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ORIGINAL RESEARCH ARTICLE

First record of resistance to flumethrin in a varroa population from Uruguay

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Varroa destructor is the most serious parasitic mite that infests the honey bee *Apis mellifera*. Different treatments with systemic acaricides are applied to control, but due to the intensive use of these chemicals, resistance to organophosphates and pyrethroids has developed worldwide. Most of the resistance episodes have been registered for fluvalinate, and only in a few cases has flumethrin resistance been reported. In Uruguay, no studies on *V. destructor* resistance to flumethrin have yet been recorded. High infestation levels of *V. destructor* are continuously detected in colonies of *A. mellifera* after treatments with flumethrin. Hence, this study aimed to estimate the possible resistance to flumethrin of a *V. destructor* population from the Colonia Department. Furthermore, the LC_{50} baseline levels for flumethrin in two Uruguayan populations were determined. The LC_{50} for flumethrin for the population from Colonia Department was 3.8 μg/Petri dish, which means an increase of 34.5 fold when compared to the corresponding baseline, suggesting the development of resistance. These results are the first report of resistance to flumethrin in *V. destructor* in Uruguay, and extend the knowledge of acaricide resistance in the country.

Primer registro de resistencia a flumetrina en una población de *Varroa destructor* en Uruguay

Varroa destructor, ácaro parásito de la abeja *Apis mellifera*, constituye uno de los mayores problemas de la apicultura mundial. El control de dicha parasitosis, es altamente dependiente de la aplicación de productos acaricidas de síntesis. Sin embargo, el uso indiscriminado e incorrecto de estos principios activos ha producido una fuerte presión de selección sobre las diferentes poblaciones de ácaros y generado la aparición de focos de resistencia en distintos lugares del mundo. En Uruguay, no existen antecedentes sobre poblaciones resistentes a la flumetrina. Altos niveles de infestación con *V. destructor* se han detectado en colmenas del Departamento de Colonia, luego de tratamientos con flumetrina, por lo tanto el objetivo del presente estudio fue estimar la posible resistencia de una población de *V. destructor* de Uruguay a la flumetrina. Además, se estimaron los niveles de base de CL_{50} para la flumetrina en poblaciones susceptibles de *V. destructor* en Uruguay. Para la población del Departamento de Colonia la CL_{50} para flumetrina fue de 3.8 μg/cápsula, lo que significó un incremento de 34.5 veces cuando se comparó con los niveles de base de la CL_{50} correspondiente, indicando que se trataría de una población resistente a la flumetrina. Estos resultados representan el primer reporte de resistencia a la flumetrina en una población de *V. destructor* de Uruguay.

Keywords: *Varroa destructor*; *Apis mellifera*; resistance; flumethrin; bioassay; Uruguay

Introduction

The honey bee, *Apis mellifera* L., is highly valued around the world for its activity as a pollinator and its production of honey. Colonies of honey bees are, however, susceptible to a number of pests and diseases which have negative economic implications for the beekeeping industry and agriculture. *Varroa destructor* (Anderson & Trueman, 2000) is considered the major pest affecting *A. mellifera* worldwide. This parasite feeds on the haemolymph of developing and adult bees, resulting in the transmission of secondary diseases by inoculation and multiplication of bee pathogens (Rosenkranz, Aumeier, & Ziegelmann, 2010). In general, *V. destructor* mites are kept under control by the use of synthetic acaricides such as coumaphos, amitraz, flumethrin, and fluvalinate. Due to the consistent and exclusive use of these acaricides it was almost inevitable that varroa mites would become resistant to them. Mites resistant to pyrethroids (specifically fluvalinate) emerged for the first time in Italy around 1991 (Lodesani, Colombo, & Spreafico, 1995; Loglio & Plebani, 1992). They then continued to spread throughout other places like France, the USA, Israel and the UK (Elzen, Eischen, Baxter, Elzen, & Wilson, 1999; Mozes-Koch et al., 2000;

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Thompson, Brown, Ball, & Bew, 2002; Vandame, Colin, Belzunces, & Jourdan, 1995). Most of the resistance episodes to pyrethroids have been registered for fluvalinate, and only in a few cases, has flumethrin resistance in varroa been reported (Goodwin, Taylor, McBrydie, & Cox, 2005; Milani, 1995; Perez Santiago, Otero-Colina, Mota Sanchez, Ramirez Guzman, & Vandame, 2000; Rodríguez-Dehaibes, Otero-Colina, Pardio Sedas, & Villanueva Jiménez, 2005; Thompson et al., 2002).

