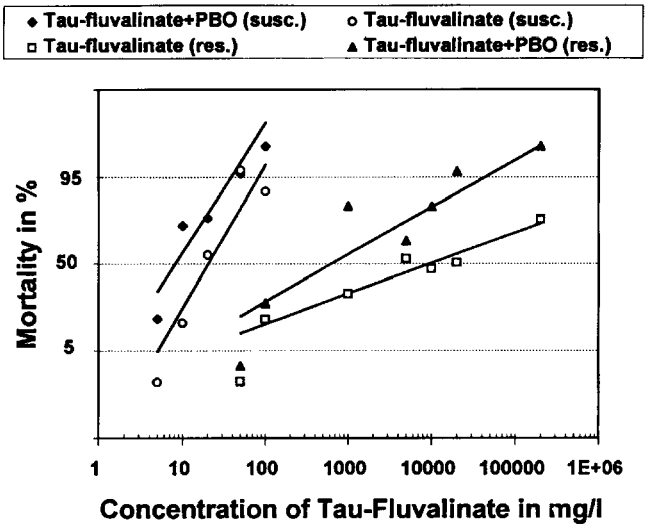

Fig. 2 Dose–mortality curves (percent corrected mortality) of susceptible and of resistant *Varroa* mites after 48 h with and without PBO to tau-fluvalinate.

strains was observed. However, the addition of DEF did not modify significantly the level of activity on susceptible as well as on resistant strains. The RR and the SF, as well as their 95% confidence limits, were calculated. In the presence of DEF, the RRs decreased to some extent (Table 5) but the SFs for the susceptible and for the resistant mite strains remained small and were not significantly different from 1. For the slopes, a similar picture was obtained which was observed for the mites treated with the synergist PBO. The slopes obtained from the susceptible mites are steeper than the ones of the resistant mite strain (see also Fig. 4).

DISCUSSION

Resistance to tau-fluvalinate in *Varroa* mites seems to be partly due to some detoxication processes. PBO has a significant effect, but does not completely reverse this type of resistance, thus indicating that some enhanced detoxication might be due to oxygenases. It is known that in insects, PBO blocks most of the oxygenases, without being completely selective (i.e. no 100% blockage of all oxygenases). Whether other oxygenases, which are not blocked by PBO, are also involved in the detoxication of tau-fluvalinate is not known yet. To answer this question, additional studies would be needed, e.g. with other synergists or biochemical methods.

Esterases seems to play a negligible role in the resistance of *Varroa* to tau-fluvalinate. The slight non-significant shift to the left of the dose–mortality curves and the small decrease of the corresponding LC_{50} values when DEF is

TABLE 2

Biological activity of tau-fluvalinate applied with and without PBO against susceptible and resistant *Varroa* mites

Strains	Trial No.	Without PBO				With PBO					
		n	LC_{50}	(95% CL)	Slope	*n*	LC_{50}	(95% CL)	Slope	SF	(95% CL)
Susceptible	1	152	20.4	(16.0–26.0)	5 ± 0.8	149	8.2	(3.6–18.5)	4.5 ± 0.8	2.5	(1.4–4.4)
	2	120	33.2	(22.9–48.0)	3.1 ± 0.7	122	12.8	(10.6–15.4)	6.7 ± 1.1	2.6	(1.7–3.9)
Resistant	3	207	9504.2	(4576.1–19 739.9)	1.1 ± 0.2	206	629.1	(131.7–3005.7)	1.6 ± 0.3	15.1	(3.7–61.2)
	4	209	1695.1	(939.1–3059.6)	1.4 ± 0.2	118	246.3	(142.8–424.9)	2.8 ± 0.5	6.9	(3.1–15.4)

The LC_{50} is given in mg l^{-1}, (95% CL) is the 95% confidence limits, *n* is the number of tested mites without the control mites, slope ± standard error and SF is the synergistic factor (i.e. the LC_{50} of the unsynergized treatment divided by the synergized treatment).

