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ORIGINAL RESEARCH ARTICLE

Determining the dose of oxalic acid applied via vaporization needed for the control of the honey bee (*Apis mellifera*) pest *Varroa destructor*

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Oxalic acid (OA) is a natural compound that has been used to control the honey bee (*Apis mellifera*) pest *Varroa destructor*. One method of OA application gaining popularity among beekeepers in the US involves vaporizing OA crystals with heat inside a closed hive. Herein, we tested different doses of OA applied via vaporization to determine the most effective amount of OA needed to reduce *V. destructor* populations below that of the negative controls. Forty experimental colonies were assigned to one of four treatment groups, with ten colonies composing each group. The four treatments were: (1) 1 g OA, (2) 2 g OA, (3) 4 g OA and (4) no OA (negative control). The OA was applied via vaporization once per week for three weeks. *V. destructor* infestation rate and colony strength assessments were estimated before, during, and after treatment applications. Colonies in the 4 g OA treatment group had significantly lower infestation rates than did those in the untreated control and 1 g OA treatment groups, but not those in the 2 g OA treatment group. The infestation rate of colonies treated three times with 1 g OA, which is the current legal limit for OA vaporization in the US, was not significantly different from that of colonies in the negative control or 2 g OA treatment groups. Colonies receiving the highest dose of OA were generally healthier than those treated at lower OA doses. Our results may lead to improved efficacy of OA vaporization, thus aiding beekeepers in their efforts to control *V. destructor*.

Keywords: *Apis mellifera*; *Varroa destructor*; oxalic acid; vaporization; dose

Introduction

Beekeepers around the world are struggling to control the honey bee pest *Varroa destructor* effectively (Beyer et al., 2018; Haber et al., 2019; Oberreiter & Brodschneider, 2020). There are several reasons that make the mite difficult to control, but a primary reason is the issue of mite resistance to many of the chemicals used against it (Rosenkranz et al., 2010). Due to the overuse of some chemical treatments, *V. destructor* has rapidly developed resistance to many once effective chemical treatments (Elzen & Westervelt, 2002; González-Cabrera et al., 2016). The lack of effective chemical controls has compelled researchers to look for alternative treatments (Huang et al., 2019; Reinbacher et al., 2018; Ziegelmann et al., 2018). However, it is unlikely we will ever discover a chemical silver-bullet capable of total *V. destructor* control without the mites eventually developing resistance. Therefore, future and existing chemical treatments should be rotated with treatments with different chemistries to ensure their effectiveness over long periods.

Oxalic acid (OA) is an organic compound that has been used to control *V. destructor* for decades (Popov et al., 1989). Despite its long history of use in Europe and North America (Johnson et al., 2010), there are no reports of *V. destructor* resistance to OA to date (Maggi et al., 2017). Beekeepers commonly apply OA to broodless colonies (Charriere & Imdorf, 2002; Gregorc & Planinc, 2001), which exposes more mites to the treatment, as the chemical will not affect mites that are inside capped brood cells (Rademacher & Harz, 2006). However, some beekeepers still treat with OA while brood is present in the hive, reapplying their treatment once per week for three weeks (Gregorc & Planinc, 2001, Maggi et al., 2016, Terpin et al., 2019). The most common methods of OA application include dissolving OA into sugar water and trickling or spraying it directly onto adult bees (Aliano & Ellis, 2008; Toomemaa et al., 2010); however, fumigating colonies with the compound is becoming increasingly more common (Jack et al., 2020; Terpin et al., 2019).

Fumigating colonies with OA involves heating pure OA or OA dihydrate crystals with an apparatus until the crystals turn into a gas. Many prefer to apply OA in this manner as it can coat the inner surface of the hives and adult bees, eliciting a grooming response that can lead to increased mite fall (Schneider et al., 2012). Often, the terms sublimation and vaporization are confused and incorrectly used. Pure OA heated to a temperature of 157 °C will sublimate, going straight from a solid to a gas (International Labour Organization [ILO], 2009). Heating OA dihydrate, which is the legal

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treatment in the US (United States Environmental Protection Agency, 2015), to a temperature of 101 °C will cause the crystals to melt to a liquid and then vaporize with continued heating (ILO, 2009). Thus, vaporization occurs when using OA dihydrate.