Currently, there is no agreement about how many times resistance has appeared. It has been suggested that mite resistance to pyrethroids has arisen only once or twice and that the major cause for the rapid spread of resistant mites has been the movement of bee colonies by beekeepers (Martin, 2004). Organisms develop resistance via reduced penetration, increased sequestration or excretion, behavioral changes, enzymatic detoxification or target site insensitivity (modifications of action site) (Onstad, 2007). In a population of *V. destructor* resistant to pyrethroids a series of mutations were found in voltage-gated sodium channels (action site of pyrethroids) (Hubert et al., 2014; Gonzalez-Cabrera et al., 2013; Wang et al., 2002). The confirmation of new resistance episodes to pyrethroids and the study of resistance mechanisms in varroa populations could help elucidate whether resistance has appeared independently several times or once and spread worldwide (Martin, 2004).

In Uruguay, the acaricides approved according to official regulations are coumaphos, amitraz, fluvalinate and flumethrin. Coumaphos resistant mite populations have already been detected (Maggi et al., 2011). This situation is aggravated because most beekeepers prefer the use of their own homemade preparations. Given the complicated scenario for Uruguay, monitoring acaricide susceptibility in *V. destructor* populations has become mandatory in order to adopt sanitary plans for mite populations in this country. Early detection of *V. destructor* resistance is crucial to reduce both colony losses and spread of a resistant mite strain.

Resistance to flumethrin in *V. destructor* mites was suspected in Colonia (Uruguay), when high infestation levels of mites were continuously detected after treatments with this pyrethroid. For this reason, this study aimed to estimate the possible resistance of a *V. destructor* population from Colonia, to flumethrin. Furthermore, the LC_{50} baseline levels for flumethrin in two susceptible Uruguayan populations were determined. To establish a baseline for *V. destructor* susceptibility to flumethrin is essential in order to compare and detect resistant mite populations.

Materials and methods

Collection of mites

Investigations were conducted between June 2014 and November 2015. *V. destructor* specimens were obtained from different apiaries from Colonia Department, Treinta y Tres Department and San José Department (Figure 1). These mite populations presented the following features: (1) a possible resistant mite population to flumethrin from Colonia Department, where failure of treatments were detected in the field; (2) a susceptible mite population from San José Department, where a rotation acaricide scheme was used across time to control infestation; and (3) a susceptible mite population from Treinta y Tres Department, where acaricides have never been used. Treinta y Tres Department possesses an important Africanization level of bees and a good tolerance to *V. destructor* (Invernizzi, Zefferino, Santos, Sánchez, & Mendoza, 2015). A history of the acaricides used in each of the apiaries under study was carried out in order to recognize the sanitary management of the mite populations (possible resistant mites and susceptible mites).

Combs with sealed brood from each apiary were brought to the laboratory, adult *V. destructor* females were taken from capped brood by opening and inspecting individual cells. They were removed with a moistened paint brush, placed in an incubator at 70% RH and 30–32 °C on bee larvae, and kept in a glass Petri dish for 1–3 h until the number of mites collected were enough.

