

Fluvalinate resistance of *Varroa jacobsoni* Oudemans (Acari: Varroidae) in Mediterranean apiaries of France

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Summary — For control of *Varroa jacobsoni* Oudemans, a parasite of the honey bee (*Apis mellifera* L), we evaluated the level of susceptibility of mite populations to the most common acaricide (fluvalinate). Mortality was recorded after 24 h exposure on the bottom of plastic Petri dishes, treated with different dilutions of the 240 g/L emulsifiable concentrate Klartan[®]. The results showed three categories of mite populations: i) susceptible with a LC50 lower than 2 ng/cm²; ii) populations, with a LC50 ranging from 2 to 20 ng/cm², that included some resistant mites; and iii) resistant with a LC50 higher than 20 ng/cm². For field surveys of resistance, a 10 ng/cm² fluvalinate concentration threshold would allow early resistance detection.

***Apis mellifera* / *Varroa jacobsoni* / acaricide resistance / fluvalinate susceptibility / resistance survey / France**

INTRODUCTION

The ectoparasitic mite *Varroa jacobsoni* Oudemans began to be a problem in the Mediterranean area in the 1970s. After a presence of 20 years in apiaries, it is always considered as the major pest of honey bee *Apis mellifera* L. Without treatment, “the

mortality in the apiaries continually increases: sporadic at the beginning, it reaches 50% and finally 100%” (Grobov, 1977). Similar concern was reported just after the discovery of the mite in the United States (Cobey and Lawrence, 1988).

The serious effect of this epizootic parasitosis is due to the parasitism of both adult

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bees and brood. Adult mated mite females settle on bee workers and drones, enter the brood cells a few hours before sealing and reproduce in close synchronization with the bee development (Martin, 1994). After piercing the bee cuticle, the mite females suck the haemolymph, taking proteins and contributing at the same time to the inoculation and the multiplication of bee pathogens, mainly in the brood (Ball and Allen, 1988).

In 1983, 1 year after its discovery near the German border of France, the mite was recognized in the Mediterranean area of France (Colin, 1984). Within 3 years, most of these Mediterranean apiaries were contaminated. Under Mediterranean climate, the presence of sealed brood even during winter time favoured mite reproduction and protected the entered mites from the action of the acaricides. Since 1990, the most common acaricide administered in the hives of the area is fluvalinate in PVC strips (Apistan[®], Zocon Company), which release the active substance for a minimum of 6 weeks. The release duration is calculated considering that the mites, entering the cells just before the hanging of the strip, are protected for the whole sealing period of the worker bee cell (about 12 days). Some mites that emerged with the new adult bee are not killed during their phoretic period and are thus able to reenter a cell. To reach the 99% optimal efficiency, the treatment must cover three capping periods, which means about 6 weeks.

Fluvalinate is an insecticide and a non-selective acaricide belonging to the pyrethroid family that acts mainly on nerve transmission [for a review see Soderlund and Bloomquist (1989)]. It is well tolerated in the honey bee colonies at the doses used for *V jacobsoni* control.

Loglio and Plebani (1992) indicated for the first time an important decrease in the fluvalinate efficiency in Italian apiaries. In the area of Lombardy, the average effec-

tiveness of Apistan ranged between 4 and 89.5% (Colombo et al, 1994; Lodesani et al, 1995). The authors also indicated a large variability among both colonies and apiaries. Because of the tendency of the mites to quickly develop resistance to insecticides or specific acaricides [for a review see Otto and Weber (1992)], the possibility that *V jacobsoni* will become resistant must be objectively considered (Gerson et al, 1991). Before using the fluvalinate acaricide, Smirnov (1979) asserted that resistances against phenothiazin and tedion were observed in Japanese apiaries. In experimental conditions, Ritter and Roth (1988) described the ability of *V jacobsoni* to become resistant.

