ACUTE CONTACT TOXICITY TEST OF OXALIC ACID ON HONEYBEES IN THE SOUTHWESTERN ZONE OF URUGUAY

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This work studies the acute contact toxicity of oxalic acid (OA) on a honeybee polyhybrid subspecies (*Apis mellifera*), which is the dominant biotype in southwestern zone of Uruguay (SWZU) and the country’s most important honey-producing region. We determined the mean lethal dose (LD50), as well as the no observed effect level (NOEL) and the lowest observed effect level (LOEL) values. We also estimated the total number of honeybees per hive in the test area. The aim was to assess the relationship between the maximum OA dose used in Uruguay (3.1 g OA per hive) and the toxicological parameters of honeybees from SWZU. The current dose of 3.1 g OA per hive corresponds to 132.8 µg OA per honeybee; our results indicate that the current dose could be increased to 9.3 g OA per hive. The results also highlight some differences between the LD50 value in SWZU honeybees (548.95 µg OA per honeybee) and some published LD50 values for other honeybee subspecies.

Key words: LD50, Varroa destructor, Uruguay.

Beekeeping is an economically important agricultural activity in Uruguay with annual exports of honey contributing more than 0.5% of gross domestic product (approximately US$28 million) according to DIEA (2009) statistics. This percentage could be increased if the various problems that adversely affect honey production in Uruguay were addressed (Mendoza et al., 2008). Currently, the main biological factor harming honey production in Uruguay is the prevalence of varroosis (the infection of hives caused by the mite *Varroa destructor*) (Mendoza et al., 2008). However, efforts to control mites with synthetic miticides (normally with xenobiotic characteristics) have led to two additional problems, that is, an increase in the risk of selecting resistant *V. destructor* strains (Lodesani et al., 1995; Elzen et al., 2000; Thompson et al., 2002; Pettis, 2004) and an increase in the risk of hive products by miticides (e.g., coumaphos) (Wallner, 1999; Tremolada et al., 2004). More specifically, the first problem has led to a progressive decrease in the efficacy of synthetic acaricides to control resistant *V. destructor* strains.

For these reasons, the study of chemical compounds with miticidal characteristics has become relevant, particularly those chemical compounds normally found in hives or essential oils, such as oxalic acid (OA) (Prandin et al., 2001; Gregorc and Planinc, 2001; 2002; Marcangeli et al., 2003, 2000; Diniz et al., 2003; Nanetti et al., 2003; Marcangeli et al., 2003, 2000; Marcangeli and García, 2004, 2006; Rademacher and Harz, 2006; Bacandritsos et al., 2007), formic acid (Calderone, 2000; Bogdanov et al., 2002; Eguaras et al., 2003), and thymol (Imdorf et al., 1995). This type of compounds do not pollute a hive’s products (Bogdanov et al., 2002) or produce resistant *V. destructor* strains. As a result, OA is one of the chemicals most often used as a complementary miticide (Prandin et al., 2001; Gregorc and Planinc, 2001; 2002; Nanetti et al., 2003; Marcangeli et al., 2003, 2000; Marcangeli and García, 2004, 2006; Rademacher and Harz, 2006; Bacandritsos et al., 2007). Nevertheless, although some studies have reported the toxic activity of OA against *V. destructor*, few have examined its toxic effect on honeybees (Gregorc and Planinc, 2002) because it is normally assumed that the doses used are of low toxicity (Marcangeli et al., 2003; Marcangeli and García, 2004). Furthermore, scientific advice about using OA is based on studies of *Apis mellifera mellifera* rather than on the honeybee polyhybrid subspecies (Diniz et al., 2003) found in southwestern zone of Uruguay (SWZU) (Figure 1). It is necessary to determine the acceptable dose of OA for the dominant SWZU polyhybrid subspecies because the acute toxicological response can differ between species (Suchail et al., 2000). The suggested OA dose is estimated per hive and based on a certain number of honeybees; however, these factors may be different in SWZU because of the local environmental conditions and the genetic mixture of *Apis mellifera scutellata* (Fellow and Bertram, 2002; Diniz et al., 2003; Carrasco-Letelier et al., 2012). There is also a lack of agreement between OA doses used and suggested in Uruguay (Campá et al., 2007; Ramallo et al., 2008) and the doses recommended...
in other studies, such as those by Aliano et al. (2006) and Martín-Hernández et al. (2007). In Uruguay, the OA dose is normally applied by trickling 5 mL per bee space (30-50 mL per hive) in a sucrose solution 1:1; a dose of 62.6 g L⁻¹ (Campá et al., 2007) is administered in summer with brood as a strategy to reduce the varroa population, avoid the use of synthetic miticides, and allow a better performance of synthetic miticides in autumn. This is similar to the practice in Argentina, country from which the OA formulation Oxavar® (Apilab, Buenos Aires, Argentina) is imported (Campá et al., 2007).

