Bulletin of Insectology ***70*** *(1): 39-44, 2017*
ISSN 1721-8861

The susceptibility of *Varroa destructor* against oxalic acid: a study case

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Abstract

Varroa destructor Anderson et Trueman is an ectoparasitic mite of the honey bee *Apis mellifera* L. and it must be controlled in managed bee colonies to maintain colony health. Taking into account that these mites are now resistant to synthetic acaricides worldwide, oxalic acid was suggested as an alternative for *Varroa* control. Oxalic acid is one of the most common natural miticides used against varroosis by spraying and sublimation administration techniques. It is a natural constituent of honey, very active against the *Varroa* mite, safe to use for beekeepers, and has no residue problems. Nevertheless, some authors have predicted that the risk of developing resistance to oxalic acid in mites is high. The objective of this research was to assess the susceptibility to oxalic acid of a *V. destructor* population belonging to a commercial apiary where 64 consecutive control treatments with this acid were performed. Bioassays to assess the oxalic acid susceptibility were performed on two mite populations: (1) a 'focal' population consisting of mites previously exposed to oxalic acid treatments, and (2) a 'naïve' population that was never exposed to this acid, which allows setting a reference in the absence of historical data on our 'focal' mites. The results reported here suggest that the *Varroa* population exposed during 8 successive years to oxalic acid treatments remains susceptible to this acid.

Key words: *Varroa destructor*, susceptibility, oxalic acid, honey bee.

Introduction

Varroa destructor Anderson et Trueman is an obligate, ectoparasitic mite of the European honey bee *Apis mellifera* L. (Anderson and Trueman, 2000). *Varroa* damage is the main factor that increases the wintering losses of bee colonies (Akyol *et al.*, 2006). High infestation levels may reduce the bee weight and lifespan; cause malformations; suppress the immune response; and decline breeding production, adult bee population and honey storage in the colony (De Jong *et al.*, 1982; Murilhas, 2002; Gregory *et al.*, 2005). Because of that, mite population levels in beehives must be maintained below the economic injury (Delaplane *et al.*, 2005) by means of acaricide treatments (Rosenkranz *et al.*, 2010). Historically, synthetic acaricides such as pyrethroids and organophosphates have been preferably chosen for *Varroa* control in the apiaries (Maggi *et al.*, 2008). From the late 1980s to the early 1990s, the efficacy of fluvalinate (pyrethroid) application was close to 100% (Herbert *et al.*, 1988). Its widespread use and, often its misuse throughout those years have exerted a strong selective pressure on mite populations. As a result, resistant populations emerged in several countries worldwide (Milani, 1995; Elzen *et al.*, 1998; Macedo *et al.*, 2002) and the treatments are no longer effective. Cross-resistance between pyrethroids was reported in *V. destructor* populations (Floris *et al.*, 2001; Thompson *et al.*, 2002). Beekeepers began applying coumaphos (organophosphate) and amitraz (formamidine) in strip formulations as an alternative treatment in areas in which the fluvalinate resistance prevailed (Elzen *et al.*, 2000; Elzen and Westervelt, 2002). However, evidence of resistance episodes to these pesticides has been also reported in Europe and in the Americas (Rodriguez-Dehaibes *et al.*, 2005; Maggi *et al.*, 2009; 2011).

Natural pesticides based on plant extracts have been suggested as an alternative for the control of this parasitosis at the emergence of resistance (Milani, 1999; Ruffinengo *et al.*, 2014). Imdorf *et al.* (1999) has proposed that, the selection pressure for resistance against these "natural miticides", should be low due to its rapid degradation inside the colony and its little use by the beekeepers which reduce the contact time with mites infesting bees. This is completely opposite to what has happened with synthetic acaricides. Therefore, the residual effect of a pesticide and their frequency of use in the hive are important factors that modulate the emergence of resistant mite populations (Medici *et al.*, 2015). In addition, most vegetable extracts or even propolis are mixtures of more than 150 components (Marcucci, 1995; Imdorf *et al.*, 1999). In this way, it is unlikely that mites generate resistance against all these components at the same time. Despite their high bioactivity on the mites in laboratory conditions, very few of them have proven effective when were applied in hives during field trials. Considerable variation in local environmental and colony conditions can affect the efficacy and make it difficult to predict the outcome of many treatments. These reasons have discouraged beekeepers to use this kind of pesticides as over synthetic ones (Imdorf *et al.*, 1999). The solution, often, has been to use an effective single component isolated from a natural extract as has occurred with thymol, formic acid, oxalic acid (OA), among others (Calderone, 1999; Floris *et al*., 2004; Maggi *et al.*, 2016).

