

ORIGINAL RESEARCH ARTICLE



## Pesticide residues in beeswax and beebread samples collected from honey bee colonies (*Apis mellifera* L.) in Spain.

### Possible implications for bee losses

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### Summary

Two experiments were carried out on professional bee hives in Spain in order to measure the levels of contamination in beeswax and beebread and its possible relation to the bee losses currently found in bees. In beeswax the main components that we found were chlorfenvinphos and tau-fluvalinate. Chlorfenvinphos was detected in 100% of the samples analyzed (n=32) with an average concentration of  $449.28 \mu\text{g kg}^{-1} \pm 708.42 \mu\text{g kg}^{-1}$  (range:  $20.45 \mu\text{g kg}^{-1} - 3182.31 \mu\text{g kg}^{-1}$ ). Tau-fluvalinate was detected in 96.8% of the samples analyzed (n=32) with an average concentration of  $996.49 \mu\text{g kg}^{-1} \pm 2384.37 \mu\text{g kg}^{-1}$  (range:  $<\text{LD} \mu\text{g kg}^{-1} - 12978.73 \mu\text{g kg}^{-1}$ ). In beebread the most frequent acaricide was chlorfenvinphos, being detected in 90.6% of the samples analyzed (n= 32) with an average concentration of  $35.9 \mu\text{g kg}^{-1} \pm 60.86 \mu\text{g kg}^{-1}$  (range:  $<\text{LD} \mu\text{g kg}^{-1} - 285.56 \mu\text{g kg}^{-1}$ ). The samples from depopulated apiaries showed higher concentrations of this compound as compared to the non-depopulated apiaries ( $74.62 \mu\text{g kg}^{-1}$  vs.  $23.84 \mu\text{g kg}^{-1}$ ;  $F = 4.254$ ;  $p = 0.048$ ). This tendency was found in the studies of the accumulated acaricides in beeswax coming from hives with both a high or low survival rate. Of the beebread samples taken 59.4% had detectable levels of other agricultural pesticides and in total 16 active ingredients were detected. If we consider acaricides and agricultural pesticides together, 100% of the samples had some kind of contamination. The possible impact of acaricides and agricultural pesticides and cocktails of these in beebread fed to bee larvae is discussed.

## Residuos de pesticidas en cera de abejas y polen ensilado en colmenares (*Apis mellifera* L.) de España. Posibles implicaciones sobre el despoblamiento de las colmenas

### Resumen

Se han realizado dos experiencias en colmenares profesionales de España, con el fin de conocer los niveles de contaminación de la cera y del polen ensilado, y observar si existe o no relación con los fenómenos de pérdida de abejas que existe en España. En la cera de abeja los principales contaminantes encontrados han sido el clorfenvinfos y el tau-fluvalinato. El clorfenvinfos ha sido detectado en el 100% de las muestras analizadas (n=32) con una concentración media de  $449.28 \mu\text{g kg}^{-1} \pm 708.42 \mu\text{g kg}^{-1}$  (rango:  $20.45 \mu\text{g kg}^{-1} - 3182.31 \mu\text{g kg}^{-1}$ ). El tau-fluvalinato ha sido detectado en el 96.8% de las muestras analizadas (n = 32) con una concentración media de  $996.49 \mu\text{g kg}^{-1} \pm 2384.37 \mu\text{g kg}^{-1}$  (rango:  $<\text{LD} \mu\text{g kg}^{-1} - 12978.73 \mu\text{g kg}^{-1}$ ). En el polen ensilado el acaricida más frecuente encontrado es el clorfenvinfos, detectado en el 90.6% de las muestras analizadas (n = 32) con una concentración media de  $35.9 \mu\text{g kg}^{-1} \pm 60.86 \mu\text{g kg}^{-1}$  (rango:  $<\text{LD} \mu\text{g kg}^{-1} - 285.56 \mu\text{g kg}^{-1}$ ). Las muestras procedentes de colmenares con mortandades superiores al 30% de las colmenas muestran tres veces más contaminación por este compuesto que las muestras procedentes de colmenares que no han sufrido pérdida de abejas ( $74.62 \mu\text{g kg}^{-1}$  versus  $23.84 \mu\text{g kg}^{-1}$ ;  $F = 4.254$ ;  $p=0.048$ ). Los resultados parecen indicar que la acumulación de los residuos en cera y su traspaso al polen ensilado pueden jugar un papel importante en los fenómenos de despoblamiento que existen en España y en la baja supervivencia de la cría que muestran las colmenas con despoblamiento. Por otro lado, el 59.4% de las muestras de polen ensilado presentan contaminación por diferentes

Colmenas. En total se han detectado 16 compuestos entre plaguicidas y fungicidas. Si consideramos conjuntamente la presencia de acaricidas y pesticidas, el 100% de las muestras presenta algún tipo de contaminación formado por 1 a 5 compuestos. Se discute el posible impacto sobre la supervivencia de las abejas, de esta acumulación de acaricidas y pesticidas hallados, así como la presencia de varios compuestos en una misma muestra.

