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ORIGINAL RESEARCH ARTICLE

The presence of synthetic acaricides in beeswax and its influence on the development of resistance in *Varroa destructor*

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After honey production, beeswax ranks second as regards hive product used in industry and cosmetics. In Argentina, the use of commercial wax adulterated with paraffin and other olefins for comb foundation is a common practice. As regards beehives, the progressive accumulation of synthetic acaricides in wax has caused adverse effects on bees, mainly on individuals at different stages of development. Another issue associated with the use of synthetic acaricides is the phenomenon of resistance. This study aimed to determine the presence of adulterants and acaricide residues in commercial wax used in Argentina. Furthermore, the relationship between coumaphos content in wax and the development of mite resistance reported in recent years in Argentina was investigated. The results demonstrate that paraffin is the most common contaminant substance present in recycled beeswax and commercial wax used for comb foundation in the country. Coumaphos was also found to be the most common acaricide present in wax; 87% in commercial and 80% in recycled wax. Fluvalinate was detected in 33% of commercial wax samples and in 27% of recycled wax. A relationship between coumaphos residues and resistance was also established. Future studies should be conducted to establish the mechanisms by which the buildup of acaricides in the beeswax affects the development of resistance in populations of varroa.

Presencia de residuos de acaricidas en cera de abejas y su influencia sobre el desarrollo de resistencia de *Varroa destructor*

La cera de abejas es el producto más importante de la colmena después de la miel, tanto para la industria apícola como para la cosmética. La adulteración con parafina y otras oleofinas es relativamente fácil y común en nuestra cera estampada. Con respecto a la colmena la acumulación progresiva de acaricidas sintéticos en la cera ha causado efectos adversos en las abejas, principalmente en los individuos en distintas etapas del desarrollo. Otro factor asociado al uso de acaricidas es el fenómeno de resistencia. El objetivo de este estudio fue determinar la presencia de residuos de acaricidas y adulterantes en la cera comercial utilizada en Argentina. Por otro lado se investigó la relación entre el contenido de cumafós en la cera y el desarrollo de resistencia en los ácaros. Los resultados mostraron que la parafina es la sustancia más comúnmente hallada tanto en ceras de recupero como comerciales. El cumafós también fue el acaricida con mayor presencia en la cera, 87% en cera comercial y 80% en la de recupero. El fluvalinato fue detectado en el 33% de las muestras de cera comercial y en el 27% de las ceras de recupero. También se pudo establecer una relación entre los residuos de cumafós y el fenómeno de resistencia. Futuros estudios deberían profundizar acerca de los mecanismos por los cuales la acumulación de residuos de acaricidas en la cera influye sobre el desarrollo de resistencia en las poblaciones de *Varroa*.

Keywords: coumaphos; fluvalinate; flumethrin; beeswax; acaricide resistance; *Varroa destructor*

Introduction

After honey production, beeswax ranks second as regards hive products used in industry and cosmetics. Immediately after being secreted, elaborated, and molded, beeswax presents a white color. With time, beeswax used in brood combs takes on a darker color, due to the addition of pollen, propolis, molts, and feces. Currently, the quality criteria applied to beeswax in the Republic of Argentina are established by the Argentine Pharmacopoeia (acid value, ester value, saponification index, etc.). The use of commercial wax adulterated with paraffin and other olefins for comb foundation, is a common practice in Argentina (Medici, 2011). The products usually used to adulterate beeswax in this country are: paraffin, animal fats, and stearin. Nevertheless, the official methods applied for the detection of these adulterations are obsolete. Another important issue faced by the cosmetic industry is the indirect contamination of wax taking place when beekeepers apply synthetic acaricides on beehives to control *Varroa destructor* (Wallner, 1997). The most common effects of these

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molecules include: congenital malformations, carcinogenesis, and mutagenesis (Vázquez Castillo, 2001). It can also act as an analog of certain hormones, affect the normal metabolic function, or generate chemical reactions in the human body, which eventually results in various disorders and diseases (Rodríguez García, 2007).

