

**COMPARING EFFECTS OF THREE ACARICIDES ON
VARROA JACOBSONI (ACARI: VARROIDAE) AND APIS
MELLIFERA (HYMENOPTERA: APIDAE) USING
TWO APPLICATION TECHNIQUES**

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ABSTRACT

Two bioassays were administered to determine the dose-lethality response of *Varroa jacobsoni* Oudemans and the honey bee, *Apis mellifera* L., to amitraz, flumethrin and fluvalinate. The first bioassay method was spraying by means of the Potter-Bourgerjon's tower. The results are expressed in mean lethal concentrations (LC_{50}).

The second method was topical application by means of microsyringe and manual applicator. The results are expressed in mean lethal doses (LD_{50}). Both LC_{50} and LD_{50} values were considerably higher in honey bees than in varroa mites, showing that a wide margin of safety exists between effective doses against mites and harmful doses for honey bees. Both methods gave similar confidence intervals; they showed a comparable sensitivity to changes in dose or concentration of pesticides.

Key Words: amitraz, bioassays, flumethrin, fluvalinate, honey bees, susceptibility, toxicity, varroa mites

RESUMEN

Se probaron dos métodos de bioensayos toxicológicos para determinar la respuesta dosis-letalidad de amitraz, flumetrina y fluvalinato sobre *Varroa jacobsoni* Oudemans y *Apis mellifera* L. El primero fue aspersión por medio de la torre de Potter-Burgerjon; sus resultados se expresan en concentraciones letales medias (CL_{50}). El segundo fue aplicación tópica por medio de microjeringa y aplicador manual; sus resultados se expresan en dosis letales medias (DL_{50}). Las DL_{50} y CL_{50} de todos los productos fueron considerablemente más altas en abejas que en ácaros, lo cual muestra que existe un amplio margen de seguridad entre dosis que son lo suficientemente tóxicas sobre los ácaros, sin llegar a ser peligrosas para las abejas. Ambos métodos de bioensayo dieron intervalos de confianza comparables y presentaron similar sensibilidad en la respuesta a los cambios de dosis y concentración aplicados.

Beekeepers in many parts of the world face severe problems because of recent introductions of a parasitic mite, *Varroa jacobsoni* Oudemans (Acari: Varroidae), known as varroa. Originally from tropical Asia and found on the Indian honey bee, *Apis cerana* Fabricius, this mite has shifted to its new host *A. mellifera* L. Owing to human activities, it has infested most of honey bee colonies around the world, causing severe losses.

Many control measures have been developed for varroa. Most include the use of chemicals. However, chemical control has the disadvantages of variable efficacy, increased costs, contamination of hives and hive products and the risk of target pest resistance. Varroa resistance to fluvalinate was documented by the first time in Italy (Lodesani et al. 1995) and soon in several European countries (Londzin & Sledzinski 1996, Moosbeckhofer & Trouiller 1996, Bruneau et al. 1997, Vandame et al. 1995). Elzen et al. (1999), by application of discriminating doses of fluvalinate, found indications that varroa mites from Florida and California were developing resistance to this acaricide.

Development of acaricide resistance by varroa is of concern. Chemical control necessarily involves contact of pesticides with bees and hives. When resistance occurs, doses should not be increased because of the risk of harming or killing bee hosts and increasing contamination in the hive environment and hive products (Lodesani et al. 1992). Toxicological bioassays can track changes in pesticide susceptibility of a population, by detecting changes in the calculated mean lethal concentrations or doses (LC_{50} or LD_{50} , respectively), compared to a maximum reference susceptibility or baseline (Georghiou 1963). Early detection of pesticide resistance is mandatory for developing a long-term strategy of chemical control, based on replacing ineffective pesticides. Bioassay methods must be sensitive to dose variations and easily repeatable, to allow comparison of lethal values (Lagunes-Tejeda & Villanueva-Jimenez 1994).

Topical application bioassays have been conducted on varroa by various researchers. Ritter & Roth (1986) determined mite susceptibility to Folbex VA (bromopropi-

late) and K79 (clorodimeformidrochloride); they found a positive correlation between lethal doses and number of previous treatments, suggesting early manifestations of resistance. Also by topical application, Abed & Ducos de Lahitte (1993) estimated LD₅₀'s of amitraz and coumaphos.

A spraying method of application for toxicological bioassays has been proposed by Colin et al. (1994), who used the Potter-Burgerjon's tower in testing lethality and behavioral effects of pesticides on varroa mites. This device sprays doses onto an area, simulating a field application. In this method, data are expressed in lethal concentrations (LC₅₀) of the material surrounding the specimen; the exact quantity of pesticide contacting the specimen is unknown. Units are mg L⁻¹, parts per million (ppm), g cm⁻² or their equivalencies.