**Keywords:** beeswax, beebread, contamination, acaricides, bee losses, *chlorfenvinphos*

## Introduction

In Spain, the beeswax industry has gone through many important changes in recent decades. The beekeeper with cork hives used to renew all the beeswax in the hives every two years and did not reuse it in the hive. This extracted beeswax was used to light up church services and for various industries such as the manufacturing of sheathing for submarine communication cables, etc. From the 1950s and 1960s this situation began to change. The logical development of mobile hives and the use of other waxes in candles meant that the beeswax industry exported a small amount of beeswax to other sectors. Most beeswax was heated, cleaned and then returned to the hive in the form of comb foundation. In this way, the beeswax is recycled every year after being subjected to a heating process. To this process is added a mere 40%-50% of pure beeswax from the rebuilding of the combs by the bees. This process is common to beekeeping throughout the whole world.

This problem may not at first seem to be serious, but since the arrival of the parasitic mite *Varroa destructor* (Anderson & Trueman) in Spain in 1985, this recycling circuit accumulates each year the acaricides used in the fight against the mite, which are fat soluble. In Spain, beeswax mainly accumulates tau-fluvalinate (26.5%) and chlorfenvinphos (88.5%), reaching values between 0.02 – 88.6 mg kg<sup>-1</sup> and 0.13 – 10.64 mg kg<sup>-1</sup> respectively during the period 1996-2006 (Orantes Bermejo, 2008).

This is a world-wide problem, and each country has its beeswax contaminated in varying degrees with acaricides used in the fight against *V. destructor*. Thus Bogdanov (2006) found levels of contamination between 0.5–5.2 mg kg<sup>-1</sup> in beeswax from Switzerland and Germany, with the most frequent acaricides being coumaphos (61%), followed by bromopropilate (54.9%) and tau-fluvalinate (37.2%). In France, beeswax is contaminated with tau-fluvalinate (61.9%), coumaphos (52.2%) and endosulfan (23.4%), reaching concentrations of 4.1 mg kg<sup>-1</sup> of coumaphos (Chauzat and Faucon, 2007). In Italy coumaphos is present in 90.9% of beeswax samples and chlorfenvinphos is present in 51.5% (Persano Oddo *et al.*, 2003). Frazier *et al.*, (2008) in the USA reported high levels of acaricide in beeswax (100% for tau-fluvalinate and coumaphos) with coumaphos reaching maximum concentrations of 204 mg kg<sup>-1</sup>. They also found other pesticides such as chlorpyrifos, chlorothalonil, endosulfan and dicofol.

In beebread the problem is similar, but there are much lower levels of contamination. In Spain between 1996-2006 up to 16 pesticides were found in beebread amongst which were various acaricides used to control *V. destructor* (chlorfenvinphos, tau-fluvalinate, amitraz), and other pesticides (cypermethrin, malathion, aldrin, fipronil) including neoticotinoids (imidacloprid) (Orantes-Bermejo, 2008). In France, 19 substances were detected, and again it was coumaphos and tau-fluvalinate which had the highest concentrations (Chauzat *et al.*, 2006). In the USA, 105 out of 108 pollen samples were found to have contaminants, the most frequent being tau-fluvalinate, coumaphos and chlorpyrifos (Frazier *et al.*, 2008).

The action of these acaricides on bees has been studied by various authors, reporting harmful effects at different levels. For example fluvalinate reduces spermatozoa production in drone honey bees (Rinderer *et al.*, 1999; Fell and Tignor, 2001). Studies on coumaphos have revealed acceptance problems in the queen brood cells (Fell and Tignor, 2001; Haarmann *et al.*, 2002) or when the beeswax is contaminated (Wallner, 1999; Pettis *et al.*, 2004). Recently Burley and Saake (2008), studied the effects on drones born in colonies treated with coumaphos, observing a large reduction in the viability of drone spermatozoa (50%) compared with a control group.

