

ORIGINAL RESEARCH ARTICLE

Towards integrated control of varroa: 2) comparing application methods and doses of oxalic acid on the mortality of phoretic *Varroa destructor* mites and their honey bee hosts

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10 In the past two decades, the parasitic mite *Varroa destructor* has become harder to control with synthetic acaricide chemicals due to genetic resistance. We determined the efficacy of the natural chemical oxalic acid (OA) in killing phoretic mites on adult worker bees under field conditions in southern England. We compared three OA application methods (trickling, spraying, and sublimation) at three or four (sublimation) doses, using 110 broodless colonies in early January 2013. Treatment efficacy was assessed by extracting mites from samples of c. 270 worker bees collected immediately before and 10 days after treatment. All three methods could give high varroa mortality, c. 93–95%, using 2.25 g OA per colony. However, sublimation was superior as it gave higher mortality at lower doses (.56 or 1.125 g per colony: trickling 20, 57% mortality; spraying 25, 86%; sublimation 81, 97%). Sublimation using 2.25 g of OA also resulted in 3 and 12 times less worker bee mortality in the 10 days after application than either trickling or spraying, respectively, and lower colony mortality four months later in mid spring. Colonies treated via sublimation also had greater brood area four months later than colonies treated via trickling, spraying, or control colonies. A second trial in December 2013 treated 89 broodless colonies with 2.25 g OA via sublimation to confirm the previous results. Varroa mortality was 97.6% and 87 (98%) of the colonies survived until spring. This confirms that applying OA via sublimation in broodless honey bee colonies in winter is a highly effective way of controlling *V. destructor* and causes no harm to the colonies.

25 **Hacia el control integrado de varroa: comparación de métodos de aplicación y dosis de ácido oxálico en la mortalidad de ácaros foréticos de *Varroa destructor* y sus abejas hospederas**

30 En los últimos años el ácaro parásito *Varroa destructor* se ha hecho más difícil de controlar debido a la resistencia a los acaricidas sintéticos utilizados. Se determinó la eficacia del ácido oxálico matando ácaros foréticos en abejas obreras adultas en condiciones de campo en el sur de Inglaterra. Se compararon tres métodos ya utilizados por los apicultores (goteo y pulverización de una solución de sacarosa y ácido oxálico, y sublimación) en tres o cuatro dosis en un experimento con 110 colonias sin cría, a principios de enero de 2013. La mortalidad de los ácaros se determinó mediante la extracción de ácaros a partir de muestras de cerca de 270 abejas obreras recogidas inmediatamente antes y 10 días después del tratamiento. Los tres métodos podrían dar una alta mortalidad de Varroa, c. 93-95%, utilizando 2,25 g de ácido oxálico por colonia. Sin embargo, la sublimación dio mayor mortalidad en dosis más bajas (0,56 o 1,125g por colonia: goteo 20, 57%; pulverización 25, 86%; sublimación 81, 97%). La sublimación utilizando 2,25 g de ácido oxálico dio lugar a 3 y 12 veces menos mortalidad de abejas obreras en los 10 días después de la aplicación que con goteo o aspersión, respectivamente. La sublimación también dio lugar a menor mortalidad de colonias cuatro meses más tarde a mediados de primavera (0/10 colonias frente a 3/10 con goteo, 6/10 con aspersión, 2/10 colonias de control). Las colonias tratadas a través de la sublimación también tenían una mayor área de cría cuatro meses después que las colonias tratadas a través de goteo y aspersión, y las colonias de control. Un año más tarde, a mediados de diciembre de 2013, 89 colonias sin larvas fueron tratadas con 2,25 g de ácido oxálico mediante sublimación para confirmar los resultados anteriores. La mortalidad de Varroa fue del 97,6% y 87 (98%) de las colonias que sobrevivieron hasta la primavera. Esto confirma que la aplicación de 2,25 g de ácido oxálico mediante sublimación en colonias de abejas sin larvas en invierno es una forma muy eficaz de control de *V. destructor* y no causa ningún daño a las colonias.

45 **Keywords:** varroa; oxalic acid; trickling; spraying; sublimation; *Apis mellifera***Introduction**

50 The mite *Varroa destructor* is native to Asia where it is a parasite of the eastern honey bee *Apis cerana* (Oudemans, 1904). Through human intervention (Anderson & Trueman, 2000; Delfinado, 1963), it has been transferred to *Apis mellifera*, and is now found on *A. mellifera* worldwide except Australia (Anderson & Trueman, 2000).

Varroa is a serious pest of *A. mellifera*. It can harm colonies and bees both directly, for example, by damaging individual worker bees during the pupal stage so that their adult lifespan and body weight (Amdam, Hartfelder, Norberg, Hagen, & Omholt, 2004; De Jong, De Jong, & Goncalves, 1982) are reduced, and indirectly, by exacerbating virus diseases (Boecking & Genersch, 2008;

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5 Brødsgaard, Ritter, Hansen, & Brødsgaard, 2000; Tentcheva et al., 2004) and causing colony mortality (Boecking & Genersch, 2008; Genersch, Evans, & Fries, 2010; Hayes, Underwood, & Pettis, 2008; Potts et al., 2010).

10 Varroa has been successfully controlled using synthetic acaricides (Alonso De Vega, Reguera, Martinez, Alonso, & Ortiz, 1990; Milani & Barbattini, 1988; Milani & Iob, 1998). However, resistance to the most effective compound, fluvalinate (the active ingredient in Apistan) is now widespread (Elzen, Baxter, Spivak, & Wilson, 15 2000; Floris et al., 2001; Milani, 1999; Milani & Barbattini, 1988; Mozes-Koch et al., 2000; Patti, James, Marla, & William, 2000). Resistance to coumaphos and flumethrin also occurs (Eizen et al., 2001, Elzen et al., 2000; Elzen & Westevelt, 2002; Milani, 1999). Many other varroa control compounds have also been tried (Rosenkranz et al., 20 2010; Wallner, 1999;), including natural organic acids such as oxalic acid (OA; Aliano & Ellis, 2008; Bacandritsos, Papanastasiou, Saitanis, Nanetti, & Roinioti, 2007; Gregorc & Poklucar, 2003; Marinelli, Formato, Vari, & De Pace, 2006; Nanetti et al., 2003; Rademacher & Imdorf, 2004; Takeuchi & Sakai, 1985), formic acid (Althen, 1979; Eizen et al., 2001; Fries, Aarhus, Hansen, & Korpela, 1991; Mahmood, Wagchoure, Raja, & Sarwar, 2012; Satta et al., 2005), and lactic acid (Emsen & Dodologlu, 2009; Koeniger, Koeniger, & Delfinado-Baker, 1983; Kraus & Berg, 1994) and essential oils such as thymol (Emsen & Dodologlu, 2009; Floris, Satta, Cabras, Garau, & Angioni, 2004; Imdorf, Bogdanov, Ochoa, & Calderone, 1999).