Study of varroa populations established in Mexico may provide useful information to other parts of the world. According to Otero-Colina & Santillan-Galicia (1996), these mites were first detected in Veracruz state in the Mexican Gulf Coast lowlands in 1992, although they probably were already present there since about 1989. Before their discovery and at least three years afterwards, they were seldom chemically treated. Thus, they have been almost free of selection pressure by pesticides for at least six years and supposedly show maximal levels of susceptibility to most acaricides.

The present study had the following objectives: a) to estimate LC₅₀ and LD₅₀ on *V. jacobsoni* and *A. mellifera* to the acaricides amitraz, flumethrin and fluvalinate, and b) to compare two toxicological bioassay methods for determining susceptibility to these pesticides of varroa mites and honey bees.

MATERIALS AND METHODS

All varroa specimens were obtained from a commercial apiary that had received a single treatment of fluvalinate (Apistan® Novartis) one year before. Adult female mites were collected manually, from CO₂ anesthetized worker bees or by uncapping parasitized worker pupae. Mites were kept at 25°C and 50% R. H. and put on pupae until they were used in the tests, up to 4 hours later. Worker bees were collected from combs of healthy (non-parasitized or with low levels of infestation) European colonies (*Apis mellifera ligustica* Spinola). In order to avoid recently emerged nurse bees and to use bees of similar age, collections were made from combs without open brood (Felton et al. 1986). Adult bees were transported to the laboratory and used in bioassays about two hours later.

All acaricides were used in commercial formulations; they were amitraz (Taktic®, 12.5%, liquid, Hoechst), flumethrin (Bayticol®, 3%, emulsifying concentrate, Bayer) and fluvalinate (Fluvalin®, emulsifying concentrate 25%, Isihara). Commercial formulations were preferred as they are easily available and because they are currently in use against varroa in many countries (Arculeo et al. 1989, Benitez-Reynoso 1998, Cardenal Galvan et al. 1989). In the spraying method, the solvent was water; in topical application, the solvent was acetone.

For each pesticide, preliminary bioassays were conducted to obtain maximal dose causing 0% mortality and minimal dose causing 100% mortality. Then, logarithmic intermediate doses were applied to obtain the LC₅₀ and LD₅₀. Four to seven intermediate doses plus extreme values were used in each replication. All dilutions were prepared immediately before being used.

Bioassays on Mites

When the spraying method was carried out, a Potter-Burgerjon's tower was calibrated for applying 1.7 mg cm⁻² (s.d. = 0.14) of acaricide solutions, by spraying 15 mL solution at a pressure of 0.703 kg cm⁻², then waiting one minute for sedimentation of

droplets. A solid cone nozzle (Cat. 1/4J-SS+SU1A-SS, Spraying Systems) was used. Groups of 14 varroa females were placed in a 14 cm diameter Petri dish containing a floor of filter paper; each group was treated by an acaricide, then transferred to another Petri dish (5 cm diameter), and incubated at $32 \pm 2^\circ\text{C}$, $70 \pm 10\%$ RH. To feed the mites, two or three worker pupae one to three days old were placed in each Petri dish. Pupal age was determined by their light yellow thorax, according to Jay (1953). Mortality data were taken 24 hours after the treatment.

For topical application, groups of 14 varroa females were stuck ventral side up on a microscope slide with Scotch® double sided tape; 0.1 mL of pesticide solution was then applied ventrally to each mite using a microsyringe and a microapplicator. This contrasted with the method proposed by Ritter & Roth (1986), who applied 0.2 mL solution. The slides were placed in an incubator at $25 \pm 2^\circ\text{C}$ and $70 \pm 10\%$ R.H. (instead of 16°C and 98% R.H., by the same authors). Mortality was recorded 24 h later. A specimen was considered dead when it did not respond to tactile stimuli. All tests comprised four replications per dose on different days; a solvent-only control was included.

Bioassays on Honey Bees

To compare results, the same bioassay methods were used on bees, with some differences owing to size, flying ability and nutritional requirements as given below. All bees were anesthetized with a stream of CO_2 ; in the spraying test, groups of 30 workers were confined in a galvanized iron cage ($15 \times 20 \times 25$ cm, 4 mm mesh) with a filter paper floor, then sprayed in the Potter-Burgerjons tower. In the topical application test, every bee in a group of 30 received 1 mL solution dorsally on the thorax. In both tests, after exposure to chemicals, the groups of bees (replications) were confined in 1 L plastic cages; they were supplied with solid food (candy) and water, and incubated at $25 \pm 2^\circ\text{C}$ and $70 \pm 10\%$ R.H. Every bioassay had three replications.