All these issues have an unquestionable effect on bee losses that are now occurring in many countries. Larvae that are born in a contaminated environment and feed on contaminated pollen, drones that are born with physiological defects, are all factors that greatly affect the viability of a hive. In field inspections, we find hives that are apparently healthy but with a very low brood cell survival rate, and which present variability in this rate within the same hive. It is within this framework that the experiments of this work are based. The bee larva rests and develops on highly contaminated beeswax, and the beebread on which the larva feeds is kept in these contaminated cells which could in turn contaminate it.

To this end, two experiments were devised to determine whether there is a difference in the level of acaricide contamination in hives which have suffered bee losses and in hives with a low survival rate of operculated brood cell. Some of these acaricides have been recently evaluated as extremely toxic to bees, such as tau-fluvalinate (US EPA's-OPP, 2005) and when its use has not been registered as a veterinary aid in beekeeping (chlorfenvinphos), although it is widely used in some European countries.

## Materials and methods

### Experiment 1

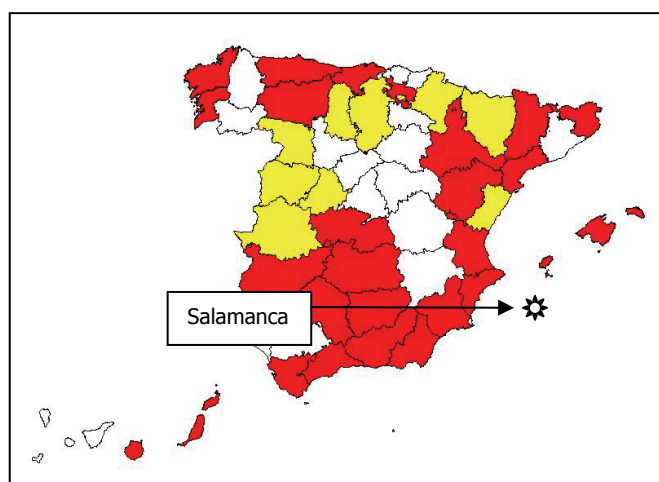
During 2006 and 2007, 36 apiaries located throughout Spain were followed and in each apiary 10 hives were marked and sampled. Samples of each hive were taken in the summer of 2006 and the beekeepers were interviewed to gather their thoughts on bee losses, noting the number of depopulated hives at the end of the study (Spring 2007). We have considered that there were bee losses when the death rate was higher than 30% of the hives. (Fig.1). A sample of beeswax and honey comb containing beebread was taken from each hive. The 10 samples of one apiary were weighed and homogenized before the analysis.

### Experiment 2

In a separate study in Salamanca, one of the areas most affected by bee losses in Spain, 101 hives were followed in five apiaries during the autumn of 2008 (Fig.1). Three of these apiaries were located in an area considered to be problematic by beekeepers (with many houses and people in the surroundings) and two apiaries were located outside this problematic area.

The 101 hives were examined individually and the brood cell survival was calculated following the method of Gomez-Pajuelo *et al.*, (2008). Brood survival was measured by counting the empty cells in representative 10 x 10 cm areas of sealed brood in each hive, classifying high and medium as 10-30% empty cells and low as >30% empty cells.

Four apiaries had hives with high and low survival rates and one apiary had a high survival rate. From the statistical point of view, this presented two groups (problem zone *vs.* no problem zone; and high survival hives *vs.* low survival hives) independent from the group they were located in.



**Fig. 1.** Study on bee losses in Spain. Red areas indicate apiaries without bee losses. Yellow areas indicate apiaries with bee losses (Experiment 1). Salamanca is marked with an asterisk (Experiment 2).

### Sample preparation

A sample of beeswax ( $n = 101$ ) and comb containing beebread was taken from each hive. The beeswax samples were processed individually. The pollen samples were put together according to their origin, thus obtaining four samples of beebread coming from the low survival rate hives and five from the high survival rate hives. In the preparation of beeswax samples we followed the methodology described of Bogdanov *et al.*, (2003), whose method was originally published by Zimmermann *et al.*, (1993) and subsequently modified (Bogdanov *et al.*, 1998). The chromatography by GC Ms/Ms was performed with Varian 3800 and Saturn 2200 equipment (Varian Scientific Equipment; Palo Alto, CA, USA). The chromatograph was fitted with a 30 m x 0.25 mm x 0.39 mm + 5 m EZ-guard CP Factorfour 9012 column from Varian. The column oven temperature program consisted of a 3.50 min at 70°C, an increase of 25°C min<sup>-1</sup> for 10 minutes reaching a temperature of 180°C (total time: 14.4 mins) and an increase of 4°C up to 300°C for 10 mins, maintaining this temperature for 30 mins (total time: 57.90 mins). Injection (10 ml) was performed with a 1079 injector (Varian). The temperature program consisted of a 0.5 min at 70°C, an increase of 100°C min<sup>-1</sup> up to 300°C. We analyzed 70 active ingredients with a LoD (Limit of Detection) of 3.0 - 10.0 mg kg<sup>-1</sup>. For the beebread we followed the method of mini-Luke, modified, (Luke *et al.*, 1975), with the same conditions for the chromatograph described above.