25 Beekeepers have been using OA against varroa for several decades (Popov, Melnik, & Matchinev, 1989) and research has shown that it can be effective. OA kills varroa phoretic on the bodies of adult bees, but not those in brood cells (Charriere & Imdorf, 2002; Gregorc & Planinc, 2001, 2002; Nanetti, Mutinelli, & Cremasco, 1995). As a result, it is more effective when applied to broodless colonies, such as in winter (Bacandritsos et al., 2007; Marinelli, Persano Oddo, De Pace, & Ricci, 2000; Nanetti & Stradi, 1997) or with a queen that has been caged for long enough to allow existing brood to reach the adult stage (Wagnitz, 2009; Wagnitz & Ellis, 2010).

30 OA appears to combat varroa in two ways. It damages varroa mouthparts and also causes increased bee to bee contact and grooming (Aliano & Ellis, 2008; Aliano, Ellis, & Siegfried, 2006; Fries, Huazhen, Wei, & Jin, 1996; Schneider, Eisenhardt, & Rademacher, 2012). Grooming dislodges mites which then fall onto the hive floor where many die from starvation (Aliano et al., 2006; Stevanovic, Stanimirovic, Lakic, Djelic, & Radovic, 35 2012). OA may potentially harm the bees (Higes, Meana, Suárez, & Llorente, 1999). It can penetrate into the body after topical or oral application, which resulted in detectable OA concentrations in different organs of caged worker bees although mortality was not measured in the honey bee colony (Nozal, Bernal, Gómez, Higes, & Meana, 2003).

65 Beekeepers apply OA to the bees in a hive either in water solution, usually combined with sucrose, or in pure form via sublimation. Two methods of applying OA solution are widely used. In the spraying method, the hive is opened, the frames are briefly removed one at a time, and the exposed bees are misted with the OA solution (Imdorf, Charriere, & Bachofen, 1997; Nanetti et al., 2003; Rademacher & Harz, 2006). In the trickling method, the hive is opened and the solution is poured onto the exposed bees and into the gaps between the frame top bars without removing the frames. In the sublimation method, pure OA is applied using a heated metal tool that causes the crystals to sublimate (Marinelli et al., 2004; Radetzki, 2001; Rademacher & Harz, 2006). The hive does not need to be opened as the tool is inserted via the entrance. This saves time and can also be carried out more easily in winter or under conditions unsuited to opening a hive. Sublimation is a more recent method (Marinelli et al., 2004; Radetzki & Bärmann, 2001b).

80 Surprisingly, previous research has not directly compared these three application methods to determine which of them, and which dose, is best. Previous research typically compared a few doses (Gregorc & Poklucar, 2003; Mahmood et al., 2012) or one or two methods (Bacandritsos et al., 2007; Imdorf et al., 1997; Marinelli et al., 2004; Nanetti et al., 1995), but no study has systematically compared all three methods across a range of doses. In addition, most previous research did not determine the proportion of phoretic mites in the colony that were killed. More typically, the number of mites killed, but not the number surviving, was determined by counting dead mites on the hive floor (Calderone & Lin, 2003; Fries et al., 1991; Mahmood et al., 2012). In addition, most previous studies did not determine possible harmful effects on the bees and colony, such as the numbers of worker bees killed at the time of application and longer term colony and queen survival or colony performance.

95 The aim of this study was therefore to compare the effect of winter application of OA to broodless honey bee colonies on the mortality of phoretic varroa mites and their host bees and colonies. We compared the three main methods (trickling, spraying, and sublimation) using the same three doses with one additional higher dose for sublimation. The study was carried out in southern England, which has a temperate climate with little or no foraging during winter, December to February, and a natural reduction in brood rearing to a low level in December and January (personal observations).

Materials and methods

Experimental setup and OA treatment

Setup of colonies

115 Trials were carried out in winter, January 2013, using 110 honey bee colonies located in 10 apiaries at or

within 20 km of the University of Sussex in southern England. The colonies were all in hives consisting of a single “commercial” brood chamber (11 frames each 43.8 25.4 cm, vol. 56.4 l), wooden bottom board with mesh floor, inner cover, and telescopic outer cover.

Hive inspections approximately four weeks before the trials were used to exclude any queenless colonies. Subsequent hive inspections approximately three weeks before the experimental trials showed that c. 90% of the colonies did not have any brood. The other 10% had small amounts of sealed or open brood, which was removed 1–2 days later using a honey fork. As a result, all varroa mites in all colonies were phoretic on the adult bees. This is the situation in which varroa can most effectively be killed by OA, and also makes it possible to quantify changes in varroa numbers by extracting mites from samples of worker bees. In particular, in broodless colonies changes in the numbers of phoretic mites on the worker bees are unaffected by mites either emerging from or entering brood cells.

Monitoring mite mortality on the hive floor

The fall of mites from the colony was monitored at two-day intervals for 8 days before, and 10 days after OA treatment (Figure 1). Each hive had a mesh floor with a sticky white plastic sheet underneath. Mites would fall through the mesh onto the plastic. Worker bees did not have access to this part of the hive and so could not clean away dead mites. We stopped counting

fallen mites after 10 days, as by then the number had been at low levels, less than three mites per day, for 4 days (see Results).