Analysis of Results

Percentages of mortality were corrected by Abbott's (1925) formula when mortality was found in the control; when mortality of one bioassay exceeded 10% of bees and 15% of mites, the results were discarded. Values of LC_{50} and LD_{50} and their confidence intervals were estimated by Probit analysis. Relative toxicity of all products was estimated in varroa and in bees, by dividing experimental lethal values by the most toxic value. Toxicity of all products was also compared on mites vs. bees, by dividing LC_{50} and LD_{50} values.

Results of aspersion and topical methods are expressed in different units and their values are not comparable. However, an attempt was made to compare these methods taking the width of confidence intervals as a measure of precision and slopes as a measure of sensitivity, the last by means of Student's t-test (Dittrich 1962). Ease of bioassay methods was also considered.

RESULTS

Susceptibility of varroa

Spraying, LC_{50} and confidence intervals are shown in Table 1. Previous studies of fluvalinate LC_{50} levels on varroa were conducted using a residual application technique, but these results are not comparable with those of the current work, because different bioassay methods were used. Milani (1995) placed varroa specimens on flu-

TABLE 1. LC₅₀, LD₅₀ AND RANGE (CONFIDENCE INTERVALS, 95%) OF FIVE ACARICIDES AGAINST *VARROA JACOBSONI*, BY SPRAYING AND TOPICAL APPLICATION, RESPECTIVELY.

Acaricide	LC ₅₀ mg L ⁻¹	Confidence int. (LV-HV)	HV ¹ /LV ² (LC ₅₀)	LD ₅₀ pg mite	Confidence int. (LV-HV)	HV/LV (LD ₅₀)
Amitraz	0.23	0.14-0.37	2.68	1.7	1.21-2.39	1.98
Flumethrin	875.08 ³	201-6554	32.61	0.46	0.36-0.59	1.62
Fluvalinate	0.19	0.13 -0.29	2.31	15.42	9.91-24.94	2.52

¹Highest value.

²Lowest value.

³Nanograms L⁻¹.

valinate-impregnated paraffin and determined a LC₅₀ of 20 mg L⁻¹ for a susceptible population from Udine, whereas a resistant population from Lombardy (both in Italy) showed a LC₅₀ higher than 200 mg L⁻¹. Vandame et al. (1995), using fluvalinate-sprayed surfaces, estimated a LC₅₀ of 0.21 mg per mL of sedimented solution in samples from Brignoles, while specimens from Draguignan (both in France) had a LC₅₀ of 2.67 mg mL⁻¹, indicating a twelve fold resistance factor.

According to the above statements, Mexican varroa populations are considered to have maximum levels of susceptibility to most acaricides, owing to their isolation from chemically-selected strains. Thus, LC₅₀ values obtained in this study are proposed as baselines for testing acaricides.

Topical application. Table 1 shows LD₅₀ against varroa. Abed & Ducos de Lahitte (1993) estimated an amitraz LD₅₀ of 2.16 pg per mite, with a confidence interval of 1.46-3.2 pg. These values are close and overlap values obtained in the current work; this fact suggests comparable levels of susceptibility in both mite populations. Baseline data expressed as LD₅₀ are proposed now as they appear in Table 1.

Susceptibility of *Apis mellifera*

Spraying. Results are shown in Table 2. There are no published data for direct comparison with our results, since most research on bee toxicology used oral and contact bioassay methods (Oomen 1986). According to a pesticide classification of Felton et al. (1986) of toxicity to honey bees, flumethrin and fluvalinate belong to Group 1, highly toxic pesticides, with LD₅₀ < 1 µg/bee. Amitraz belongs to Group 2, moderately toxic, with LD₅₀ 1-10 µg/bee.

TABLE 2. LC₅₀, LD₅₀ AND RANGE (CONFIDENCE INTERVALS, 95%) OF FIVE ACARICIDES AGAINST *APIS MELLIFERA*, BY SPRAYING AND TOPICAL APPLICATION, RESPECTIVELY.

Acaricide	LC ₅₀ µg L ⁻¹	Confidence int. (LV-HV)	HV ¹ /LV ² (LC ₅₀)	LD ₅₀ µg/bee	Confidence int. (LV-HV)	HV/LV (LD ₅₀)
Amitraz	1636	983.79-2825	2.32	2.55	1.57-4.32	2.75
Flumethrin	46.87	21.15-95.61	4.52	0.05	0.03-0.09	3.26
Fluvalinate	1601	1429-1803	1.26	0.97	0.57-1.66	2.91

¹Highest value.

²Lowest value.