Our aim in this study was to determine the upper limit of the OA dose that could be used on the dominant polyhybrid subspecies in SWZU without generating toxic problems for this type of honeybee. We determined a lethal dose (LD₅₀) value for OA with a 48-h bioassay with newborn bees of the SWZU biotype; we also determined the no observed effect level (NOEL), the lowest observed effect level (LOEL), number of bees per hive, and the data required to define an adequate dose per hive.

**MATERIALS AND METHODS**

The honeybee we used in this study belongs to the polyhybrid subspecies of *A. mellifera* from SWZU. Bees were obtained from experimental apiaries kept by the Beekeeping Unit of the Experimental Station Alberto Boerger, INIA La Estanzuela, Colonia, Uruguay (34°20′22.20″ S, 57°41′14.93″ W). The honeybees in the bioassays were newborn bees (aged 1-7 d) from hive frames isolated with plastic mesh bags (square cells, 1 x 1 mm) in the hives with no treatment against varroosis. The honeybees were closely monitored after treatment and then observed for mortality and signs of intoxication after 48 h.

The bioassay of acute toxicity was developed in accordance with criteria from the United States Environmental Protection Agency (US EPA, 1996): 48 h in the dark, 60% humidity, and temperature-controlled (25 °C) conditions. Five doses of OA (200, 400, 600, 800, and 1000 µg OA per bee, all with a diluent of sucrose solution 1:1 v/v) were tested in the bioassay with five replicates for each one. Each replicate consisted of 10 honeybees. Each dose of OA (C₂H₂O₄·2H₂O, Biopack, Buenos Aires, Argentina) was applied on the honeybee’s thorax with a micropipette. Five hives were randomly selected from an apiary of 50 hives; two honeybees were taken from each selected hive to make up the group of 10 for each replicate. The formation of each group and the thoracic dose were carried out by anesthetizing the honeybees with CO₂ (g) (US EPA, 1996). Each group of 10 honeybees was kept in a glass Petri dish (I.D. 10 cm) lined with clean filter paper and containing a feeder with 1 mL sucrose 50% w/v for *ad libitum* consumption. A procedure similar to the one described above was carried out for the control treatment with five replicates, but the thoracic dose of OA was replaced with acetone.

We selected five other hives from the same apiary to estimate the number of honeybees per hive. Hives were closed at night and the bees killed by saturating the hive with diesel vapors. The next morning, all the bees in each hive were collected and weighed; two subsamples were also weighed and manually counted to estimate the number of honeybees in each hive. All the experiments were carried out in the summer of 2009 (January and February). The colonies were not infested by varroa at a level higher than 1% or by Nosema.

The LD₅₀ was determined by the nonparametric trimmed Spearman-Karber test (Hamilton et al., 1977; Hamilton, 1979). This test was run with the TSK version 1.5 software from the US EPA (2006) in DOS emulation with DOSEMUs (DOSEMUs.org, 2010) on a GNU/Linux operating system (Canonical, 2010). NOEL and LOEL were determined by one-way ANOVA after assessing the normal distribution and homogeneity of variance by the Shapiro-Wilk and Levene tests, respectively. The determined mean LD₅₀ value was compared with those reported by Aliano et al. (2006) (372.01 µg OA per honeybee) and Martín-Hernández et al. (2007) (530 µg OA per honeybee). This was compared by Student’s *t* test for a single sample after assessing the normal distribution and homogeneity of variance by the Shapiro-Wilk and Levene tests, respectively.

All the statistical tests were run with the statistical package R version 2.12.0 (R Development Core Team, 2010) for the platform i486-pc-Linux-gnu (32-bit) with R Commander 1.5-4 (Fox, 2005; R Development Core Team, 2010) on a GNU/Linux operating system (Canonical, 2010).

**RESULTS**

The results of the bioassay agreed with the criteria of the US EPA for a mortality rate in the control treatment (US
Moreover, the characteristics of the results satisfied the requirements of the trimmed Spearman-Karber test (Table 1). The determined LD$_{50}$ was in the range of 446.42-736.87 µg OA per honeybee with a mean value of 548.95 µg OA per honeybee, a standard deviation of 114.55 µg OA per honeybee, and a 95% confidence interval of 406.72-691.19 µg OA per honeybee (Table 1).

Analysis of the mortality rates showed homogeneity of variance ($p = 0.3411$), but not a normal distribution ($p = 0.0004$). Therefore, we performed a Kruskal-Wallis rank variance test which revealed significant statistical differences ($\chi^2 = 23.0747$, df = 5, $p = 0.0003$). For this result, the mortality rate of the control treatment was compared with values obtained using different doses of OA and the Wilcoxon signed-rank test. This allowed us to determine NOEL and LOEL as 400 µg OA per honeybee ($p = 0.1797$) and 600 µg OA per honeybee ($p = 0.0431$), respectively.

