

Monitoring *Varroa jacobsoni* resistance to pyrethroids in western Europe

Jérôme Trouiller*

Dipartimento di Biologia Applicata alla Difesa delle Piante
Udine University, Viale delle Scienze, 208-I-33100 Udine, Italy

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Abstract – Since 1991, *Varroa jacobsoni* resistance to pyrethroids has been reported to occur in several European countries. Monitoring surveys were performed in 1995, 1996 and 1997 in Europe using a laboratory assay to localise possible foci of resistance. A resistant strain seems to have originated in Italy, and spread to Slovenia, Switzerland, France, Belgium and Austria. The areas where *Varroa jacobsoni* resistance to pyrethroids have been detected are indicated in this paper.
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***Varroa jacobsoni* / acaricide / monitoring / pyrethroid / resistance**

1. INTRODUCTION

In much of the world, the mite *Varroa jacobsoni* Oudemans is potentially the main threatening parasite for *Apis mellifera* L. If an infested colony is not treated, it will generally die within a few years. Only *Apis cerana* Fabricius, the original host of the *V. jacobsoni* mite, African subspecies of *A. mellifera* and Africanized honeybees of the new world, are known to maintain the mite population at a harmless level. When *V. jacobsoni* reached Europe at the end of the 1970s/beginning of the 1980s, it spread

to most countries causing heavy colony losses [18].

It was not until the end of the 1980s that a satisfactory control of the mite was obtained with long term contact acaricide treatments. Consequently most of the European countries used pyrethroid acaricides (τ -fluvalinate and flumethrin) which have a high efficacy against the *V. jacobsoni* mite and a low toxicity for bees. However since the beginning of the 1990s, resistance of the mite to pyrethroids has been claimed in different parts of Europe, including Italy,

* Correspondence and reprints: SWARM, 2, côte de la Jonchère, 78380 Bougival, France
E-mail: swarm@wanadoo.fr

Switzerland, France, Spain, Hungary and Austria. In some regions a high level of mortality was reached, even for treated colonies [5, 7, 11, 12].

The existence of *V. jacobsoni* resistance to pyrethroids (acrinathrin, flumethrin and τ -fluvalinate) has been demonstrated in Italy using a laboratory assay [13]. Because of the risk of mite reinfestation of the colonies at the end of the treatment [9], and the necessity to use long term control treatment when brood is present, field efficacy tests cannot demonstrate with certainty the presence or absence of resistance. Unsatisfactory efficacy, which leads to the suspicion of resistance, can also be caused by the improper use of acaricides by beekeepers [8]. Since no external morphological features are able to distinguish between susceptible and resistant mites [15], it was then necessary to use a reliable methodology to perform monitoring survey in Europe to localise the possible centres of *V. jacobsoni* resistance to pyrethroids.

Several laboratory methodologies have been developed to test *V. jacobsoni* resistance to pyrethroids [6, 13, 20] and acaricides in general [1, 16, 19]. We chose to use Milani's methodology applied to τ -fluvalinate because of its accuracy and easy handling [13].

To monitor *V. jacobsoni* resistance to τ -fluvalinate, we first validated the methodology. We first standardised the methodology according to possible geographical variations in mite susceptibility to τ -fluvalinate. Standard susceptible and resistant populations were chosen and accepted as standard according to field efficacy and laboratory assay data. According to laboratory data, we chose a discriminating concentration. We studied the relationship between field treatment efficacy and the data obtained in laboratory assay at the discriminating concentration.

The evolution of resistance has been followed across several European countries. The absence of resistance was in fact based

on the detection threshold of 10 % of resistance (according to our laboratory assay definition) with a risk of 5 % of not detecting it. To lessen the side effects of a long transportation on mite samples, the methodology was conducted in several laboratories: Udine University (Italy), THI of Freiburg (Germany), CNEVA of Sophia-Antipolis (France), and CAR of Guadalajara (Spain).