Monitoring bee fall

Dead worker bees were also monitored for 8 days prior to treatment and for 10 days after. Dead bees that had fallen onto the mesh floor were removed and counted. Dead bees that were removed from the hive by undertaker bees were caught in a dead bee trap. This was a 50 30 cm fine-mesh net attached to the bottom board at the two corners on either side of the hive entrance and to bamboo canes pressed into the soil below the hive stand at the other two corners. As the study was carried out in winter during cold weather (day time high temperatures before and after OA treatment were 9 °C (average of 8 days), 5 °C (day of treatment), and 3 °C (average of 10 days after treatment), respectively), the bees were not flying, so that undertaker bees (Visscher, 1983) did not fly away with the dead bees. Temperature and humidity are considered important when applying OA (Aliano & Ellis, 2009), and outdoor temperatures in the range 4–16 °C are recommended, depending on application method (Rademacher & Harz, 2006).

OA application methods

The three methods we used (trickling, spraying, and sublimation) are widely used by beekeepers. We followed

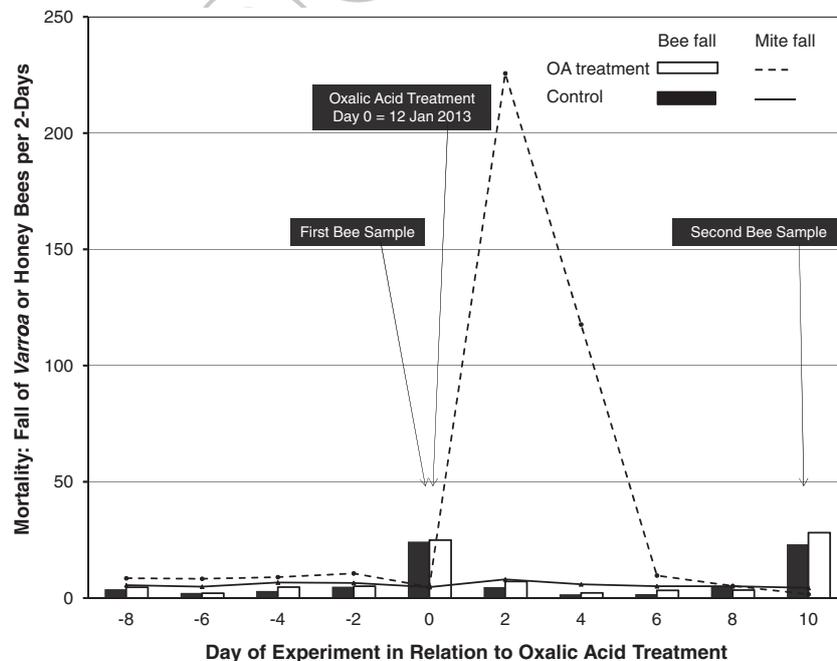


Figure 1. Time course of experiment showing the average number of dead varroa on the hive floors and the number of dead worker bees on hive floors and dead bee traps combined for 8 days before treatment and 10 days after, per 2 days. OA treatments were made on day 0. Control colonies (untreated) N = 10. Treatment colonies combined (N = 10 colonies for each of 10 dose application methods).

standard application procedures as used by beekeepers and previous researchers (Imdorf et al., 1997; Mahmood et al., 2012; Marinelli et al., 2004; Nanetti et al., 2006; Rademacher & Harz, 2006).

In the trickling method, we followed existing protocols (Brødsgaard, Jensen, Hansen, and Hansen, 1999; Imdorf et al., 1997) using a plastic bottle connected to a syringe pump (Vacc 5 ml V grip syringe- M3090) to dispense 50 ml of a water solution of OA and sucrose per colony in a narrow stream. Approximately, half was applied in an equal layer onto the exposed bees on the top bars of the frames, and half into the gaps between the top bars of the frames where the cluster of bees was located.

In the spraying method, we used an applicator of the type used to spray plants by hand to apply 50 ml of solution in a fine mist directly onto the bees on both sides of each frame, which were removed one at a time from the hive. In this way, most of the bees were directly contacted by the solution.

In the sublimation method, we used a commercially available applicator used in beekeeping (Varrox M3080) obtained from a UK beekeeping equipment supplier (E H Thorne (Beehives Ltd.); Wragby) powered by a 12 V car battery to heat a metal dish, diameter 3.5 cm, containing OA to cause sublimation. This part of the applicator was inserted into the hive entrance. Previous trials of the applicator had shown us how long it would take to sublimate different amounts of OA, and that it was possible to insert the tool into the hive before any vapor was produced. During application, the hive entrance was closed with plastic foam to prevent any vapor from escaping. As an additional safety precaution, the operators wore approved respiratory masks (Chap-Smith R300 Series, with a filter for organic gases).

OA doses

Based on previous research, we chose three OA doses that would cover the critical range from low to high varroa mortality (Gregorc & Poklucar, 2003; Martín-Hernández et al., 2007, Nanetti et al., 2003; Radetzki, 1994). We used dehydrated OA, purity 99.6% (Sigma-Aldrich obtained from Riedel-de Haën, Enologia Apicultura). The solutions of OA used in the trickling and spraying methods were the same, .8, 1.6, and 3.2% (3.2% means that 4.5 g of OA crystals were dissolved and added to sugar solution and made up to 100 ml, and is a .5 M solution of OA). By applying 50 ml of solution, the actual dose per colony was thus .56, 1.125, and 2.25 g. The sucrose solution was itself made up using one kilogram sucrose per one liter water. It is standard beekeeping procedure to apply OA in strong sucrose solution (50%W/W). The solution was prepared 12–18 h before application.

The sublimation method used pure OA. We applied the three equivalent doses (.56, 1.125, and 2.25 g) plus an additional higher dose (4.5 g). We decided to use a

fourth higher dose because there was less background information on varroa mortality using sublimation. In addition, our pilot research had shown that this high dose appeared not to cause high bee mortality, meaning that it could, if necessary, be used to control varroa in hives.