Oxalic acid is an organic acid, natural constituent of the honey and very effective against the *Varroa* mite (Rademacher and Harz, 2006). It has been used by the beekeepers in the USA and Europe and the EU regulations have permitted its utilization in biological beekeeping practices (EU Council Regulation, No. 1804/1999). The OA is simple to use, cheap, safe for beekeepers, has no residue problems, is well tolerated by bees and it is especially applied in broodless colonies (Mutinelli *et al.*, 1997). These features make that this acid is widely and frequently being used in some regions. In this way, Milani (2001) has postulated that the risk of emergence of resistance for OA is high if its use is prolonged in time. In some European countries, it has already been used intensively and with scarce rotation with other miticides (Charriére and Imdorf, 2002; Nanetti *et al.*, 2003).

Although the mode of action against *Varroa* mites is not yet clearly understood, it seems that a direct contact with the low pH of OA solutions has a deleterious effect on the mites and it would be a key factor (Nanetti and Stradi, 1997; Nanetti, 1999; Nanetti *et al.*, 2003). The OA has been conventionally applied in hives against the varroosis by spraying and sublimation administration techniques. It is known that by mixing OA in sugar water, the solution becomes attractive and is better distributed by bees. Thus, it comes into contact easily with bees and mites throughout the colony. In addition, Aliano and Ellis (2008) performed a clever experiment: By splitting single colonies with various dividers and applying OA only on one side, they found that it is apparently transferred through the colony by contact among bees, rather than by trophallaxis, and it is well distributed by this way (80% of killed mites on treated side, and 65% on untreated). Recently, in Argentina it has been approved a new commercial formulation based on OA which is applied in cellulose strips that remain 42 days within the colonies. Thus, by this application mode and action mechanism, it would be identical to the formulations based on synthetic acaricides that many foci of resistant mites have generated (Maggi *et al.*, 2016). High efficacy, ease of use, extremely low cost, lack of temperature-dependence, and the shortage of alternatives currently make that OA treatments become popular (Nanetti *et al.*, 2003; Maggi *et al.*, 2016).

To date, there are no published data supporting resistance to any organic acid in *V. destructor*. There is also little information on the susceptibility of mite populations that have ever been in contact with OA. This kind of data would be useful for the design of studies estimating possible changes of susceptibility to this acid in *Varroa* populations as it has occurred with the synthetic miticides. For example, the available data on the toxicity to coumaphos and amitraz in mites from never treated populations (Maggi *et al.*, 2008; 2011) has made possible to detect resistance to these acaricides in exposed mite populations (Maggi *et al.*, 2009; 2010; 2011) before bee losses are reported.

Theoretical and empirical works have been carried out to develop tactics to delay the emergence of resistance in insects or mites (Denholm and Rowland, 1992). In the case of *V. destructor*, tactics meant to preserve susceptible mites (moderation tactics) avoiding an excessive killing of mites, could be more suitable for slowing the selection of resistant mites. The success of these strategies depends on the balance between the selection pressure resulting from the application of acaricides and the disadvantage associated with resistance (Milani, 1999), although this last is very variable and often small (Roush and Daly, 1990). Both factors would cause that the frequency of resistance genes is reduced in the interval between treatments. However, for *Varroa* populations, where several generations of mites take place during a brief period of time, even a fitness decrease in the order of a few percent per generation would produce an appreciable disadvantage (Milani, 1999). On the other hand, the selection pressure increases dramatically when the efficacy of treatments approaches 100% and the same acaricides are used repeatedly, or for prolonged periods, reducing correspondingly the effect of any disadvantage of the resistant strain. An example was the high number of resistance phenomena to coumaphos reported in Argentina and Uruguay, due the abusive use of this organophosphate (Maggi *et al.*, 2009; 2011; Medici *et al.*, 2015).

For this study, we have selected a commercial apiary where a high frequency of treatments with oxalic acid was exerted during 8 consecutive years. For that, our main objective was determinate the susceptibility to oxalic acid in this ‘focal’ mite population comparing it with a *Varroa* population never exposed to this compound. We hypothesize that the ‘focal’ population suffered a high selection pressure for OA during the 8 years.