## Results

### Experiment 1

Of the 36 apiaries sampled, nine suffered bee losses at the end of the study with mortality rates of between 30%-80% of the hives (Average: 32.2%). Twenty-seven did not suffer bee losses and had mortality rates between 0%-20% (average: 7.3%). To study the multi-residues in the beeswax, 320 samples were taken and examined from 32 apiaries. Eight corresponded to apiaries with colony collapse and 24 to apiaries without colony collapse.

In beeswax the main components that we found were chlorfenvinphos and tau-fluvalinate. Chlorfenvinphos was detected in 100% of the samples analyzed ( $n = 32$ ) with an average concentration of  $449.28 \mu\text{g kg}^{-1} \pm 708.42 \mu\text{g kg}^{-1}$  (range:  $20.45 \mu\text{g kg}^{-1} - 3182.31 \mu\text{g kg}^{-1}$ ). Tau-fluvalinate was detected in 96.8% of the samples analyzed ( $n = 32$ ) with an average concentration of  $996.49 \mu\text{g kg}^{-1} \pm 2384.37 \mu\text{g kg}^{-1}$  (range:  $<LD \mu\text{g kg}^{-1} - 12978.73 \mu\text{g kg}^{-1}$ ).

Other components detected were bromopropilate, which showed up in 87.9% of the samples with an average concentration of  $14.41 \mu\text{g kg}^{-1} \pm 1.03 \mu\text{g kg}^{-1}$  (range:  $<LD \mu\text{g kg}^{-1} - 22.6 \mu\text{g kg}^{-1}$ ), Coumaphos was detected in 9.3% of the samples ( $n = 32$ ), with an average concentration of  $3.98 \mu\text{g kg}^{-1} \pm 2.85 \mu\text{g kg}^{-1}$  (range:  $<LD \mu\text{g kg}^{-1}$ ).

**Table 1.** Summary of the main acaricides detected in beeswax and beebread.

	<b>beebread (n=32)</b>	<b>beeswax (n=32)</b>
<b>chlorfenvinphos</b>	90.6 % positive samples	100 % positive samples
<b>Mean ±SD</b>	35.9 µg kg <sup>-1</sup> ± 60.86 µg kg <sup>-1</sup>	449.28 µg kg <sup>-1</sup> ± 708.42 µg kg <sup>-1</sup>
<b>Range</b>	(<LD µg kg <sup>-1</sup> – 285.56 µg kg <sup>-1</sup> )	(20.45 µg kg <sup>-1</sup> – 3182.31 µg kg <sup>-1</sup> )
<b>tau-fluvalinate</b>	43.75 % positive samples	96.8 % positive samples
<b>Mean ±SD</b>	200.68 µg kg <sup>-1</sup> ± 563.11 µg kg <sup>-1</sup>	996.49 µg kg <sup>-1</sup> ± 2384.37 µg kg <sup>-1</sup>
<b>Range</b>	(<LD µg kg <sup>-1</sup> – 2273.16 µg kg <sup>-1</sup> )	(<LD µg kg <sup>-1</sup> – 12978.73 µg kg <sup>-1</sup> )
<b>coumaphos</b>	9.3 % positive samples	9.3 % positive samples
<b>Mean ±SD</b>	6.04 µg kg <sup>-1</sup> ± 25.3 µg kg <sup>-1</sup>	3.98 µg kg <sup>-1</sup> ± 2.85 µg kg <sup>-1</sup>
<b>Range</b>	(<LD µg kg <sup>-1</sup> – 130.74 µg kg <sup>-1</sup> )	(<LD µg kg <sup>-1</sup> – 88.55 µg kg <sup>-1</sup> )
<b>bromopropilate</b>	6.25 % positive samples	87.9 % positive samples
<b>Mean ±SD</b>	1.31 µg kg <sup>-1</sup> ± 6.3 µg kg <sup>-1</sup>	14.41 µg kg <sup>-1</sup> ± 1.03 µg kg <sup>-1</sup>
<b>Range</b>	(<LD µg kg <sup>-1</sup> – 35.2 µg kg <sup>-1</sup> )	(<LD µg kg <sup>-1</sup> – 22.6 µg kg <sup>-1</sup> )

**Table 2.** Average concentration of chlorfenvinphos in beeswax in apiaries which showed bee losses compared to apiaries that did not show this. No-significant differences (F=0.652; p=0.426).

