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Original article

The resistance of *Varroa jacobsoni* Oud to pyrethroids: a laboratory assay

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Summary — A bioassay has been developed to assess the susceptibility of *Varroa* mites to fluvalinate, flumethrin and acrinathrin. No significant differences were found among groups of mites from the same origin but from different brood stages (capped larvae, pupae with white eyes, pupae with dark eyes and white or pale bodies), while a rather variable response was observed when mites from adult bees were assayed. The LC_{50} of mites from areas where treatments with fluvalinate are no longer effective was about 25–50 times higher than that of susceptible mites; an even larger increase in the LC_{95} was found. The LC_{50} of flumethrin and acrinathrin on mites surviving Apistan treatments increased 10–60 times.

pyrethroid / *Varroa jacobsoni* / resistance / laboratory assay

INTRODUCTION

Since 1992, a reduction in the effectiveness of Apistan against *Varroa jacobsoni* Oud in a large, rapidly expanding area of northern Italy has been reported (Loglio and Plebani, 1992; Astuti *et al*, 1995; Lodesani *et al*, 1995). In many cases this was revealed by a serious weakening or collapse of colonies still heavily infested after the treatment and resulted in dramatic bee losses.

The effectiveness of Apistan can be checked by using another highly effective, chemically unrelated acaricide in the absence of capped brood. However, this technique is time consuming and is more easily applied (at least under the climatic conditions of northern Italy and most European regions) in late summer or in early autumn. At this time of the year, the results can be influenced by the increased reinfestation rate (Greatti *et al*, 1992) and, in addition, many colonies are already weakened and cannot recover.

The development of a laboratory test to assay the susceptibility of the *Varroa* mite to fluvalinate would help clarify to what extent failures in the control of *V jacobsoni* using Apistan are due to the spread of

strains resistant to fluvalinate and possibly other pyrethroids. A reliable laboratory assay is essential to study some aspects of the resistance of *V jacobsoni* to pyrethroids (heritability, biochemical mechanisms, fitness of the resistant strains and possible reversion).

For this reason, we developed a bioassay to measure the susceptibility of the *Varroa* mite to pyrethroids, refining the previously used technique (Milani, 1994; Milani *et al*, 1995). The method chosen takes into account the mechanism of action of Apistan, Bayvarol and other varroacide products (*eg,* Gabon PA-92; Vesely, 1993) formulated in plastic strips: the active ingredient contained in the strips contaminates the cuticular lipids of the bees and is progressively taken up by the mite by indirect contact. Thus, the action of the acaricide is more easily counteracted by detoxifying mechanisms before the lethal dose is built up (D Bassand, personal communication), than if the same amount of active ingredient were applied all at once (Ritter and Roth, 1988; Abed and Ducos de Lahitte, 1993).

The technique was tested on strains of *Varroa* mites believed to be susceptible; these came from apiaries that had never been treated with pyrethroids or from areas where Apistan had been used for several years but with no reduction of effectiveness. Strains of mites surviving Apistan treatments, and thus supposed to be resistant, were also used.

MATERIALS AND METHODS

Pyrethroids tested

More extensive investigations were carried out on τ-fluvalinate (Sandoz), using the standard supplied by the manufacturer (rs-flu-101990, 92.3% purity). Assays were carried out also using flumethrin (Bayer), standard supplied by the manufacturer (920107dor01, 87.8%) and acrinathrin (Hoechst), kindly supplied by Prof V Veselý, VUVč, Dol, Czech Republic; the concentration of the latter sample was assumed to be 100%.

Origin of the mites

Mites were sampled from infested colonies in the following localities (fig 1): a) Udine (Friuli, north-eastern Italy), where Apistan has been used by most beekeepers since 1989, but no reduction in effectiveness has been reported so far (Greatti, unpublished data); b) Tirano (Lombardy, northern Italy), where treatment with Apistan had been unsatisfactory; a single colony, heavily infested a few months after treatment with Apistan, was brought to Udine in spring 1994 and kept in a flightroom; c) Lunz-am-See and Randegg (Niederösterreich), from apiaries never treated with pyrethroids (the former has been kept in an isolated area, without any chemical treatment since 1986) (Pechhacker, personal communication); d) Como (Lombardy), Chiavenna (Lombardy) and Varallo Pombia (Piedmont, but right on the border with Lombardy) from apiaries previously treated with Apistan up to 4 d before or being still treated and heavily infested.

The mites from colonies belonging to the same apiary were pooled, owing to the continuous exchange of mites that takes places between colonies of the same apiary (Sakofski and Koeniger, 1988).

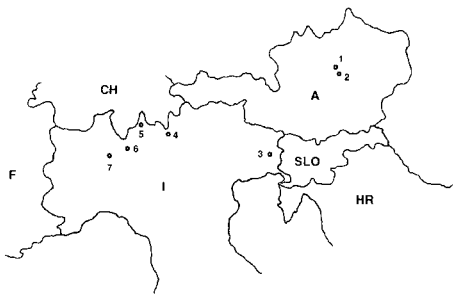

Fig 1. Localities in which *Varroa* mites were sampled: 1, Randegg; 2, Lunz-am-See; 3, Udine; 4, Tirano; 5, Chiavenna; 6, Como; 7, Varallo Pombia.