Materials and methods

A commercial apiary composed by 54 colonies of a regional ecotype of *A. mellifera* located at Federal (30°57'4.42"S 58°47'55.78"W: Entre Rios province, Argentina) was selected as biological model for the assays. In this apiary, the OA was topically applied as the sole option for *Varroa* control during eight consecutive years (2000-2008). Thus, this *V. destructor* population was considered as the ‘focal’ population in our study. An average of eight treatment applications (± one) per year was made. The treatment solution was composed of 4% (w/v) oxalic acid (64 g oxalic acid dehydrate, 500 g sugar, and distilled water to 1000 mL). The average dosage was 50 mL per hive (5 mL per comb covered by bees) applied on the top of each frame. According to the beekeeper’s personal observation, the colonies managed by applying only OA treatments must be maintained with a mite infestation level below than 2% to avoid colony weakness and honey production reduction. Thus, the monitoring of *Varroa* infestation levels was monthly performed following the protocol of Marcangeli (2000). The method involves collecting among 200 to 300 bees taken from both sides of three brood frames of each hive, in a container with water and detergent. After the sample shaking, mites are separated from bees. The percentage of infestation is obtained by dividing the number of mites by the total number of sampled bees per one hundred.

A *V. destructor* population from Mar del Plata (38°10'06"S 57°38'10"W: Buenos Aires province, Argentina) which had never been treated with OA neither other organic acid was used as control group. Thus, these mites constituted a 'naïve' population. *Varroa* control in this apiary was always performed using coumaphos, flumethrin, or amitraz in a rotation scheme. The apiaries were at 900 km distance between them but nevertheless both had similar geographic and climatic conditions. The queens, which were provided by a regional beekeeping house, do not stop egg laying during winter in these regions. Both apiaries had an inter-localities distance higher than 5 km with regard to other apiaries, minimizing the bee drifting and the possibility of re-infestations.

To perform the bioassays, five colonies from each apiary were selected during spring 2008. Capped brood combs were removed from these colonies and transported to the laboratory. Mites were collected by opening and inspecting individual brood cells. Only mature female mites were sampled and transferred with a slender moistened paint brush to a plastic Petri dish with bee larvae as food for mites. Thus, they were kept at 28 ± 1 °C and 70% RH for 1-3 h until used. A pool of mites from each population was exposed to increasing concentrations of OA (Cicarelli Laboratory, Argentina, Pro-analysis) solutions (0.05; 0.1; 0.2; and 0.4% w/v) prepared in 55% (w/v) ethanol solution. These treatment concentrations were selected according to Toomemaa *et al.* (2010). For treatment, 200 µl of each specified concentration of OA was applied over six mites that remain on a piece of filter paper (3 × 3 cm). After 45 s, the contact with the solution was stopped by removing the mites from the paper to a new clean Petri dish (Damiani *et al.*, 2010). Three adult bees were added to each of these new dishes as food for mites. Controls were made by treating mites with a 55% (w/v) ethanol solution during the same contact time. Five replicates for each experimental unit were run. All treatments were carried out at room temperature (22-24 °C) and the treated mites were incubated at 28 ± 1 °C and 60% RH. After 24 h, mortality was evaluated by gently prodding each mite with a narrow paintbrush under a dissecting microscope; lack of response to consecutive stimulus over 1 min was considered an indication of death. Bioassays for the 'naïve' mite population (Mar del Plata) and the 'focal' mite population (Federal) were conducted simultaneously. A χ^2 test was carried out to determine statistical differences on mite mortality between both populations. This method has already been successfully applied to evaluate the toxicity of propolis and ethanol botanical extracts against populations of *V. destructor* (Damiani *et al.*, 2010; 2014).

Results

Oxalic acid control on field

The infestation level of *V. destructor* on adult bees before and after applications of OA in the commercial apiary from Federal city during the year preceding the bioassay are reported in table 1.

Bioassays

The percentage of dead mites recorded 24 h after oxalic acid treatments for both mite populations is reported in figure 1. For all the tested acaricide concentrations, the mortality of mites from the 'focal' population (Federal city) was higher than from the 'naïve' mite population (Mar del Plata city) ($p < 0.01$, figure 1). These mortality percentages were between 2.8-fold and 7.2-fold higher for the 'focal' mite population than for the control mite population. Mite mortality in controls was always below than 10%.

Table 1. Infestation levels of *V. destructor* on adult bees before and after treatment applications of oxalic acid in the 'focal' apiary studied.