Resistance is defined as "a genetic change in response to selection by toxicants that may impair control in the field" (Sawicki 1987). Taking into account the difficulty in rearing *V jacobsoni* mite strains in controlled conditions, we cannot isolate resistant and susceptible strains and proceed to crosses or backcrosses [see Otto and Weber (1992) for a review]. From a methodological point of view the LD50 determination was chosen rather than the survival time (Faucon et al, 1996) according to Finney (1985). The latter observed that the definition of the median lethal time introduced serious confusion with the natural mortality. Thus, the aim of the present work is twofold:

- i) to adapt techniques for detection of resistance in arthropods other than the mite *V jacobsoni*;
- ii) to investigate the reason for the decrease in efficiency of fluvalinate in some French apiaries, following the example of Italy (Milani, 1995).

MATERIALS AND METHODS

V jacobsoni mites

Mites were sampled from apiaries:

i) where the beekeeper did not observe brood diseases in the presence of mites and abnormal mortalities of bee colonies (Brignoles and Alpilles areas);

ii) placed near apiaries with mites resistant to fluvalinate or where the beekeeper only observed one or two mites on adult bees just after the end of the treatment (Draguignan and Aix areas);

iii) where numerous mites were visually detected during the treatment, often with brood diseases, sometimes responsible for the loss of the colony (Le Muy and Toulon areas). Mites were taken about 2 weeks after the end of the treatment.

In each apiary, one colony was chosen in order to produce a minimum of 300 mites. The adult mites were caught after opening the capped cells with consideration of the immature bee stage (Milani, 1995), if a large number of infested cells were present. If not, dark brown mites were also taken from pupae with pigmented bodies.

Contamination technique

Topical application on the ventral mite surface (Ritter and Roth, 1988) or on the dorsal surface (Abed and Ducos de Lahitte, 1993) were difficult, because the droplets did not remain on the mite scutum. In order to simulate the contact of mites with fluvalinate-contaminated surfaces, such as wax cell walls or insect cuticle, we used freshly treated Petri dishes (Grafton-Caldwell et al. 1989; Thistlewood et al. 1992) made of polystyrene (Greiner®), instead of films of paraffin wax mixed with fluvalinate (Milani, 1995). The deposit on the surface of a solid support is more closely related to the mode of action of the fluvalinate-impregnated PVC strip.

Small Petri dishes (35 mm in diameter) were sprayed in a Potter-Burgerjon tower (Burgerjon, 1956) so that the deposit of the spray ranged from 1.5 to 2 mg/cm² (Hassan et al. 1985) at the considered concentrations of the agrochemical formulation containing 240 g/L of tau-fluvalinate (Klartan emulsifiable concentrate®, Sandoz Agro).

Test protocol

Before spraying, the dish wall was coated with Fluon®, containing silicone, to avoid escape of the mites. Twelve dishes per concentration were sprayed with dilutions of 0.25 to 25 mg (AI)/L in

distilled water for testing the susceptible strains and from 2 to 128 mg (AI)/L for the suspected resistant strains. If the number of collected mites was sufficient, more than four dilutions were used to frame the LC50. The sprayed dishes were dried in an incubator (34 °C) for 1 h. The same day, five mites were dropped in each dish and left for about 1 h at room temperature (20 °C), so that 60 mites were tested per concentration. Then, we carefully put a living bee pupa with white eyes into the dish to provide mites with food, if necessary. Immediately after the deposit of the pupa, the dishes were stored at 33 ± 2 °C and 50 ± 10% RH until needed. The mites moved freely in the dishes, sitting or not on the pupa.

At 24 and 48 h, the mites were observed at × 12 magnification under a dissecting microscope to count the dead mites. Mites were considered as dead when they exhibited “no movements after prodding” (ffrench-Constant and Roush, 1992).

Statistical analysis

According to Abbott (1925), we plotted values corrected for the natural mortality.

Considering that the mite population in one colony is more probably a mixture of at least two phenotypes (resistant and susceptible), “the calculation of only one straight log dosage–probit mortality relationship analysis is not possible” (Otto et al. 1992; Thistlewood et al. 1992). With regards to the dose–mortality relationship, we simply consider the regression curve (Computer Associates, 1993), which fit the experimental data ($r^2 \geq 0.88$) to calculate the LC50 and LC90 (Scherrer, 1984). According to Otto et al (1992), we also used the spline regression analysis to calculate the LC50 separately for both the susceptible and resistant phenotypes when they were clearly distinguished (Toulon’s apiary). In the case of the most susceptible population (Brignoles’ apiary) the LCs are also calculated by the probit regression, because we suppose that the population is formed with susceptible mites only and consequently normally distributed.