2. MATERIALS AND METHODS

2.1. Standardisation of the laboratory methodology

We first considered as pure susceptible *V. jacobsoni* (standard susceptible), mites present in regions where good control of mite populations was obtained with pyrethroid treatments at the time of the experimentation. The susceptibility to fluvalinate of susceptible populations from three regions was compared. We collected resistant *V. jacobsoni* in regions where pyrethroid treatments were no longer able to control mite population. Pure resistant populations (standard resistant) were obtained by the selection of resistant population with 8 weeks Apistan® treatment in an isolating flight room. The susceptibility towards fluvalinate of several resistant mite populations was compared. Our definition of susceptible and resistant population was then based on field treatment and a laboratory assay. However, it was not possible to exclude the possibility of some resistant individuals among susceptible population.

Experiments were conducted in 1995 in two laboratories (University of Udine (Italy) and THI of Freiburg (Germany)). The methodology used for laboratory analysis was based on the laboratory assay described by Milani [13]. It was slightly modified in the preparation of the capsules using a longer period (1.5 h) to remove hexane residues, and modifying the storage of the capsules (at 33 °C). The τ -fluvalinate (95 % purity) was supplied by Sandoz-SPC.

The susceptibility of mites to τ -fluvalinate was measured in 1995 on standard susceptible strains from different areas (Udine, Italy; Valais, Switzerland; Freiburg, Germany), and on various standard resistant strains (in 1995 at Sondrio (Italy), Rome, and in Tessin (Switzerland), and in 1996 at Udine). The resistant mites from Udine were selected the following year from another site.

Mites were taken from capped bee brood at the larva, pre-pupa, white pupa and non-pigmented dark eyed pupa stages. Five to six doses were tested for each strain of susceptible mites ($n = 19$ to 98 mites/dose, total 917 mites excluding the controls) and seven to nine doses for each strain of resistant mites ($n = 12$ to 65 mites/dose, total 1 078 mites excluding the controls). This first step allowed us to define a discriminating concentration.

2.2. Correlation between laboratory assay and field efficacy test

The experiment was conducted in Friuli (Italy) in autumn 1995. Thirty-one colonies were selected in seven apiaries. For each colony, some infested brood was sampled at the start of the experiment and the mites analysed for their susceptibility to τ -fluvalinate with the laboratory assay at the discriminating concentration of 200 mg·kg⁻¹ (a concentration at which 99.7 % of the susceptible mites are killed) with control at 0 mg·kg⁻¹ (respectively $n = 15$ –63, mean 50 and $n = 15$ –36, mean 32). The laboratory efficacy or corrected mortality is: $CM = (TM - M) / (1 - M)$ where TM and M are respectively the mortality at 200 and 0 mg·kg⁻¹.

The colonies were then treated with two Apistan® strips for 6 to 9 weeks, depending on the natural disappearing period of the brood. The fallen mites were recovered from a bottom board covered with paraffin oil and isolated from the colony by a screen allowing mites to fall through but preventing the bees from making contact. The fallen mites were counted every week. After 5 weeks the number of mites falling during Apistan® treatment was very low, and we considered the Apistan® treatment as completed at 6 weeks. At the end of the treatment, when brood naturally disappeared because of wintering, the Apistan® strips were removed and two treatments of Perizin® (5 mL of diluted Perizin® in water (1:50) per occupied comb per treatment (active ingredient organophosphorous coumaphos)) were carried out with a 1 week interval. The last fallen mite count was carried out 1 week after the last Perizin® treatment. Because very few mites had fallen following the second treatment, we considered that, in the absence of brood, two Perizin® treatments were adequate to recover the large part of the remaining mites. For each colony, the efficacy of the Apistan® treatment was then calculated. The efficacy (%) is: $FA / (FA + FP) \times 100$, where FA is the number of

mites fallen during the Apistan® treatment and FP the number of mites fallen following the Perizin® treatments.