Treatment application event	*V. destructor* infestation on adult bees (%) Before treatment [a]	After treatment [b]	Date of treatment application
1	2 (0.98)	1.7 (1.1)	08/23/2006
2	1.7 (0.65)	0.9 (0.45)	08/30/2006
3	1.6 (1.1)	1.1 (0.58)	01/24/2007
4	1.1 (0.62)	0.4 (0.3)	01/31/2007
5	8.1 (3.57)	6 (2.6)	04/15/2007
6	6 (3.1)	4.8 (2.35)	04/22/2007
7	4.8 (2.35)	1.2 (0.73)	04/29/2007
8	3.9 (2.5)	0.5 (0.15)	06/15/2007

Varroa infestation rates were estimated in 10 of the 54 colonies each time. The mean values reported here represent the mite infestation in the apiary managed only with OA treatments during 8 successive years. Standard deviations are reported in brackets.

[a] The same day of the treatment application.

[b] One week after treatment.

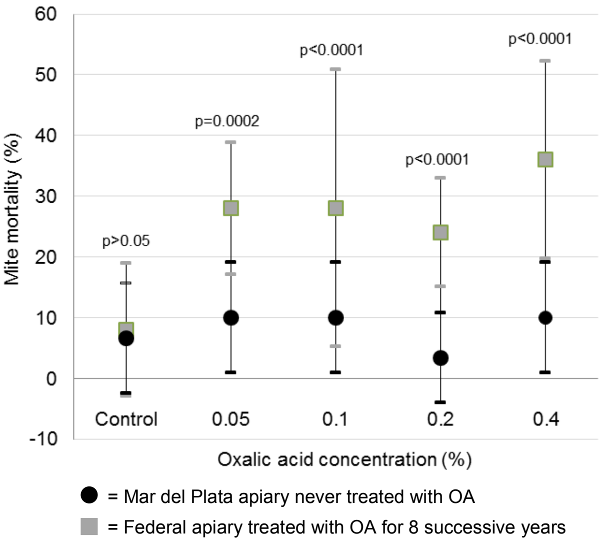

Figure 1. Mortality of *V. destructor* from both mite populations subjected to increased concentrations of oxalic acid. Mite mortality is expressed as percentage of dead individual ± standard deviation after 24 h of oxalic acid treatment. Five replicates for each experimental unit were run (N = 125). *P* value from χ^2 test compare mite mortality in both populations within the same treatment concentration.

Discussion and conclusions

In this study, we found an ideal biological framework to test the hypothesis of Milani (2001). This author postulated that the risk of emergence of resistance for the oxalic acid is high if its use is prolonged in time. Here, we have compared the susceptibility of a *Varroa* population exposed to 64 consecutive treatments with OA ('focal' mite population) versus the susceptibility of a *Varroa* population never exposed to it ('naïve' mite population) in order to evaluate if resistance episodes to the OA happened.

The methodology employed in the present study for conducting the bioassay allowed us to satisfactorily assess the bioactivity of the OA on treated mites. Furthermore, it proved be quick and easy to implement when it was previously used for the evaluation of mortality by drugs against *V. destructor* populations. The mite mortality in control treatments did not exceed 10%, thus complying with the standard norms for trials set forth by OECD (1998a; 1998b) and EPPO (2001).

In our bioassay, the acaricide toxicity of the OA was higher for mites from the 'focal' apiary (Federal city) than for those mites belonging to the 'naïve' population never exposed to the OA (Mar del Plata city). These results indicate that the *Varroa* population from the Federal apiary remained susceptible to the OA despite its prolonged use in time. It was also interesting that this 'focal' population resulted more susceptible to the acid than the 'naïve' population with mites never exposed to it. A possible explanation for these results is that mite populations on which the toxic effects from a chemical are analyzed can show different degrees of natural susceptibility, a feature inherent of any population with respect to a given variable: different mite populations may yield different susceptibility levels to pesticides depending on geographic location (Watkins, 1997). For example, although the LC_{50} values for amitraz on the mites from the study of Elzen *et al*. (2000) was 164 times higher than for mites from the Maggi *et al.* (2008) study, both *Varroa* populations were considered susceptible to the acaricide. Nevertheless, it is important to mention that we only were able to estimate the susceptibility to OA of the *Varroa* population from Federal apiary, after 8 years of continuous exposition to this acaricide (we could not sample in previous years). It would be interesting to have a study of susceptibility in this population, at time zero and year by year. Probably, with this kind of data, we might have been able to detect a possible change in the toxicity of OA on the mites through the years. But even if this had happened, we hold that this mite population would be even susceptible to the OA, taking into account the bioassay results reported here and the efficacy in field conditions (table 1) that allowed *A. mellifera* colonies to survive year after year. If the resistance phenomenon to the OA has happened in the 'focal' apiary, we should find a significant change in the susceptibility compared to the 'naïve' mites. Several studies have reported that susceptibility to an acaricide is abruptly decreased when the *Varroa* mites develop resistance to it. A decreased susceptibility to amitraz around the order of 175 times was reported in Mexico (Rodriguez-Dehaibes *et al*., 2005). For Europa, similar results related to a reduction in the susceptibility to fluvalinate were published (Milani, 1995, Thompson *et al*., 2002). In this context, our results indicate that there were no changes in susceptibility to OA in the 'focal' mite population during the field treatments, and consequently, our main hypothesis could not be accepted: "the risk of emergence of resistance for the OA is high if its use is prolonged in time" (Milani, 2001).