Experimental design

We used 10 apiaries within 20 km of the University of Sussex, each of which had 11 experimental colonies. At each site one colony was a control, untreated with OA or syrup but opened and inspected and used to recover dead bees and mites in the same way as the treatment colonies. Each of the other 10 colonies at each apiary was used for 1 of the 10 experimental treatments (dose application). The 10 treatments and control were applied at random to the colonies within an apiary. Colonies were also inspected one day before treatment and collection of the first and second worker bee sample to verify that none had sealed brood. Colony strength, in terms of number of frames of bees, was also quantified during this final inspection.

Time taken to apply OA

The time taken for two people to treat each colony was noted, including the time needed to open the hive in the trickling and spraying methods.

Estimating varroa and bee mortality at the time of application, and colony survival and strength in spring

Determining varroa mortality by extraction from samples of worker bees

To estimate the proportion of varroa mites killed, we collected one sample of worker bees per colony immediately before treatment and another 10 days after treatment. Samples were sufficiently large (mean = 266.9 worker bees, range 256–302) to contain sufficient mites for meaningful analysis (Dietemann et al., 2013). The samples were frozen. Subsequently, the mites were washed off the bees using a jet of water (warm water for about 5 min) and caught in a fine metal screen. A pilot study that checked three samples of 300 worker bees under a microscope after washing had shown that 100% of the mites were extracted. The number of bees per sample was also counted. The proportion of varroa killed was then calculated as $1 - d/e$, where e is the number of mites per 100 worker bees before treatment and d the number after.

Determining varroa efficacy from mite fall onto hive floor

Most previous studies used mite fall onto the hive floor or bottom board to measure varroa mortality (Dietemann et al., 2013) using the formula: $(a - b)$

100/($a + b$), where a = number of mites falling per day after using OA and b = number of mites falling per day before using OA (bottom board) (Calderone & Lin, 2003; Fries et al., 1991; Gregorc & Jelenc, 1996; Ritter, 1981). However, this method does not determine the proportion of varroa that have been killed. We used this method to link our study to previous research and to show the relationship between absolute varroa mortality and mortality estimated from the increase in mite fall.

Quantifying worker bee mortality

Dead bees were collected from the dead bee trap and from the mesh above the bottom board every 2 days for 8 days before and 10 days after OA treatment.

Quantifying effects on colonies after 4 months

Each colony was inspected in mid-spring, 3 May 2013, 111 days after treatment with OA on 12 January, to determine whether it was still alive and if alive, whether it had a queen. The number of frames (counting .5 per side with brood) of sealed and unsealed brood was also determined in the colonies with a queen.

Confirming high varroa mortality, colony survival and strength

A second trial was carried out in the period 12–21 December 2013 using 89 bee colonies located in nine apiaries within 20 km of the University of Sussex, most being colonies and apiaries also used in the first trial. As before, the colonies were all broodless or made broodless and in hives consisting of a single “commercial” brood chamber. All colonies were treated with 2.25 g OA via sublimation. The aim was to test this particular dose and method, which was the best combination as shown by the results of the first trial, to verify that the results were replicable and to firmly establish the result using a large number of colonies, rather than the 10 in the first trial.

The 89 bee colonies treated in December 2013 with OA were inspected on 31 March 2014 for survival and brood amount, to compare with results of the efficacy, strength, and colony mortality from the previous year. Colony inspections were carried out earlier than in the previous year because spring 2014 was approximately one month in advance of spring 2013.

Statistical analysis

Data were analyzed using the SPSS statistical program version 20. If necessary, we log or arcsine transformed the response variable to meet the assumptions of ANOVA (Grafen & Hails, 2002; Zuur, Ieno, & Elphick, 2010). We then used two-way ANOVA to test for differences between the effects of OA application method

(treatments) and dose efficacy against varroa and side effects on cute bee mortality. We then used Tukey's *post hoc* tests to compare varroa mortality and effects on bees between OA application methods or doses. $p < .05$ is defined as significant. Descriptive statistics are given as mean \pm standard error.

Results

Varroa fall onto the hive floor before and after treatment

After OA treatment, the number of dead mites on the hive bottom board increased greatly in comparison to pre-treatment levels, and then decreased to a low level to six days after treatment (Figure 1). This shows that the killing effect was considerable, and occurred at or soon after treatment. It also shows that extracting surviving mites from a sample of worker bees collected 10 days after treatment was appropriate to measure the number of surviving varroa, and from this to calculate the proportion of mites killed by OA treatment.

Effect of OA on varroa and bee mortality

Determining varroa mortality from samples of worker bees

We first tested whether varroa mortality depended on colony strength (number of frames of adult bees). As this had no significant effect ($F = 1.027$; $p = .44$), we removed it from the model. We found a significant effect of application method ($F = 22.53$, $p < .001$), dose ($F = 38.13$, $p < .001$), and their interaction ($F = 9.59$, $p < .001$). We then compared the effect of each dose and method *post hoc* with Tukey's test (Table 1, Figure 2a).

All treatments gave significantly greater varroa mortality than the control, except for the lowest dose (.56 g) via both spraying and trickling (Figure 2a). All three methods gave high (c. 93% or above) and statistically similar levels of mortality at the 2.25 g dose. Both trickling and spraying showed clear and statistically significant dose response effects. With sublimation, varroa mortality was high, 81%, even at the lowest dose .56 g, and higher still at the three higher doses. However, the *post hoc* tests showed that these differences among doses were not statistically significant. Overall, the trickling method was the least effective and sublimation the most effective in terms of dose mortality. The sublimation method gave high mite mortality at all doses used. Dose differences were significant when the lowest sublimation dose was compared to the three highest doses combined ($F = 12.89$, $p = .001$).

Effect of dose and application method on varroa fall onto the hive floor

As above, we found no effect of colony strength, but a significant effect of method ($F = 18.93$, $p < .001$), dose

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Table 1. *p* Values for pairwise *post hoc* Tukey tests comparing varroa mortality based on numbers of mites in samples of worker bees from hives with different OA application methods and doses before and after treatment.

