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ORIGINAL RESEARCH ARTICLE

The concentration effect of selected acaricides present in beeswax foundation on the survival of *Apis mellifera* colonies

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Summary

Acaricides used for the control of *Varroa destructor* are a major source of pollution in a honey bee (*Apis mellifera*) hive. Because they involve a slow release, they must be present for a period of up to 45 days inside a hive to be effective. The aim of this study was to determine whether the presence of acaricides in beeswax affects the survival of breeding bees, and if this effect is greater at higher concentrations. Three types of recycled beeswax foundation containing paraffin wax in different proportions (0%, 20% and 40%) were used. Brood survival rate was calculated in each treatment (Nº. pupae / Nº. Eggs * 100). Acaricide content in wax sheets was determined by multi-residue analysis by GC-ECD. Survival rate was higher when using beeswax adulterated with paraffin. Recycled beeswax without added paraffin wax (0%) had high levels of coumaphos and fluvalinate contamination, and when paraffin wax was added in different percentages (20%, 40%) the concentration of these components was lower. The presence of acaricides in beeswax adversely affected brood survival. When the pesticide concentration decreased, an improvement in the survival rate was found. Larvae developed in beeswax foundation without paraffin wax, exposed at higher concentration of pollutant residues were more vulnerable to the toxic effects of the acaricides.

Efecto de la concentración de diferentes acaricidas presentes en la cera de abejas sobre la supervivencia de las colonias de *Apis mellífera*

Resumen

Los acaricidas utilizados para el control de *Varroa destructor* son la mayor fuente de contaminación en las colmenas; ya que implican una liberación lenta del producto, que debe estar presente hasta 45 días en la colmena para ser efectivo. El objetivo de este estudio fue determinar si la presencia de acaricidas en la cera afecta a la supervivencia de la cría de *Apis mellifera* y si este efecto es mayor a mayores concentraciones. Se usaron tres tipos de cera estampada reciclada que contenía parafina en diferentes proporciones (0%, 20% y 40%). El ratio de supervivencia de la cría fue calculado para cada tratamiento (Nº. pupas / Nº. huevos * 100). El contenido de acaricida en las hojas de cera fue determinado por análisis de múltiples residuos mediante GC-ECD. El ratio de supervivencia fue mayor usando cera adulterada con parafina. La cera reciclada sin adición de parafina (0%) tuvo mayores niveles de contaminación de cumafos y fluvalinatos, y cuando se añadió parafina en diferentes porcentajes (20, 40%), la concentración de estos componentes fue menor. La presencia de acaricidas en la cera afecta negativamente a la supervivencia de la cría. Cuando la concentración de los pesticidas disminuye, aparece una mejora en el ratio de supervivencia. Las larvas que se desarrollan en cera estampada sin parafina y expuesta a mayores concentraciones de residuos de contaminantes, fueron más vulnerables a los efectos tóxicos de los acaricidas.

Keywords: Honey bees, acaricides, brood, beeswax, survival

Introduction

Honey bees (*Apis mellifera*) are social Hymenoptera that build nests composed of wax combs parallel to each other in which their offspring develops and food is stored for the colony (Seely and Visscher, 1985). Although beeswax is used for the manufacture of many products such as candles or cosmetics, much of it is recycled by the beekeeping industry and returned to the hives into sheets of wax foundation. This use of beeswax sheets is an essential management practice in commercial beekeeping, whether conventional or organic.

Acaricides used for the control of *Varroa destructor* are a major source of pollution in a hive. Because they involve a slow release, they must be present for a period of up to 45 days inside the hive to be effective. Hydrophilic substances are retained in the honey whilst lipophilic ones will accumulate in wax after repeated treatments (Bogdanov *et al.*, 1999).

The stability of residues in beeswax can trace the history of the treatments carried out in hives because they cannot be removed without compromising the physical and chemical characteristics of the wax. (Piro *et al.*, 2000). These residues are in the range of 0.5 to 10 mg / kg (ppm). Numerous studies concluded that the residual wax concentration increases with successive applications of acaricides and the decline after ceasing their use is very slow. The action of these pesticides presents different effects depending on the individual's role in the hive. For example fluvalinate reduces sperm production in drones (Rinderer *et al.*, 1999). Other studies found that coumaphos causes problems in queen cell acceptance (Fell and Tignes, 2001). Some authors have reported in field studies that apparently healthy hives showed very low survival rates of bee larvae (Orantes-Bermejo *et al.*, 2010) probably caused by a very high acaricide concentration in the wax. The aim of this study was to determine whether the presence of acaricides at different concentrations in beeswax affects the survival of breeding honey bees.