Collection of the mites

Infested combs (or pieces of comb) were brought to the laboratory and adult *Varroa* females were taken from capped brood by opening and inspecting individual cells; the mites were kept in a glass Petri dish with a paper towel in the base for 1–3 h, on bee larvae, until a sufficient number of mites was collected.

In the case of the colonies kept in Udine (either free-flying or in the flightroom: a and b above), the combs were brought to the laboratory immediately before the experiment. In the remaining cases (c and d), the combs were brought to the laboratory in an insulating box and kept in an incubator at 34.5 °C, 70% RH until needed (for not more than 2 d).

As a rule, *Varroa* mites from brood of different ages were assayed separately. In the case of the Udine colonies (a) and the colony kept in the flightroom (b), the age of the larvae was determined by marking the cells at the capping; the following groups were considered: mites from larvae 0–15 h after capping *(l5);* from pupae with white eyes *(pw),* 96–120 h after capping; from pupae with dark eyes *(pd),* 168–192 h after capping. This was not possible when the mites were taken from colonies kept elsewhere (c and d), so the approximate age of the brood was inferred on the basis of the morphology and pigmentation of the larva or the pupa; the following groups were considered: mites from spinning larvae (*l5*) and stretched larvae (*sl*), from pupae with white eyes (*pw*) and with dark eyes and white or pale body (*pd*). The use of the same name does not imply that the age was necessarily within the time intervals indicated for the Udine mites. *Varroa* mites from pupae with pigmented body (9 or more days after capping) or from adult bees about to emerge were not used, because newly moulted adult mites can be present in the cells at that time and sometimes cannot be easily distinguished from the parent mite; their response might not be uniform, as hardening of the cuticle is still in progress at that time.

Varroa mites from different brood stages were pooled only when their number was too small to carry out separate assays and previous results had indicated that no difference among them was to be expected.

Varroa mites were also taken from adult bees from the Udine apiary, both by dusting the bees with flour and by picking up infested bees by hand, when they were seen on the combs.

Mites found on dead or clearly diseased bee larvae, and the few mites which seemed weak or otherwise abnormal, were discarded.

The assays were carried out between June and the beginning of November 1994; about 5 500 mites were assayed with fluvalinate, 1 500 with flumethrin and 1 000 with acrinathrin.

The bioassay

Shallow capsules, made of 2 glass disks (62 mm diameter) and 1 or 2 stainless steel rings (56 mm inner diameter, 3–5 mm total height) were prepared. The interior of these capsules (including the rings) was coated with paraffin wax (Merck 7151, melting point 46–48 °C) containing a known concentration of the active ingredient. Four grams of paraffin wax was melted in a Petri dish kept in a water bath heated to 60°C and then the required amount of pyrethroid, dissolved in 2 ml hexane (Sigma H9379), was added. Hexane only was added to the control. The mixture was stirred for 1 min and the hexane was allowed to evaporate for at least 10 min. The steel rings were immersed into the molten paraffin wax and one side of the glass disks was coated by lowering the disk onto the molten paraffin; in a series of 14 capsules, the total weight of paraffin was in the range 1.6–2.0 g. The concentration used are reported in table I.

The capsules were then kept open for at least 24 h at room temperature to allow hexane residues to evaporate. The capsules were used for 1 month after they had been prepared; when not in use, they were kept at room temperature (23–29°C). Some assays, however, were carried out using capsules prepared about 2 months earlier, to assess the effects of ageing.

Ten or 15 *Varroa* females were introduced into each capsule; after 6 h they were transferred to a clean glass Petri dish (60 mm diameter) with respectively 2 or 3 worker larvae taken from cells 0–24 h after capping.

The mites were observed under a dissecting microscope 6 (when transferred to the Petri dish), 24 and 48 h after the introduction into the capsule and classified in the following categories: i) mobile mites: when they could move when put on their legs and stimulated if necessary, though

sometimes they were affected by the treatment to a varying degree and their movements were more or less uncoordinated; ii) paralysed mites: when they could move one or more appendages, but they could not progress; and iii) dead mites: when they did not react to stimulation repeated 3 times.

Mites lost or accidentally killed (*eg,* crushed between the steel rings) were not included in the counts. The mites inside the capsules coated with paraffin and in the Petri dishes were kept in an incubator at 32.5 °C and 70% RH.

In an experiment aimed at assessing the influence of the temperature, assays were carried out at 20 and 26°C, 70% RH, on mites from the Udine apiary.

As a rule, the assay was repeated 2 or 3 times on mites from a given origin and a given brood stage, until 30 mites per concentration were assayed. However, assays were not repeated at concentrations higher than those which were expected to give 100% mortality, or when mortality not exceeding that of the controls was expected at a higher concentration, since these data give little information. In a few experiments, when the number of mites was limited, more mites were assayed at concentrations around the median lethal concentration (Finney, 1971).

Further details on the bioassay can be obtained from the author.

The assays whose results are reported here are summarised in table II.

Precautions to avoid contamination

Precautions were taken to avoid contamination, due to the extremely large range of concentrations used (up to 1:50 000).