Bioassays

The lethal concentration that kills 50% of the exposed mites (LC_{50}) to flumethrin was determined by using a toxicity method (Maggi, Ruffinengo, Gende, Eguaras, & Sardella, 2008; Maggi, Ruffinengo, Negri, & Eguaras, 2010). Technical grades (micrograms) of flumethrin (Sigma Aldrich) were diluted in 1 ml of hexane (Ciccarelli Laboratory, Argentina, Pro-analysis) and directly applied to the inner bottom of the glass Petri dish (90 × 20 mm) (1 ml of concentration per Petri dish). For the Treinta y Tres and San José populations, concentrations of 0, 0.5, 1 and 2 μg/ml were applied to the bottom of the glass Petri dish (1 ml of concentration per Petri dish). The same was done for Colonia population, using concentrations of 0, 2.5, 5, 10 and 20 μg/ml. Petri dishes were then kept open for one hour at room temperature to allow hexane residues to evaporate. Once the solvent had evaporated, five female mites were placed in each Petri dish. After one hour three bees were added as a food source to each dish. Candy (made up of powdered sugar and water, 3:1) was provided to feed the bees. Five replicates were done for each concentration and for the control treatment (which consisted of 1 ml of hexane). Throughout the experiments, glass Petri dishes were kept in an incubator at 29 °C and 61.5% relative humidity. Mite mortality counts were taken 24 h later. Specimens were considered dead if they did not move or respond to tactile stimulus.

Figure 1. Geographical locations from Uruguay where *V. destructor* populations were studied.
Notes: Black circles represent the Departments from Uruguay where *A. mellifera* colonies with susceptible mite populations were sampled. The black triangle represents the Department of Colonia, where *A. mellifera* apiaries with possible resistant mite populations to flumethrin were sampled (34° 25′ 31.37′′ S and 57° 08′ 58.7′′ W; 33° 15′ 22.54′′ S and 54° 25′ 35.63′′ W; 34° 20′ 23.72′′ S and 57° 41′ 39.48′′ W).

Statistical analysis

Calculations of LC_{50} values and 95% fiducial limits, as established by USEPA (1986), were conducted using EPA software (version 1.5) as recommended by Lindberg, Melathopoulos, and Winston (2000). Briefly, the data observed in each treatment (registered as percentage of mite mortality) are used by the software to estimate the LC50 by means of algorithms. Mortality values were adjusted in accordance with Abbott (1925) as a function of natural mortality. LC_{50} values and resistance indexes carried out among data were compared between the susceptible population used in this study and the possible resistant mite population. Resistance index was calculated as LC_{50} "resistant" mites/LC_{50} susceptible mites and statistically analyzed with Chi-squared test.

Results

The acaricides used for *V. destructor* control in the apiaries studied are detailed in Table 1. Mites from Treinta y Tres population were never exposed to treatment with synthetic acaricides. The percentages of mite mortality for each flumethrin concentration and controls for susceptible and resistant populations of *V. destructor* are listed in Table 2.

LC_{50} values obtained for the studied populations are shown in Table 3. The LC_{50} for Treinta y Tres mite population was 0.11 μg/Petri dish (0.001729 $\mu g/cm^2$) while the LC_{50} for mite population from San José was 0.36 μg/Petri dish (0.005658 $\mu g/cm^2$). For mites from Colonia the flumethrin LC_{50} was 3.8 μg/Petri dish (0.059729 $\mu g/cm^2$), which means an increased at 34.5 fold when compared to the LC_{50} from Treinta y Tres population and 10.6 fold from LC_{50} San José population, indeed suggesting the development of flumethrin resistance in Colonia population.

Discussion

The current study documents a resistance to flumethrin in a population of *V. destructor* mites of Colonia and extends the knowledge of *V. destructor* resistance to acaricides in Uruguay. In 2011 Maggi and co-workers carried out the first bioassays in order to establish the LC_{50} baseline for flumethrin from Uruguay's populations which had never been exposed to synthetic acaricides. The LC_{50} of flumethrin was below 0.3 μg/Petri dish (0.004715 $\mu g/cm^2$) and could not be calculated given the high mite mortality registered in the minor concentrations assayed (Maggi et al., 2011). The LC_{50} baseline for flumethrin is essential in order to compare with other

Table 1. Acaricide history of apiaries used as source of mites for the bioassays. All treatments reported in each year were done in autumn. Mites from Treinta y Tres population were never exposed to acaricides (synthetic or organic).

