Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/sjar

Spanish Journal of Agricultural Research 2007 5(4), 474-480
ISSN: 1695-971-X

Short term negative effect of oxalic acid in *Apis mellifera iberiensis*

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Abstract

The toxic effect of oxalic acid (OA) on *Apis mellifera iberiensis* was studied using field and laboratory assays. Bee deaths were higher in OA treated hives than in control hives. Pathological repercussions of topical application of 10% OA were observed in different internal honeybee organs. After 24 h, there were severe alterations in the ventricular epithelial layer while by 48 h there was clearly seen degeneration of the rectal epithelium. Irreversible lesions appeared at 48 h in different bee organs with increased cellular damage after 72 h. Indications are that the effect of the OA continues after initial contact and causes permanent lesions in digestive and excretory organs. Tissue distribution of the acid in different bee organs, after topical administration, suggests that some of the acid is ingested, in some way, by the bee.

Additional key words: honeybee, insect toxicity, internal lesions.

Resumen

Efectos negativos a corto plazo causados por el ácido oxálico en *Apis mellifera iberiensis*

En este trabajo se demuestra el efecto tóxico de la aplicación de ácido oxálico (AO) en *Apis mellifera iberiensis* en ensayos de campo y de laboratorio. La mortalidad de las abejas fue mayor en las colmenas tratadas frente a los grupos controles. Se observaron lesiones patológicas en diferentes órganos internos tras la administración tópica de AO al 10%. A las 24 h de la administración, las lesiones fueron graves en el epitelio del ventrículo, mientras que a las 48 h también pudo observarse la degeneración epitelial en el recto. El efecto tóxico del AO continúa tras el contacto inicial y es capaz de producir lesiones permanentes en el aparato digestivo y excretor. La distribución tisular del AO tras la aplicación tópica señala que parte se ingiere por la abeja, quizás en el proceso de autolimpieza.

Palabras clave adicionales: abejas melíferas, lesiones internas, toxicidad.

Introduction

Oxalic acid (OA, $H_2C_2O_4$) is an organic acid with known acaricidal properties and is present in different plant genera such as *Rumex*, *Oxalis* and *Tamarindus*. It has recently been shown to act against ticks (Chungsamarnyart and Jansawan, 2001). However, most studies have focussed on its use for the control of varroosis in *Apis mellifera* L. (Mutinelli *et al.,* 1997; Milani, 2001). Most tests have shown its high efficacy and a good bee tolerance (Imdorf *et al*., 1997). A negative long-term effect of OA has been described (Higes *et al*., 1999) and, although acute toxicity has not been proved, there are several reports that a high OA concentration, administered in autumn by trickling, weakens the colony with a corresponding negative effect in the following spring (Charriere and Imdorf, 1999; Nanetti, 2001). These negative effects seem to appear when the most effective solution of 4.2% OA is used, but 3.6% OA is also not well tolerated by bees when 50 mL are trickled on. It seems that at higher concentrations, the high acidity affects bees soaked with the solution (Anchling, 2001), although some authors did not find

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Received: 15-11-07; Accepted: 22-10-07.

more dead bees post treatment in bee traps (Arculeo, 2000; Gregorc and Planinc, 2001) or by direct observation (Mariani *et al.,* 2002).

The aim of this work was to study the effects of OA on adult Spanish bees (*Apis mellifera iberiensis)* in a controlled field trial, to determine the LD_{50} and histologically study alterations present in structures and tissues.

Material and Methods

Controlled field trial

Thirty homogeneous hives were randomly assigned into three groups of ten. They were located at the experimental apiary of the *Centro Apícola Regional*, in Marchamalo, Guadalajara, Spain. The site is in the centre of the Iberian Peninsula (at an altitude of 680 m, with a warm Mediterranean climate). One group (G_1) was treated with 3 g of OA in two applications of 1.5 g of OA with a 3-week interval, in autumn, during the broodless period (the first treatment was applied in mid October). All colonies had 8-9 frames covered by bees. The treatments were carried out by trickling 50 mL of a freshly prepared OA solution per hive. The solution was prepared by dissolving the acid in a 50% water/sugar solution (w/w). Hives of one control group (G_2) were treated twice with Perizin® (coumaphos), following the manufacturer's recommendations. The second application in the G_1 and G_2 groups was applied at the same time. The third group, which received no treatment, was the control for natural deaths (G_3) of bees and varroa mites. Fallen mites were collected and counted three times a week, to evaluate acaricide effectiveness, and for two weeks prior to the first application. To determine the number of surviving mites after treatment, all colonies (including the controls) were treated with fluvalinate (Apistan®) three weeks after the second applications of the chemicals. One month after, the strips were removed, and an additional treatment with coumaphos (Perizin®) was applied to all hives, with the exception of the G_2 hives, which received no additional treatment. Treatment efficacy (E) was estimated using the following formula (Higes *et al.,* 1999):