Materials and methods

Study area and experimental design

The study was conducted on 12 Langstroth hives located in Buenos Aires Province, Argentina (37° 56' S, 57° 40' W) during the summer season (January 2009). Each hive was composed of 10 standard frames, each one covered by bees, and a one year old queen. Previously, the apiary received appropriate sanitary management. Varroa control treatment consisted in the application of 6.25 g / strip of Amitraz acaricide (AMIVAR®; APILAB SRL), using two strips per hive and removed 30 days after placement. For Nosema treatment, 60 mg of 2% Fumagillin (NOSEMIX Fumagillin-B®; Solemar SA) was administered per hive in three applications every seven days. Periodic examinations showed no presence of American foulbrood or European foulbrood. The products used were approved by the National Agroalimentary Health and Quality Service (SENASA, Argentina), for its use in apiculture. During the course of the experiment the colonies were not fed.

Three types of beeswax foundation containing paraffin wax (Ciccareli®, CAS Nº 8002-74-02, melting point: 56-58ºC) in different proportions (0%, 20% and 40%) were used. Foundation was produced by a professional manufacturer (Mr Rodolfo Danieli, Entre Ríos, Argentina) following a protocol established by our laboratory. Recycled wax was melted and separated into three groups: the first group was applied directly without any addition. To the second and third group, paraffin wax was added before stamping in a proportion of 20% and 40%, respectively.

In order to ensure that the sheets of beeswax contained only the specified adulterations and not any other components that could affect the outcome of the experiment, the following analyses were performed: 1. determination of the paraffin wax added; 2. acidity index (FCC, 1981); 3. saponification index (Bernal *et al.*, 2005); 4. ester index; 5. ester-acid rate; and 6. melting point. We followed the protocols established by Maidana (2005) in all cases except for the acidity and saponification indexes.

In order to avoid any effect of frame position, two experimental frames were placed in contact with the brood nest in each hive, one on the left and the other on the right of the brood (rear view of the hive), in the third and eighth positions (counting the first position from the right). The sheets of beeswax were divided transversely, and in each experimental frame a section of pure wax and one of wax with paraffin wax were placed. Thus, each frame was composed of sheets with: a. 0%, 20%; and b. 0%, 40% of paraffin wax, alternating its position in the hive: Left-Right, and inside the frame: Front-Back according to Castro *et al.* (2010). A random combination was assigned for each hive in the apiary.

Brood survival analysis

The combs were built in their entirety and eggs were laid by the queen. The number of worker honey bee cells with eggs was counted over a rectangular area of 7 x 6 cm^2 (138 cells) marked on each wax section in both sides. This record was repeated at intervals of four days, counting the number of pupae. The brood survival rate in each treatment (Nº. of pupae / Nº. of eggs * 100) was estimated as Vera Graziano *et al.* (2002) and were compared by one way ANOVA (percentage of paraffin in wax), using the XLSTAT© v 8.02 program, 2007. Subsequently, a Tukey test was performed in order to detect in which treatments differences occur. In both cases the value of α considered was 0.05.

Analysis of contaminants residues in wax

Wax acaricide extraction

Detection of coumaphos, fluvalinate and flumethrin was performed by the multiresidue method proposed by the International Honey Commission (Bogdanov *et al.*, 1997). This methodology allows the simultaneous detection of coumaphos, fluvalinate and flumethrin as well as several pyrethroid pesticides, organochlorines and organophosphates. Once the extraction technique was set-up, the products obtained were analysed with GC-ECD chromatography.

Chromatography GC-ECD

Capillary column used was an Vf 5-ms 30m x 0.25 mm ID x 0.25 mm. (Factor Four; Varian Inc) The injection was done 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 μg/kg for coumaphos, 100 μg/kg for fluvalinate and 300 μg/kg for flumethrin. The recovery rate varied between 90-97%. Statistical analysis was performed using the package XLSTAT© v 8.02, 2007.

Results

Wax quality analysis

Paraffin analysis determination confirmed that the amount of paraffin wax added to the wax samples (0%, 20% and 40%) was as planned. The physicochemical parameters obtained from the wax samples for each treatment are shown in Table 1.