In each test, controls and capsules with lower concentrations were examined first. A sheet of paper was spread out on the bench and changed after processing each batch of cells. A different brush for each active ingredient and concentration was used to manipulate mites.

All the equipment that had come into contact with pyrethroids was washed separately; if it could withstand the treatment, it was immersed in concentrated KOH or NaOH (~10%) for some hours at least, before washing at 75°C in an automatic washer using an alkaline detergent. The preparation of the capsules was carried out in a separate room.

Statistical analysis

The data were analysed using the probit transformation; the natural mortality rate was taken into account using the iterative maximum likelihood approach, according to Finney (1949). As a starting estimate of the natural mortality (which is to some extent arbitrary), both the proportion of mites dead in the control capsules in that experiment (m_0) and the weighted mean of m_0 and of the proportion of mites dead in the controls of that series of experiments (with weights $\sqrt{(n_0)}$ and to $\sqrt{(n_t/10)}$, n_0 and n_t being the number of mites assayed in the controls of that test and in the whole series of tests) were used. The latter often allowed a faster convergence of the iterative computation, especially when n_0 was small. Three iterations were computed, although the first usually gave a satisfactory approximation, except for a single case, when 6 iterations were necessary. The fiducial limits were calculated according to Finney (1971), including a heterogeneity factor when appropriate. The heterogeneity was com-

Table I. Concentrations used in the bioassays (ppm).

Fluvalinate	0, 1*, 2*, 5, 10, 20, 50, 100, 200, 500, 1 000, 2 000, 5 000, 10 000, 20 000, 30 000*, 50 000
Flumethrin	0, 0.1*, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1 000
Acrinathrin	0, 0.1*, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500

The concentrations marked with an asterisk were used only in some assays.

puted using the χ^2 statistics. In a few cases, large χ^2 resulted from contributions of groups with small or very small expectations, leading to an overestimation of the heterogeneity. In these cases, the χ^2 was recalculated combining the groups with small expectations, until at least an expectation of 4 (or more) dead or surviving mites was reached.

Experimental values plotted in the graphs are corrected for the natural mortality.

RESULTS

Methodological aspects

The conditions of the mites at different times, the influence of the sampling technique, the effects of temperature and the ageing of the capsules were investigated using mainly flu-

Table II. Assays carried out on *Varroa* mites from different origins.

ai	*Origin of mites*	*Honeybee stage*	*Mites per concentration*	*Notes*
A) Assays carried out at 32.5°C				
fv	Udine	*l5*	30	
fv	Udine	*pw*	30	
fv	Udine	*pd*	30	
fv	Udine	*a*	25–30	Mites taken by dusting adult bees; 15 mites in the control
fv	Udine	*a*	3 x 30	Infested bees picked up by hand
fv	Tirano	*l5*	30	
fv	Tirano	*pw*	30	
fv	Lunz-am-See	*pw*	15	
fv	Lunz-am-See	*pd*	30	
fv	Randegg	*pw*	25	
fv	Randegg	*pd*	30	
fv	Como	*sl + pd*	30	15 mites from *sl*, 15 from *pd*
fm	Udine	*l5*	45	
fm	Varallo Pombia	*pd*	25	15 mites at concentration ≤ 5 ppm or ≥ 200 ppm
fm	Chiavenna	*va*	30	15 mites at concentration ≤ 0.1 ppm or ≥100 ppm
fm	Lunz-am-See	*pd*	30	
ac	Udine	*l5 + sl*	30	
ac	Lunz-am-see	*pd*	30	15 mites at concentration ≤ 0.5 ppm
ac	Como	*va*	10	
ac	Chiavenna	*pd*	10	
ac	Varallo Pombia	*va*	15	
B) Using 2-month-old capsules				
fv	Como	*sl + pd*	30	15 mites from *sl*, 15 from *pd*
fv	Udine	*va*	45	Brood stages: *l5* and *sl*
fv	Udine	*va*	30	Brood stages: *l5* and *sl*
C) Assays carried out at different temperatures				
fv	Udine	*va*	45	20°C
fv	Udine	*va*	30	26°C

Active ingredients: fv, fluvalinate; fm: flumethrin; ac: acrinathrin; honeybee stage: *l5, sl, pw, pd:* see text; *a*: adult bees; *va*: mites taken from brood of various ages.

valinate. Only results obtained with this ai are reported here.

Conditions of the mites at different times

At 6 h, when the *Varroa* mites were transferred to Petri dishes, a large proportion, usually increasing with the concentration, was affected to a varying degree. The proportion of dead mites was quite variable even within a given strain. The classification of surviving mites into the categories 'mobile' and 'paralysed' was difficult and in a few cases partly subjective, since these categories are the extremes of a continuum of conditions. The conditions of the mites (table III) was much clearer at 24 h and especially at 48 h, when most mites had either recovered or were dead, often after having regurgitated a conspicuous droplet of fluid. At 48 h, most mobile mites did not show any sign of intoxication, and the few paralysed individuals were usually moribund. At 20 and 26°C the proportion of paralysed mites at 48 h was higher, with a maximum of 20 and 24% respectively at 50 ppm. The relationship between the proportion of dead mites at 6 and 48 h was very loose. In contrast, the sum of dead and paralysed mites at 6 h (or at 24 h) was close to the number of mites dead at 48 h in several samples, but there were variations in both directions (fig 2).