$$E = (V_{OA}/ V_T) \times 100,$$

where V_{OA} = Varroa mites killed by OA treatment; V_T = = Total varroa mites killed by OA and control treatments (with Apistan® and Perizin®).

The bee deaths in the treatments were recorded at the hive entrance at least twice weekly (a total of 19 determinations) by hive entrance traps (Perez *et al.,* 2001). Results were compared with bee deaths in the untreated group (G3). Mean bee deaths in all three groups were compared by a Kruskal-Wallis test (Significance level $\alpha = 0.05$) using SPSS 11.5 software program (SPSS Inc., Chicago ILL, USA).

Laboratory assays

European and Mediterranean Plant Protection Organization (Anonymous, 1992) and the United States Environmental Protection Agency for Prevention, Pesticides and Toxic Substances (Anonymous, 2002) recommendations were followed.

In all trials, uniform adult worker bees (*A. mellifera iberiensis*) were used. They were picked from no-brood frames at the *Centro Apícola Regional* apiary (Marchamalo, Spain). Bees were collected in late spring at 9 a.m. by brushing them into specially prepared cardboard boxes and were taken immediately to the laboratory. The bees were immobilized with carbon dioxide for easier handling and were randomly assigned to different groups. After product application they were placed in metal cages.

LD_{50} determination

A 20% OA (Panreac) + 1% Tween® 20 (Panreac) solution (w/w) was applied at 37 ± 2°C to anaesthetized adult worker bees. Dosages of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µL were administered on the thorax and abdomen of each bee using a micropipette. The volumes used correspond to 0, 0.22, 0.44, 0.66, 0.88, 1.10 and 1.32 mg of OA bee^{-1} respectively. Three µL of the solution, without OA, was applied to control bees at the same temperature. There were three replicates of 12 bees $dose^{-1}$. During the assay, the bees were kept in an incubator at 25 + 0.36°C and at a relative humidity of 87.4 ± 5.9%. They were given syrup in water ad lib, which was renewed daily. Bees were examined at 24, 48 and 72 h. Dead bees were counted and removed.

Pathological study

Ten groups of 12 bees were caged separately: five of them for a test-time sequential study and the other

five as controls for each sequential group. Bees from the test-time study were given a sublethal dose of 10% (w/w) OA in solvent (water and 1% Tween® 20) by topical application of a 3 µL drop on the thorax and 3 µL on the abdomen (equivalent to 0.66 mg OA bee^{-1}). Control bees received the same volume of solvent. The 10% dilution and dose selection were based on previous studies, close to LD_{50} values, to produce lesions on at least 50% of the bees. Control and test groups were supplied daily with fresh 50% sugar/water solution *ad lib* that was changed daily. Test and control groups were killed 6, 12, 24, 48 and 72 h after topical treatment. Dead or immobile bees were not included in the pathological study. Bees were killed with ethyl acetate. After death, they were examined for external alterations and complete dissections were done on all, except one bee. Samples taken were: hypopharyngeal glands, ventriculus, Malpighian tubules, rectal and thoracic cuticle. Samples were fixed overnight in Bouin's fluid and then placed in 40% ethanol. Previous studies had shown that other fixatives (e.g. buffered 10% formalin) were not suitable for these insect tissues. Once fixed, samples were washed twice in distilled water and embedded in paraffin wax and cut into 4 µm thick sections and stained following routine techniques such as haematoxylin-eosin, Masson's trichromic and Schiff periodic acid. Finally, sampled were dehydrated and mounted. One bee from each group was routinely prepared for examination by SEM at the Electron Microscopy Service of the Veterinary Faculty, Madrid.

Tissue distribution of OA

Six groups of 12 bees were used to measure the amount of OA in different bee organs after topical administration. A batch of three groups was dosed topically with a 20% OA (w/w) solution diluted in ddH_2O + 1%Tween® 20, administered as a 3 mL drop application on the thorax and 3 µL on the abdomen (equivalent to 1,320 µg a.i. bee^{-1}). Another batch of three groups were controls and received only solvent. The OA solution was heated at 37ºC to avoid crystallization at the moment of application. Control and test groups were supplied daily with fresh sucrose solution as described above.