The dose required to be effective for treating honey bee colonies via OA vaporization is somewhat questionable. For U.S. beekeepers, the current legal rate of OA vaporization is1 g per brood chamber for standard 10-frame Langstroth hives (United States Environmental Protection Agency, 2015). Some researchers have observed a significant decrease in *V. destructor* populations at a dose of 1 g OA (reviewed by Rademacher and Harz (2006) and Gregorc et al. (2016)). Rademacher and Harz (2006) reviewed many unpublished European studies and reported that 1 g OA applied via sublimation was sufficient for treating small colonies maintained in small hives. However, the same authors noted that 2 g is needed for larger colonies maintained in larger hives, such as the standard deep Langstroth hive. This finding is supported by others who have observed the need to use higher doses (Al Toufailia et al., 2015, 2018). Al Toufailia et al. (2015) found the lowest effective dose of OA sublimation in the UK was 2.25 g per brood chamber, which is more than twice the labelled rate in the US. In the US, Jack et al. (2020) showed that even repeated treatments of 1 g OA applied via vaporization did not affect *V. destructor* levels or increase the survival of treated colonies. Therefore, it is likely that an increased dose of OA is necessary to be effective, though caution is required as high doses of OA can have negative effects on individual bees (Rademacher et al., 2017; Strachecka et al., 2012). Thus, there is a critical need to determine the effective dose of OA vaporization that will effectively reduce *V. destructor* infestations below that of untreated controls without damaging the honey bee colony.

The primary objective for this project was to determine the effective dose of OA dihydrate vaporization needed to reduce *V. destructor* populations below that of untreated controls in managed honey bee colonies. Dose limit tests of OA applied via vaporization have been conducted before (reviewed by Rademacher and Harz (2006) and Al Toufailia et al. (2015)); but to our knowledge, no one has tested this in the US. The second objective was to evaluate the effects of these doses on colony health by measuring various colony health parameters. We hypothesized that higher doses of OA would decrease *V. destructor* populations but would also negatively impact measured colony strength parameters (number of adult bees, brood cells, honey cells and pollen cells).

Materials and methods

Experimental design

In October 2017, 40 honey bee colonies of European-derived honey bee stock were maintained at the University of Florida Honey Bee Research and Extension Laboratory (Entomology and Nematology Department, Gainesville, FL, USA, 29°37′38″N 82°21′23″W). During the experimental period, brood was present in all honey bee colonies and the average temperature and humidity were 21.7 °C (71 °F) and 80%, respectively. All colonies were infested with *V. destructor* and managed in 10-frame Langstroth hives consisting of a single deep hive body and a solid bottom board. Colonies were equalized prior to the start of the experiment to ensure that each colony was of similar size and strength (∼nine frames of bees and six frames of brood). After equalization, no brood combs were shared between colonies, even within treatments, to prevent the transfer of *V. destructor*; otherwise, all colonies were managed according to standard management practices for the region. Each colony was randomly assigned to one of four treatment groups such that there were ten colonies per group. The treatment groups were as follows: (1) 1 g OA, (2) 2 g OA, (3) 4 g OA and (4) untreated negative control. Colonies receiving OA were treated once per week for three consecutive weeks.

OA application

OA was administered to treated colonies as a vapour per the label instructions (United States Environmental Protection Agency, 2015). This process involves heating a metal plate (vaporizer) using a 12-volt car battery causing the OA dihydrate to vaporize inside of the honey bee hive. We used the commercially available Varrox®-Vaporizer (OxaVap LLC, Manning, SC, USA) apparatus to vaporize the OA dihydrate (Sigma Aldrich, St. Louis, MO, USA). The vaporizer was inserted into the colony entrance and the OA was vaporized as the plate heated for 2–4 min. During vaporization, the hive entrance and all cracks around the nest were closed in order to limit the escape of the OA vapour. After the OA was completely vaporized, the vaporizer was removed from the hive entrance, but the hive remained sealed for an additional ten minutes to ensure sufficient exposure to the chemical. Applicators were properly equipped with personal protective equipment, including protective eyewear and respirators with cartridges to filter organic gases, per product label instructions.