The mortality in the controls ('natural mortality') of experiments using fluvalinate was rather low when mites were taken from brood, varying from an average of 1.6% at 6 h, 4.4% at 24 h, to 5.8% at 48 h; 10% mortality was exceeded only in 2 capsules, in different experiments. Preliminary results indicated a faster increase after 2 d, and thus the observations were limited to the 48 h period. The natural mortality was rather variable, and generally higher, up to 71% in a capsule, when mites were taken from adult bees.

For these reasons, the mortality of the mites at 48 h will be reported and discussed in the next sections.

Comparison of *Varroa* mites from different brood stages and from adult bees

A good fit using the probit regression was found in nearly all the cases and the results of the replications were consistent. Except when otherwise noted, no heterogeneity was found using the χ^2 test.

The susceptibility of mites from different brood stages (larva just after capping, pupa with white eyes, pupa with dark eyes and white or pale body) was similar in all the *Varroa* strains tested. The differences in the median lethal concentration (LC_{50}) between groups of mites of the same strain but different brood stages were rather small and

Table III. Paralysed mites at 6, 24 and 48 h (percentage of the total).

	T (°C)	*6 h*	*24 h*	*48 h*
Susceptible strains	32.5	46.3%	8.2%	1.1%
Resistant strains	32.5	34.9%	4.3%	1.5%
Susceptible strain	26	67.6%	12.0%	5.8%
	20	58.6%	39.4%	5.3%

No comparison between the susceptible and resistant strains can be made, since the number of susceptible mites tested at concentrations higher than 200 ppm was smaller.

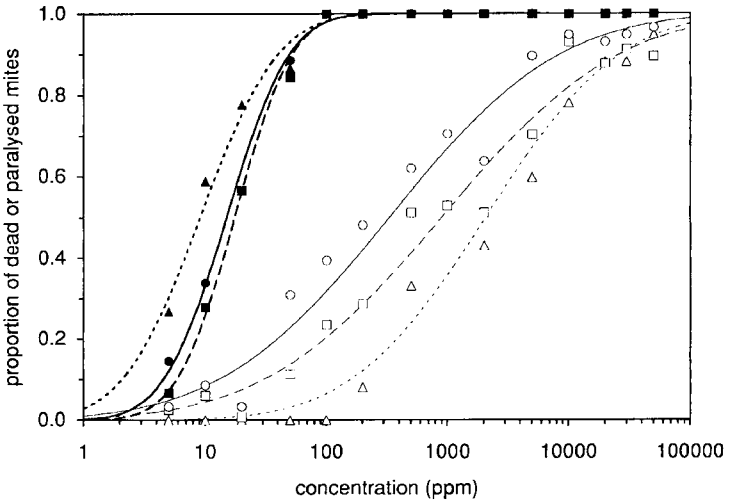

Fig 2. Proportion of dead or paralysed *Varroa* mites at different times (triangles: 6 h; squares: 24 h; circles: 48 h) in the assays carried out on mites from Udine (black; $n \approx 90$ mites per point, except for those giving 100% mortality) and Tirano (white; $n \approx 60$ mites per point) and regression lines obtained using the probit transformation. Data corrected for natural mortality (Udine mites: 0, 0, 2.2% at 6, 24, 48 h; Tirano mites: 1.7, 5 and 5%, respectively).

with broadly overlapping confidence intervals (table IV).

In contrast, the response of the mites taken from adult bees was rather variable. The mites fallen following dusting adult bees with wheat flour at the end of September (when brood was still present in the colonies) showed a somewhat higher sus-

Table IV. Median lethal concentration (LC_{50}) of fluvalinate and its fiducial limits for mites from different brood stages.

Origin	*Stage*	LC_{50}	*Fiducial limits (95%)*	
Udine	*l5*	15.6	11.8	20.0
	pw	19.2	14.0	25.3
	pd	13.0	9.9	16.5
Lumz-am-See	*pw*	17.5	11.5	24.8
	pd	19.6	12.8	27.3
Randegg	*pw**	14.9	5.0	31.1
	pd	19.3	12.3	27.4
Tirano	*l5*	435	260	688
	pw	331	154	629

* A heterogeneity factor was used to compute the fiducial limits, since $P(\chi^2) \approx 0.05$ (Finney, 1971).

ceptibility. However, this result left some doubt whether selection of somehow weaker *Varroa* females, *ie* more easily removed when adult bees are dusted, had taken place. The small number of mites assayed in the control gives little information. However, when mites were collected later in the season (October 17) taking infested bees from the combs, the mortality was even higher: in the control it reached 17% by 24 h, with a highly significant difference from that of mites taken from brood. This trend was confirmed by the successive assays (on October 27 and November 4), when about 50% of the mites died in the control within 24 h. In the period of time during which the assays were carried out, the amount of brood in the colonies rapidly decreased and (especially in the latest assays) more *Varroa* females were found between the abdominal sternites rather than on other parts of the bodies of the honeybees. Many of these mites were thinner than those taken from brood. The proportion of dead mites could not be corrected for the natural mortality using the so-called Abbott's formula (Tattersfield and Morris, 1924), since the mortality caused by fluvalinate might not be independent of natural mortality, and the data were not analysed using the probit regression.