A group of bees from each batch was killed with ethyl acetate after 24, 48 and 72 h post treatment, decapitated to obtain the haemolymph, the Malpighian tubules, the ventriculus and the rectum. The OA concentration was determined by the method of Nozal *et al*. (2003) with a detection limit of 0.05 mg L^{-1} and a determination limit of 0.18 mg L^{-1}, after dividing the final sample concentration and the number of bee organs included.

Results

Controlled field trial

Good efficacy (Table 1) was obtained with OA (> 80%) although it was slightly lower than the reference coumaphos (96%). The honeybee deaths in the field assays are shown in Figure 1. The mean number of dead bees (Table 2) was significantly higher in the group treated with OA than in the two control groups ($p < 0.05$). Bee deaths in hives treated with Perizin® were significantly higher ($p < 0.05$) than in untreated hives (natural deaths) (Fig. 1) but was significantly lower than in the OA group ($p < 0.05$).

LD_{50} determination

At 24 h, the LD_{50} was 0.62 mg bee^{-1} (fiducial limits 0.57-0.70). Results at 48 h were similar (0.53 mg bee^{-1}; fiducial limits 0.48-0.62), and at 72 h (0.53 mg bee^{-1}; fiducial limits 0.48-0.57). The 72 hour LD_{50} was the most reliable due to the narrower fiducial limits.

Table 1. Efficacy of oxalic acid treatment and control. Fallen varroa in group G3 are also indicated at the same time, coinciding with the 1st and 2nd applications in treated groups for comparison

Treatment	Mean daily varroa fall before treatment	Varroa fall		Total varroa fall	Efficacy
		First application	Second application		
G_1-Twice 1.5 g of oxalic acid	27 ± 19	1,422 ± 307	982 ± 786	2,913 ± 1,129	84.6 ± 11.1
G_2-Coumaphos	58 ± 5	1,911 ± 642	403 ± 34	2,391 ± 435	96.6 ± 1.2
G_3-Control	15 ± 11	452 ± 362	552 ± 370	4,419 ± 2,335	21.8 ± 4.8

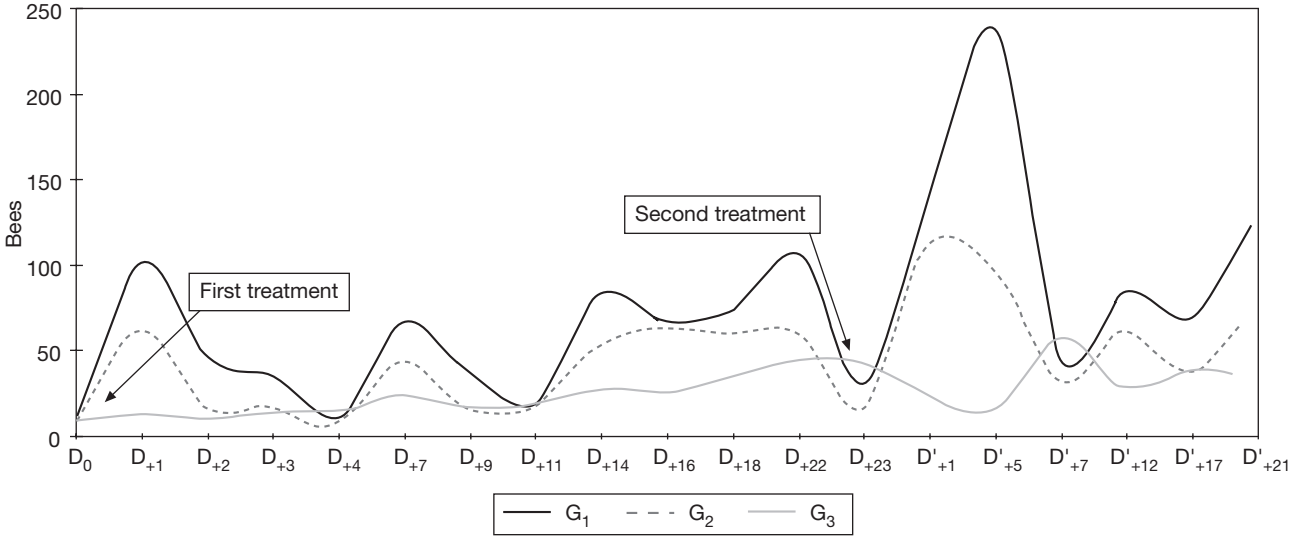

Figure 1. Bee deaths in field assays. G_1:two applications of 1.5 g of oxalic acid. G_2: two treatments with coumaphos. G_3: control (natural deaths). D_0 = day of 1st application. D_{+23} = day of 2nd application (23 d after first application). D' = days after 2nd application.