	<b>Depopulated apiaries n=8</b>	<b>Non-depopulated apiaries n=24</b>	<b>Total n=32</b>
<b>chlorfenvinphos</b>			
<b>Mean ±SD</b>	625.38 µg kg <sup>-1</sup> ± 1072.71 µg kg <sup>-1</sup>	390.58 µg kg <sup>-1</sup> ± 558.41 µg kg <sup>-1</sup>	449.28 µg kg <sup>-1</sup> ± 708.42 µg kg <sup>-1</sup>
<b>Range</b>	(20.47 µg kg <sup>-1</sup> – 3182.31 µg kg <sup>-1</sup> )	(20.45 µg kg <sup>-1</sup> – 2089.19 µg kg <sup>-1</sup> )	(20.45 µg kg <sup>-1</sup> – 3182.31 µg kg <sup>-1</sup> )

**Table 3.** Average concentration of chlorfenvinphos in beebread in apiaries showing bee losses compared to those which did not show this. Significant differences (F=4.254; p=0.048).

	<b>Depopulated apiaries n=7</b>	<b>Non-depopulated apiaries n=25</b>	<b>Total n=32</b>
<b>chlorfenvinphos</b>			
<b>Mean ±SD</b>	74.62 µg kg <sup>-1</sup> ± 112.46 µg kg <sup>-1</sup>	23.84 µg kg <sup>-1</sup> ± 31.31 µg kg <sup>-1</sup>	34.95 µg kg <sup>-1</sup> ± 60.51 µg kg <sup>-1</sup>
<b>Range</b>	(1.27 µg kg <sup>-1</sup> – 285.56 µg kg <sup>-1</sup> )	(<LD µg kg <sup>-1</sup> – 146.98 µg kg <sup>-1</sup> )	(< LD µg kg <sup>-1</sup> – 285.56 µg kg <sup>-1</sup> )

kg<sup>-1</sup> – 88.55 µg kg<sup>-1</sup>). In one case, malathion was detected (34.4 µg kg<sup>-1</sup>) (Table 1).

In the case of chlorfenvinphos, it is interesting to note that the samples of beeswax coming from depopulated hives had higher concentrations of this component than the non-depopulated hives (625.38 µg kg<sup>-1</sup> vs. 390.58 µg kg<sup>-1</sup>) although the differences were not significant (F = 0.652; p = 0.426) (Table 2). 320 samples from 32 apiaries were processed to find the multi-residues in beebread. Seven corresponded to apiaries with colony collapse and 25 corresponded to non-depopulated apiaries.

For beebread the situation is more complicated and quite a few components were detected (Table 1). The acaricides detected were chlorfenvinphos, tau-fluvalinate, bromopropilate and coumaphos. Chlorfenvinphos was the most common active ingredient, being detected in 90.6% of the samples analyzed (n = 32) with an average

concentration of 35.9 µg kg<sup>-1</sup> ± 60.86 µg kg<sup>-1</sup> (range: <LD µg kg<sup>-1</sup> – 285.56 µg kg<sup>-1</sup>). As with chlorfenvinphos, the samples from the depopulated apiaries showed higher concentrations of this compound as compared to the non-depopulated apiaries (74.62 µg kg<sup>-1</sup> vs. 23.84 µg kg<sup>-1</sup>) and there were significant differences in these averages (F = 4.254; p = 0.048) (Table 3).

Nineteen samples (59.4%, n = 32) of beebread had detectable levels of other agricultural pesticides; in total 16 active principles were detected, with the most frequent being formothion (5 - 24 µg kg<sup>-1</sup>; n=7), acephate (9 - 17 µg kg<sup>-1</sup>; n=5), dimethoate (12 - 34 µg kg<sup>-1</sup>; n=4), terbutylazine (12 - 51 µg kg<sup>-1</sup>; n=3), carbaryl (18 - 24 µg kg<sup>-1</sup>; n=3), malathion (5 - 11 µg kg<sup>-1</sup>; n=2); cypermethrin (7 - 13 µg kg<sup>-1</sup>; n=2); fenthion (21 µg kg<sup>-1</sup>; n=1); parathion-ethyl (15 µg kg<sup>-1</sup>; n=1); endosulfan (32 µg kg<sup>-1</sup>; n=1); chlopyrifos-ethyl (11 µg kg<sup>-1</sup>; n=1); deltamethrin (41 µg kg<sup>-1</sup>; n=1); permethrin (4 µg kg<sup>-1</sup>; n=1); captan

**Table 4a.** Experiment 2 (Salamanca). Average concentration of chlorfenvinphos and tau-fluvalinate in beeswax in apiaries located in the problem and non-problem area. No-significant differences ( $F=0.070$ ;  $p=0.792$ ). No-significant differences ( $F=0.738$ ;  $p=0.392$ ).