Table 2. Survival rates for each treatment. X ± SD (n = 7). The same letters in the same column indicate that rates are not statistically different from each other ($p > 0.05$) by Tukey test (XLSTAT© v 8.02, 2007).

Treatment	Survival (%)
0% paraffin	65 ± 8.6 [a]
20% paraffin	68 ± 12.4 [ab]
40% paraffin	78.8 ± 6.5 [b]

Brood survival

Brood survival rates are shown in Table 2. The proportion of paraffin embedded in wax foundation was a factor that caused differences in brood viability ($F = 4.070$, $P = 0.035$). Survival rates (Table 2) in wax sheets with 40% paraffin wax were significantly higher than in pure wax ones ($P = 0.036$). By contrast, survival rates did not differ significantly between sheets with 0% and 20% of paraffin wax ($P = 0.823$), nor between sheets with 20% and 40% of paraffin wax ($P = 0.114$). The survival rate was higher when using beeswax adulterated with paraffin wax.

Acaricide content in wax

The chromatograms obtained for each type of wax are shown in Fig. 1. Results presented in Table 3 show that the recovered wax without added paraffin wax (0%) had high levels of coumaphos and fluvalinate contamination, and when paraffin wax was added in different proportions (20%, 40%) these values decreased.

Table 1. Physicochemical parameters for pure beeswax (FCC, 1981) and values observed in samples with 0%, 20% and 40% of paraffin. x ± SD (n).

Determination	Reference values for pure beeswax (FCC, 1981)	Recycled beeswax (paraffin 0 %)	Recycled beeswax (paraffin 20 %)	Recycled beeswax (paraffin 40 %)
Acidity index	17-24	18.38 ± 1.83 (5)	15.76 ± 2.36 (6)	15.03 ± 1.54 (5)
Ester index	72-79	75.19 ± 1.42 (3)	47.97 ± 18.63 (3)	45.43 ± 8.94 (3)
Saponification index	87-103	100.57 ± 10.73 (4)	59.13 ± 6.32 (4)	68.62 ± 11.43 (4)
Ester-acid relation	3.3-4.2	3.89 ± 0.29 (3)	3.16 ± 0.78 (3)	2.89 ± 0.51 (3)
Melting point	62ºC - 64ºC	63ºC	60.5ºC	58.9ºC

Table 3. Residue concentrations of fluvalinate, coumaphos and flumethrin in pure wax and in samples with 0%, 20% and 40% of paraffin wax. x ± SD (n). Different letters indicate significant differences.

Sample	**Concentration in beeswax (µg / kg)**		
	coumaphos	**fluvalinate**	**flumethrin**
100% virgin beeswax	0	0	0
0% paraffin recycled beeswax	6311.64 ± 757.39^{a}	204 ± 30.75^{a}	0
20% paraffin recycled beeswax	1224.5 ± 122.50^{b}	104 ± 17.68^{b}	0
40% paraffin recycled beeswax	826 ± 114.64^{c}	0^{c}	0