	T .56 g	T 1.125 g	T 2.25 g	Sp .56 g	Sp 1.125 g	Sp 2.25 g	Su .56 g	Su 1.125 g	Su 2.25 g	Su 4.5 g
Control	.81	<.001	<.001	.48	<.001	<.001	<.001	<.001	<.001	<.001
T .56 g		.007	<.001	1.00	<.001	<.001	<.001	<.001	<.001	<.001
T 1.125 g			.003	.03	.025	<.001	.15	<.001	.005	<.001
T 2.25 g				<.001	1.00	1.00	.97	1.00	1.00	.92
Sp .56 g					<.001	<.001	<.001	<.001	<.001	<.001
Sp 1.125 g						.98	1.00	.97	1.00	.58
Sp 2.25 g							.72	1.00	1.00	.99
Su .56 g								.66	.98	.18
Su 1.125 g									.99	.99
Su 2.25 g										.88

Notes: Bold values are significant ($p < .05$). T, Sp, and Su refer to OA application via trickling, spraying, and Sublimation, respectively. Numbers, such as 2.25 g, refer to the amount of OA in the 50 ml of syrup applied or to the weight of OA applied directly via sublimation, per hive.

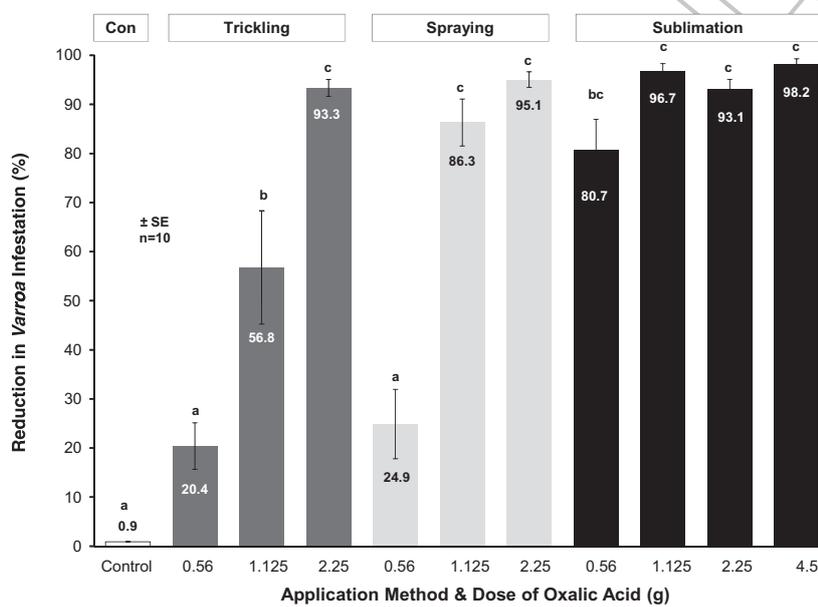


Figure 2a. *Varroa* mortality as determined by the numbers of mites in the samples of worker bees taken immediately before and 10 days after OA treatment. Histogram bars with different letters indicate significant differences, $p < .05$. Error bars show the standard error. Numbers on the x-axis refer to the weight in grams of OA applied to each hive, either in 50 ml of sucrose syrup (trickling or spraying methods) or directly via sublimation.

($F = 24.75$, $p < .001$) and their interaction ($F = 15.56$, $p < .001$), and carried out *post hoc* analysis with Tukey tests (Table 2). The number of varroa falling to the hive floor (Figure 2b) showed a very similar pattern to the proportion of varroa killed (Figure 2a). In particular, trickling and spraying showed a dose response effect, but sublimation did not. In addition, the lowest trickling dose did not differ from the control. The similarity between the two measures is expected, but is also reassuring given that they are independent data sets relevant to the same thing: varroa mortality.

Effect of application method and dose on bee mortality

We found no significant effect of colony strength on the number of dead bees ($F_{1,109} = 1.495$; $p = .106$) so we removed this from the model. We found a significant

effect of application method ($F = 4.56$, $p = .013$), but no significant effect of dose ($F = 1.35$, $p = .262$) or dose-method interaction ($F = 1.33$, $p = .265$). We then carried out Tukey *post hoc* tests (Table 3a).

Figure 3 shows that sublimation caused lower bee mortality than spraying, and was similar to the control. There was also a trend towards higher mortality with increasing dose in the trickling and spraying methods, but not with sublimation.

Effect of OA on colony strength and survival

Effect of dose and application method on colony and queen mortality

Across all doses, more colonies died following spraying (11/30, 37%) than for trickling (5/30, 17%) and sublimation (2/40, 5%), respectively (Figure 4). Two of the 10

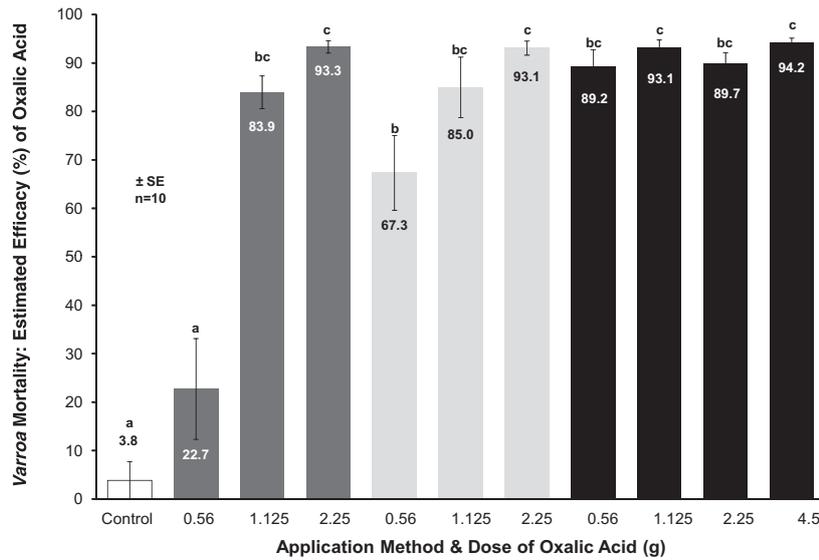


Figure 2b. Varroa mortality as determined from the numbers of mites counted on the hive bottom board before and after OA treatment (using formula: $(a - b) 100 / (a + b)$ (Calderone & Lin, 2003; Fries et al., 1991; Gregorc & Jelenc, 1996; Ritter, 1981). a = number of mites falling per day after using OA. b = number of mites falling per day before using OA. Mites were counted every 2 days for 8 days before and 10 days after OA treatment, and the numbers averaged. Histogram bars with different letters indicate significant differences, $p < .05$. Error bars show the standard error.