With regard to beehives, the progressive accumulation of synthetic acaricides in wax has deleterious effects on bees, mainly on individuals at different stages of development (Johnson, Pollock, & Berenbaum, 2009; Medici, Castro, Sarlo, Marioli, & Eguaras, 2012; Orantes-Bermejo, Gomez Pajuelo, Megías, & Torres Fernández-Piñar, 2010). Beekeepers also become economically harmed when they acquire commercial wax containing acaricide residues. Medici (2011) studied wax contamination by fluvalinate and coumaphos in colonies of *Apis mellifera* from Buenos Aires Province, Argentina, reporting high levels of acaricide residues in beehives where synthetic acaricides had never been applied. This has become a worldwide concern, and several studies have reported serious issues related to acaricide use. Due to the deleterious effects of synthetic acaricides produced in the beekeeping industry, some countries like USA have prohibited the use of coumaphos. It is worth mentioning that acaricide residues were detected in colonies where they had been applied seven years before.

Studies conducted in Spain have demonstrated a higher accumulation of tau-fluvalinate (26,5%) and chlorphenvinphos (88,5%) in beeswax, accounting for values between 0,02 and 88,6 mg/kg and 0,13 and 10,64 mg/kg, respectively (Orantes-Bermejo, 2008). These results are explained by the fact that these drugs were the only ones approved by the Spanish authorities for varroa control. Bogdanov (2006) reported the presence of residues of coumaphos (61.9%), bromopropilate (54.9%), and tau-fluvalinate (37.2%) in Swiss and German bee wax. A similar situation was reported in France, where tau-fluvalinate (61.9%); coumaphos (52.2%) and endosulfan (23.4%) were detected in wax (Chauzat & Faucon, 2007); and also in Italy, where coumaphos and chlorphenvinphos were detected in beeswax (Persano Oddo, Pulcini, Morgia, & Marinelli, 2003). In USA, all wax samples analyzed featured acaricide residues of coumaphos and fluvalinate in concentrations above 204 mg/kg (Frazier, Mullin, Frazier, & Ashcraft, 2008). In Argentina, there is no information available in this respect.

Another problem related to the use of synthetic acaricide is the resistance phenomena. Insecticide resistance is defined as a heritable change in the sensitivity of a pest population reflected in repeated failures of the product efficacy when used according to label recommendations (Milani & Della-Vedova, 2002). This is usually ascribed to the "abuse" or "misuse" of the insecticide in the control of a pest, resulting in the selection of resistant individuals and the subsequent spread of the resistant populations. In Argentina, resistance phenomena to synthetic acaricides were reported for coumaphos and amitraz (Maggi, Ruffinengo, Gende, Eguaras, & Sardella, 2008; Maggi et al., 2011). Added to the issue of resistant mite populations, are the negative effects of acaricide use on bees. Fell and Tignos (2001) related coumaphos use to a low queen acceptance the colonies. Orantes-Bermejo et al. (2010) reported that in field studies of apparently healthy hives in which a very low brood survival rate had been found, concentration values of acaricides in wax were very high. Medici et al. (2012) remarked that the increasing concentrations of fluvalinate and coumaphos in wax gradually decreased offspring survival. By the other hand, Pettis, Collins, Wilbanks, and Feldlaufer (2004) described that queens failed to develop when they were reared in beeswax containing 1000 mg/kg of coumaphos, and greater than 50% of the queen cells were rejected at the 100 mg/kg concentration. Additionally, they state that queens that survived exposure to 100 mg/kg coumaphos weighed significantly less than control queens.

The aim of this study was thus to determine the presence of adulterants and acaricide residues in commercial wax used in Argentina. Furthermore, the relationship between coumaphos content in wax and the development of mite resistance reported in recent years in Argentina were investigated.

Materials and methods

Determination of acaricides and adulterants in comb foundation and recovered wax

To carry out this trial, 15 Argentine commercial waxes used for comb foundation were utilized. These beeswax samples represent the 80% of the waxes commercialized in our country. Also, 27 samples of recycled wax from commercial apiaries located across Argentina were collected. Samples of recycled wax (250 g/apiary) were obtained and placed in a clean nylon pocket. In each wax sample, several parameters were assessed according to the Argentine Pharmacopeia: (a) determination of paraffin or grease added, (b) acidity index (Bianchi, 1996), (c) saponification index (Bernal, Jiménez, del Nozal, Toribio, & Martín, 2005), (d) ester index, (e) ester-acid rate, and (f) melting point, following the protocols established by Maidana (2005) in all cases (except for the acidity and saponification indexes).