Biomass assessment in SWZU hives revealed a range of 1384.8-2780.7 g of honeybees per hive (gbh h$^{-1}$), a mean value of 2382.1 gbh h$^{-1}$, and a standard deviation of 564.8 ggbh h$^{-1}$. When this assessment was performed for the population in SWZU hives, the results showed a range of 15 508.6-27 618.9 honeybees per hive (hb h$^{-1}$), a mean value of 23 350 hb h$^{-1}$, and a standard deviation of 4640.1 hb h$^{-1}$ (Table 2). The measurements had a 95% confidence interval of 1680.8-3083.5 ggbh h$^{-1}$ for the biomass mean value and 17 588.1-29 111.0 hb h$^{-1}$ for the population mean value.

**DISCUSSION**

The LD$_{50}$ mean value determined in our study is not statistically different from zero when compared with the value reported in Martín-Hernández et al. (2007). However, it is statistically different ($P = 0.0260$) when compared with the value in Aliano et al. (2006). These statistical comparisons suggest that honeybees in SWU have a similar toxicological response to Apis mellifera iberiensis used by Martín-Hernández et al. (2007), but not to A. mellifera L. used by Aliano et al. (2006) and in other studies (Gregorc and Planinc, 2001; Aliano and Ellis, 2009). Therefore, we suggest that research using OA in varroosis control associated with A. mellifera iberiensis should be considered as a guide to manage the SWU honeybee polyhybrid subspecies.

We can extrapolate these results to the trickling method that is commonly used in Uruguay by considering the product of NOEL (400 µg OA per honeybee) and the mean number of bees per hive (23 350 hb h$^{-1}$) to suggest that the maximum dose of OA should be 9.3 g per SWZU hive (i.e., without expecting a significant loss of honeybees). Based on the range of the number of bees per hive, the maximum dose of OA could vary from 7.0 to 11.6 g per hive. The suggested 3.1 g per hive dose in Ramallo et al. (2008) is therefore 6.2 g lower than the maximum OA dose calculated in this study based on the NOEL value. These results imply that there could be further opportunities to control V. destructor in SWZU hives with OA and suggest that higher doses of OA might be used along with concomitant reductions in the dose and frequency of synthetic miticide applications (fluvinate, amitraz, coumaphos, and flumethrin). Making such changes could lead to improved management and risk reduction associated with resistant V. destructor strains and pollution by synthetic miticides (Tremolada et al., 2004). Furthermore, changes in practices based on these results could facilitate Uruguayan honey exports to Europe where the use of OA is permitted and there are no

| Hive Nº | Biomass (g) | Subsample weight | Honeybees per sample | Honeybees per hive |
|---------|-------------|-------------------|----------------------|---------------------|
| 13      | 2624.3      | 383.73            | 272.35               | 25 561.6            |
| 31      | 1384.8      | 205.88            | 140.33               | 15 508.6            |
| 47      | 2574.8      | 313.98            | 239.73               | 23 482.5            |
| 57      | 2780.7      | 296.75            | 299.43               | 27 618.9            |
| 129     | 2546.1      | 258.95            | 377.58               | 24 575.9            |

Mean: 2382.1, SD: 564.84, Mean ± SD: 23 349.5 ± 4 640.11

**Table 1. Mortality rate, mean lethal dose (LD$_{50}$), and 95% confidence intervals of acute toxicological bioassay with newborn honeybees from southwestern Uruguay.**

**Table 2. Number and biomass of honeybees per hive in a representative apiary in southwestern Uruguay.**

Dose (g oxalic acid per bee) | Bioassay result | Mean | SD |
|---------------------------|-----------------|------|----|
| 200                       | 0.2             | 0.0  | 0.0|
| 400                       | 0.1             | 0.1  | 0.4|
| 600                       | 1.0             | 0.7  | 0.3|
| 800                       | 1.0             | 1.0  | 0.8|
| 1000                      | 0.7             | 0.9  | 1.0|
| LD$_{50}$ calculated by TSK | 501.58          | 486.96 | 446.42 | 736.87 | 572.94 | 548.95 | 114.55 |
| Upper limit 95% CI        | 538.86          | 534.48 | 543.15 | 875.29 | 648.74 |
| Lower limit 95% CI        | 466.00          | 436.33 | 366.92 | 620.35 | 506.00 |
| SK-Trim (%)               | 14.8 ± 3.51     | 0     | 38.89 | 5.36 |

TSK: Trimmed Spearman-Karber test; SK-Trim: Spearman-Karber trim; CI: confidence interval; SD: standard deviation.
Based on the results of this study, we conclude that the maximum dose for OA with no effect (NOEL) is 9.2 g per SWZU hive in acute toxicity conditions, which indicates the maximum range that should be used in future chronic field trials. Moreover, LD$_{50}$ for OA in the dominant honeybee polyhybrid subspecies in SWZU is 548.95 µg OA per honeybee.

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**CONCLUSIONS**

maximum OA residue levels (Bogdanov, 2006). However, it must be noted that this study was conducted under acute toxicity conditions, and the results cannot be used to determine the response of hives under chronic exposure, such as those studied by Higes et al. (1999).

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**PALABRAS CLAVE:** DL$_{50}$, *Varroa destructor*, Uruguay.
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