Pathological results

Images from the SEM did not show any change in the external surface of treated bees although a white deposit could be seen on test bees (Fig. 2). Gross lesions were not observed on external structures or internal organs. There was higher fragility while dissecting the digestive tube and the Malpighian tubes in bees killed after 48 and 72 h but not in corresponding control bees.

Pathological changes were present in epithelial layers covering the ventriculus, the rectum and the Malpighian tubules of all bees killed 24 h post dosing. Lesions were more severe in bees killed at 48 h and even more severe in the 72 h group. No alterations were seen in control bees. Some variations were seen in control and test bee hypopharyngeal glands. This was probably due to technical problems and therefore is not included in this study.

At the microscopic level, the epithelia of the ventriculus appeared to have a degree of hydropic degeneration including the presence of multiple vacuoles around the nucleus in bees killed at 24 h after treatment (Fig. 3B). Cytoplasmatic structures were altered after 48 h especially in the regeneration crypts (Fig. 3C). At 72 h, the mucous layer was severely injured. There were wide denuded areas or zones a few cells with a picnotic nucleus could be seen (Fig. 3D).

Cells from control bees had an eosinophilic granular cytoplasm without vacuoles (Fig. 4A). Lesions in the rectum at 24 h after treatment were similar to those in the ventriculus but were to a lesser degree (Fig. 4B). Mucous cells had slight hydropic degeneration with cytoplasmatic tumefaction due to small vacuoles. At 48 h more severe cellular damage was confirmed by the dilution of organelles. Part of them could be seen close to the nucleus, which usually appeared in the basal area of the cell (Fig. 4C). Seventy two hours after OA administration cells had a polygonal morphology, the cytoplasm

Table 2. Daily bee deaths before and after treatment (mean ± sd)

Treatment	Bee deaths	
	Before treatment	After treatment
G_1	10 ± 4.03	30.1 ± 10.6
G_2	7 ± 5.6	18.2 ± 7.4
G_3	8 ± 4.9	10.2 ± 6.8

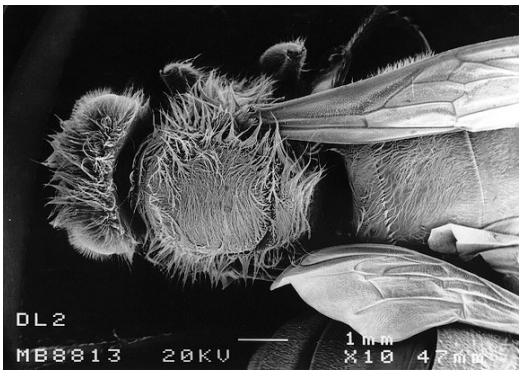

Figure 2. Absence of superficial lesions in a bee treated with topical 10% oxalic acid (SEM image).

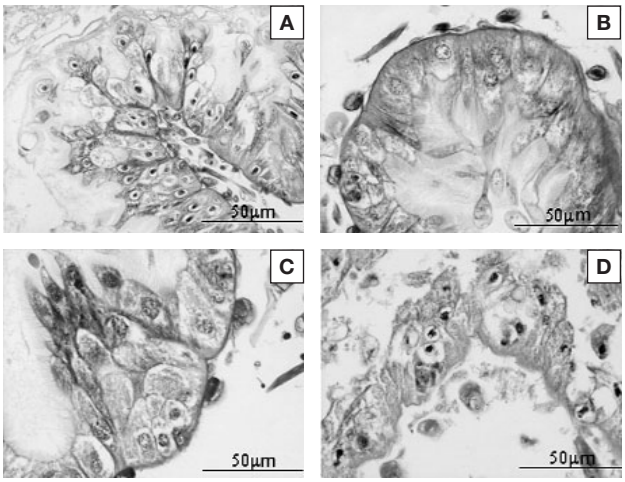

Figure 3. Microscope images of the epithelial layer of the ventriculus of a control bee (A) and at 24 h (B), 48 h (C) and 72 h (D) after treatment. Stain: haematoxylin and eosin.

was optically empty and the nucleus picnotic displaced to the basal area («ballooning cell»), which showed the maximum level of alteration (Fig. 4D).