2.3. Monitoring of mite resistance

Experiments were conducted in spring and summer 1995, 1996 and 1997. Tests were performed in several European countries (Italy, Slovenia, Austria, Switzerland, France, Spain, Hungary, Belgium and Germany). The samples were chosen by local veterinary services or bee-keeping organisations. Each sample of living infested brood was taken from one to three colonies from an apiary. The samples were sent by courier and analysed within 3 days of sampling. To lessen the risk of using mites with a low fitness, we kept them 24 h in an incubator (34 ± 1 °C, 80 ± 10 % RH) in presence of just capped worker larvae, before testing. Only mites with a normal moving behaviour were used for the test. From each sample, the mites were tested at the discriminating concentration of 200 mg·kg⁻¹ of τ -fluvalinate and at 0 mg·kg⁻¹ to detect a possible decrease in fitness. When mortality in the control was higher than 5 %, the sample was discarded (five instances). The maximum sample size was 176 mites. When no resistant mite was detected, samples of less than 32 mites were discarded (11 instances) because of their insufficient reliability.

3. RESULTS

3.1. Standardisation

Whatever the area of origin of the susceptible mites, the toxicity of τ -fluvalinate did not significantly differ (χ^2 test, five standard susceptible samples tested one against one and grouped) (figure 1). The data were then pooled (five samples, 917 mites) to give a standard curve of susceptibility. The LC50 was 25 mg·kg⁻¹ (fiducial limits 22–29 mg·kg⁻¹) and the LC95 was 105 mg·kg⁻¹ (fiducial limits 87–133 mg·kg⁻¹). The equation of the probit was: $y = 2.66 \times x + 1.27$.

Except for one sample (which was only tested at lower doses (no more than 5 000 mg·kg⁻¹)) the resistant mites did not differ in their susceptibility towards τ -fluvalinate. The standard curve of susceptibil-

ity (five standard resistant samples, 1 078 mites) presented a LC50 of 9 234 mg·kg⁻¹ (limits 6 580 to 13,135 mg·kg⁻¹) and the LC95 was of 810 539 mg·kg⁻¹ (limits 310 524–1 000 000 mg·kg⁻¹). The equation of the probit was $y = 0.85 \times x + 1.64$. The ratio between the LC50 of resistant and susceptible mites was 369.

According to the reference curves, the death rate of resistant mite at 200 mg·kg⁻¹ was 8.3 % (experimental 18.3 %, $n = 75$). At that concentration the mortality for susceptible mites was 99.9 % (experimental 99.3 %, $n = 152$). This concentration was used as the discriminating concentration for the further tests. Taking into account that some resistant mites are killed at 200 mg·kg⁻¹ (ref-

erence curve; 8 %), an approximation of the proportion of resistant mites (PRM) of a sample was obtained with the formula: $PRM = CM \times 1.08$ where CM is the corrected mortality at 200 mg·kg⁻¹. This transformation was used for the monitoring data.

3.2. Correlation between laboratory assay and field efficacy test

The infestation of the colonies was from 549 to 9 465 mites (mean 2 928). The field efficacy was in the range 37–100 %. The range of mite mortality in the laboratory test (at 200 mg·kg⁻¹) was 25–93 % (figure 2). Unfortunately in this test we were not able to find purely susceptible mite populations.

