A scientific note on the effect of oxalic acid on honey bee larvae

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Abstract – The approval of oxalic acid as a treatment for *Varroa destructor* infestation of honey bee hives gives beekeepers an additional option for controlling this devastating parasite and disease vector, but the effects of oxalic acid on developing bees are not completely understood. In this study, we find that doses of oxalic acid not reported to be toxic to adult bees are toxic to larval bees. While it has been recommended that oxalic acid only be used during broodless periods because it does not penetrate cappings and is only effective in killing phoretic mites, it is tempting to use it at other times of the year because of the dearth of effective treatment options. Knowing whether oxalic acid is toxic to larvae and at what doses is important for beekeepers as they manage their colony population throughout the year.

Varroa destructor / oxalic acid / honey bee / *Apis mellifera*

The mite *Varroa destructor* is a major pathogen of honey bees and a leading cause of colony collapse. Mites have evolved resistance to first-generation treatments such as amitraz, fluvalinate, and coumaphos (Sammataro et al. 2005) prompting researchers to explore alternatives such as oxalic acid (OA) which was approved for use in honey bee hives by the EPA in 2015. OA is acutely toxic to mites and is also toxic to adult bees at high concentrations (Aliano et al. 2006; Martín-Hernández et al. 2007; Toomemaa et al. 2010). There are some data that suggest OA is toxic to honey bee larvae (Hatjina and Haristos 2005), possibly by inducing cell death (Gregorc et al. 2004), but the extent of OA toxicity to larvae is not known. It is recommended that OA be applied in the fall or winter when larvae levels are low, but many beekeepers are so desperate to reduce mite burden that they are using it at other times of the year (personal communications). The goal of this study is to determine the mortality rates of OA and associated lethal concentration (LC50) for honey bee larvae. Larval culture and toxicity

assays were conducted using a guidance document protocol developed for repeated exposure (OECD 2016).

Six groups of larvae were tested: one control (0.00%) and five treatments of increasing concentrations of OA (0.01%, 0.05%, 0.1%, 0.5%, and 1.0%). Table 1 describes the dose per larva ($\mu\text{g}/\text{larva}$) associated with each concentration. The experiment was replicated 12 times. Two larvae were assigned to each instar age and OA concentration. Larvae were collected from the Georgia Tech apiary located on the roof of the Clough Undergraduate Learning Commons in Atlanta, Georgia (33.775215, –84.396436) between May and June 2018. On Day 1 (D1), a Chinese grafting tool was used to remove larvae from the comb. The larvae were sorted into second, third, and fourth instar stages by observing the space occupied by the larva in the cell (Human et al. 2013). Larvae were placed into sterile 48-well plates containing 40 μl of artificial diet composed of 50% w/v royal jelly (Stakich Inc., Troy, MI) and 50% sugar/yeast solution made of 9% w/v D-glucose (VWR, Radnor, PA), 9% w/v D-fructose (VWR, Radnor, PA), and 2% w/v bacto-yeast (VWR, Radnor, PA) in deionized water. Grafting was restricted to 60-min duration to limit larval stress. Larvae were cultured at 35 °C and 90% humidity for the duration of the experiment (D1 to D5). Larvae were not fed on D2 as this

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Table 1. Mortality of honey bee larvae treated with oxalic acid (OA). Mortality and standard error are calculated using the Kaplan–Meier method with the log-rank test with censored data. Combined data accounts for both dosing events (D4 and D5)

2nd instar (young) larvae									
OA conc. (%)	D1 No. of larvae alive	D3 No. of larvae alive	D4 Mortality	D4 StdError	D4 No. of failed	D5 Mortality	D5 StdError	D5 No. of failed	Combined
0.00	24	23	0.0672	0.0216	9	0.1154	0.028	15	0.0603
0.01	24	23	0.126	0.0296	7	0.2229	0.0372	13	0.1174
0.05	23	22	0.1558	0.0332	3	0.3154	0.0428	10	0.1616
0.10	22	22	0.2453	0.0436	7	0.4037	0.0473	8	0.2264
0.50	22	22	0.4511	0.0598	12	0.5826	0.0544	12	0.3714
1.00	22	22	0.8753	0.0509	17	0.9621	0.0261	20	0.7238
3rd instar (middle) larvae									
OA conc. (%)	D1 No. of larvae alive	D3 No. of larvae alive	D4 Mortality	D4 StdError	D4 No. of failed	D5 Mortality	D5 StdError	D5 No. of failed	Combined
0.00	24	24	0.0211	0.0121	3	0.0423	0.0169	6	0.0211
0.01	24	24	0.0211	0.0121	0	0.0504	0.0186	1	0.0239
0.05	24	24	0.0315	0.0158	1	0.0807	0.0249	3	0.0377
0.10	24	23	0.0869	0.0307	4	0.1726	0.0399	7	0.0881
0.50	24	24	0.2229	0.0542	7	0.4895	0.0636	18	0.2498
1.00	23	23	0.8986	0.055	20	1	0	23	0.7173
4th instar (old) larvae									
OA conc. (%)	D1 No. of larvae alive	D3 No. of larvae alive	D4 Mortality	D4 StdError	D4 No. of failed	D5 Mortality	D5 StdError	D5 No. of failed	Combined
0.00	24	23	0.0074	0.0073	1	0.0294	0.0145	4	0.0123
0.01	24	22	0.0337	0.0166	3	0.0638	0.0219	4	0.0327
0.05	24	24	0.0549	0.022	2	0.1152	0.0305	5	0.0575
0.10	23	23	0.0973	0.0318	3	0.2869	0.0493	13	0.1325
0.50	24	22	0.2204	0.0542	6	0.5786	0.0604	18	0.2902
1.00	24	22	0.5393	0.0878	9	1	0	22	0.6236
									Combined
									StdError
									No. of failed
									5
									7
									7
									16
									24
									31