RESULTS

The mortalities in the different control groups varied from an average of 0 to 23% at 24 h.

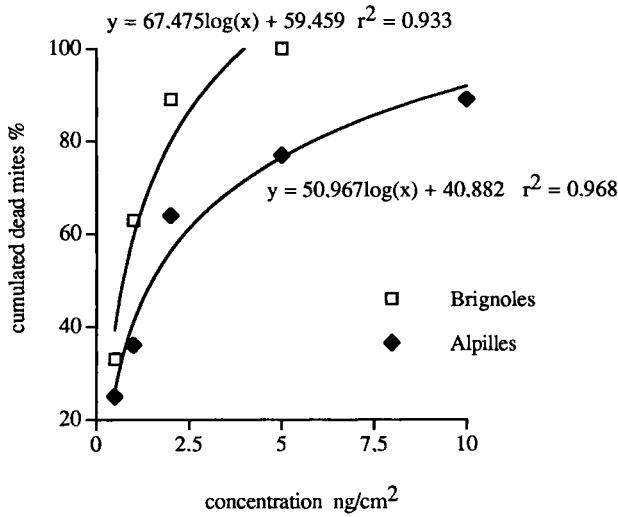


Fig 1. Fluvalinate dose–letality relationship for the group where the beekeeper did not observe abnormalities in the colonies. Abscissa: concentrations deposited on the Petri dish. Ordinate: mean percentage of mortality [corrected following Abbott (1925)].

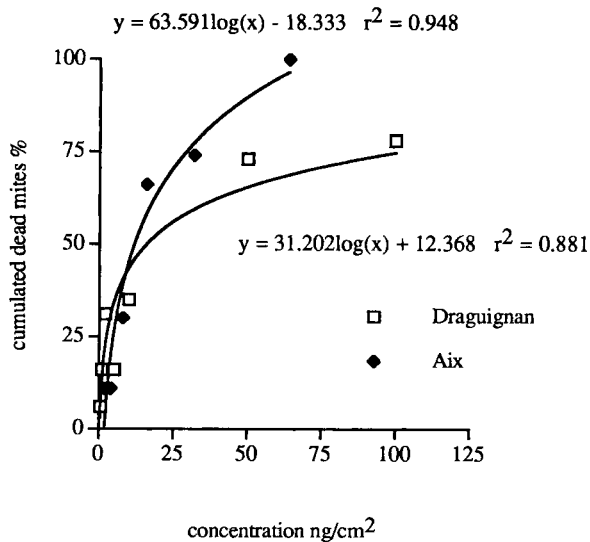


Fig 2. Fluvalinate dose–letality relationship for the group where the colonies were placed near apiaries with resistant mites or where the beekeeper only observed some mites just after the end of the treatment. Abscissa: concentrations deposited on the Petri dish. Ordinate: mean percentage of mortality [corrected following Abbott (1925)].

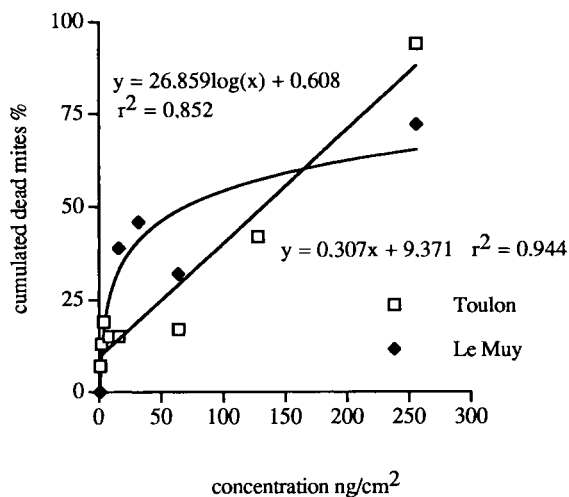


Fig 3. Fluvalinate dose–lethality relationship for the group where numerous mites were visually detected during the treatment, often with brood diseases, sometimes responsible for the loss of the colony. Abscissa: concentrations deposited on the Petri dish. Ordinate: mean percentage of mortality [corrected following Abbott (1925)].