Treatment efficacy

V. destructor infestation levels were determined using alcohol washes of 200–300 bee samples collected in all treatments prior to treatment application and every week following OA applications (avg. # mites/100 adult bees = infestation). Samples of bees were collected from brood areas containing emerging adult honey bee workers per Dietemann et al. (2013). Mite fall data were also collected by placing sticky boards (Mann Lake, Product # DC-681, Minnesota, USA) at the bottom of each hive twice per week for 72-h periods.

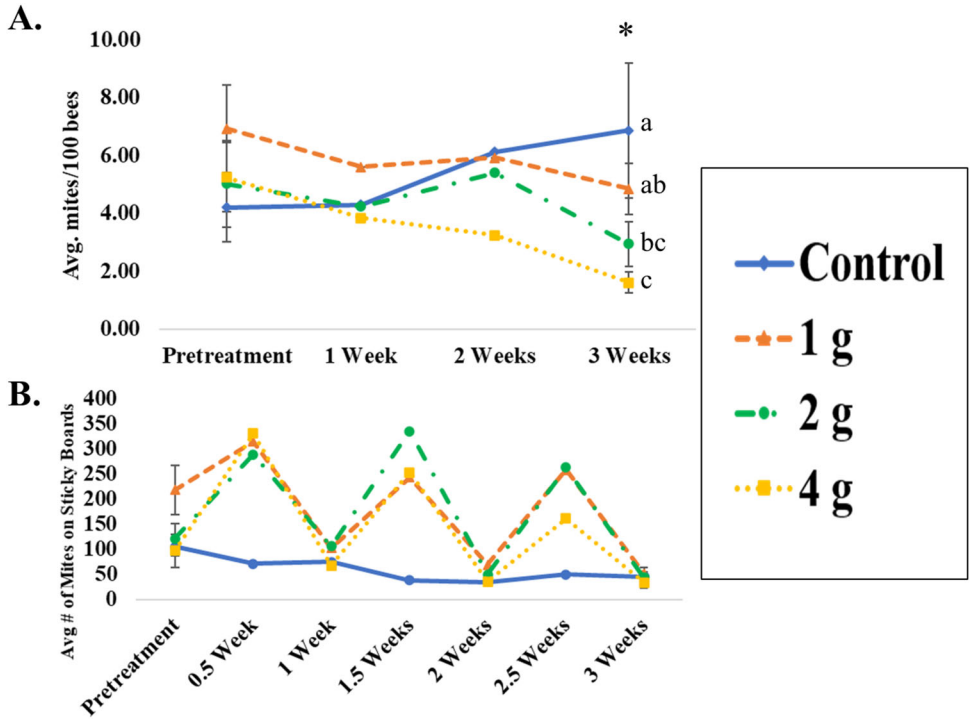

Figure 1. (A) The average mite infestation (number of mites/100 bees) and (B) 72 h mite fall (using sticky boards) of test colonies at each evaluation period. Statistical analyses were conducted on Pretreatment and end-of-treatment (3 weeks) data. Data from weeks 1 and 2 are included only for informational purposes. Error bars represent the standard error. $N = 7$, 9, 9 and 10, for Negative Control, 1, 2 and 4 g OA groups, respectively. The asterisk indicates a significant impact of treatment on the test parameter ($p \leq 0.05$) and significant differences between means are indicated with different letters (pairwise *t*-tests).

Colony strength parameters

Colony strength was evaluated using visual estimates of adult bees, brood, honey and pollen as described by Delaplane et al. (2013). Briefly, colonies were opened and a single observer visually estimated the percentage of comb surface covered by bees, brood cells, honey cells or pollen cells. The percentage of surface covered by bees was converted into an area (cm^2) covered by bees using the surface area of deep (880 cm^2) or medium (665 cm^2) frames used in the US. Then, the cm^2 bees was converted into the appropriate bee density (1.38 bees/cm^2). Similarly, the percentages of brood, honey, and pollen cells were also converted into an area estimate and converted to an estimate of number of cells using the average cell density of 3.7 worker cells/cm^2. Strength estimates were made prior to the start of the experiment and again one week following every OA treatment.