Effect of the temperature

The assays carried out with *Varroa* mites from Udine at 20 and 26°C gave results similar to those carried out at 32.5°C; the mortality curve differs only slightly and not significantly from that obtained at 32.5°C, with a somewhat increased LC_{50} (20.2 ppm at 20°C, 24.7 ppm at 26°C). However, the χ^2 test reveals a significant heterogeneity between the replications, at both temperatures. In assays carried out at these temperatures an unusually large number of paralysed mites (table III) was counted at 48 h, especially at concentrations ranging from 10 to 50 ppm. If the sum of the paralysed and dead mites is considered instead, there is no heterogeneity between the replications and the agreement with the results at 32.5°C is even better (the concentration at which 50% of the mites are dead or paralysed are 15.0, 17.8 and 15.1 ppm at 20, 26 and 32.5°C respectively).

Effect of ageing of the capsules

The results obtained in successive assays carried out on the same strain within 1 month of the preparation of the capsules are not statistically different, though in some cases a slight increase in the mortality of mites was observed as the age of the capsules increased. The mortality obtained in the assay carried out on the Como strain with capsules prepared 75 d earlier was higher than that obtained using capsules prepared 2 weeks earlier; a significant, 6-fold decrease of the LC_{50} was observed and a highly significant heterogeneity ($\chi^2 = 46$, 16 *df*) between the 2 series of data was found. Two further tests carried out on the susceptible strain from Udine and thus on a limited range of concentrations confirmed this result, using freshly prepared capsules and capsules prepared 50 d before. About a 2-fold reduction of the LC_{50} in the latter and a highly significant heterogeneity between the series of data were observed.

Comparison of the susceptibility to pyrethroid acaricides of Varroa *mites from different origins*

Fluvalinate

The susceptibility to fluvalinate of mites from Udine and the 2 Austrian localities (combining the data on mites from different brood stages) are quite similar (fig 3), and no significant differences were found. The LC_{50} lies between 15.9 and 18.5 ppm (table V),

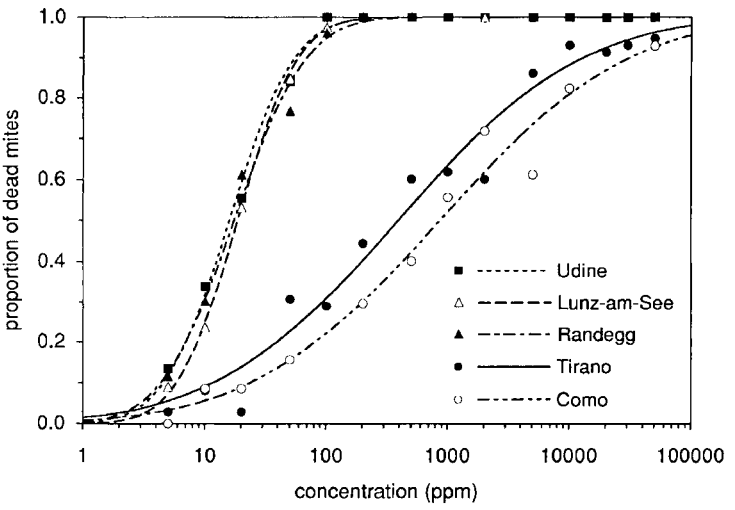

Fig 3. Proportion of dead *Varroa* mites at 48 h in the assays carried out on mites from different localities (approximate number of mites per point for each sample: Udine, 90; Randegg, 55; Lunz-am-See, 45; Tirano, 60; Como, 30) and regression lines obtained using the probit transformation. Data obtained using mites from brood of different ages have been combined. Data corrected for natural mortality (Udine, 2.2%; Randegg, 6.2%; Lunz-am-See, 8.9%; Tirano, 5%; Como, 6.7%).

with broadly overlapping fiducial intervals. The probit curves fit very closely the experimental data.

The mites from Lombardy (Tirano and Como) showed a more variable response (fig 3), making the fit less close, even if the heterogeneity χ^2 is not significant, and an increased LC_{50} (about 24 and 54 times higher, respectively, than that of the Udine strain). Moreover, the regression lines deviate considerably from parallelism and thus the LC_{95} (though its estimate is affected by a large error) is even more increased (about 440 and 1 100 times higher, respectively, than that of the Udine strain; table V); 100% kill was not obtained in the assays even at the highest concentration tested (50 000 ppm).

Table V. LC_{50} and LC_{95} of fluvalinate and their fiducial limits for mites from different localities.

Origin	*LC_{50}*	*Fiducial limits (95%)*		*LC_{95}*	*Fiducial limits (95%)*	
Udine	15.9	13.5	18.4	78	62	104
Lunz-am-See	18.5	13.7	23.8	81	59	126
Randegg	17.3	13.2	21.9	101	73	158
Tirano	385	250	576	34 400	20 000	65 500
Como	857	426	1 571	86 500	30 400	369 000

Flumethrin

The overall pattern of the mite mortality observed in assays carried out with flumethrin was very similar to that obtained using fluvalinate, except that, at the same concentration, flumethrin is more active against *Varroa* than fluvalinate.