TABLE 3

Resistance ratios of susceptible and resistant *V. jacobsoni* strains with and without the synergist PBO

Treatment	Trial Nos.	RR	(95% CL)
Without PBO	1 and 3	467	(216–1009)
	2 and 3	287	(126–650)
	1 and 4	83	(44–158)
	2 and 4	51	(25–103)
With PBO	1 and 3	77	(21–281)
	2 and 3	49	(15–164)
	1 and 4	30	(14–63)
	2 and 4	19	(11–34)

RR, resistance ratio (i.e. the LC_{50} resistant strain divided by the LC_{50} of the susceptible strain) and (95% CL) is the 95% confidence limits.

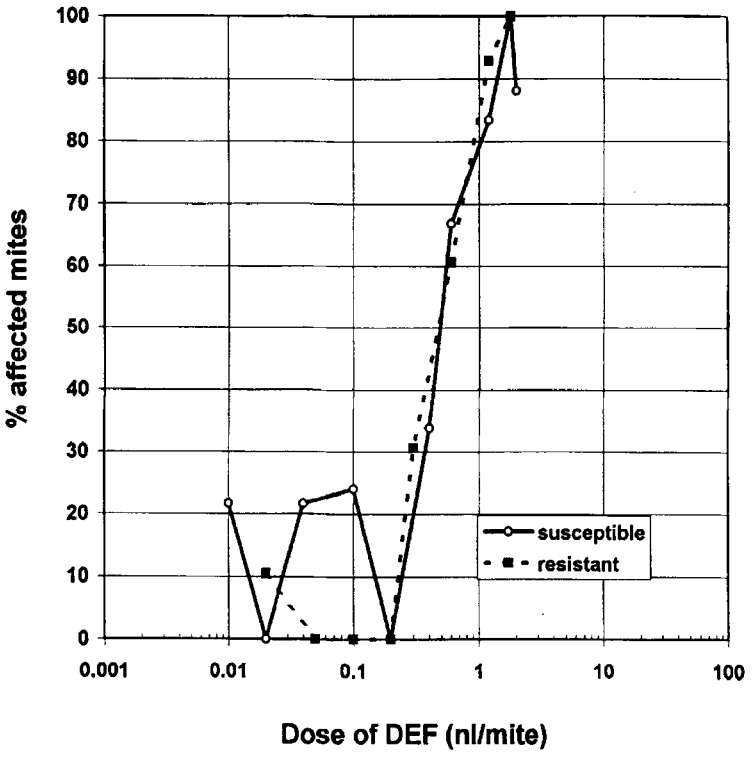

Fig. 3. Percentage of affected susceptible and resistant *Varroa* mites (dead and paralysed mites) after 48 h treatment with the synergist DEF. Susceptible mites, $n = 211$; resistant mites, $n = 242$.

TABLE 4

Biological activity of tau-fluvalinate applied with and without DEF against susceptible and resistant *Varroa* mites

Strains	Trial No.	Without DEF				With DEF					
		n	LC_{50}	(95% CL)	Slope	*n*	LC_{50}	(95% CL)	Slope	SF	(95% CL)
Susceptible	1	79	18.6	(14.0–24.8)	5 ± 1.1	61	12.4	(8.9–17.3)	5.6 ± 1.3	1.5	(1.0–2.3)
	2	60	12.2	(8.2–18.2)	3.8 ± 1.0	60	6.8	(4.1–11.1)	2.9 ± 1.3	1.8	(1.0–3.4)
Resistant	3	209	1695.1	(939.1–3059.6)	1.4 ± 0.2	210	558.5	(125.8–2479.0)	1.5 ± 0.2	3.0	(0.8–10.9)

The LC_{50} is given in mg l^{-1}, (95% CL) is the 95% confidence limits, *n* is the number of tested mites without the control mites and SF is the synergistic factor (i.e. LC_{50} of the unsynergized treatment divided by the synergized treatment).

TABLE 5

Resistance ratios of susceptible and resistant *V. jacobsoni* strains with and without the synergist DEF

Treatment	Trial Nos	RR	(95% CL)
Without DEF	1 and 3	91	(47–176)
	2 and 3	139	(68–284)
With DEF	1 and 3	45	(14–147)
	2 and 3	83	(24–286)

RR, resistance ratio (i.e. the LC_{50} of the resistant strain divided by the LC_{50} of the susceptible strain) and (95% CL) is the 95% confidence limits.