Salamanca	Problem area n=57	Non-problem area n=44	Total n=101
<b>chlorfenvinphos</b>			100 % positive samples
<b>Mean <math>\pm</math>SD</b>	1062.23 $\mu\text{g kg}^{-1} \pm 696.7 \mu\text{g kg}^{-1}$	1111.09 $\mu\text{g kg}^{-1} \pm 1144.69 \mu\text{g kg}^{-1}$	1983.52 $\mu\text{g kg}^{-1} \pm 914.25 \mu\text{g kg}^{-1}$
<b>Range</b>	(60.83 $\mu\text{g kg}^{-1}$ – 2878.25 $\mu\text{g kg}^{-1}$ )	(146.27 $\mu\text{g kg}^{-1}$ – 4752.99 $\mu\text{g kg}^{-1}$ )	(60.83 $\mu\text{g kg}^{-1}$ – 4752.99 $\mu\text{g kg}^{-1}$ )
<b>tau-fluvalinate</b>			97.01 % positive samples
<b>Mean <math>\pm</math>SD</b>	550.39 $\mu\text{g kg}^{-1} \pm 1798.64 \mu\text{g kg}^{-1}$	309.75 $\mu\text{g kg}^{-1} \pm 524.01 \mu\text{g kg}^{-1}$	445.56 $\mu\text{g kg}^{-1} \pm 1394.31 \mu\text{g kg}^{-1}$
<b>Range</b>	(< LD – 13781.17 $\mu\text{g kg}^{-1}$ )	(< LD – 3526.30 $\mu\text{g kg}^{-1}$ )	(< LD – 13781.17 $\mu\text{g kg}^{-1}$ )

**Table 4b.** Experiment 2 (Salamanca). Average concentration of chlorfenvinphos and tau-fluvalinate in beeswax in apiaries with high and low brood cell survival rate. No-significant differences ( $F=0.230$ ;  $p=0.633$ ). No-significant differences ( $F=0.965$ ;  $p=0.328$ ).

Salamanca	Hives with high brood cell survival rate n=54	Hives with low brood cell survival rate n=47	Total n=101
<b>chlorfenvinphos</b>			100% positive samples
<b>Mean <math>\pm</math>SD</b>	1042.67 $\mu\text{g kg}^{-1} \pm 977.95 \mu\text{g kg}^{-1}$	1130.46 $\mu\text{g kg}^{-1} \pm 843.16 \mu\text{g kg}^{-1}$	1083.52 $\mu\text{g kg}^{-1} \pm 914.25 \mu\text{g kg}^{-1}$
<b>Range</b>	(146.27 $\mu\text{g kg}^{-1}$ – 4752.99 $\mu\text{g kg}^{-1}$ )	(60.83 $\mu\text{g kg}^{-1}$ – 4293.40 $\mu\text{g kg}^{-1}$ )	(60.83 $\mu\text{g kg}^{-1}$ – 4752.99 $\mu\text{g kg}^{-1}$ )
<b>tau-fluvalinate</b>			97.01% positive samples
<b>Mean <math>\pm</math>SD</b>	318.39 $\mu\text{g kg}^{-1} \pm 481.24 \mu\text{g kg}^{-1}$	591.67 $\mu\text{g kg}^{-1} \pm 1979.56 \mu\text{g kg}^{-1}$	445.56 $\mu\text{g kg}^{-1} \pm 1394.31 \mu\text{g kg}^{-1}$
<b>Range</b>	(<LD – 3526.3 $\mu\text{g kg}^{-1}$ )	(< LD – 13781.17 $\mu\text{g kg}^{-1}$ )	(< LD – 13781.17 $\mu\text{g kg}^{-1}$ )

**Table 5a.** Experiment 2 (Salamanca). Average concentration of chlorfenvinphos in beebread in apiaries located in the problem and non-problem area. No-significant differences ( $F=0.041$ ;  $p=0.846$ ).