The detection of coumaphos, fluvalinate and flumethrin was performed by the multiresidue method proposed by the International Honey Commission (Bogdanov, Kilchenmann, & Imdorf, 1997). This methodology allows the simultaneous detection of coumaphos, fluvalinate, and flumethrin as well as of several pyrethroid pesticides, organochlorines, and organophosphates. Once the extraction technique was setup, the products obtained were analyzed with GC-ECD chromatography. Virgin beeswax was used as negative control because it is free of acaricide residues. The capillary column used was an Vf 5-ms 30 m 0.25 mm ID 0.25 m (Factor Four, Varian Inc). The injection was applied manually using the injector split/splitless of the equipment. Calibration was performed at five points using the external standard technique. The detection limit obtained was 50 g/kg for coumaphos, 100 g/kg for fluvalinate, and 300 g/kg for flumethrin, respectively. The recovery rate varied between 90 and 97%. The statistical analysis was performed using the package Xlstat. 2007 v. 8,02, 2007 v. 8,02, 2007.

Bioassays for mite susceptibility to coumaphos and wax residues

In autumn 2008, four commercial beekeepers from Entre Ríos Province, Argentina, complained of high *V. destructor* infestation following treatment of their colonies with coumaphos. To determine if acaricide resistance was involved, bioassays were conducted on mite populations from Entre Ríos Province, Orense (Buenos Aires province), Buenos Aires, Tres Arroyos (Buenos Aires province), and compared to data from susceptible mite populations of Mar del Plata city, Buenos Aires Province, Argentina (Maggi et al., 2008). *V. destructor* mites were obtained from different infested *A. mellifera* combs from each apiary. Mites were taken from capped brood by opening and inspecting individual cells. Mature mites were removed from bee broods with a slender moistened paint brush, placed in an incubation stove at 70% RH and 33–34 °C on bee larvae, and kept in a glass Petri dish for 1–3 h until the number of mites collected was sufficient. LC_{50} for coumaphos was determined using a toxicity method (Maggi et al., 2008). Technical grades of coumaphos (Pestanal, Sigma–Aldrich) were diluted in 1 ml of hexane (Ciccarelli Laboratories, Argentina, Pro-analysis).

Concentrations increasing from 0 to 400 g/ml were applied to the bottom of the dish for the acaricide. Five replicates were done for each concentration and control. 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). Mortality values were adjusted in accordance with Abbott (1925) as a function of natural mortality. The LC_{50} values and resistance indexes (RI) estimated in this study were compared with the baseline reported by Maggi et al. (2008). The resistance index was calculated as LC_{50} "resistant" mites/LC_{50} susceptible mites, and statistically analyzed with the Fisher exact test (APHA, 1992). From each mite population (resistant or susceptible), wax samples from brood combs were analyzed for coumaphos residues based on the protocols described above.

Results

Acaricide residues and adulterants presence in recycled wax

The physicochemical parameters obtained for each sample are listed in Table 1. These results indicate that 55.5% of the total samples analyzed were not suitable for consideration as pure bee wax, according to the Argentine Pharmacopoeia. A high percentage of paraffin and fats in wax can also be noticed. In relation to adulterants presence in recovered wax, 18% (n = 5) of samples presented paraffin concentrations between 15 and 25%, while only 13% (n = 3) were contaminated with fats. The highest paraffin percentage found in wax was 40% (n = 1). The results indicated that 80% of the waxes tested were contaminated with coumaphos residues. Coumaphos residues were in the range of 132.4–14,840.8 g/kg (Table 4), reaching values higher than those observed in commercial wax used for comb foundation (Table 3). With regard to fluvalinate residues, 27% of waxes presented this active principle. Flumethrin residues were not detected in any sample.

Acaricide residues and adulterants presence in commercial wax used for comb foundation

Table 2 lists the results of adulterants present in commercial wax used for comb foundation. Sixty seven percent of the commercial wax analyzed in this research presented paraffin contamination (range: 15–45%). No wax contamination by fats was detected.

The results obtained from the analysis of 15 samples of Argentine commercial wax are presented in Table 3. Coumaphos and fluvalinate residues were detected in 13 (87%; range: 291–7470 g/kg) and in 5 samples (33%; range: 102.8–405.6 g/kg), respectively. Flumethrin residues were not detected in any of the samples analyzed. Only two samples of the total were acaricide free. All samples with fluvalinate also presented coumaphos residues.