Why a *Varroa* population does not modify their susceptibility against such selective pressure is an interesting focus for future investigations. It is possible that the mode of action of this acaricide and stability inside the colony are the key to understanding the results presented here. A combination between the deleterious effects on mites by means of the low pH in aqueous solution (Nanetti and Stradi, 1997; Nanetti, 1999; Nanetti *et al*., 2003) and its fast degradation within the colony (Maggi *et al*., 2016) generate that the selection pressure exerted in the 'focal' *Varroa* population could be still lower compared with the selection pressure generated by a synthetic drug such as fluvalinate or coumaphos. Compared to OA, these acaricides possess a high stability and residual effect inside honey bee colonies and a different mode of action, killing mites by an over stimulation of its central nervous system (Wang *et al*., 2002; Johnson *et al*., 2009). A recent study has established a positive correlation between coumaphos residues in wax and the development of *Varroa* resistance (Medici *et al*., 2015). However, residues of OA are not detected in wax after acaricide treatments with this acid (Maggi *et al*., 2016). Further investigations are needed to elucidate and understand the mechanism of *Varroa* tolerance to the OA.

In the case of *V. destructor*, tactics meant to preserve susceptible mites ("moderation tactics"), avoiding an excessive death of mites, are suggested for slowing the selection of resistant mites (Milani, 1999). The success of these tactics depends on the balance between the selection pressure resulting from the application of acaricides and possible disadvantages associated with the resistance, which would cause that the frequency of genes for resistance decreases in the interval between treatments. The OA applied in sucrose solution would be a reliable tool for a moderation tactic. First, if OA is applied only in one dose (as it was applied in the apiary studied here) its efficacy is rarely higher than 90%. Several researchers have documented that OA should be applied more than two times to obtain higher efficacy (reviewed in Rademacher and Hartz, 2006). For the other hand, the efficacy could be even lower accordingly of the climate and the presence of brood, further reducing the aggressiveness of treatment against mites (Bacandritsos *et al.*, 2007). Second, the fast degradation and the low residual of the OA make this product an interesting alternative acaricide. By this way, organic miticides exert lower selection pressure against *Varroa* populations compared to synthetic compounds. In Argentina, the low selection pressures exerted by organic miticides is due to that this kinds of products is not used very often by beekeepers, even when resistance phenomena to synthetic chemicals have been widely reported (Maggi *et al.*, 2009; 2010).

Although no changes in susceptibility were detected in this research, it is strongly recommended to rotate this acaricide with other miticides and with nonchemical control techniques applied in different seasons of the year, each one acting for a restricted period of time. In Italy, since the spread of fluvalinate-resistant *V. destructor* strains, many beekeepers control this mite with a combination of essential oils at the end of summer and a single treatment with an organophosphorus acaricide or the OA in late autumn. A similar treatment schedule based on acaricide rotation aimed to avoid resistance episodes to the OA could be applied here. Regional integrated pest management programs against *V. destructor* should be performed in Argentina considering the use or misuse of the available acaricides. Implicit in this approach remain expectations that multiple tactics (1) reduce the likelihood that pests evolve resistance to any one miticide, or (2) interact such that control is enhanced or that a compensatory control can be provided if one component fails (Ellis *et al.*, 2001; Rinderer *et al.*, 2004; Sammataro *et al.*, 2004; Delaplane *et al.*, 2005).

Acknowledgements

The authors would like to thank the Universidad Nacional de Mar del Plata (UNMDP) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). This research was supported by a grant of ANPCyT, PICT 2011 to Matías D. Maggi. None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper. Matías D. Maggi and Natalia Damiani contributed equally to this work.

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Received April 18, 2015. Accepted November 17, 2016.