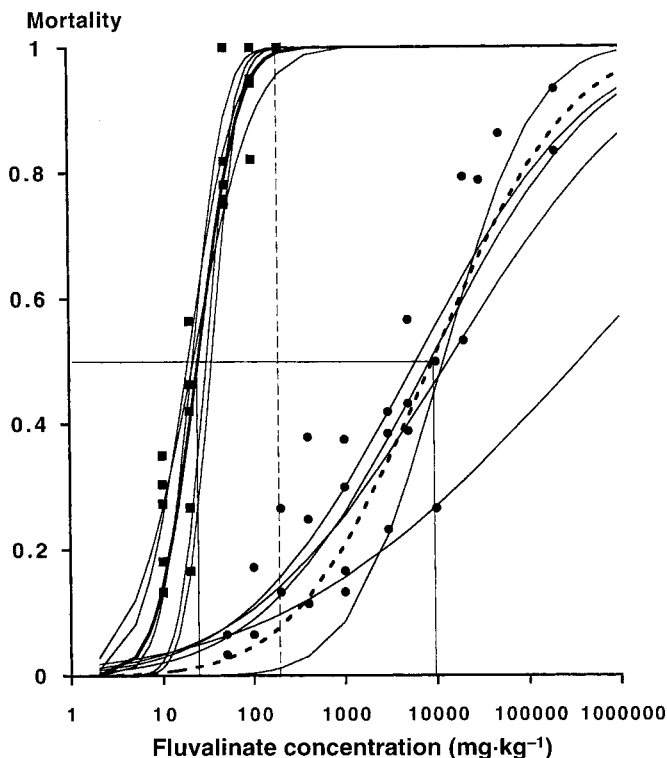


Figure 1. Mortality of different populations of *Varroa jacobsoni* following exposure to τ -fluvalinate. X-axis: fluvalinate concentration (mg·kg⁻¹). The experimental values are represented (susceptible mites: closed squares; resistant mites: closed circles), with the corresponding regression lines (thin curves). The global regression lines are represented by the thick curves (susceptible: continued curve; resistant: dotted curve).

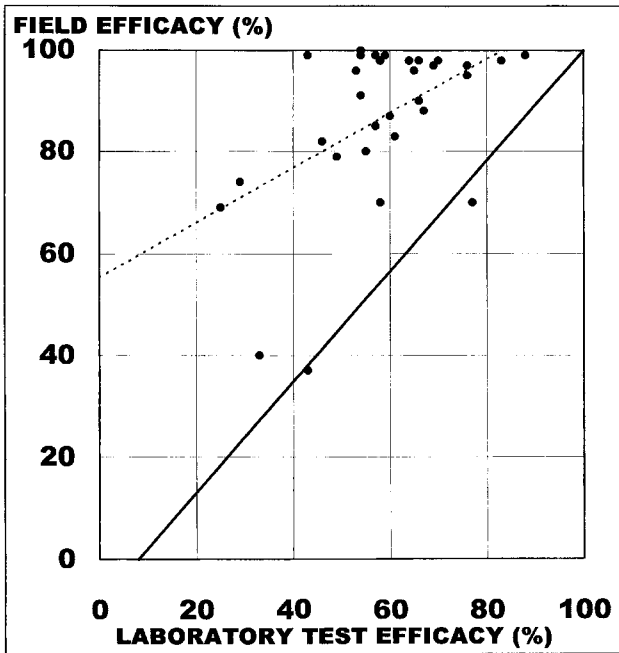


Figure 2. Relationship between laboratory efficacy (corrected mortality) at 200 mg·kg⁻¹ and field efficacy. Experimental values (closed circles), theoretical relationship (thick line), and regression line (thin dotted line).

The correlation between laboratory and field efficacy was significant ($R^2 = 0.28$, $t = 3.44$, $df = 29$, $P = 0.01$). There was a tendency towards a higher efficacy in the field compared to what should be expected with a direct correlation with the laboratory test data (theoretical relationship). The regression curve: field efficacy = $(0.53 \times \text{lab. efficacy}) + 53.03$.

