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ORIGINAL RESEARCH ARTICLE

Varroa destructor: when reversion to coumaphos resistance does not happen

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The ectoparasitic mite *Varroa destructor* is potentially the greatest threat to honey bee colonies of *Apis mellifera* worldwide. Acaricide rotation is widely accepted to mitigate development of resistance to synthetic acaricides. When a population has developed resistance to a compound, cessation of its use can result in some degree of susceptibility. This study aimed to follow changes in the susceptibility of mites to coumaphos in a population not treated with the acaricide over many generations. The LC_{50} increase over the years from 2 to 8-fold. Considering that coumaphos had not been used for nine years in the studied apiary, the results provided here lead to reflections about the problem of the resistance phenomenon and its populations dynamics.

***Varroa destructor*: cuando la reversión de la Resistencia a cumafós no ocurre**

El ácaro ectoparásito *Varroa destructor* constituye una de las mayores amenazas en el mundo para las colmenas de *Apis mellifera*. La rotación de acaricidas es una medida ampliamente aceptada para mitigar el desarrollo de resistencia de acaricidas sintéticos. Se ha observado que, cuando se deja de aplicar un acaricida a una población resistente, luego de un periodo de tiempo la población se puede volver susceptible al mismo. El objetivo del presente trabajo fue estudiar si la susceptibilidad al cumafós de una población de *Varroa destructor* no expuesta a dicho acaricida durante varios años, decrece a lo largo del tiempo. Los valores de CL_{50} se fueron incrementando a lo largo de los años, este incremento fue desde 2 a 8 veces. Considerando que el apiario estudiado no ha sido expuesto al cumafós durante nueve años, estos resultados llevan a la reflexión sobre el problema de la resistencia a acaricidas de síntesis y su dinámica en las poblaciones.

Keywords: *Varroa destructor*; *Apis mellifera*; resistance; coumaphos; reversion

Introduction

Managed honey bee colonies are decreasing worldwide over the last decades because of many biotic and abiotic factors such as pests and diseases, pesticides, loss of forage, and beekeeping practices (Maggi et al., 2016; Neumann & Carreck, 2010). The ectoparasitic mite *Varroa destructor* is potentially the greatest threat for honey bee colonies of *Apis mellifera* worldwide (Nazzi & Le Conte, 2016). The acaricides currently used for *Varroa* control are fluvalinate, flumethrin, coumaphos, and amitraz. However, mite populations have become resistant to these molecules in many countries, such as Italy, Israel, the United Kingdom, Argentina, and the United States (Elzen et al., 1998; Maggi, Ruffinengo, Damiani, Sardella, & Eguaras, 2009; Maggi et al., 2011; Milani, 1995; Mozes-Koch et al., 2000; Sammataro, Untalan, Guerro, & Finley, 2005; Spreacifico, Eördegh, Bernardinelli, & Colombo, 2001; Thompson, Brown, Ball, & Bew, 2002). In Argentina, the resistance phenomena to synthetic acaricides have been reported for coumaphos and amitraz (Maggi et al., 2009; Maggi, Ruffinengo, Negri, & Eguaras, 2010).

There are several factors associated with resistance development: (1) reinfestation due to the presence of resistant populations in nearby apiaries (Greatti, Milani, & Nazzi, 1992; Sammataro et al., 2005); (2) abuse or misuse of the acaricide that can lead to selection of resistant individuals and the subsequent spread of resistant population (Maggi et al., 2011); (3) recent studies have revealed that mite resistance can be attributed to the intense and continuous contact with sublethal doses of acaricides present in wax of brood cells (Medici et al., 2016; Onstad, 2008). If we consider that resistance happens due to genetic mutations (Onstad, 2008; Van Leeuwen, Vontas, Tsagkarakou, Dermauw, & Tirry, 2010; Wang et al., 2002), the last

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two factors together would explain the mention pattern as an artificial selective pressure, in favor of resistant individuals over the susceptible ones within a population (Maggi et al., 2011).

As seen in many other agricultural pests that have developed resistance, avoiding the use of certain compounds could lead to a reversion of resistance, this should cause a decline in the frequency of resistant genotypes necessarily follow when use of a compound is stopped (Georghiou & Taylor, 1986). The reversion of *Varroa* resistance to fluvalinate has been observed in populations not exposed to pyrethroids for four years in several localities from Italy. The variation in susceptibility over three years was highly significant in each locality (Milani & Della Vedova, 2002). The results reported by Milani and Della Vedova (2002) were also consistent with results published by Elzen and Westervelt (2004); where these authors reported significant changes in *Varroa* susceptibility to fluvalinate after nine months avoiding specific exposure.