Table 2. p Values for pairwise *post hoc* Tukey tests to determine whether varroa mortality determined from mite fall onto the hive bottom board differed from that determined from numbers of mites extracted from samples of worker bees before and after OA treatment.

	T .56 g	T 1.125 g	T 2.25 g	Sp .56 g	Sp 1.125 g	Sp 2.25 g	Su .56 g	Su 1.125 g	Su 2.25 g	Su 4.5 g
Control	.14	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
T .56 g		.007	<.001	<.001	<.001	<.001	<.001	<.001	<.001	<.001
T 1.125 g			.92	.64	1.00	.93	.99	.93	.99	.87
T 2.25 g				.027	.99	1.00	1.00	1.00	1.00	1.00
Sp .56 g					.35	.031	.15	.029	.15	.018
Sp 1.125 g						.99	1.00	.99	1.00	.98
Sp 2.25 g							1.00	1.00	1.00	1.00
Su .56 g								1.00	1.00	.99
Su 1.125 g									1.00	1.00
Su 2.25 g										.99

Notes: bold values are significant, $p < .05$. T, Sp, and Su refer to OA application methods trickling, spraying, and sublimation, respectively. Numbers, such as 2.25 g, refer to the amount of OA in the 50 ml of syrup applied or to the weight of OA applied directly via sublimation, per hive.

Table 3a. p Values for pairwise *post hoc* Tukey tests comparing the number of dead bees from the bee trap and mesh above the bottom board in different OA application methods.

	Trickling	Spraying	Sublimation
Control	.89	.34	.99
Trickling		.53	.56
Spraying			.03

Note: Bold values are significant.

control colonies (20%) also died. We found a significant effect of method ($F = 4.98, p = .009$) on colony mortality, but no significant effect of dose ($F = 1.49, p = .22$) and no interaction between these two factors ($F = 1.53, p = .199$). To determine the effect of method we made

post hoc Tukey tests (Table 3b). The difference between sublimation and spraying was significant ($p = .01$) (Figure 5).

Effect of dose and application method on colony strength

We found a significant effect of application method ($F = 16.37, p < .001$), but no significant effect of dose ($F = .253, p = .859$) or dose-method interaction ($F = .971, p = .429$) on colony strength (number of frames of brood) four months after treatment. *Post hoc* test analysis using Tukey's test showed that colonies treated by sublimation had significantly more brood than colonies treated using the other methods and also the control colonies (Table 3c).

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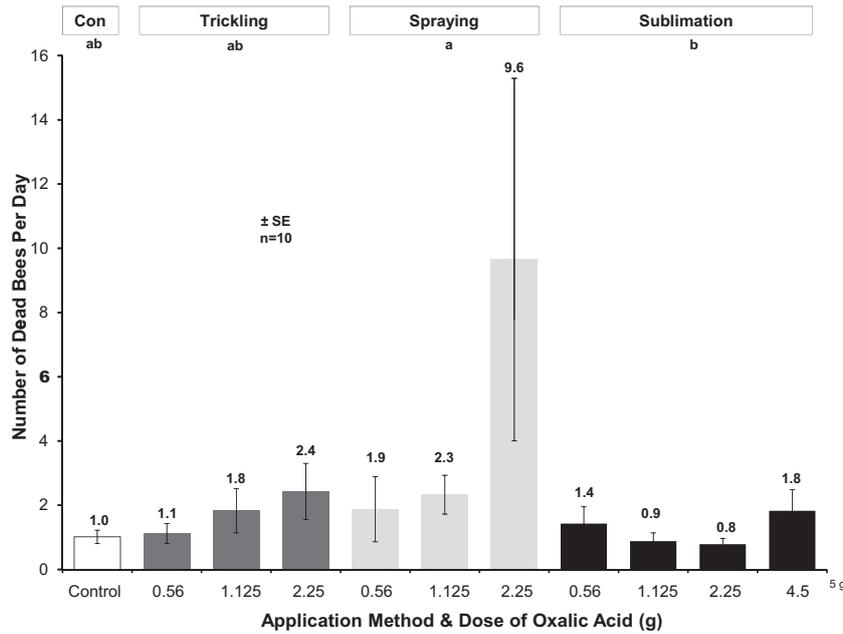


Figure 3. Mean numbers of dead bees in each treatment group in the dead bee trap and on the mesh above the bottom board combined. Bees were counted every 2 days for 8 days before and 10 days after treatment. Histogram bars with different letters indicate significant differences, $p < .05$. Error bars show the standard error.