Mite susceptibility to coumaphos and acaricide residues in brood combs

Coumaphos LC_{50} for resistant mite and RI are shown in Table 5. The mite populations studied were compared to a susceptible mite population (Maggi et al., 2008), yielding resistance index of 559. Table 6 depicts the values obtained for each acaricide residue found in brood combs where mite resistant populations were obtained. Virgin wax was utilized as a negative control. All wax samples presented coumaphos residues, but in none of them pyrethroid residues (fluvalinate and flumethrin) were detected. A positive relationship was noticed between RI and coumaphos residues in wax obtained from brood combs (Figure 1).

Discussion

The results reported in this research have demonstrated that paraffin is the most contaminant substance present in recovered and commercial wax used for comb foundation in Argentina. The contaminant values detected in wax were higher than those set forth by the

Table 1. Physicochemical parameters and adulterant's presence in recycled beeswax.

Sample Nos.	Acid value	Fits Pharmacopeia (17-24)	Ester value	Fits Pharmacopeia (72-79)	Ester/ Acid Index	Fits Pharmacopeia (3,2-4,2)	Saponification index	Paraffin (%)	Fats (%)	Pure beeswax?
1	23.68	YES	72.47	YES	3.1	NO	91	0	0	NO
2	22.33	YES	71.36	NO	3.2	NO	102.5	20	0	NO
3	11.83	NO	123.93	NO	10.5	NO	158.8	40	10	NO
4	20.4	YES	74.86	YES	3.7	YES	87.08	0	0	YES
5	15.89	NO	68.49	NO	4.3	NO	75.1	30	0	NO
6	18.47	YES	76.86	YES	4.2	YES	90.05	0	0	YES
7	19.93	YES	78.24	YES	3.9	YES	90.63	0	0	YES
8	23.37	YES	74.21	YES	3.2	NO	92.9	0	0	NO
9	21.37	YES	74.5	YES	3.5	YES	116.5	10	10	NO
10	20.16	YES	71.6	NO	3.6	YES	102.79	0	0	NO
11	17.75	YES	73.55	YES	4.1	YES	79.88	0	0	YES
12	19.63	YES	72.11	YES	3.7	YES	91.88	0	0	YES
13	20.76	YES	72.85	YES	3.5	YES	93.43	0	0	YES
14	17.11	YES	72.36	YES	4.2	YES	96.81	0	0	YES
15	20.86	YES	71.98	NO	3.5	YES	93.5	15	0	NO
16	24.14	YES	72.84	YES	3.0	NO	106.4	0	0	NO
17	20.94	YES	74	YES	3.5	YES	97.9	0	0	YES
18	21.01	YES	71.9	NO	3.4	YES	170.7	25	15	NO
19	20.13	YES	75.7	YES	3.8	YES	102.7	0	0	YES
20	19.09	YES	73.55	YES	3.9	YES	86.2	20	0	NO
21	19.24	YES	67.72	NO	3.5	YES	91.42	0	0	NO
22	20.83	YES	74.98	YES	3.6	YES	86.2	0	0	NO
23	21.04	YES	75.65	YES	3.6	YES	99.2	0	0	YES
24	17.94	YES	78.5	YES	4.4	NO	88.32	0	0	NO
25	21.3	YES	75.14	YES	3.5	YES	88.44	0	0	YES
26	18.53	YES	79.45	NO	4.3	NO	100.63	25	0	NO
27	17.65	YES	74.4	YES	4.2	YES	90.58	0	0	YES

Table 2. Presence of paraffin and/or fats in commercial beeswax from Argentina.

Sample Nos.	Paraffin content (%)	Fats content (%)
1	0	0
2	0	0
3	25	0
4	15	0
5	20	0
6	0	0
7	20	0
8	0	0
9	15	0
10	35	0
11	0	0
12	20	0
13	15	0
14	45	0
15	30	0

Argentinian Pharmacopeia. The presence of these substances in beeswax could explain combs melting when temperatures are higher in the field, new wax rejection for comb foundation by bees and/or extra combs construction by bees inside the nest.

If we compare quality parameters and the outcomes from adulterants presence, it can be noticed that, in general, the samples falling outside the acceptable values for the Argentinean Pharmacopoeia correspond to different adulterant values. The ester/acid relationship is rather unreliable as a wax quality estimator, since, as it can be observed in sample 8, despite the fact that the acid and ester values are within the normal range, the relationship between them falls outside the acceptable values. Samples 10 and 22 present an ester value below the established range, but reveal no adulterants presence, thereby indicating that it is likely that these waxes had been excessively heated during the recycling process.