Currently in Argentina, *Varroa* resistance to coumaphos is widely disseminated and as a consequence, beekeepers stop using this organophosphate (Maggi, 2010). Moreover, Medici et al. (2016) reported that coumaphos acaricide was the main beekeeping plaguicide contaminating commercial waxes in the country. Despite all that was mentioned above, in Argentina resistance to coumaphos was early detected (Maggi et al., 2009). As a consequence, its use by beekeepers had fallen into disuse in recent years. In this work, we monitored changes in the susceptibility of mites to coumaphos in a population not treated with coumaphos over many generations to determine changes in LC_{50}.

Materials and methods

Collection of mites

Experiments were conducted between April 2014 and April 2017. The sampling points for the trials were: April 2014, April 2016, December 2016, and April 2017. In all cases, the tests made in April were carried out before the cure of mite populations. *V. destructor* specimens were obtained from Santa Paula experimental apiary located on the route 226, km 10, Mar del Plata, Buenos Aires, Argentina (37° 56′ 0.69″ S; 57° 40′ 40.53″ O). Table 1 shows the last 10 years of acaricide history of the analyzed apiary, where failures were detected during the last coumaphos control, with an efficiency less than 75% (2008) (Maggi, 2010).

Brood combs, at least two per colony (at minimum from three different colonies) (Milani & Della Vedova, 2002), were taken and brought to the laboratory. For each trial, a pool of mites from the different colonies of the apiary were used. Adult *V. destructor* females were taken from capped brood by opening and inspecting individual cells. They were removed with a paint brush, placed in an incubation stove at 70% RH and 30–32 °C, and kept in a Petri dish glass for 1–3 h on bee larvae, until all the mites needed for the assay were collected, approximately, between 130 and 180 for each one.

Bioassays

Bioassays were carried out using a toxicity method (Maggi, Ruffinengo, Gende, Eguaras, & Sardella, 2008; Maggi, Ruffinengo, Negri, & Eguaras, 2010). Technical grade (micrograms) of coumaphos (Sigma Aldrich) was diluted in 1 ml of hexane. For the tests conducted in April 2014 and 2016, concentrations of 0, 0.5, 1, and 2 μg/ml were applied on the bottom of the Petri dish (1 ml of concentration per dish). The same procedure was repeated for the trial conducted in April 2016, using concentrations of 0, 5, 10, and 20 μg/ml, and for the tests conducted in April 2017, using concentrations of 0, 1, 5, 10 μg/ml. These changes in coumaphos concentrations used on trials respond to the hypothesis that changes of mite susceptibility can occur across time. The dishes were kept open for one hour at room temperature to allow the hexane residues evaporation. Then, five female mites were placed in each Petri dish. After one hour three bees were added as food source to each dish. Candy (made up of powdered sugar and water, 3:1) was provided to feed the bees.

Five replicates for each concentration and a control were done (which consisted of 1 ml of hexane). Throughout the experiments, Petri dishes were kept in an incubator at 29 °C and 61.5% of relative humidity. Mite mortality was measure 24 h later. Specimens were considered dead if they did not move or respond to tactile stimulus.

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. Mortality values were adjusted in accordance with Abbott (1925) as a function of natural mortality. LC_{50} values and resistance indexes obtained in the present study were compared with LC_{50} baseline values obtained by Maggi et al. (2008). Resistance index was calculated as LC_{50} "resistant" mites/LC_{50} susceptible mites and LC_{50} values were statistically analyzed with Kendall's tau-b in order to establish a correlation coefficient, which is a measure of the strength and direction of association that

Table 1. Acaricide history of Santa Paula apiary.

2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Coumphos **FD** Amitraz	Flumethrin	Amitraz	Flumethrin	Amitraz	Flumethrin	Amitraz	Flumethrin	Ac oxalic	Ac oxalic

Notes: All treatments reported in each year were done in autumn (March). **FD**: Failures detected in the last control.

Table 2. Coumaphos concentration (μg/Petri dish) and mite mortality rate (%) after 24 h of acaricide exposition for *V. destructor* mites in each test performed in different years.

	0.25 μg/ Petri dish	0.5 μg/ Petri dish	1 μg/ Petri dish	2 μg/ Petri dish	5 μg/ Petri dish	10 μg/ Petri dish	20 μg/ Petri dish	Control
April 2014	48	56	44	48	–	–	–	0
April 2016	21	44	60	71	–	–	–	20
December 2016	16	46	32	64	64	79	–	18
April 2017	–	–	28	–	58	69	88	12

Notes: In all the cases where "–" symbol appears, it means that this concentration was not tested in the corresponding year. Coumaphos concentrations used on trials from different years were modified hypothesizing that changes of mite susceptibility can occur across time. No significant differences were observed between years (chi-square, $p > 0.05$).

Table 3. LC_{50} of coumaphos for *V. destructor* and 95% confidence intervals estimated using bioassays at different years.