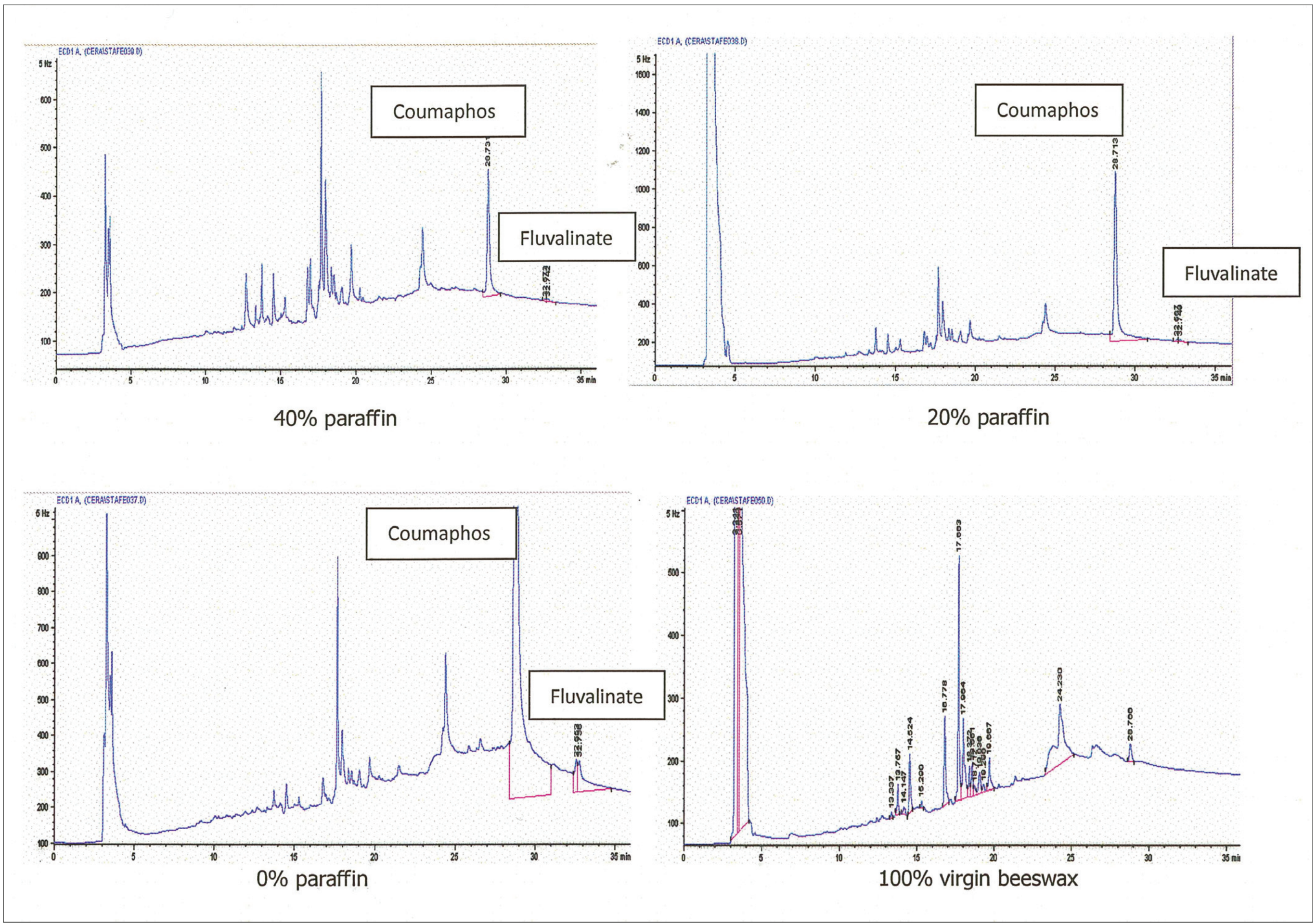

Fig. 1. Chromatograms obtained from multi-residue analysis by GC-ECD for each type of wax used in this work. 100% virgin beeswax was used as negative control for the presence of acaricides.

Discussion

The presence of acaricides in the beeswax affected adversely brood survival. When the pesticide concentration decreased an improvement in the survival rate was found. Coumaphos was the acaricide found in higher concentrations. Fluvalinate was found in low concentrations. This result is consistent with the fact that in Argentina this acaricide has not been used for at least five years. No flumethrin residues were found in the samples studied, probably because this acaricide has only recently been used in beekeeping in this area and also because the detection limit of this compound with this methodology is high.

Paraffin wax acts as a diluent of residual contaminants present in recycled wax. The use of this hydrocarbon is not recommended in beekeeping practice because it is considered to be an adulteration.

Studies carried by Castro *et al*. (2010) showed that the addition of paraffin wax to beeswax foundation decreased the area built in the combs and also reduced the queen acceptance for oviposition.

The larvae more exposed to the toxic effects of acaricides were those developed in foundation without paraffin wax, where pollutant residues were more concentrated. The high coumaphos levels found in the recovered wax may be due to the continued and exclusive use of this product by beekeepers, with the consequence of being accumulated every year in the wax. On the other hand, the presence of fluvalinate (an acaricide not currently used in the country) may be due to its use in the past and the high persistence in the wax.

Bee brood developed in a contaminated environment is more sensitive to the effects of these acaricides than adult bees. *V. destructor* resistance to acaricides promotes the increase of applications and on the concentration of chemicals in the hive. Recent studies based on the synergistic effects of fluvalinate and coumaphos showed that there is a marked increase in fluvalinate toxicity on younger bees previously treated with coumaphos, suggesting that bee mortality may occur during the application of sub-lethal doses when coumaphos and fluvalinate were found simultaneously in wax (Johnson *et al*., 2009). Further studies are needed for determining whether this effect on brood survivorship is caused by either of the two acaricides found, or if it is a result of a synergistic effect of both.

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