24 Hours Post-Treatment Instar Survival

Figure 1. Instar larval mortality 24 h post initial feeding (D4). Graphs show mortality 24 h after first feeding. The horizontal axis (abscissa) is the percent concentration of oxalic acid (OA) versus vertical (ordinate) percentage larval survival. Error bars represent the calculated standard error.

served as an environmental acclimation period. On D3, larvae were checked for movement via a dissecting microscope. Larvae demonstrating movement of any kind were deemed alive and continued in the experiment. On D3 and D4, all live larvae were fed artificial diet with the appropriate concentration of OA for their treatment group. The volume of diet varied by instar stage in order not to suffocate the larvae. Larvae in the second instar stage were fed 20 μ l, third instar larvae were fed 50 μ l, and fourth instar larvae were fed 100 μ l.

After 24 h, on D4, larvae were checked for movement as an indicator of survival. All surviving larvae were again fed their corresponding OA concentration diet. The experiment concluded on D5.

Statistical analysis was conducted using SAS JMP Pro version 14.1.0 software. The Kaplan-Meier method with the log-rank test and censored data was used for comparing survival curves. The Cox proportional hazards regression was used to examine risk ratios between covariates. Significance

48 Hours Post-Treatment Instar Survival

Figure 2. Instar larval mortality 48 h post initial feeding (D5). Graph depicts mortality 48 h after first feeding. Larvae were fed on D3 and D4. The horizontal axis (abscissa) is the percent concentration of oxalic acid (OA) versus vertical (ordinate) percentage larval survival. Error bars represent the calculated standard error.

Figure 3. Kaplan-Meier predicted mortality rates by instar described by OA percentage concentrations.

was defined as *p* values <0.05. The median lethal dose (LC50) of combined dose exposure was with confidence limits of 95% using a generalized regression dose-response.

In total, 423 larvae were grafted on D1. On D3, the 412 surviving larvae continued into the study. After 24 h of treatment (D4), all instar groups showed decreased survival at each OA concentration higher than 0.05% (Figure 1). Older larvae were

more resistant to treatment than younger larvae. After 48 h of treatment, survival rates of larvae in all instar phases were lower than at 24 h and older larvae had higher survival rates than younger larvae (Figure 2).

Mortality rates of the second, third, and fourth instar groups for the 12 replicates are shown in Table I, and Figure 3 depicts the associated Kaplan-Meier predicted mortality. While the effects of dosing on instar by

Table II. Cox proportional hazard model establishing the hazard risk ratios associated with random effects of the single and double dosing on instar mortality, the added effect of OA concentrations, and the binary effects of OA concentration on each instar by dosing

Effects of instar					
Level 1	Level 2	Risk ratio	Prob > Chisq	Lower 95%	Upper 95%
4th instar	3rd instar	1.0327	0.6819	0.8855	1.2043
2nd instar	3rd instar	1.1098	0.2059	0.9443	1.3043
2nd instar	4th instar	1.0747	0.3864	0.9130	1.2651
Effects of instar and OA percentage concentration percentages					
Term		Risk ratio		Lower 95%	Upper 95%
OA conc.		1.7625		1.4160	2.1779
Level 1	Level 2	Risk ratio	Prob > Chisq	Lower 95%	Upper 95%
4th instar	3rd instar	1.0140	0.8595	0.8693	1.1828
2nd instar	3rd instar	1.0780	0.3636	0.9168	1.2675
2nd instar	4th instar	1.0631	0.4623	0.9031	1.2515
Effects of the binary interactions of instar and dose concentration percentages					
Term		Risk ratio		Lower 95%	Upper 95%
OA conc.		3.9993		3.0704	5.2092
Level 1	Level 2	Risk ratio	Prob > Chisq	Lower 95%	Upper 95%
4th instar	3rd instar	1.2015	0.3044	0.8464	1.7056
2nd instar	3rd instar	2.0618	< 0.0001	1.5050	2.8245
2nd instar	4th instar	1.7160	0.0003	1.2804	2.2997

Table III. Estimated lethal concentration (LC50) of oxalic acid for honey bee larvae