Date of experiment	LC_{50} (μg/Petri dish)	95% Confidence interval	Resistance index
Base line – Maggi et al. (2008) (a)	0.57	0.25–0.79	–
April 2014	No estimated	–	–
April 2016 (b)	1.14	0.69–2.53	2 (b/a)
December 2016 (c)	2.81	1.59–5.52	4.9 (c/a)
April 2017 (d)	4.58	2.18–7.41	8 (d/a) 4 (d/b)

Notes: Resistance index was calculated as LC50 "resistant" mites/LC50 susceptible mites. In each case, the letters "a", "b", "c" and "d" indicate the reference population for the calculation.

exists between two variables. Finally, mite mortality for each concentration tested across time was compared by using a chi-square test (Di Rienzo ei al., 2017).

Results

Table 2 shows the percentages of mite mortality from different tests performed during the study. It can be observed that in the year 2014 the percentage of mite mortality is low even at the highest coumaphos concentration used in the bioassay. Mite mortality levels were statistically equivalent across years ($p > 0.05$). Table 3 shows LC_{50} values and confidence intervals from tests performed in different years. LC_{50} was 1,14 μg/Petri dish and 4,58 μg/Petri dish for 2016 and 2017, respectively, indicating an increase of 4 fold with respect to LC_{50} values for 2014. Comparison of LC_{50} values obtained in the present study with LC_{50} baseline value (Maggi et al., 2008) revealed a considerable increase from 2 to 8-fold among the different years.

A positive correlation was noticed between LC_{50} values and the different years in which tests were done (correlation= 0.83; Kendall's Tau-b p value: 0.04154), demonstrating that LC_{50} of coumpahos was increasing over time in the studied *Varroa* population.

Discussion

This study constitutes the first report of resistance/susceptibility dynamics to coumaphos in different mite populations throughout years. The results obtain to demonstrate that mite population shows a decrease in susceptibility to coumaphos in the Santa Paula experimental apiary. Despite what was mentioned above, it is important to remark that the studied apiary has not been in direct exposure to coumaphos since 2009, however an increase in LC_{50} values was detected throughout time, so that, it is clear that somewhat another process is affecting mite susceptibility to coumaphos. In addition, a similar pattern was publish by Maggi et al. (2011), where coumaphos resistance was detected in apiaries from Uruguay even though suitable mite population management strategies had been adopted for *Varroa* control.

Medici et al. (2016) reported that 87% of the commercial wax used by beekeepers in Argentina for comb foundation and 80% of the recycled wax bears high concentrations of coumaphos residues. These authors show a great amount of beeswax contamination in Argentine commercial wax. Hence, the *Varroa* mite population studied here could have been continuously exposed to sublethal doses of coumaphos residues present in wax, with the consequent change in their drug susceptibility. In fact, Medici et al. (2016) demonstrated a positive relationship between coumaphos residues and *Varroa* resistance. Thus, the results reported in our study could be understood under the light of this scenario.

Previous data suggest a decrease in resistance to pyrethroids over time in *Varroa* populations from Italy, where levels of pyrethroid resistance diminished over a three years period (Milani & Della Vedova, 2002). Similar results were obtained in a *Varroa* population from Florida, USA, where a significant increase in susceptibility over time was observed in a short period of time (Elzen & Westervelt, 2004). Milani and Della Vedova (2002) hypothesized that resistance reversion could happen due to reproductive fitness cost associated with pyrethroid resistance. However, Martin, Elzen, and Rubink (2002) demonstrated that there is little, or no reproductive fitness cost associated with pyrethroid resistance in *V. destructor* in Texas, and they proposed that the reversion previously observed in Florida, USA, and possibly Italy, were caused by the influx of susceptible mites into the resistant population. In our study,

this migration is unlikely, as the apiary studied was located too far from another apiary (at least 5 kilometers).

Stability of acaricide resistance has been studied for several compounds in others species of mites, it has been observed a wide diversity of response, cases in which reversion occur and situations in which resistance remains stable overtime (Inoue, 1980; Omoto, Dennehy, McCoy, Crane, & Long, 1995; Overmeer, Van Zon, & Helle, 1975; Sato et al., 2004, 2005). These differences can be explained by the type of mechanism associated with its resistance. It is possible that different populations of mites have developed different mechanisms of resistance which confer different levels of fitness costs. So, it is important to study both, the mechanism of mite resistance and its biological cost as proxies to understand dynamics in pyrethroids susceptibilities (Martin et al., 2002).

The results presented in this study, together with previous studies reported by our research team (Medici et al., 2016), demonstrate that reversion of resistance to coumaphos in *V. destructor* is a phenomenon that should be deeper explored. Monitoring acaricide susceptibility by using bioassays and residue detection studies in apiaries where *Varroa* resistance is detected become of paramount importance in order to establish appropriate epidemiological interpretations. Therefore, this contribution points out the *Varroa* resistance phenomenon as a matter of serious concern to all beekeepers and governmental authorities, taking into account the continuous evidence in Argentina and Uruguay presented across time (Maggi et al., 2009, 2010, 2011; Medici et al., 2016; Mitton et al., 2016). Further research should be focus on the process and management behind pyrethroid resistance in mites.

Disclosure statement

No potential conflict of interest was reported by the authors.

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