3.3. Monitoring

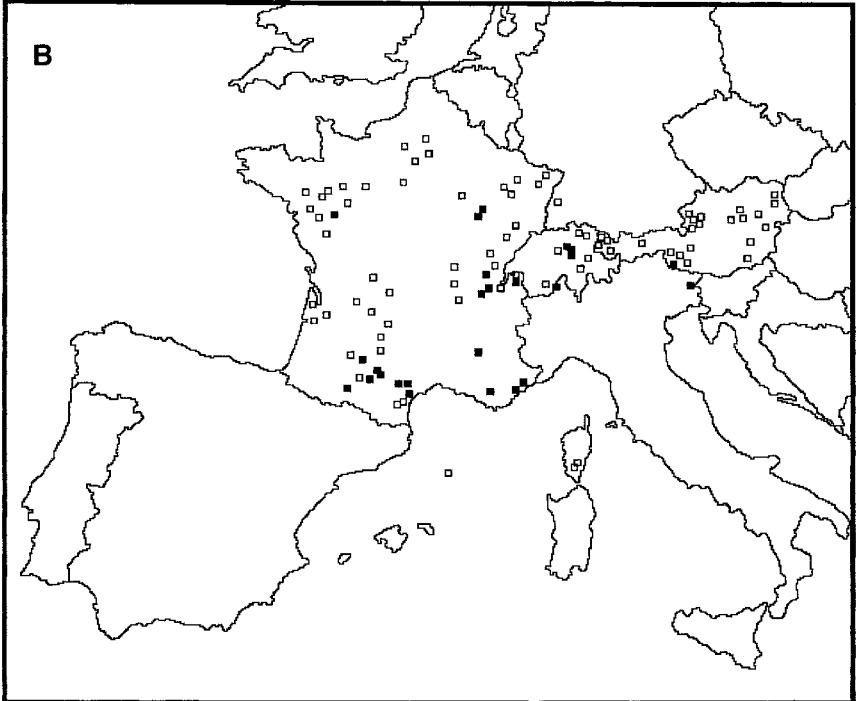
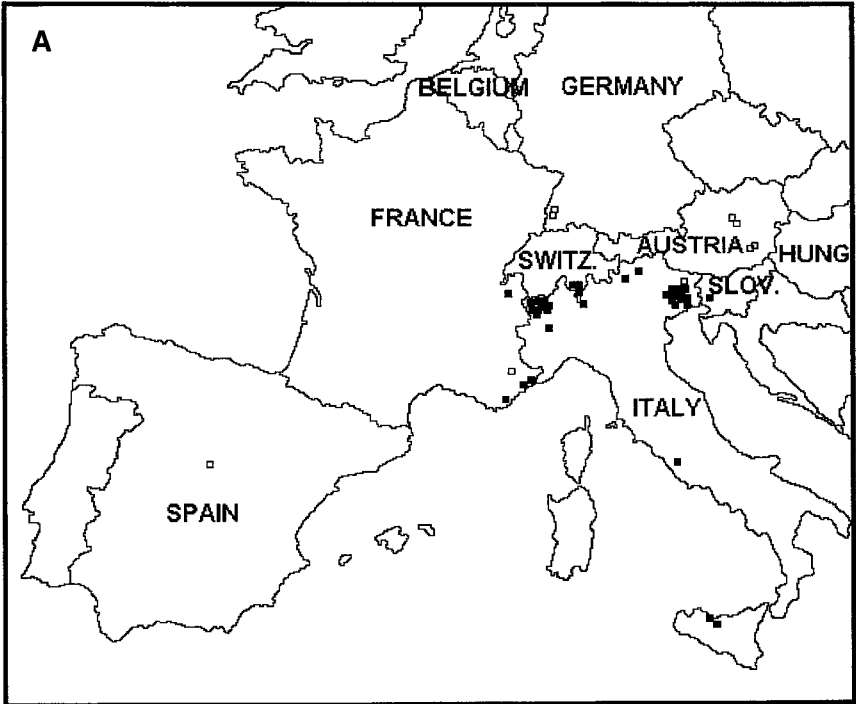
3.3.1. 1995 (figure 3a)

In Italy, except for one sample, all 42 samples tested showed resistance (5–97 % resistance). In Switzerland, the two apiaries located at the southern side of the Alps showed the presence of resistance (74–100 % resistance) whereas one sample from Valais did not show any resistance. In Slovenia the

sample analysed showed the presence of resistance (18 % resistance). In France, one sample out of five did not show any resistance (6–83 % resistance). In Austria, the four samples analysed did not show any resistance. In Spain and Germany, the samples analysed did not show the presence of resistance.