Instar class	Predicted OA concentration	Lower 95%	Upper 95%
2nd	0.649	0.553	0.759
3rd	0.972	0.853	1.118
4th	0.959	0.838	1.105

concentration were established, mortality data was fitted with the Cox proportional hazard model to further establish the hazard risk ratios associated with random effects of the single and double dosing on instar mortality, the added effect of OA concentration, and the binary effects of OA concentration on each instar by dosing. Table II depicts the results which show that while second instar mortality is more likely relative to the third and fourth instars after double dosing (see Risk Ratio), p values are not significant. Adding the continuous predictor of OA percentages and removing the effects of instar groups, larvae mortality is 1.76 times more from increasing OA percentages. After removing the effects of OA concentration at any given time, larvae mortality by instar remains relatively constant with insignificant p values. However, increasing OA percentages indicates a 3.99 greater likelihood of mortality across instars. The second instar is 2.06 ($p < 0.0001$) times more likely to die relative to the third instar and 1.71 ($p < 0.0003$) times relative to the fourth instar. Table III shows the estimated OA lethal concentration (LC50) for each instar. The third and fourth instar larvae showed higher LC50 values compared with the second instar larvae following the trends found in Table II.

Because larvae are protected in brood cells, it is unlikely that they are exposed to the full 3–5% concentration of OA recommended by the EPA for treatment of mites by the solution method of administration. The lower concentration ranges of oxalic acid (0.01–1.0%) used in this study tested may more closely match levels larvae experience due to adsorption of the surrounding colony matrix in response to trickling, spraying, sublimation, or transport of the pesticide through the hive by tracking of adult bees. After a single exposure, the third and fourth instar larvae had higher survival rates in each treatment group compared with the second instar larvae indicating that older larvae have a greater tolerance for the pesticide. A significant decrease in survival in all larvae 48 h post treatment was documented.

Sources of variability in mortality could result from back-to-back OA feedings, time and order of grafting (control groups grafted first allowing for more exposure

to environment), larvae classified as live/dead with no intermediate morbidity defined or recorded, and no change in diet composition as the larvae age. Another consideration is that while adult pesticide studies are reported as oral or dermal application, no distinction is proven as larvae are immersed in the solution containing the prescribed OA percentage concentration. Further work is needed to determine if the mortality rates resulted from repeated exposure of double dosing of OA, or simply the presence of OA in the diet after single intervention. That experimental design could include intermittent feeding such as 24 h and 72 h.

While oxalic acid at EPA-recommended treatment levels of 5 ml at 3–5% of OA in sugar water has been found to be safe for adult bees, this study shows that oxalic acid is very toxic to honey bee larvae. The current study provides a reasonable model of larval exposure to OA in treated beehives. Beekeepers should be aware that using OA for mite control while larvae are present could have a negative downstream effect on the colony population size and wintering capability.

AUTHORS' CONTRIBUTION

All authors have contributed equally to this work. Funding information

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Une note scientifique sur l'effet de l'acide oxalique sur les larves d'abeilles

Varroa destructor / acide oxalique / *Apis mellifera*

Eine wissenschaftliche Notiz über den Effekt von Oxalsäure auf Honigbienenlarven.

Varroa destructor / Oxalsäure / Honigbiene / *Apis mellifera*

REFERENCES

- Aliano, N.P., Ellis, M.D., Siegfried, B.D. (2006) Acute Contact Toxicity of Oxalic Acid to *Varroa destructor* (Acari: Varroidae) and Their *Apis mellifera* (Hymenoptera: Apidae) Hosts in Laboratory Bioassays. *J. Econ. Entomol.* **99** (5), 1579–1582
- Gregorc, A., Pogacnik, A., Bowen, I.D. (2004) Cell death in honeybee (*Apis mellifera*) larvae treated with oxalic or formic acid. *Apidologie.* **35** (5), 453–460

- Hatjina, F., Haristos, L. (2005) Indirect effects of oxalic acid administered by trickling method on honey bee brood. *J. Apic. Res.* **44** (4), 172–174
- Human, H., Brodschneider, R., Dietemann, V., et al. (2013) Miscellaneous standard methods for *Apis mellifera* research. *J. Apic. Res.* **52** (4), 1–53
- Martín-Hernández, R., Higes, M., Pérez, J.L., Nozal, M.J., Gómez, L., Meana, A. (2007) Short term negative effect of oxalic acid in *Apis mellifera iberiensis*. *Span. J. Agric. Res.* **5** (4), 474–480
- OECD (2016) Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure, Series on Testing and Assessment No. 239. Organization for Economic Cooperation and Development. ENV/JM/MONO(2016)34
- Sammataro, D., Untalan, P., Guerrero, F., Finley, J. (2005) The resistance of varroa mites (Acari: Varroidae) to acaricides and the presence of esterase. *Int. J. Acarol.* **31** (1), 67–74
- Toomemaa, K., Martin, A.-J., Williams, I.H. (2010) The effect of different concentrations of oxalic acid in aqueous and sucrose solution on Varroa mites and honey bees. *Apidologie.* **41** (6), 643–653

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