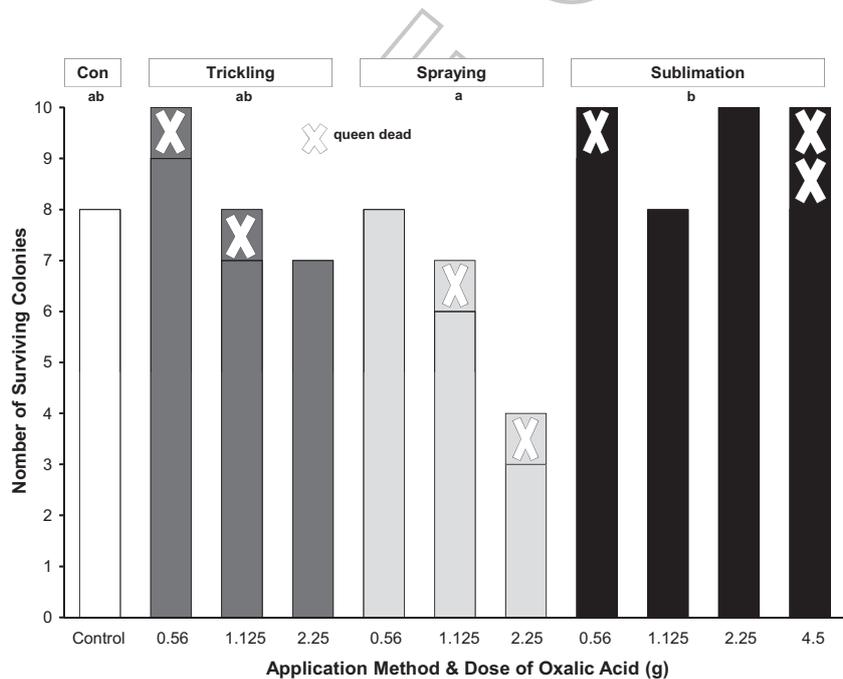


Figure 4. Colony survival on 3 May, 111 days after treatment. Crosses represent colonies that were alive but queenless. Treatment groups with different letters indicate significant differences, $p < .05$.

Effect of application method on time taken to treat a colony

Figure 6 shows the amount of time taken for two people working together to treat a colony, or in the case of the control, to open the hive for one minute. As

expected, spraying is the most time consuming method, as the frames with bees on them need to be removed from the hive. The sublimation method takes longer with higher doses as it takes longer for the OA to completely sublimate.

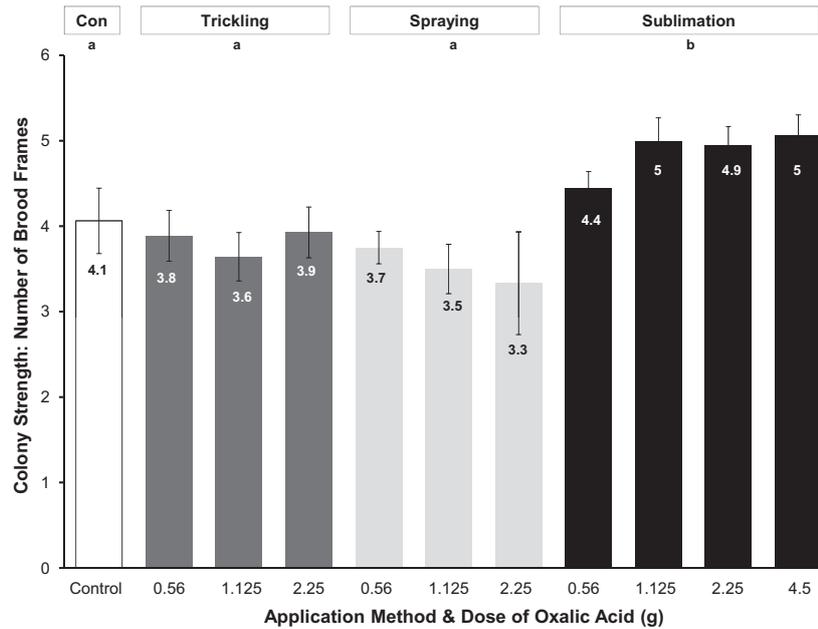


Figure 5. Colony strength four months after OA treatment, quantified as the mean number of frames (.5 per side) with either sealed or open brood cells in the surviving colonies with a queen in each treatment group. Histogram bars with different letters indicate significant differences, $p < .05$. Error bars show the standard error.

Confirming the effectiveness of the best method, 2.25 g sublimation

Determining varroa mortality from samples of worker bees

Sublimation treatment with 2.25 g of OA in December 2013 gave mean varroa mortality of 97.6%. This is significantly higher than the mortality for 2.25 g via sublimation in January 2013 (93.1%, $p = .006$), but not higher than for the three highest doses, (1.125, 2.25, and 4.5 g) combined (96.0%, $p = .101$).

Table 3b. p Values for pairwise *post hoc* Tukey tests comparing colony survival four months after treatment by different OA application methods.

	Trickling	Spraying	Sublimation
Control	.99	.41	.96
Trickling		.25	.70
Spraying			.01

Notes: Bold values are significant.

Table 3c. p Values for pairwise *post hoc* Tukey tests of colony strength in terms the number of frames of brood in mid-spring four months after the treatment with different OA application methods.

	Trickling	Spraying	Sublimation
Control	.87	.47	.44
Trickling		.76	<.001
Spraying			<.001

Note: Bold values are significant.

Quantifying midterm effects on colony mortality and strength

The treated colonies had high survival 98% (87/89) after 109 days. All were queenright and had an average of 4.75 frames with brood.

Discussion

Our results show clearly that OA can be highly effective at killing varroa mites under beekeeping conditions in broodless hives in winter. However, varroa mortality is affected by application method and dose. In addition, bee and colony mortality and colony performance are also affected by application method and dose. The results show that sublimation is the best method, in that it gives greater varroa mortality at lower doses, and results in no harm to the colonies. In fact, colonies treated via sublimation had significantly more brood in spring than controls, and lower winter mortality, although this difference was not significant.

Varroa mortality showed a dose effect in all three application methods, and in all three methods, one or more of the higher OA doses gave mortality of 93% or more (Figures 2a and 2b). Sublimation gave higher varroa mortality at lower equivalent doses (i.e., the same amount of OA per colony) than trickling or spraying. At lower doses, sublimation was the most effective method and spraying was more effective than trickling (e.g. significantly higher mortality at the 1.125 g dose (1.6% OA)). This is probably because spraying results in more of the bees, and hence mites, being contacted by the solution than with trickling. Previous research also indicated that all three methods can give high varroa