Table I. LC50s, resistance level and LC90s of mite populations coming from Mediterranean bee colonies.

Origin	LC50 (ng/cm ²)	95% fid int (ng/cm ²)	Résist level ^a	LC90 (ng/cm ²)	95% fid limits	
					Inf	Sup
Brignoles	0.73	0.24	—	2.83	2.16	3.72
Alpilles	1.50	0.26	4.23	9.18	6.40	13.21
Aix	11.92	3.48	16.56	50.34	37.82	67.52
Draguignan	16.07	1.06	22.31	> 100 ^b		
Le Muy	69.04	3.62	95.89	> 256 ^b		
Toulon	131.54	24.08	182.7	259.52	236.58	288.70

^a Calculated as LC50 considered population ÷ LC50 most susceptible population; ^b the calculated value is outside the experimental points.

Calculation of the LC50s and LC90s

The ranges of the experimental concentrations frame the 50% lethality points. The LC50 values are obtained by calculation from a logarithmic regression, supported by a regression coefficient superior to 0.88 (figs 1–3). The estimated LC50s at 24 h

range between 0.73 and 131.54 ng/cm². Due to the high mortalities in the control groups at 48 h, we consider that it is hazardous to calculate LC50 at this time. When the LC90s fall within the experimental points, they range between 2.83 and 236.58 ng/cm² (table I).

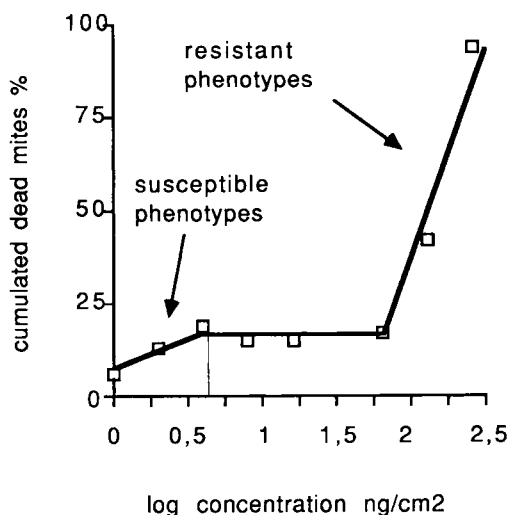


Fig 4. Spline regression analysis of the dose-lethality relationship for the group where numerous mites were visually detected during the treatment. The spline regression analysis allows us to separate two phenotypes in the mite population as indicated by the arrows. Abscissa: log of the concentrations deposited on the Petri dish. Ordinate: mean percentage of mortality [corrected following Abbott (1925)].

When the probit analysis is applied to the data of the most susceptible population coming from Brignoles ($\chi^2 = 0.1372$, $df = 1$), the LC50 is 0.73 ng/cm² and the LC90 is 2.15 ng/cm². These values are very close to that given by the logarithmic regression. Keeping in mind that the design guidelines for precise estimation of the LD values described by Finney pertain to the LD50 and not necessarily to other dose levels (Robertson et al. 1984), the LC99 is 5.16 ng/cm² and the 95% fiducial limits are 3.59 and 9.27 ng/cm².

In two cases (Le Muy and Toulon), the probit analysis is strongly rejected ($\chi^2 = 16.26$, $df = 2$ and $\chi^2 = 89.31$, $df = 6$, respectively) and the logarithmic regression does not fit well with the experimental values from Toulon. The linear regression gives a r^2 coefficient of 0.944, which allows us to calculate a LC50 of 131.54 ng/cm². When the spline regression analysis is applied (Otto

et al, 1992), the LC50s for the susceptible and resistant phenotypes are, respectively, 2 and 128 ng/cm² and the given frequency of the susceptible phenotypes is 18% (fig 4). For the resistant phenotype, the LC50s calculated by the two ways are identical. The value of the susceptible phenotype (2 ng/cm²) is consistent with the LC50 of the mites coming from Brignoles or Alpillès (0.73 and 1.50 ng/cm²). The presence of a large plateau is not clear when analysing the data of Le Muy by the same method. The lower value of the Le Muy LD50 could be explained by the presence of a lower frequency of resistant phenotypes.