3.3.2. 1996 (figure 3b)

In France, 64 samples were analysed among which 19 presented resistance (3–60 % resistance). In Austria out of 23 samples only one from a site close to the Italian border showed the presence of resistance (15 % resistance). In Switzerland out of nine samples resistance was detected in one from south of the Alps and in three from north of the Alps (40–100 % resistance).



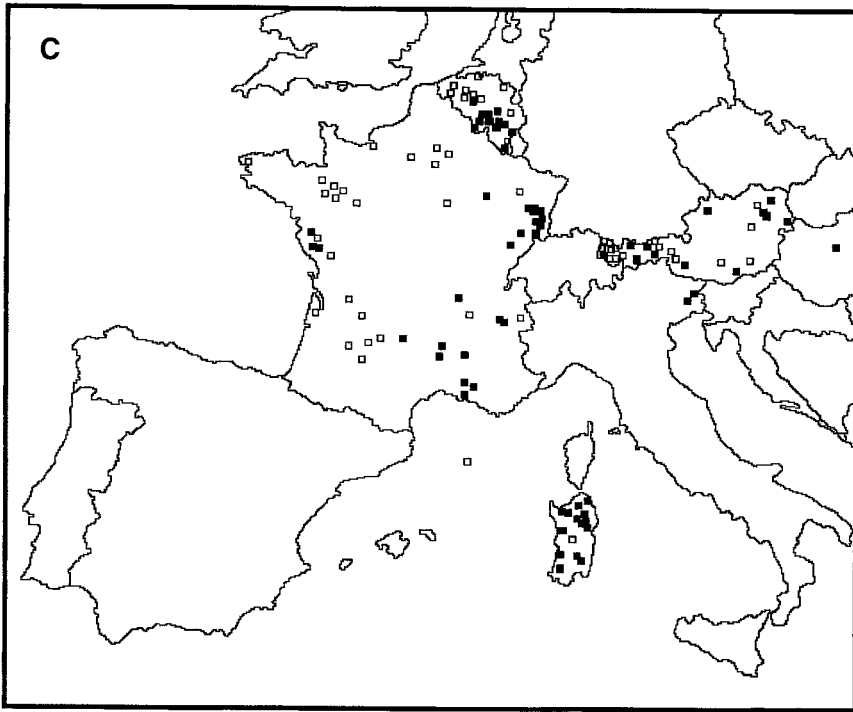


Figure 3. Data of the sample analysed in the laboratory for resistance to τ -fluvalinate. Open square: no resistance detected; closed square: resistance detected. **A:** 1995; **B:** 1996; **C:** 1997.

3.3.3. 1997 (figure 3c)

In France, 50 samples were analysed among which 26 presented resistance (2–100 % resistance). In Austria, out of the 25 samples analysed, 12 presented resistance (7–27 % resistance). In Hungary, the only sample analysed presented resistance (40 % resistance). In Belgium, 22 samples were analysed and 13 presented resistance (1.7–20 % resistance). In Sardinia, only one sample out of the 14 analysed did not present resistance (3–96 % resistance).

4. DISCUSSION

4.1. Validity of standard populations

Our data differed from those of Milani who found for the susceptible strain a

slightly lower LC50 of 13–24 mg·kg⁻¹ (no significant difference) [13]. The susceptibility to τ -fluvalinate were identical in several countries and with Milani data. Since these values were identical to the susceptibility of mites never treated with pyrethroids [13], it seems that a unique basic pyrethroid susceptibility existed before the spread of resistance within Europe.