Statistical analyses

The effect of OA dose (nothing, 1 g, 2 g or 4 g), time (pre-treatment and post-treatment), and the interaction between the two on *V. destructor* infestation, 72 h mite fall, and colony strength were determined using generalized linear mixed models methodology as implemented in SAS PROC GLIMMIX (SAS/STAT 14.1; SAS Institute, Cary, NC). A negative binomial distribution functioned its canonical link was chosen over a Poisson because of over dispersion, manifesting itself in a large Pearson χ^2/DF ratio (>10) fit statistic, except for mite infection, which was modelled as a Poisson. In this study, we had extremely large *F*-values for time, which are highly suspect according to Stroup (2013). Modelling over dispersion appropriately reduced all Pearson χ^2/DF ratio to <1.0 as well as drastically reducing the *F*-value for time and the treatment $\times$ time interaction. Treatment (OA dose), time (pre-treatment and post-treatment) and their interaction were considered fixed effects; colony within treatment was the sole random effect. Pairwise *t*-tests were used to compare treatment means within time (pre-treatment and post-treatment) without any adjustment for multiple comparisons based on the recommendations made by Milliken and Johnson (2009) and Saville (2015). Several experimental colonies were in the process of collapsing due to severe *V. destructor* infestations at the onset of the project. These extreme cases were considered outliers. Therefore, only colonies surviving the entire three-week experimental period were included in the analyses.

Results

Treatment efficacy

There was a significant interaction between OA dose and time for *V. destructor* infestation ($F_{3,\ 31} = 6.507$, $p = 0.002$) so analyses were run separately by time. Prior to treatment with OA, there were no significant

Table 1. The average mite infestation (mites/100 bees), mite fall (mites on sticky board after 72 h), and number of adult bees, brood cells, honey cells and pollen cells for all treatment groups pre- and post-treatment.

		Mite infestation	Mite fall	Adult bees	Brood cells	Honey cells	Pollen cells
Negative control	Pre-treatment	4.21 ± 1.2	105.4 ± 18.2	3929.5 ± 205.6	8744.7 ± 992	11930.9 ± 856.9	2986.2 ± 430
	Post-treatment	6.87 ± 2.3[a]	45.1 ± 9.2	3131.4 ± 360.4	4698 ± 1307.9	11558.8 ± 497.4	2697.8 ± 279.4
1 g OA	Pre-treatment	6.93 ± 1.5	217.9 ± 49.2	4129 ± 378.4	8320.9 ± 928.7	11287.5 ± 1440.9	3418.9 ± 916.5
	Post-treatment	4.85 ± 0.9[a,b]	51.1 ± 11.8	2843.1 ± 400	5336.2 ± 861.4	11052.3 ± 1270.8	3617.8 ± 740
2 g OA	Pre-treatment	5.02 ± 1.5	121.4 ± 30.3	3909 ± 201.1	8791.2 ± 512.7	10961.9 ± 1198.5	2080.2 ± 298.2
	Post-treatment	2.94 ± 0.8[b,c]	43.9 ± 8.6	3346.3 ± 206.8	4902.1 ± 456	12246.2 ± 1256.9	2026 ± 250.3
4 g OA	Pre-treatment	5.24 ± 1.2	97.2 ± 32.8	4074.3 ± 211.1	8325.6 ± 625.2	12389.1 ± 900.1	1882 ± 355.5
	Post-treatment	1.61 ± 0.4[c]	33.6 ± 10.3	3351.7 ± 180.3	5567.8 ± 354.9	11851.8 ± 804.1	2653.6 ± 230.3

Data are mean ± SE ($N = 7$, 9, 9 and 10, for negative control, 1, 2 and 4 g OA, respectively). For mite infestation within post-treated colonies only, means with different letters are different at $\alpha \leq 0.05$ (pairwise *t*-tests).

differences in mite infestations among treatment groups ($F_{3,\ 31} = 0.98$, $p = 0.414$). There were, however, significant differences in mite infestations across the treatment groups post treatment ($F_{3,\ 31} = 4.68$, $p = 0.008$) (Figure 1A, Table 1). Collectively, colonies in the 4 g OA treatment group had a significantly lower infestation rate at the end of the experiment than did colonies in the untreated control and 1 g OA treatment groups ($p < 0.05$), but not colonies in the 2 g OA treatment group ($p = 0.333$). Colonies in the 1 g OA treatment group, which is the current legal limit for OA vaporization in the US, had an infestation rate that was not significantly different from that of colonies in the negative control ($p = 0.303$) or 2 g OA treatment ($p = 0.223$) groups.