Topical application. LD₅₀ and confidence intervals appear in Table 2; previous data were obtained by Oomen (1986) for amitraz: LD₅₀ > 16 LD₅₀ µg/bee, and by Bornek (1989) for fluvalinate: LD₅₀ = 4.66 µg/bee, using Mavrik; LD₅₀ = 9.12 µg/bee, using Klartan. Amitraz and fluvalinate LD₅₀ values estimated herein are lower than those obtained by both authors; however, data cannot be accurately compared because of different experimental conditions and analytical methods.

Relative Toxicity

Tables 3 shows relative toxicity values for all acaricides used on varroa mites and honey bees. Consistently, flumethrin was the most toxic product, while fluvalinate and amitraz showed a lesser similar toxicity.

Comparative Susceptibility

The rates of bee LC₅₀ or LD₅₀ divided by mite LC₅₀ or LD₅₀ are presented in Table 4. These data show that all products have acaricidal, rather than insecticidal action; different toxicity ranges from 500 fold to more than one million fold. This indicates a wide safety margin between lethal levels against mites and toxic levels for honey bees.

Comparison of bioassay methods

As a measure of sensitivity, slopes resulting from spraying and topical application were analyzed. In most cases they attained the quality criteria proposed by Ibarra & Federici (1987) for toxicological bioassays. Table 5 shows a comparison of slopes for spraying vs. topical application (Student t test, $\alpha = 0.05$). Significantly higher slopes for spraying method occurred only in amitraz and fluvalinate applied to honey bees, representing their greater sensitivity to spraying.

DISCUSSION AND CONCLUSIONS

Precision, as estimated by means of the confidence intervals, is shown in Tables 1 and 2. Although in several cases the quotient HV/LV exceeded the value of 2 (proposed by Ibarra & Federeci 1987, as the highest permissible limit), sufficiently accurate es-

TABLE 3. RELATIVE TOXICITY OF ACARICIDES ON VARROA JACOBSONI AND *APIS MELIFERA*.

Acaricide	on varroa		on bees	
	Spraying	Topical	Spraying	Topical
Flumethrin	1	1	1	1
Amitraz	262.83	3.7	34.9	51
Fluvalinate	217.12	33.52	34.16	19.4

TABLE 4. COMPARATIVE LC₅₀ AND LD₅₀ FOR ACARICIDES USED AGAINST VARROA JACOBSONI AND APIS MELLIFERA.

Acaricide	LC ₅₀ bee/LC ₅₀ varroa	LD ₅₀ bee/LD ₅₀ varroa
Amitraz	7113.04	1.5*10 ⁶
Flumethrin	5360.81	1*10 ⁵
Fluvalinate	8426.31	6.3*10 ⁴

timates of LC₅₀ and LD₅₀ were obtained in both aspersion and topical methods. An important exception is the large confidence interval shown by spraying of flumethrin on varroa; no explanation for this fact can be given.

Samples included mixed specimens obtained from adult bees and uncapped pupae, which constituted a potential source of variation (Milani & Della Vedova 1996), and no attempt was made to detect differences in susceptibility between such origins. However, obtaining female mites from a single source was a difficult task, and confidence intervals may reflect this possible variation.

The spraying method has the advantage of treating all insects or mites at the same time; sticking individual mites to a slide as well as topical application to honey bees and mites are very laborious procedures. In addition, by using the Potter-Burgeron's tower, the amount of applied droplets could be narrowly regulated. Thus spraying proved to be more practical for testing on varroa mites, regardless of the need to regularly calibrate the spraying nozzle.

Although both application methods can be useful, spraying showed a more sensitive response of honey bees and it is easier in both species. So we consider it the best choice.

Fluvalinate has been widely used and, as expected, mites have developed resistance to it in many localities. Reproduction of whole bioassays as well as use of their estimated LC₉₀ or LD₉₀ as a discriminant screen will aid to decide its eventual replacing in a local or regional basis. Like fluvalinate, flumethrin is a pyrethroid. Thus, a risk exists of cross-resistance, as shown by Milani (1995). Its useful life is expected to be shorter and so early detection of resistance is important. Since amitraz is not chemically related to the pyrethroids, if an efficient and environmentally acceptable acaricide containing amitraz is available to beekeepers, it could be an option for alternating with pyrethroid treatments.

TABLE 5. COMPARISON OF SLOPES (β) FOR THE DOSE-LETHALITY RELATIONSHIP OF SPRAYING (S) VS. TOPICAL (T) TESTS CONDUCTED ON *V. JACOBSONI* AND *A. MELLIFERA*, BY MEANS OF A STUDENT T TEST ($A = 0.25$). H₀: BS = BT.

Acaricide	On Varroa β_s	On bees Results of t test
Amitraz	2.03	=
Flumethrin	1.01	=
Fluvalinate	1.87	=

¹> H₀ rejected, bs > bt.²= H₀ accepted.

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