Coumaphos was also found to be the most common acaricide present in wax, both in commercial and recovered wax. This acaricide yielded the highest acaricide concentration registered in the study (14.840, 8 g/kg), which is consistent with other studies conducted in Europe (Orantes-Bermejo et al., 2010). Our results suggest that coumaphos residues found in the waxes analyzed not only come from the repetitive application of treatments against *Varroa* in beehives, but also come from the recycle of the waxes containing the active ingredient. Thus, recycled wax may have higher concentrations of coumaphos due to its commercial recycling. This could become serious concern if government agencies do not take concrete actions since coumaphos residues will increase in the wax used for comb foundation, and, therefore, exert undesirable effects on bee larvae (Medici et al., 2012). In Argentina, coumaphos treatments are applied in two different ways: (a) by dusting over the frames and/or (b) embedded into plastic or wood strips.

Table 3. Acaricide content (g/kg) found in commercial beeswax.

Sample N°	Coumaphos (g/kg)	Tau-Fluvalinate (g/kg)	Flumethrin (g/kg)
1	682.76	ND*	ND*
2	291	ND*	ND*
3	458	ND*	ND*
4	4563	ND*	ND*
5	ND*	ND*	ND*
6	1470	ND*	ND*
7	ND*	ND*	ND*
8	346.5	ND*	ND*
9	1128	184.2	ND*
10	3611.5	405.6	ND*
11	7470	ND*	ND*
12	268	102.8	ND*
13	968	203.5	ND*
14	2400	165	ND*
15	435	ND*	ND*

Notes: Mean CV (%) 15. Values shown as ND are those below method's detection limit.
*ND: No residue detected.

Table 4. Acaricide content (g/kg) found in Argentinian-recycled beeswax.

Sample Nos.	Coumaphos (g/kg)	Tau-Fluvalinate (g/kg)	Flumethrin (g/kg)
1	ND*	ND*	ND*
2	ND*	ND*	ND*
3	1468.9	ND*	ND*
4	3510.0	ND*	ND*
5	559.8	103.2	ND*
6	ND*	ND*	ND*
7	547.0	ND*	ND*
8	14840.8	ND*	ND*
9	3122.3	ND*	ND*
10	2290.7	200.2	ND*
11	10298.1	105.9	ND*
12	132.4	ND*	ND*
13	826.8	ND*	ND*
14	1224.5	104	ND*
15	5879.6	ND*	ND*

Notes: Mean CV (%) 15. Values shown as ND are those below method's detection limit.
*ND: No residue detected.

Table 5. LC_{50} and RI estimated for each mite population studied. (LC: Lethal Dose).

Mites origin	LC_{50} (g/Petri dish)	Resistance index (RI)
Entre Ríos	319	559
Buenos Aires 1	22.8	40
Buenos Aires 2	4.9	8.59
Tres Arroyos	3.5	6.25
Orense	0.76	1.35
Mar del Plata (Maggi et al., 2008)	0.56	1.00

Then the final amount of the product distributed inside the honeybee is dependent on the manner and the quantity of the drug (Chauzat & Faucon, 2007). Additionally, when coumaphos is used in strips its dissemination inside the colony depends on bee contact and treatment time.

Fluvalinate was found in 33% of commercial wax samples and in 27% of recovered waxes. Beekeepers ceased using this acaricide in 2000, after the resistance phenomena to this drug was hypothesized by researchers. Residues presence confirms the statements above and ascertains the liposolubility and persistence of this pyrethroid in wax. In 2009, flumethrin was approved as an acaricide for *Varroa* control in Argentina. In this study, no residues of this pyrethroid were found. This could be ascribed to the high detection limit of the methodology utilized as well as to its short term use in apiculture.

Table 6. Tau-fluvalinate, flumethrin, and coumaphos content in the studied beeswaxes.

Sample Nos.	Origin	Coumaphos (g/kg)	Tau-Fluvalinate (g/kg)	Flumethrin (g/kg)
1	Virgin beeswax	ND*	ND*	ND*
2	Entre Ríos	14480 ± 1316.8	ND*	ND*
3	Buenos Aires 1	6610 ± 698.4	ND*	ND*
4	Buenos Aires 2	1130 ± 147.9	ND*	ND*
5	Tres Arroyos	1542 ± 295.4	ND*	ND*
6	Orense	638 ± 75.9	ND*	ND*

Notes: $x \pm$ SD. Values shown as ND are those below method's detection limit.
*ND: Not detectable.