The percentage of paralysed mites decreased from 44% at 6 h, to 8% at 24 h and 1% at 48 h. No mortality in the controls was observed at 6 h, while it was 1% at 24 h and reached 3.1% at 48 h.

The LC_{50} of mites from Udine and Lunz-am-See are very similar (fig 4 and table VI). The LC_{50} of mites from Chiavenna and Varallo Pombia, which had survived an Apistan treatment, was 30–60 times higher. The LC_{95} was also greatly increased.

Acrinathrin

The overall pattern of the mite mortality was very similar to that observed using the other pyrethroids. The proportion of paralysed mites decreased from 35% at 6 h to 15% at 24 h and 3.4% at 48 h. No mortality was observed in the controls at 6 and 24 h, while it reached 1.2% at 48 h.

The LC_{50} was intermediate between those of fluvalinate and flumethrin. Mites from Udine and Lunz-am-See do not differ significantly, while the LC_{50} of mites from several origins in Lombardia is much higher (fig 5, table VII). The LC_{50} of Como and Varallo Pombia mites and heterogeneity between the 2 series of data were detected using the χ^2 test and do not significantly differ; the samples were rather small. On the

Table VI. LC_{50} of flumethrin, its fiducial limits and LC_{95} for *Varroa* mites from different origins.

Origin	*LC_{50}*	*Fiducial limits (95%)*		*LC_{95}*
Udine	0.36	0.26	0.46	2.45
Lunz-am-See	0.28	0.18	0.39	3.4
Chiavenna *	20.4	11.4	33.1	172
Varallo Pombia	11.4	5.9	20.1	356

$p\ (\chi^2) \approx 0.03$ and so a heterogeneity factor was used to compute the fiducial limits.

Table VII. LC_{50} of acrinathrin, its fiducial limits and LC_{95} for *Varroa* mites from different origins.

Origin	*LC_{50}*	*Fiducial limits (95%)*		*LC_{95}*
Udine	3.0	2.1	4.4	81
Lunz-am-See	5.0	2.9	9.0	53
Como	49.4	23	130	3 429
Varallo Pombia	79.1	48	133	726
Chiavenna	729	225	16 058	66 570

$p\ (\chi^2) \approx 0.03$ and so a heterogeneity factor was used to compute the fiducial limits.

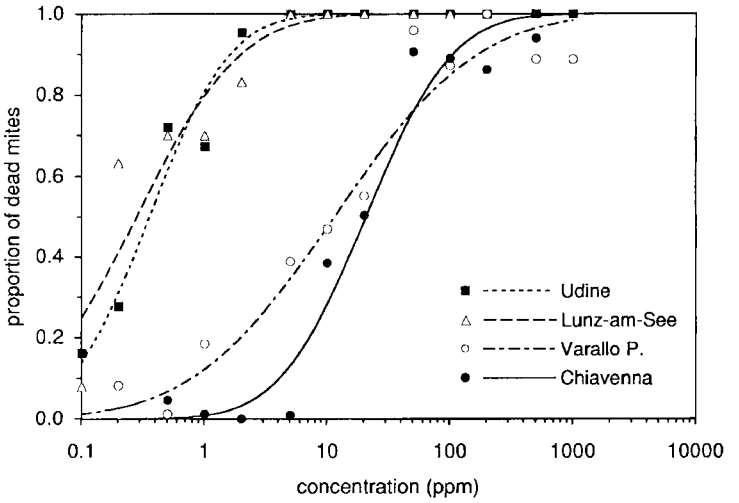

Fig 4. Proportion of dead *Varroa* mites at 48 h in the assays carried out using the ai flumethrin on mites from different localities (approximate number of mites per point for each sample: Udine, 45; Lunz-am-See, 30; Varallo Pombia, 25; Chiavenna, 30) and regression lines obtained using the probit transformation. Data corrected for natural mortality (Udine, 8.9%; Lunz-am-See, 0%; Varallo Pombia, 6.7%; Chiavenna, 0%).

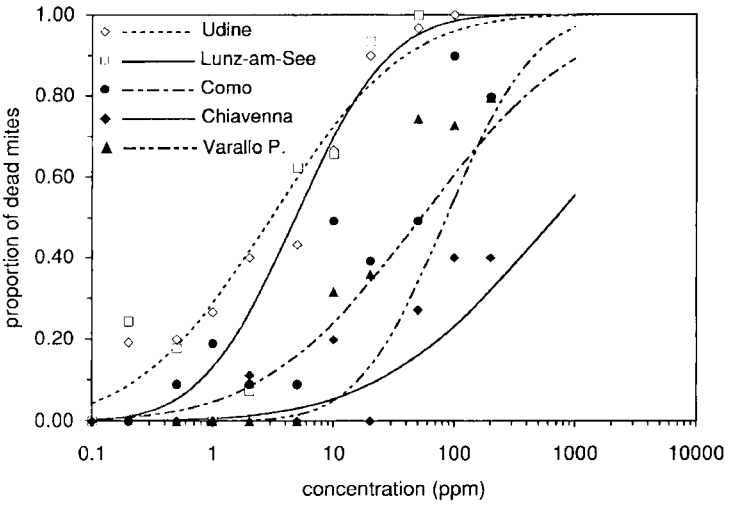

Fig 5. Proportion of dead *Varroa* mites at 48 h in the assays carried out using the ai acrinathrin on mites from different localities (approximate number of mites per point for each sample: Udine, 30; Lunz-am-See, 30; Como, 10; Chiavenna, 10; Varallo Pombia, 15) and regression lines obtained using the probit transformation. Data corrected for natural mortality (Varallo Pombia: 6.7%; 0% in the remaining cases).