There was no significant interaction between OA dose and time ($F_{3,\ 31} = 0.69$, $p = 0.566$) for mite fall. The only significant effect occurred at time ($F_{1,\ 31} = 50.64$, $p < 0.001$). Mite fall reduced from pre-treatment to post-treatment for all treatments (Figure 1B, Table 1).

Colony strength

There was no significant interaction between OA dose and time for any of the measured colony strength parameters. This was true for adult bees ($F_{3,\ 31} = 2.06$, $p = 0.126$), brood cells ($F_{3,\ 31} = 0.25$, $p = 0.863$), honey cells ($F_{3,\ 31} = 1.97$, $p = 0.139$) or pollen cells ($F_{3,\ 31} = 2.82$, $p = 0.055$). Colony strength was appropriate for the season and location where the experiment was conducted.

Discussion

To our knowledge, this is the first study designed to test the effective dose of OA applied via vaporization in the US. Our results support an earlier finding that OA vaporization at the current label rate in the US of 1 g per brood chamber is ineffective at reducing *V. destructor* populations significantly, even after multiple applications (Jack et al., 2020). Furthermore, our results show that repeated application of OA at 4× the label rate did not have significantly detectable negative impacts on colony health within the three-week testing period. Although we found no significant differences between the amount of OA vaporized and mite fall, our mite infestation results suggest that repeated applications of 2–4 g OA vaporizations will significantly reduce colony mite loads. Thus, determining treatment efficacy by measuring mite infestation levels via sampling adult honey bees is more accurate than measuring mite fall. Our findings emphasize the lack of *V. destructor* control achieved under the current label rate for OA applied via vaporization.

In this study, we observed an inverse relationship between the *V. destructor* infestations and OA dose. As the OA dose increased, we observed a decrease in *V. destructor* infestation, though differences between similar doses (doses that were adjacent to one another in this study) were never statistically different from one another (Figure 1A). Similarly, Al Toufailia et al. (2015) tested a range of doses applied via OA sublimation and observed no significant differences in *V. destructor* infestation between 2.25 and 4.5 g OA. However, they did observe a slight increase in colony mortality in colonies treated with 4.5 g OA several months after treatment (Al Toufailia et al., 2015). While we did not observe any negative impacts on colony health with a repeated application of 4 g OA, longer research studies are needed to understand fully the long-term effects of higher doses of OA on colonies, especially when the OA is applied via vaporization.

While we believe the research presented herein is important at furthering our knowledge about OA vaporization, we recognize that many additional questions should be answered before recommendations on dose can be made with confidence. For instance, the effect of repeated applications of OA vaporization at higher doses on queen health is not well understood and should be explored. As our study was conducted in a single location within specific climatic conditions, there is a need for additional research in different locations and at different seasons.

OA is most commonly applied to colonies during periods of broodlessness to increase the potential exposure to *V. destructor* that is on the bodies of adult bees (Charriere & Imdorf, 2002; Gregorc & Planinc, 2001; Gregorc et al., 2016, 2017). However, some use OA with multiple applications spaced about a week apart while brood is present in the colony (Bacandritsos et al., 2007;