Calculation of the resistance level

The resistance level is generally expressed as the ratio of the LC50 of a mite strain or field population on the LC50 of a normal susceptible reference strain or at least the

most susceptible field population. For these field populations, the resistance level increases to 182.7 (Toulon's colony) (table I).

DISCUSSION

Comparison with Milani's results

The direct comparison with the values of LC50 given by Milani is difficult because of the chosen contamination technique. In Milani's protocol, the active substance is included in paraffin which coats a glass disk and the mites stay on the impregnated paraffin for 6 h without feeding source. In the present work, the active matter, in agrochemical form, is sprayed and dried on a solid surface, then the mites stay on the treated surface only 1 h without feeding source and for 24 h with the possibility of feeding. Thus, the contact between the active matter and the mite surface is quite different. However, the comparison between the resistance levels remains possible. In both studies, the resistance levels can explain the ineffectiveness of the fluvalinate treatments observed in the apiaries. The maximum resistance level is higher in this study: 183 versus 54 in Milani. One explanation, partially contributing to this difference, is the use of the probit transformation by Milani in the case of the natural population coming from apiaries with resistant mites. This transformation does not allow us to estimate the LC50 of the resistant phenotypes of the natural population.

The resistance in the field

Between the susceptible (resistance level lower than 10) and the resistant populations (resistance level higher than 90), an intermediate stage (Draguignan and Aix apiaries) can be found. Following the hypothesis of a

population formed with resistant and susceptible phenotypes, the intermediate stage is probably made of a low frequency of resistant individuals, but the presence of a few resistant mites represents the beginning of the resistance but not the immediate ineffectiveness of the treatment.

The detection of resistant mites

As suggested by the positive results collected after a control treatment in some Mediterranean apiaries (Faucon et al, 1995), the resistance of *V. jacobsoni* against fluvalinate is present in south-east France far from the Italian border, so we have to develop a rapid bioassay to detect fluvalinate resistant mite populations. The present protocol can be simplified by using only one fluvalinate concentration discriminating the intermediate and susceptible populations. Considering that the superior limit of the LC99 of the most susceptible population (Brignoles) is 9.27 ng/cm², and following the hypothesis supported by the spline regression analysis, the dose of 10 ng/cm² (log = 1) kills more than 99% of the susceptible phenotypes, whereas 100% of the resistant phenotypes survive. Thus, the beekeeper would have to change the acaricide in the near future if mites survive at this concentration. However, further experiment with suitable protocol is needed to make valid this concentration.

In conclusion, the detection of the resistance first relies primarily on the chosen detection method. The application of a control treatment with another acaricide in the apiaries is not sensitive enough. The most reliable methods are performed in controlled conditions when the active substance, at a defined concentration, comes directly into contact with mites extracted from the brood. By choosing a convenient concentration, we have at our disposal a rapid bioassay to manage a survey of the spread of the fluvalinate resistance in the apiaries.

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Résumé — La résistance au fluvalinate dans les ruchers méditerranéens français.

La relation dose de fluvalinate–mortalité de l'acarien a été construite à partir de parasites prélevés sur trois types de ruchers : i) ruchers où l'apiculteur n'observe pas de parasites après traitement ; ii) ruchers où l'apiculteur ne remarque que quelques parasites après traitement ou ruchers placés à proximité de ruchers dans lesquels le traitement au fluvalinate a été inefficace ; iii) ruchers où la varroose demeure grave malgré la répétition des traitements avec cet acaricide. Sept dilutions de la formulation commerciale Klartan (240 g/L de fluvalinate), espacées selon une progression géométrique de raison deux (figs 1, 2 et 3 en abscisse), ont été pulvérisées sur le fond de boîtes de Pétri pour obtenir un dépôt de 1,5 à 2 mg par cm². Soixante parasites, extraits du couvain, ont été mis en contact avec chaque dilution, pour une colonie. La mortalité a ensuite été relevée après 24 et 48 h d'incubation à 33 °C et 50 % d'humidité relative. Le type de la régression dose–léthalité a été choisi en fonction du meilleur ajustement aux données expérimentales puisque la population parasitaire d'une colonie est vraisemblablement composée des deux phénotypes résistant et sensible. Dans un cas (figs 3 et 4), nous avons appliqué la régression segmentaire d'Otto. Les CL₅₀ à 24 h varient entre 0,73 et 131,54 ng de fluvalinate par cm² et les CL₉₀ à 24 h entre 2,83 et 259,52 ng/cm² (tableau I). Lorsqu'on utilise la régression segmentaire d'Otto, la CL₅₀ des parasites les plus sensibles est de 2 ng/cm² et de 128 ng/cm² pour les résistants (fig 4). L'hypothèse de l'existence des deux phénotypes (résistant et sensible) dans la population parasitaire d'une colonie est