other hand, the curve for the Chiavenna mites is very different and the LC_{50} is exaggerated. A closer inspection of the data showed that in this sample an unusually large number of mites occurred at 48 h at the highest concentrations tested (4 out of 10 mites at 500 ppm) and thus the mortality alone underestimates the toxic effects of the active ingredient. If paralysed mites are added to dead mites, this anomaly disappears.

The mortality curves of the susceptible and the resistant strains tend to be more parallel for flumethrin and acrinathrin than for fluvalinate. In fact, there is no significant departure from parallelism between some of these curves.

DISCUSSION

The observations carried out at 48 h ensured more reliable results, since the proportion of paralysed mites was usually negligible, and no uncertainty in the classification of the mites could arise. Caution in the interpretation of the data is necessary in the few cases when a !arge proportion of mites shows symptoms of unrecoverable intoxication but is still alive at 48 h. This can lead to an underestimate of the toxic effects if the mortality alone is considered. At 6 h, the number of dead mites gave little information, while the sum of the number of dead and paralysed mites was closer to the number of mites dead at 48 h.

The temperature at which the assays were carried out did not play a critical role, at least with susceptible mites, except that at 20 and 26°C a large number of mites are still paralysed at 48 h. This result is somewhat unexpected, because the toxicity of several pyrethroids is affected by the temperatures (Eesa and Moursy, 1993).

The capsules coated with paraffin can be used for 1 month after their preparation; they must be discarded later. A possible explanation for the increase in the mite mortality observed using capsules about 2 months old is that the surface of the paraffin wax was progressively enriched in fluvalinate during ageing.

No significant differences were found among mites from different brood stages (excluding pupae with dark bodies, when newly moulted adult mites may be present). For this reason, the results of assays carried out on *Varroa* females from different brood stages could be combined, and in some later tests, when the number of mites was too low to assay them separately, mites taken from different stages could be pooled.

In contrast, a different and rather variable response and an increased natural mortality were observed when mites from adult bees were assayed. Thus mites taken from adult bees should not be used, at least at the beginning of autumn. The different responses of the mites from adult bees could be due to a different physiological condition; Bruce and Needham (1995) noted that *Varroa* mites removed from the bees during the winter weigh less, lose water faster and thus die earlier. No mites taken from adult bees in spring or summer were assayed and further experiments in these seasons, in colonies with and without brood, are necessary.

The technique could detect differences in the susceptibility of *V jacobsoni* to some pyrethroids. The LC_{50} of mites from areas where treatments with fluvalinate are no longer effective was about 25–50 times higher than that of areas where the mites are susceptible; an even larger increase in the LC_{95} was found. This can explain the failures in the control of *Varroa* using Apistan. The similarity in the LC_{50} of mites from the Austrian localities where no pyrethroids have been used and from Udine shows that no selection of resistant mites (or even partially resistant mites) has taken place so far in Udine, despite the fact that Apistan had been used as almost the sole varroacide product for 5 years.

It is not known to what extent the larger variability of the resistant strain is due to some mixing with susceptible mites, but it cannot be entirely explained on the basis of this factor, since the Como colonies had been treated with Apistan until a few days before the brood from which the mites were collected was taken. This further treatment could account for the slight, insignificant increase in the tolerance towards fluvalinate observed in the latter strain. While satisfactory results could be obtained assaying 30 mites or even less at 3–5 concentrations, plus the control with susceptible strains, in the case of resistant strains a larger number of mites per concentration might be needed to obtain a comparable accuracy.

The increase in the LC_{50} of flumethrin and acrinathrin on mites surviving Apistan treatments indicates the presence of cross-resistance between fluvalinate and these pyrethroids, although it has not been possible to assay the same strain both with fluvalinate and these pyrethroids, due to the lack of a sufficient number of mites from suitable brood stages. The presence of cross-resistance is not unexpected, due to the similarity in the molecules of the ai and in particular of their alcohol moieties (*cf* AW Farnham in Denholm and Rowland, 1992). The small size of the samples, the scarcity of information on possible effects of impurities at high concentrations of the ai and especially the lack of comparisons on the same mite strain do not allow us to attach too much significance to the different shapes of the mortality curves for the resistant strains.

These results highlight the risk of basing the strategies for the control of *Varroa* on chemical treatments alone.