Salamanca	Problem area n=5	Non-problem area n=4	Total n=9
<b>Chlorfenvinphos</b>			
<b>Mean <math>\pm</math>SD</b>	80% positive samples 18.91 $\mu\text{g kg}^{-1} \pm 19.72 \mu\text{g kg}^{-1}$	75% positive samples 16.43 $\mu\text{g kg}^{-1} \pm 16.17 \mu\text{g kg}^{-1}$	17.81 $\mu\text{g kg}^{-1} \pm 17.15 \mu\text{g kg}^{-1}$
<b>Range</b>	(< LD – 51.87 $\mu\text{g kg}^{-1}$ )	(< LD – 38.20 $\mu\text{g kg}^{-1}$ )	(< LD – 51.87 $\mu\text{g kg}^{-1}$ )

**Table 5b.** Experiment 2 (Salamanca). Average concentration of chlorfenvinphos in beebread in apiaries with high and low brood cell survival rate. No-significant differences ( $F=1.335$ ;  $p=0.286$ ).

Salamanca	Hives with high brood cell survival rate n=5	Hives with low brood cell survival rate n=4	Total n=9
<b>Chlorfenvinphos</b>			
<b>Mean <math>\pm</math>SD</b>	80% positive samples 12.02 $\mu\text{g kg}^{-1} \pm 7.65 \mu\text{g kg}^{-1}$	75% positive samples 25.05 $\mu\text{g kg}^{-1} \pm 24.09 \mu\text{g kg}^{-1}$	17.81 $\mu\text{g kg}^{-1} \pm 17.15 \mu\text{g kg}^{-1}$
<b>Range</b>	(< LD – 19.55 $\mu\text{g kg}^{-1}$ )	(< LD – 51.87 $\mu\text{g kg}^{-1}$ )	(< LD – 51.87 $\mu\text{g kg}^{-1}$ )

(17  $\mu\text{g kg}^{-1}$ ; n=1); iprodione (33  $\mu\text{g kg}^{-1}$ ; n=1) and methamidophos (29  $\mu\text{g kg}^{-1}$ ; n=1).

If we consider acaricides and agricultural pesticides together, 100% of the samples showed the presence of some of the contaminants studied, in a cocktail made of up to five different contaminants.

## Experiment 2 (Salamanca)

The study of the beeswax collected in Salamanca showed similar levels of chlorfenvinphos and tau-fluvalinate (Tables 4 a and b) without any differences between the area that the beekeepers considered problematic or non-problematic, or between the hives with high or low survival rate. There was, however, a tendency for greater contamination in hives with bee losses or with low survival rates. In this area, we only found chlorfenvinphos in the beebread. We did not find any significant differences in the amount of chlorfenvinphos between the problematic and non-problematic areas, nor between the high or low brood cell survival rate, although the latter group had twice the amount of contamination (Tables 5 a and b).

## Discussion

Bee larvae develop on beeswax that is highly contaminated by residues of acaricides used in the fight against *V. destructor*, with each country having specific problems according to the acaricides used (Chauzat and Faucon, 2007). We have learnt in Spain that the bee larvae are fed on pollen bread which contains between one and five active ingredients at different concentrations, not only of acaricides but also of agricultural pesticides. The levels of acaricide contamination in beebread are lower than those found in beeswax, and probably the contamination reaches this pollen bread by the transfer of the contaminants to the fatty part of the pollen. The pollen becomes more or less contaminated probably as a function of its botanical origin or its fat content.

Chlorfenvinphos is an acaricide which is being used in Spain, Portugal, France and Italy. Together with tau-fluvalinate, this is the acaricide which is being detected more and more in Spanish beeswax and pollen bread. We have not found studies on the effects of chlorfenvinphos on the bee larvae, but it is an organophosphorous insecticide like coumaphos, whose adverse effects on bees at different levels have been studied by: Fell and Tignor (2001); Haarmann *et al.*, (2002); Wallner (1999); Pettis *et al.*, (2004); and Burley and Saake (2008).

Many of the acaricides and pesticides detected in this study have had toxicity studies made before registration. There are, however, few studies on toxicity through prolonged exposure to the bees, nor of toxicity on larvae, or how these larvae develop when they have been brought up on highly contaminated beeswax. We, therefore, know

little of the synergy between the different pesticides that appear together. Tau-fluvalinate has recently been considered to be highly toxic to bees (US EPAS's-OPP, 2005; Frazier *et al.*, 2008). The possible impacts of acaricides and agricultural pesticides and cocktails of these in beebread fed to larval bees should therefore be studied.