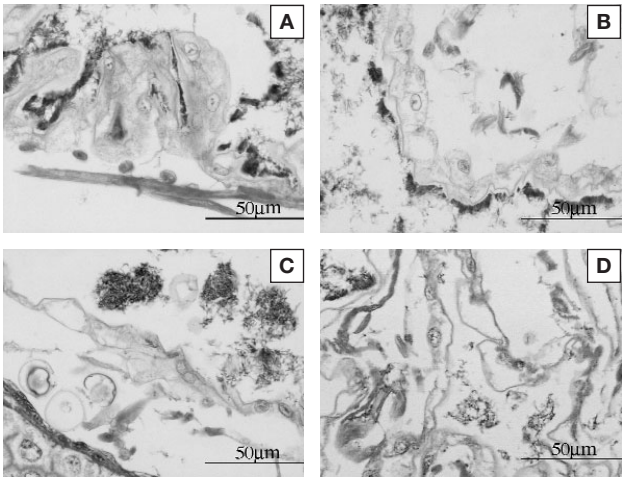

Figure 4. Microscope images of the epithelial layer of the rectum of a control bee (A) and at 24 h (B), 48 h (C) and 72 h (D) after treatment. Stain: haematoxylin and eosin.

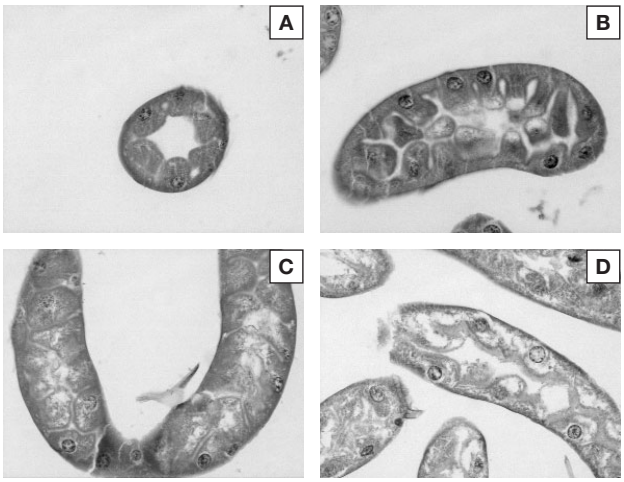

Figure 5. Microscope images of the epithelial layer of the Malpighian tubules of a control bee (A) and at 24 h (B), 48 h (C) and 72 h (D) after treatment. Stain: haematoxylin and eosin.

Malpighian tubules were also affected from 24 h. Initially a small supranuclear vacuole could be seen while the cytoplasm was similar to that of control bees (Figs. 5A and 5B). At 48 h, the cytoplasm appeared tumefacted (Fig. 5C), but it was not until 72 h that several small vacuoles in the cytoplasm and picnotic nuclei were present (Fig. 5D).

Tissue distribution

The OA concentrations in different organs are shown in Table 3. Death rates are also given. After topical administration of 20% OA, high bee deaths were observed (64.3%). The OA concentration in different organs was higher at 24 h than later.

The relationship between the OA administered and OA detected in bees is expressed as a percentage of the total equivalent active ingredient administered (Table 4). The highest relative concentration was at 24 h in all organs, mainly in the rectum after 24 h while

Table 3. Oxalic acid (OA) concentration in bee organs (µg honeybee^{-1}) and mortality rates (MR) in treated (T)[1] and control (C)[2] groups at 24, 48 and 72 h

	Haemolymph		Malpighian tub		Ventriculus		Rectum		MR	
	T	C	T	C	T	C	T	C	T	C
24 h	0.074	0.005	0.076	0.005	0.075	0.001	0.384	0.006	64.3%	0%
48 h	0.026	0.004	0.003	0.002	0.003	0.001	0.003	0.004	0%	0%
72 h	0.024	< 0.001	0.009	0.001	0.006	< 0.001	0.017	0.004	0%	2.2%

[1] Contact administration of 1,320 µg OA bee^{-1}, administered as 3 µL on the thorax and abdomen of a 20% OA solution (N = 36).
[2] Treated only with solvent (N = 36).