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Résumé — Résistance de *Varroa jacobsoni* Oud aux pyréthroïdes : mise au point d'un test. On a mis au point une méthode pour étudier la sensibilité de l'acarien *Varroa* au fluvalinate, à la fluméthrine et à l'acrinathrine. Les acariens ont été prélevés à partir de couvain operculé (jusqu'au stade de nymphe aux yeux noirs et au corps clair), et, dans le cas des *Varroa* d'Udine, également sur des ouvrières adultes. Des capsules formées de 2 disques en verre (de 62 mm de diamètre) et d'un anneau en acier inox (de 56 mm de diamètre interne et 3–5 mm de haut) ont été recouvertes intérieurement avec de la paraffine contenant une concentration connue du composé actif. Les acariens ont été conservés dans ces capsules pendant 6 h et transférés ensuite dans une boite de Pétri propre (60 mm de diamètre) où ils ont pu se nourrir sur des larves d'abeille ; ils ont été observés 6 h, 24 h et 48 h après leur introduction dans les capsules, et classés en 3 catégories : mobiles, paralysés (quand ils pouvaient bouger les appendices sans pouvoir se déplacer), morts. Les observations réalisées à 48 h (quand la proportion d'acariens paralysés est négligeable) donnent les résultats les plus fiables. Les acariens ont été échantillonnés dans différentes localités : Randegg et Lunz-am-See (Autriche), dans des ruchers jamais traités avec des pyréthroïdes ; Udine (nord-est de l'Italie), où le fluvalinate a été largement utilisé depuis 1989 et était encore efficace ; 4

localités de Lombardie (nord de l'Italie) où Apistan n'est plus efficace. Aucune différence significative n'a été trouvée entre des acariens provenant de la même localité mais issus de stades de couvain différents (larves operculées, nymphes aux yeux blancs, nymphes aux yeux noirs et au corps blanc), alors que des réponses variables ont été obtenues avec des acariens provenant d'ouvrières adultes. La LC_{50} des *Varroa* provenant de régions où le fluvalinate n'est plus efficace était environ 25–50 fois plus élevée que celle provenant de régions où les acariens sont encore sensibles ; une même augmentation existe également pour la LC_{95} (fig 3 et tableau V). La LC_{50} des *Varroa* d'Udine et d'Autriche n'était pas différente, montrant ainsi qu'aucune sélection d'acariens résistants ne s'est mis en place à Udine. Une augmentation de la LC_{50} de la fluméthrine et de l'acrinathrine a été observée chez les acariens survivants au traitement à l'Apistan (figs 4 et 5).

pyréthroïdes / *Varroa jacobsoni* / résistance / test de laboratoire

Zusammenfassung — Resistenz von *Varroa jacobsoni* Oudemans gegen Pyrothroide: ein Labortest. Um die Empfindlichkeit der *Varroa* Milben gegenüber Fluvalinat, Flumethrin und Acrinathrin abzuschätzen, wurde ein neuer Labortest entwickelt. Die Milben wurden aus verdeckelter Brut (Entwicklungsstadien bis zu hellen Puppen mit dunklen Augen) gewonnen und von Adulten gesammelt. Es wurden Versuchskapseln hergestellt, die aus 2 Glasscheiben bestanden (d = 62 mm), die durch einen Halter aus rostfreiem Stahl (innerer Durchmesser d = 56 mm, Höhe 3–5 mm) in einem Abstand von 3–5 mm gehalten wurden. Sie wurden auf der Innenseite mit Paraffinwachs überzogen, das den jeweiligen Wirkstoff in bekannten Konzentrationen enthielt. Die Milben wurden 6 Stunden in diese Kapseln gesperrt und danach in eine saubere Petrischale mit frischen Bienenlarven gesetzt, von denen sie Hämolymphe saugen konnten. Sechs, 24 und 48 Stunden nach Einsetzen in die Kapseln wurden sie kontrolliert und in 3 Kategorien eingeteilt: bewegliche, unbewegliche (wenn sie noch Körperanhänge bewegten, ohne daß eine Fortbewegung erfolgte) und tote Milben. Die Beobachtungen nach 48 Stunden, bei denen der Anteil der unbeweglichen Milben vernachlässigt werden kann, ergaben die zuverlässigsten Werte. Die Milben wurden an verschiedenen Orten gesammelt (Abb 1): in Randegg und Lunz am See (Niederösterreich) von Ständen, die nie mit Pyrethroiden behandelt wurden; in Udine (nordöstliches Italien), wo seit 1989 regelmäßig Fluvalinat angewendet wurde, und an 4 Stellen in der Lombardei (Norditalien), wo Apistan keine ausreichende Wirkung mehr hat. Zwischen Milben vom gleichen Ort, die von verschiedenen Brutstadien (verdeckelten Maden, Puppen mit weißen Augen, puppen mit dunklen Augen und weißem oder leicht pigmentiertem Körper) stammten, zeigten sich keine statistischen Unterschiede. Die Ergebnisse mit Milben von adulten Bienen waren dagegen sehr variabel. Die LC_{50} der Milben aus Gebieten mit unwirksamer Apistan-Behandlung war 25–50 mal höher als die LC_{50} der Milben aus Gebieten, in denen die Milben noch empfindlich sind. Bei der LC_{95} war der Anstieg noch höher (Abb 3 und Tabelle V). Die LC_{50} der Milben von Udine und Österreich unterschieden sich nicht. In Udine sind also noch keine Resistenzen selektiert worden. Eine höhere LC_{50} bei Flumethrin und Acrinathrin wurde bei Milben beobachtet, die Apistan Behandlungen überleben (Abb 4, 5).

Pyrethroide / *Varroa jacobsoni* / Resistenz / Labortest

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