As we have seen, beeswax is contaminated with a random and variable range of compounds, which confers on this problem a large random factor (chance), similar to the situation of bee losses in Europe or to "Colony Collapse Disorder" (CCD) in the USA. In order to understand the current phenomena of bee losses, it is necessary to be informed about the impact of pesticides on the bees and their environment. The results of this study reveal a clear relation between the presence of acaricides and low survival rates of the brood cell. The effects of pesticides and acaricides studied by the various authors quoted have an unquestionable relevance to the study of current bee losses. This could be an important factor in the worsening of the bee loss problem during periods of bad weather, when this symptom has most been noted in Spain (Gómez-Pajuelo *et al.*, 2008). We are seeing that bee losses are a problem of adult bees that disappear from the colony. However, do these adult bees always die in the countryside (through age, pesticides, etc.), or should we look at this problem from another perspective? The fact remains that we do not have an adequate renewal rate of the hive.

Stevenson (1978) tested the oral and contact toxicity of chlorfenvinphos to worker honey bees. The oral and contact LD<sub>50</sub> values were 0.55 and 4.1  $\mu\text{g}/\text{bee}$  classifying chlorfenvinphos as highly and moderately toxic, respectively. For concentrations found in beeswax and referring to the bibliography on a lethal dose of 50% by contact, (Stevenson, 1978) beeswax that contained above 2.4 mg/kg of chlorfenvinphos could present problems for the brood. 10.65% of Spanish waxes present this concentration. Closely related to beeswax contamination is the contamination of beebread, in contact with the beeswax. See the bibliography on the consumption of pollen by the larvae and the adult bees, (Babendreier *et al.*, 2004; Keller *et al.*, 2005) and oral LD<sub>50</sub> (Chauzat and Faucon, 2007; Frazier *et al.*, 2008; Stevenson, 1978). For all of the pesticides found, however, a bee would have to live and consume the pollen for a period of more than a year to reach the estimated oral LD<sub>50</sub>. However, for the acaricides tau-fluvalinate and chlorfenvinphos the LD<sub>50</sub> can be reached in less than 90 days and 22 days respectively for the maximum concentrations found.

Similar conclusions were reached by vanEngelsdorp *et al.*, (2009 a) in what they called "entombed pollen" which is a new condition in honey bee colonies associated with increased risk of colony mortality. Undoubtedly the old pollen that remains stored in the hive for a long time is likely to absorb and concentrate larger amounts of contaminants. vanEngelsdorp *et al.*, (2009 b), did not find differences in the contaminants found in the beeswax of colonies suffering from CCD or controls, but they did find high concentrations of the

acaricides tau-fluvalinate ( $41737 \mu\text{g kg}^{-1} \pm 23748 \mu\text{g kg}^{-1}$ ) and coumaphos ( $6398 \mu\text{g kg}^{-1} \pm 1815 \mu\text{g kg}^{-1}$ ). They did, however, find much higher concentrations of tau-fluvalinate and coumaphos in the beebread of apiaries suffering from CCD compared to control apiaries. For example, for coumaphos they found  $18.5 \mu\text{g kg}^{-1} \pm 7.7 \mu\text{g kg}^{-1}$  vs.  $3.6 \mu\text{g kg}^{-1} \pm 3.6 \mu\text{g kg}^{-1}$  in CCD and control apiaries respectively, and for tau-fluvalinate  $276 \mu\text{g kg}^{-1} \pm 162.5 \mu\text{g kg}^{-1}$  vs.  $68 \mu\text{g kg}^{-1} \pm 56 \mu\text{g kg}^{-1}$  in CCD and control apiaries respectively (vanEngelsdorp *et al.*, 2009 b).

We must wonder why acaricides and agricultural pesticides accumulated in beeswax have now become a problem for the survival of the brood cell, but were not before. The clearest answer lies in the dramatic change in the commercial use of beeswax. Currently most of the market for beeswax comes from the same beekeepers, as the beeswax is recycled in a closed circuit. And from the coming of *V. destructor*, with the consequent treatment by acaricides (most of them lipophilic), these then accumulate in this closed circuit. And after 22 years of fighting the parasitic mite (in the case of Spain) we are reaching high levels of contamination in beeswax without knowing the real impact of all of its effects on the hive.

The beeswax industry needs to decontaminate beeswax before it goes back into the circuit. In the laboratory, filtration of the beeswax through non-discolouring diatomaceous earth and active carbon are giving good results (Orantes Bermejo, 2010).

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