Table 4. Percentage of the total dose of oxalic acid administered in bee organs following topical administration

	Hemolymph	Malpighian tubules	Ventriculus	Rectum
24 h	0.006	0.006	0.006	0.029
48 h	0.002	< 0.001	< 0.001	< 0.001
72 h	0.002	0.001	< 0.001	0.001

the haemolymph showed a continuing high concentration of OA at 48 and 72 h. Minute amounts of OA were detected in control bee organs, which degraded over time.

Discussion

This study reports an acute toxicity of OA to bees. Administration of coumaphos and OA to hives increased bee deaths over the natural rate of death. However, in the group of bees treated with OA the number of dead bees was higher than in the group treated with the commercial product. Higher deaths after the second treatment could be due to lower temperatures because the proximity of winter.

This study also demonstrated the pathological effects of administration of OA to different internal organs of *A. mellifera iberiensis* organs. The SEM images and histological observations of the cuticle showed an absence of lesions after application of OA. Epithelial degeneration seemed to intensify as time went by and could be the origin of metabolic malfunction causing bee deaths observed in the field assays. Although the conditions of the field and laboratory assays were not the same, the existence of a degree of insecticidal effect of OA on Spanish bees, in both situations, was demonstrated. This agrees with field observations of bee deaths and negative effects on treated hives. Variation in the methodology could explain the different results found in the physiological OA effects on pupae and newly emerged bees through valuation of glucation S-transferase activity (Brodsgaard *et al.,*1999).

The increased cellular damage over time leads to the assumption that the effect of OA continues after initial contact. Pathological changes observed after 48 h were severe and irreversible; this implies that OA can cause permanent lesions perhaps dose and time related. As recommended field doses of OA are much lower than those used in here, this possible negative effect could be delayed over time. This would explain the absence of reports of acute bee mortality (Charriere and Imdorf, 1999; Arculeo, 2000; Gregorc and Planinc, 2001; Mariani *et al.*, 2002) and could explain the negative long-term effects (Higes *et al.*, 1999). Gregorc *et al.* (2004) support these results. They found a long term effect of administration of OA to larval digestive cells, cell death appeared after 50 h of acid nebulization of healthy larvae. The use of standard modified traps could have also helped to detect previously underestimated bee deaths.

The discrepancy in toxicity of OA observed by different research groups could be due to the diverse bee breeds being employed as was recently cited in relation to drug repercussions (Suchail *et al.*, 2000). The 48 h-LD_{50} value obtained was slightly higher than that recently reported by Aliano *et al.* (2006). However, in their work the OA was dissolved in acetone and the different solvent used could have influenced OA absortion.

High bee deaths in the tissue distribution assay at 24 h after topical administration of 20% OA was probably due to the elevated OA concentration used. The concentration was selected to obtain a very high dose (1,320 µg a.i. bee^{-1}) to ensure detection of low levels of the acid in all organs. In fact, these traces were detected. The extremely high acidity of the solution could explain the acute deaths, a process similar to the mode of OA action against *Varroa* (Nanetti, 2001).

The similar level of OA in the ventriculus and in the haemolymph or Malpighian tubes after 24 h of topical dosing seems to indicate that part of the OA is ingested, in some way, by the bees, probably during grooming behaviour. Localization of OA in abdominal internal organs after topical administration was demonstrated by Nanetti (2001) after application of a ^{14}C marked OA solution, although maximum activity was recorded two days after treatment. Perhaps the absence of a high amount of OA in the rectum at 48 h in this study could be due to bee defecation, indicated by the low value obtained.

The presence of OA in honeybee internal organs was also shown by Nozal *et al*. (2003). The concentration was higher in the first hour in the digestive tract, in the Malpighian tubules and in the haemolymph, demonstrating honeybee acid ingestion. The detection of small amounts of OA in the organs of control bees could indicate vestiges of OA naturally present in regional honeys which can vary from 21.0 to 239 mg kg^{-1} of honey (Nozal *et al*., 2003).

Although field conditions are not quite the same as in this study, a degree of insecticidal effect of OA has

been demonstrated on Spanish bees. This agrees with previous empirical observations of bee deaths and negative effects on treated hives and puts a constraint on its use as a pesticide.

Acknowledgments

This research was carried out with the financial support from the Spanish *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria* (INIA, Programa API nº 98-012). The authors thank P. Anguitar, E. Díez and A. Sanz for technical help.

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