donc confortée. Le coefficient de résistance le plus élevé est de 182,7 (tableau I), ce qui rend inutile toute augmentation de dose thérapeutique lors d'une baisse d'efficacité du traitement. L'émergence de la résistance serait soupçonnée si des parasites provenant d'une même colonie et soumis 24 h à la concentration de 10 ng/cm², demeuraient vivants.

Apis mellifera / *Varroa jacobsoni* / sensibilité résistance / acaricide / fluvalinate / France

Zusammenfassung — Fluvalinat - Resistenz von *Varroa jacobsoni* Oud (Acari: Varroidae) in Bienenständen am französischen Mittelmeer. Zur Untersuchung der Beziehung zwischen Fluvalinat-Dosis und *Varroa*-Sterblichkeit wurden Milben von 3 unterschiedlichen Bienenständen gesammelt: i) von Bienenständen, in denen der Imker nach der Behandlung keine Milben mehr gefunden hat; ii) von Bienenständen, in denen der Imker nur wenige Milben direkt nach der Behandlung gefunden hat oder von Bienenständen, die sich in der Nähe von Bienenständen befanden, bei denen die Fluvalinatbehandlung keinen Effekt hatte; iii) von Bienenständen, die trotz wiederholter Behandlung mit diesem Akarizid einen hohen Befall aufwiesen. Sieben verschiedene Verdünnungen des kommerziell gehandelten Klartans (240g/L Fluvalinat) wurden getestet: in einer geometrischen Abstufung von jeweils der doppelten Menge (Abszisse in Abb 1, 2 und 3) wurde der Wirkstoff auf den Boden von Petrischalen gestäubt, um ein Menge von 1,5 bis 2 mg pro cm² zu erhalten. Pro Volk wurden 60 Milben aus der Brut jeder Verdünnung ausgesetzt. Sodann wurde die Mortalität nach 24 und 48 Stunden der Einwirkung bei 33 °C und 50% relativer Luftfeuchte bestimmt. Die Art der Regression der Beziehung von dosisabhängiger Sterblichkeit wurde nach einer Funktion betrachtet, die

den experimentellen Daten am besten angepaßt ist, da die Population der Parasiten sehr wahrscheinlich aus den beiden Phänotypen 'resistent und empfindlich' zusammengesetzt ist. In einem Fall (Abb 3 und 4) haben wir die bereichsweise Regression nach Otto angewendet. Die LC50 Werte nach 24 Stunden schwanken zwischen 0,73 und 131,54 ng Fluvalinat pro cm² und die LC 90 zwischen 2,83 und 259,52 ng Fluvalinat pro cm² (Tabelle I). Wenn man die bereichsweise Regression von Otto benutzt, liegt die LC 50 der empfindlichsten Milben bei 2 ng/cm² und bei 128 ng/cm² bei den resistenten (Abb 4). Die Hypothese der Existenz von 2 Phänotypen (resistent und empfindlich) in einer Milbenpopulation in einem Volk ist damit unterstützt. Der höchste Resistenzkoeffizient beträgt 182,7 (Tabelle 1), damit erübrigt sich jede Erhöhung der therapeutischen Dosis auf Grund des Nachlassens der Wirksamkeit der Behandlung. Ein Auftreten der Resistenz kann angenommen werden, wenn die Milben in demselben Volk vorkommen und nach einer 24 stündigen Behandlung mit 10 ng/cm² am Leben bleiben.

***Apis mellifera* / *Varroa jacobsoni* / Akarizid Resistenz / Empfindlichkeit gegen Fluvalinat / Überblick über Resistenz / Frankreich**

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