For the resistant strain, Milani found a much lower LC50 of 250–1 571 mg·kg⁻¹ [13]. For pure resistant mites the mortality at 200 mg·kg⁻¹ was about 18 % in our experiments, whereas it was much higher in Milani experiments (30–40 %) [13]. The difference between Milani's and our data was probably not caused by the slight modification of the test. It is more probable that the resistant mites tested had not undergone the same degree of selection. Starting with

a known resistant population, we used a longer Apistan® treatment than Milani, consequently the actual dose of τ -fluralinate and the duration of contact were higher.

4.2. Laboratory methodology sensitivity

This type of laboratory test allows the detection of individuals which can survive a standard treatment but not of those possessing some resistant genes which then could produce some fully resistant offspring (additive or polygenic resistance). Second, the main limitation of the method is the number of mites that need to be tested. The detection of a 1 % resistance rate, with a 5 % risk of not detecting it, requires 325 mites to be analysed. However, for a 10 % resistance, only 32 mites are necessary for an identical risk. Consequently among the samples where no resistance was detected, it is possible that some had in fact a low proportion of resistant mites.

The laboratory test is more sensitive than a field efficacy test. Even if a normal efficacy is obtained, resistance could be present. In general, the field efficacy was higher than the efficacy predicted by a simple and direct relationship with the laboratory assay data. The means of application of toxin in the laboratory test and the field treatment are different, and because there is a strong natural mortality of *V. jacobsoni* mites during autumn, the efficacy is higher than expected. Roughly half of the resistant mites will die naturally during an autumn treatment. A similar study (N. Milani, pers. comm.) also showed a correlation between the two methods and a close value for the regression.

4.3. Spreading resistance?

Did resistance appear from one site and then spread, or did resistance appear in several regions at different periods? The precise process leading to the appearance of

mite resistance to pyrethroid remains uncertain. Some laboratory evidence such as the presence of an identical or closely related slope of susceptibility for resistant mite populations coming from different areas, does not support the idea of independent origins. Moreover some laboratory assays on resistance mechanisms showed that whatever the site of sampling, the resistant mites share some identity of resistance mechanisms, indicating a possibility of kinship between the samples [10]. Giving a definitive answer to this question would need a large scale study involving genetics and a biochemical determination of resistance mechanisms.

Information obtained from beekeeping organisations seems to indicate that the resistance built up, at least in 1991, in one or two sites in Italy (Sicily or/and Lombardy). Later the resistance spread throughout Italy. Those areas were linked by important migrating beekeeping and colony trade activities. The same connection between resistance and bee movement was observed in France [17]. The next countries where resistance was detected were southern Switzerland, Slovenia and southeastern France, whereas at the same period Austria, northern Switzerland, and in general, northern Europe seemed to be free of resistance. It seems that the northern regions were protected for some time by the Alps, which may have constituted a natural barrier which was crossed only lately. In southeastern France and Slovenia, the low altitude of the Alps did not act as an efficient barrier.

Spain, if resistance is confirmed, could be the site of another primary resistance centre independent from the Italian strain. However, tests conducted in central Spain in 1996 did not show the presence of resistance (M. Higes, pers. comm.).

Studies performed in Friuli in the provinces of Udine and Pordenone [14] tend to show that when the front of resistance reaches a region, almost every apiary is infected by the new resistant strain of *V. jacobsoni*. However, in mountainous

areas the spread can be more irregular. Obviously in a region some isolated apiaries can be affected earlier by the bee trade and migratory beekeeping, these apiaries then forming secondary spreading centres for resistance.

Laboratory tests are sensitive despite the fact that they are not able to detect very low levels of resistance, and as the spread of resistance affects most of the apiaries in a particular region it may be detected even analysing only a few samples. In many instances, the monitoring campaigns were the early means of detecting resistance.

4.4. Importance of resistance monitoring

A few legal synthetic varroacides are available in Europe (amitraz, bromopropylate, coumaphos, cymiazole, flumethrin, and τ -fluvalinate) and they do not share the same efficacy, ease of handling and toxicity to bees and human. It is not likely that other acaricides will be developed in the near future. Among the varroacides used in general, resistance has been claimed in many instances, though it has rarely been investigated and clearly demonstrated. These include: amitraz in Serbia [4] and coumaphos in Italy [3]. The incorrect use of varroacides will probably accelerate the process of mite resistance selection [2, 21]. In the future, there is a risk that only the less satisfactory solutions will be available to control *V. jacobsoni*.