	2008	2009	2010	2011	2012	2013	2014	2015
Apiary from Colonia Department	Flumethrin **FD**	Coumaphos	Amitraz	Flumethrin	Amitraz	Flumethrin **FD**	Amitraz	–
Apiary from San José Department	Coumaphos	Coumaphos	Coumaphos	Amitraz	Flumethrin	Amitraz	Flumethrin Oxalic acid. (spring)	Amitraz
Apiary from Treinta y Tres Department	–	–	–	–	–	–	–	–

Note: **FD**: Failures detected in the *Varroa* control with flumethrin.

Table 2. Concentration (micrograms per capsule) and mite mortality rate after 24 h (%) for flumethrin applied to susceptible and resistant mite populations from the Departments of Treinta y Tres, San José and Colonia, respectively.

	0.25	0.5	1	2	2.5	5	10	20	Control
Apiary from Treinta y Tres Department	40	84	84	100	–	–	–	–	24
Apiary from San José Department	80	88	92	100	–	–	-	–	20
Apiary from Colonia Department	–	–	–	–	37	62	98	100	10

Table 3. LC_{50} and 95% confidence intervals estimates for flumethrin, of susceptible and resistant mite populations.

Mites origin	Degree of susceptibility to flumethrin	LC_{50}	95% confidence interval	Resistance index
Apiary from Treinta y Tres Department (a)	Susceptible- population never exposed to synthetic acaricides	0.11 μg/Petri dish (0.001729 μg/cm^2)[a]	0.0001–0.25	–
Apiary from San José Department (b)	Susceptible population	0.36 μg/Petri dish (0.005658 μg/cm^2)[a]	0.21–0.49	–
Apiary from Colonia Department (c)	Potential resistant population- Failures detected in treatment with flumethrin	3.8 μg/Petri dish (0.059729 μg/cm^2)[b]	3.1–4.5	34.5 (c/a) 10.5 (c/b)

Notes: Different letters indicate significant differences among LC_{50} (X^2 = 3.84 with *df* = 1 and α = 0.005).

values of Uruguayan populations were resistance episodes are suspected, moreover considering that different mite populations may yield different susceptibility levels to pesticides depending on geographic location (Watkins, 1997). The LC_{50} baseline value estimated for Treinta y Tres varroa population was 0.11 μg/Petri dish (0.001729 μg/cm^2) while for the San José varroa population, was 0.36 μg/Petri dish (0.005658 μg/cm^2). The LC_{50} of flumethrin obtained for the Colonia population was 3.8 μg/Petri dish (0.059729 μg/cm^2) which corresponds to an increase at 34.5 fold when compared to the LC_{50} from Treinta y Tres population and 10.6 fold from LC_{50} San José population. This results plus the treatment failure detected on field reported by beekeepers, confirm the resistance phenomena in this *V. destructor* population.

There are very few previous records in which flumethrin resistant populations were detected. The majority of the studies were conducted to identify fluvalinate resistant populations. Table 4 shows the present records for flumethrin resistant mite populations. If the resistance indexes reported in Table 4 are analyzed, we might conclude that resistance indexes from Uruguay (present study) are more similar to Europe resistance indexes.

Mites from México were those which showed the highest resistance index. The origin of the varroa resistance to flumethrin in Colonia is unclear. Is this focus of resistance newly originated in Colonia, or is it due to the importation of infested bees from another neighbor country harboring flumethrin resistant mites? According to Martin (2004), the appearance of resistant mite populations in a territory is generally correlated with the movement of bee populations from one place to another. Future studies should consider the origin of mite populations in the area, to see if they were already resistant, considering that Uruguayan honey bees (and their parasites) have been introduced from Europe.

In addition, mites from México were sourced in Africanized bees, which could explain in part, the differences found between the resistance indexes reported in Table 4. Future researches involving molecular studies would be very useful in order to monitor the source of resistance episodes and how the spread of this phenomenon to other regions can be stopped.

Table 4. LC_{50} and 95% confidence interval of susceptible and resistant mite populations worldwide estimates for flumethrin.