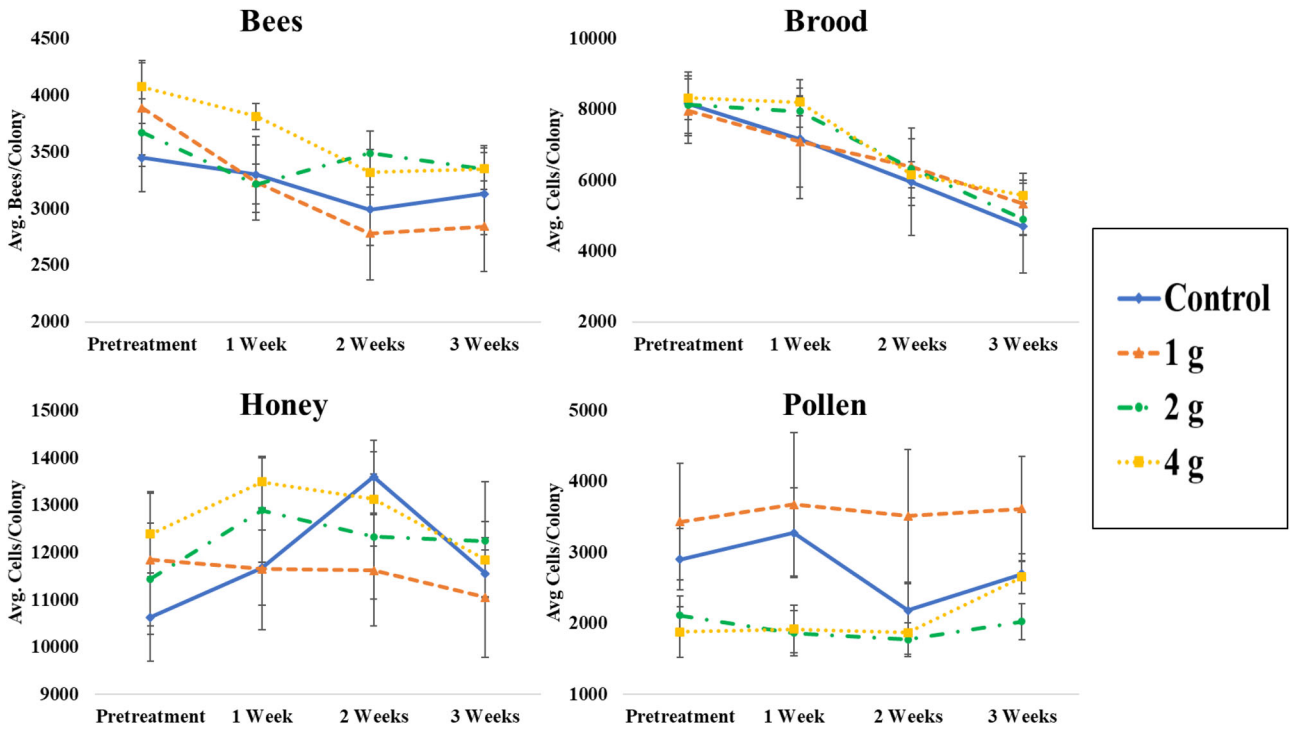

Figure 2. The average number of adult honey bees and number of cells containing brood, honey or pollen at each colony evaluation period. Error bars represent the standard error. $N = 7$, 9, 9 and 10, for negative control, 1, 2, and 4 g OA, respectively.

Gregorc & Planinc, 2001; Maggi et al., 2016), believing the mites will contact the chemical at least once during the treatment period. Some research has demonstrated the toxicity of OA to honey bee larvae and queens after treatment (Gregorc et al., 2004; Hatjina & Haristos, 2005; Higes et al., 1999; Terpin et al., 2019); however, OA was never applied to whole colonies via vaporization in these studies. Within the time frame of this study, we did not observe any effects of repeated applications of OA vaporization on the brood or total colony strength (Figure 2, Table 1). In addition, forcing broodless conditions to support OA treatment may not be a suitable option for beekeepers in every location (Jack et al., 2020). Thus, multiple applications of 2–4 g OA via vaporization while brood is present may be a useful treatment alternative to forcing broodless periods or using low doses that are not efficacious. However, it is necessary to reiterate the importance of rotating chemical treatments to prevent possible *V. destructor* development of resistance to OA.

As beekeepers around the world are desperate for effective *V. destructor* control options, our data provide valuable information about an increasingly popular treatment. However, there are more questions that should be answered before OA vaporization can be fully optimized. Future research efforts should be devoted to finding the upper limit of OA vaporization that will reduce *V. destructor* populations but not have negative effects on colony health. Furthermore, the long-term effects of these high doses on queen and brood health should be investigated. Nevertheless, OA vaporization appears to be effective at reducing mite infestations as long as at least 2 g of OA are used and can be rotated into appropriate chemical treatment regimens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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