The intensity of bee exchanges involves the risk of spreading within Europe the existing resistant *V. jacobsoni* strains, and to further complicate resistance management in the absence of clear information.

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Résumé – Surveillance de la résistance de *Varroa jacobsoni* aux pyréthri-noïdes en Europe occidentale – Depuis 1991 on a signalé dans différents pays européens l'existence d'une résistance de *Varroa jacobsoni* aux pyréthri-noïdes. Afin de localiser les régions où l'acarien est devenu résistant, on a entrepris en Europe des études de surveillance en 1995, 1996 et 1997 à l'aide du test de laboratoire décrit par Milani [13]. Des échantillons d'acariens vivants ont été prélevés et leur sensibilité au τ -fluvalinate analysée. Les acariens ont été laissés en contact avec des concentrations connues de τ -fluvalinate dans de la paraffine durant 6 h et la mortalité à 24 et 48 h a été enregistrée. Les acariens sensibles provenant des différents pays européens ont présenté la même sensibilité au τ -fluvalinate et les souches résistantes des diverses régions, le même niveau de sensibilité réduite (figure 1). Une corrélation significative a été montrée entre l'efficacité au champ de l'Apistan® et l'efficacité du test de laboratoire. La sensibilité du test de laboratoire a été plus forte que celle des essais au champ (figure 2). La figure 3 donne les régions où la résistance aux pyréthri-noïdes a été décelée. Il semble qu'une souche résistante se soit créée en Italie puis étendue à la Slovénie, la Suisse, la France, la Belgique et l'Autriche. © Inra/DIB/AGIB/Elsevier, Paris

***Varroa jacobsoni* / résistance / pyréthri-noïde / acaricide / surveillance**

Zusammenfassung – Überprüfung der *Varroa* – Resistenz gegen Pyrethroide in Westeuropa. Seit 1991 wurde in einigen europäischen Ländern über das Auftreten von Resistenz gegen Pyrethroide bei Varroamilben berichtet. Um die Gebiete mit pyrethroid – resistenten Milben zu erfassen, wurde 1995, 1996 und 1997 eine flächen-deckende Überprüfung mit dem Labortest

von Milani [13] in Europa durchgeführt. Hierzu wurden Proben lebender Milben auf ihre Empfindlichkeit gegen τ -Fluvalinat im Labor untersucht. Die Milben hatten 6 Stunden Kontakt mit festgelegten Konzentrationen von τ -Fluvalinat in Paraffin, die Mortalität wurde nach 24 und 48 Stunden kontrolliert. Die Empfindlichkeit nicht-resistenter Milben aus verschiedenen europäischen Ländern zeigte keine Unterschiede (Abb. 1). Ebenso wiesen die resistenten Linien aus verschiedenen Gebieten die gleiche verringerte Empfindlichkeit gegen τ -Fluvalinat auf (Abb. 1). Zwischen der Wirkung im Labortest und der Wirksamkeit der Behandlung von Völkern im Freiland mit Apistan® bestand eine signifikante Korrelation. Die Genauigkeit der Laborversuche war allerdings höher als die der Feldversuche (Abb. 2). Die Gebiete mit nachweislich pyrethroid-resistenten Milben sind in dieser Arbeit auf einer Karte eingetragen (Abb. 3). Es scheint, daß eine resistente Linie in Italien entstanden ist und sich von dort nach Slowenien, in die Schweiz, nach Frankreich, Belgien und Österreich verbreitet hat. © Inra/DIB/AGIB/Elsevier, Paris

Varroa jacobsoni /Akarizid / Überprüfung / Pyrethroid / Resistenz

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