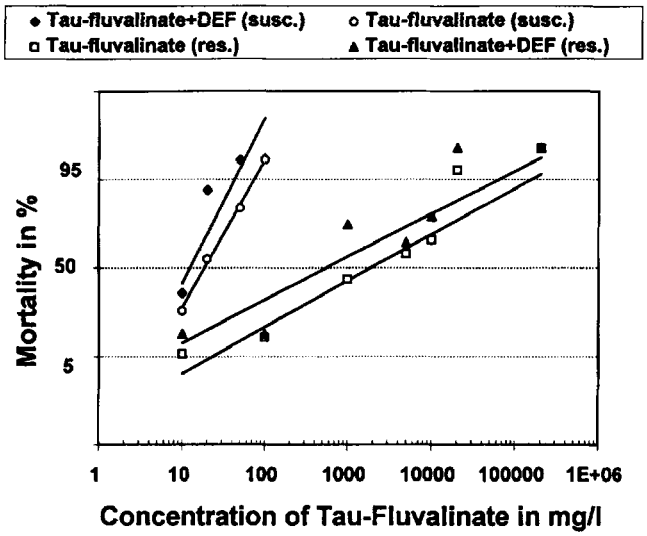

Fig. 4. Dose–mortality curves (percent corrected mortality) of susceptible and resistant *Varroa* mites after 48 h with and without DEF to tau-fluvalinate.

applied, may be due to biological random variability or can be explained by the type of synergist. However, DEF which blocks mainly esterases can also block oxidases at high concentrations (Scott, 1990). Since detoxication by oxygenases is partly involved in this type of resistance, the observed slight shift of the dose–mortality lines towards higher sensitivity might be explained by this side-effect of DEF. Whether glutathione-*S*-transferases are involved in the resistance of *Varroa* mites to tau-fluvalinate is not yet known.

The cause of the observed differences between the slopes of the dose–mortality lines obtained with susceptible and resistant strains, is still unclear. However, the

following hypothesis can be considered. The shape of the slope, i.e. the steepness of the dose–mortality lines, is influenced by the genetic variability of the strains. Homozygous susceptible or resistant strains exhibit steeper dose–mortality lines than populations consisting of a mixture of susceptible (homozygous), heterozygous and resistant (homozygous) strains (Lande, 1981; Rosenheim and Hoy, 1986; Falconer, 1989). However, this hypothesis is still a matter of controversy (Chilcutt and Tabashnik, 1995). Moreover, less than 30% of the phenotypic variation can be explained by genetic variability; the rest is caused by environmental factors such as the age of the test organisms, temperature, bioassay methodology, etc. (Omer *et al.*, 1993; Tabashnik and McGaughey, 1994).

It should also be kept in mind, that a resistance mechanism may have a polygenic origin or that several mechanisms of resistance may be present in a population. As a consequence, this complex situation will increase the variability in the population and, ultimately, result in a flatter dose–mortality line.

From a practical point of view, the question might arise, whether the use of PBO is combination with tau-fluvalinate in order to restore, at least partly, the susceptibility of resistant mites, is justified. Such combinations, using PBO and pyrethrins, for instance, are already on the market to control household pests. In the case of *Varroa* such a mixture cannot be recommended because the tolerance of honeybees to tau-fluvalinate is partly based on the detoxication by the cytochrome P450 monooxygenases in the honeybee (Bassand, unpublished data). Therefore, such a mixture would probably be highly detrimental to honeybees.

In conclusion it can be stated that oxygenases appear to be important for the resistance of *Varroa* mites to tau-fluvalinate, whereas the influence of esterases appears to be negligible. Metabolism studies are needed to confirm these results.

Apparently, other mechanisms, such as the reduced uptake of active substance or modification of the binding site, could also be involved. Further work will be needed to obtain more information on these resistance mechanism(s). Their knowledge will be essential to the development of an appropriate resistance management strategy.

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