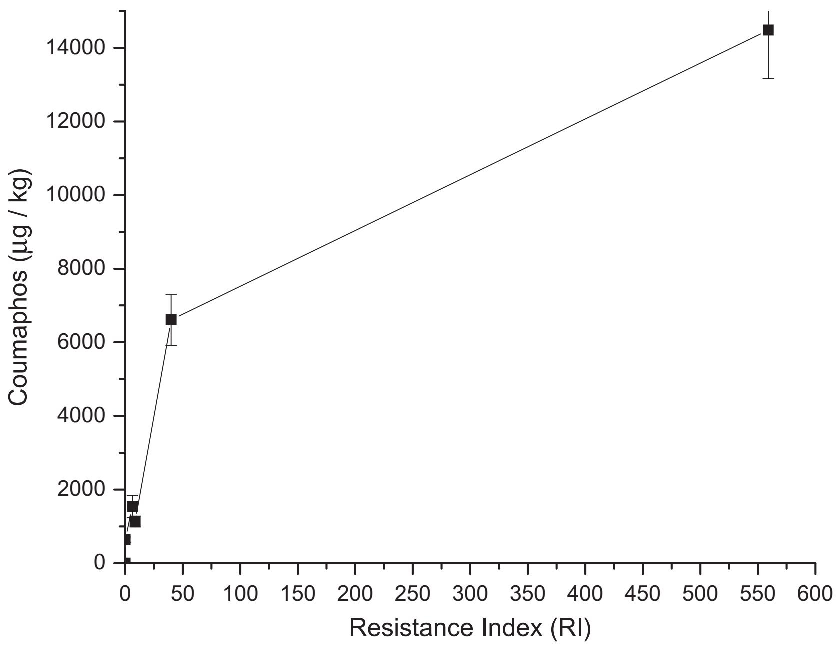

Figure 1. Relationship between coumaphos concentration and the Resistance Index (RI) obtained for each sample.

The results reported in this study show the high contamination level present in beeswax in Argentina. This is mainly explained by the methods used to control *Varroa*. Argentina should devise a method to eliminate these residues, as no fixed detection limits have been specified for these drugs so far. Keeping wax acaricide free could prevent future problems with export and cushion the impact of these pollutants in beekeeping.

On the other hand, the continuous use of coumaphos could lead to residues accumulation in wax. A relationship between these residues and the resistance phenomena was also detected. Resistance to coumaphos in *Varroa* has been reported in Argentina (Maggi et al., 2009). These same authors detected *Varroa* resistances to coumaphos in colonies where acaricide rotation had been applied (Maggi et al., 2010). Resistance was attributed to the use of homemade preparations. This paper postulates a new way to produce acaricide resistance. Mite resistance to coumaphos could result from the intense contact with sublethal doses of acaricides inside brood cells added to the lack of acaricide rotation scheme. This hypothesis could support the results described by Maggi et al., 2010 in Uruguay. The authors found populations of resistant mites in apiaries in which proper health management strategies had been adopted for *Varroa* control (rotation of three active principles with different mechanisms of action). When the study data was published, Maggi et al. (2010) had no concrete explanation for this finding. Considering the results reported in this study, the population of resistant mites detected in Uruguay may have been continuously exposed to sublethal doses of coumaphos with the consequent change in their susceptibility to the drug. This phenomenon could be understood in the light of the explanation provided by Sammataro, Untalan, Guerrero, and Finley (2005). Acaricide resistance is rarely a dominant condition (Carrière, 2003), but it can be expressed in different levels in heterozygous individuals, especially if resistance confers a gain of function (i.e. chemical detoxification by enzymes, penetration reduction, and a high-toxin excretion). Resistant females of *V. destructor* have two copies of the resistant allele (RR) and always produce homozygous progeny. Heterozygous females produce 50% of heterozygous and 50% of susceptible homozygous individuals if they copulate with susceptible mites. Conversely, if heterozygous females copulate with resistant males, their progeny will be 50% resistant homozygous and 50% of heterozygous. The homozygous state is not affected by inbreed, and its frequency increases with each generation. Under this circumstance, it is not surprising to find an increased frequency of resistant individuals over time, especially if acaricides pressure (either by application or by accumulation in wax) eliminate susceptible homozygous individuals from the population. Future studies should be conducted to establish the mechanisms by which the buildup of acaricides in the beeswax affect the development of resistance in populations of *Varroa*.

Disclosure statement

No potential conflict of interest was reported by the authors.

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