	LC_{50} (95% confidence interval)	Methodology used	Resistance index (b/a)	Reference
Susceptible population (México)	0.00087508 µg/ml **(a)**[1](95% CI: 0.000201–0.006554)	Burgerjon tower (Burgerjon, 1956)		Perez et al. (2000)
Resistant population (México)	0.286 µg/ml **(b)**[1] (95% CI: 0.255–0.321)	Burgerjon tower (Burgerjon, 1956)	**327**	Rodríguez-Dehaibes et al. (2005)
Susceptible population (York, Inglaterra)	0.47 mg/kg **(a)** (95% CI: 0.25–0.73)	Paraffin wax (Milani, 1995)		Thompson et al. (2002)
Resistant population(Devon, Inglaterra)	6.3 mg/kg **(b)** (95% CI: 3.9–10)	Paraffin wax (Milani, 1995)	**13.4**	Thompson et al. (2002)
Susceptible population (Udine, Italia)	0.36 mg/kg **(a)** (95% CI: 0.26–0.46)	Paraffin wax (Milani, 1995)		Milani (1995)
Susceptible population (Lunz- am-See, Italia)	0.28 mg/kg **(a)** (95% CI: 0.18–0.39)	Paraffin wax (Milani, 1995)	**Between 30 and 70**	Milani (1995)
Resistant population (Chiavenna, Italia)	20.4 mg/kg **(b)** (95% CI: 11.4–33.1)	Paraffin wax (Milani, 1995)		Milani (1995)
Resistant population (Varallo Pombia, Italia)	11.4 mg/kg **(b)** (95% CI: 5.9–20.1)	Paraffin wax (Milani, 1995)		Milani (1995)
Resistant population (New Zeland)	12 mg/kg **(b)** (95% CI: 7.6–17)	Paraffin wax (Milani, 1995)	**Between 30 and 40**[2]	Goodwin et al. (2005)
Present results, Department of Treinta y Tres(Uruguay)	0.11 µg/ml **(a)** (95% CI: 0.0001–0.25)	Contact method (Maggi et al., 2008)		
Present results, Department of San José (Uruguay)	0.36 µg/ml **(a)** (95% CI: 0.21–0.49)	Contact method (Maggi et al., 2008)	**Between 10.5 and 34.5**	This study
Present results, Department of Colony (Uruguay)	3.8 µg/ml **(b)** (95% CI: 3.1–4.5)	Contact method (Maggi et al., 2008)		

[1]Correspond to values of LD50 by the methodology used.
[2]The comparison was made with susceptible populations from Italy (Milani, 1995).

The discovery of flumethrin resistance in an apiary in which this acaricide was combined with other acaricides, demonstrates the potential of *V. destructor* to continuously adapt to the selection pressures imposed by beekeepers. The same situation was also reported for a resistant mite population to coumaphos in Uruguay (Maggi et al., 2011). Moreover, it should be noted that Uruguayan beekeeping practices are characterized by applying the same acaricide treatment when *V. destructor* control is performed in the country (which is coordinated by the official regulator DILAVE), instead of the subsequent acaricide rotation in order to avoid resistance phenomena. Nevertheless, in the region from which the apiary studied belongs to, all treatments were done in autumn. However, it must be considered that in apiaries in which mites had been found after spring sampling, treatments with the same acaricide used before were applied in the spring.

Also, the results provided here trigger the alert of the future of bee colonies in places where acaricide resistance is detected and subsequently, only few acaricide options will be available. Maggi et al. (2011) reported coumaphos resistance in Uruguay in 2011 and, as a consequence, the use of this acaricide was regulated by the government. During subsequent years, only few acaricide formulations options would be available for beekeepers. To curb this issue, the introduction of integrated programs for resistance management is required, including monitoring of mite population, selection of mite-tolerant bees, non-chemical control methods, and acaricide rotation, either natural or synthesized. The inclusion of organic acids in rotation schemes of acaricides should be a point to consider. Some advantages of using them are that residues in honey are slight and toxicologically insignificant, mite resistance is unlikely to happen and they are generally cheap (Eguaras & Ruffinengo, 2006).

Disclosure statement

No potential conflict of interest was reported by the authors.

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