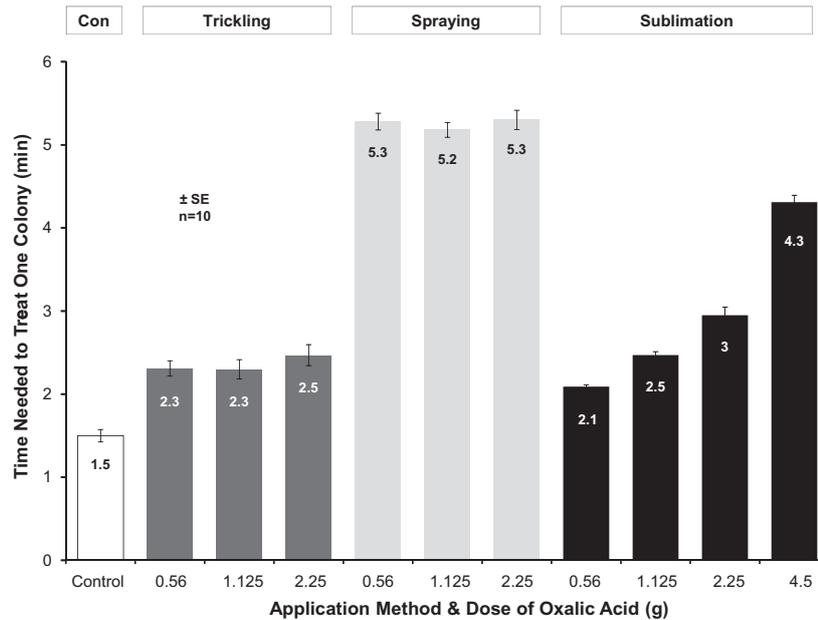


Figure 6. Time needed per hive to apply OA. Control hives were opened for 1 min. Error bars show the standard error.

mortality >90%, although this was based on mite fall (Gregorc & Planinc, 2001; Imdorf et al., 1997; Nanetti et al., 2003; Rademacher & Harz, 2006; Radetzki & Bärman, 2001a) not on the proportion of mites killed, and that spraying gives greater mite mortality than trickling (Nanetti et al., 1995) although not to a significant degree. In terms of the method used to quantify varroa mortality, with trickling and spraying, the intermediate 1.125 g, dose appeared to be more effective when considering mite fall vs. the proportion of mites killed based on extracting mites from samples of worker bees. This shows the importance of quantifying the actual proportion of varroa killed rather than the number that fall onto the hive floor.

The number of dead bees falling onto the hive floor and collecting in the dead bee trap in the 10 days after OA application was low in all treatments, with a maximum of 9.6 bees per day at the highest dose (2.25 g) with spraying. Although this was approximately 10 times higher than the control, it is still a low absolute number given that a colony in the winter will contain c. 5–10,000 bees. Thus, 10 days of mortality at 10 bees per day would be only 1–2% of the workers. Sublimation gave significantly lower bee mortality than spraying. Across all doses, sublimation gave mortality rates of .8–1.8 bees per day, similar to the control. Trickling at all three doses and spraying at the two lowest doses also gave rates similar to the control (Figure 3). High mortality following spraying has been shown in some previous studies (Higes et al., 1999) but not in others (Rademacher & Harz, 2006). Trickling causes low bee mortality (Aliano & Ellis, 2009). We are unaware of any previous data on the effect of OA sublimation on bee mortality.

Differences among application methods on colony performance were observed in mid-spring, 3 May, 111 days after application (In the study area foraging began in March in 2013). Of the control colonies, 8/10 (80%) survived, and all had a queen. This shows that background colony survival was less than 100%. Of the sublimation colonies, 38/40 (95%) survived and 35/40 (88%) had a queen. Although this difference in colony survival is not significant ($p = .43$, $hi^2 2 \times 2$ test) the trend is for the sublimation-treated colonies to have higher survival. Survival of the colonies sprayed with OA solution was significantly lower than for sublimation (19/30, 63%; $p = 0.01$) (Figure 4). Survival of colonies dribbled with OA solution (25/30, 83%) was similar and not significantly different to control or sublimation colonies ($p = .13$, $hi^2 2 \times 2$ test). Previous studies have also reported colony mortality following OA spraying, but not significantly greater than control colonies (Higes et al., 1999; Toomemaa, Martin, Mänd, & Williams, 2010). We are unaware of any previous research that has quantified the effect of OA treatment via trickling or sublimation on colony mortality.

The amount of brood after four months is also of interest. In particular, sublimation resulted in significantly more brood than controls, at 4.8 (average of the four treatment means) vs. 4.1 frames. The numbers of frames of brood was not different among control, trickling, and spraying colonies. However, there was a trend towards lower amounts of brood with higher doses for both trickling and spraying. Previous research also reported a negative effect on brood rearing following the application of OA via spraying and trickling (Higes et al., 1999; Rademacher & Harz, 2006).

Sublimation is clearly the best method overall, as it is the best in all three criteria studied. Firstly, it requires the least amount of OA to give high varroa mortality and gives high mortality over the widest range of doses. Our results from the second trial (97.7% varroa mortality with 2.25 g OA) confirmed the high mite kill that can be achieved via sublimation. Secondly, sublimation resulted in no harm to the bees, either at the time of treatment or four months later in mid-spring. In fact, it actually resulted in stronger spring colonies. We do not have any firm explanation for why this may be the case. One possibility is that by killing mites, OA treatment increases colony performance but that this benefit is counteracted in the trickling and spraying methods via harm to the bees, but not with sublimation. In this respect, it is worth noting that the amount of brood in the sublimation colonies was lowest at the lowest dose of OA. If this effect is found in further studies, it would be worthwhile to determine the underlying reason.

The third advantage of sublimation is that it is the simplest method, and quick. In particular, because it does not need the beekeeper to open the hive, it is less work and is well suited for use in winter (Radetzki & Bärman, 2001a, 2001b), when colonies are broodless but are not normally opened for inspection. It could be applied, for example, on rainy or cool days when opening a hive is not good beekeeping practice (Crane, 1990; Gould & Gould, 1988). The time taken to apply 2.25 g of OA via sublimation, which we consider to be a recommendable dose, is under three minutes per colony. This is slightly more, by about half a minute, than for trickling but less than for spraying.

Sublimation has two small disadvantages. First, it requires the use of a 12 volt car battery, and the special purchase of a mask and heated application tool. But these can be used many times. Second, OA is considered harmful if breathed (Gumpp, Drysch, Radjaipour, & Dartsch, 2003), although in our experience, all the vapor was contained within the hive. In part, this was because we sealed the hive entrance with foam immediately after inserting the sublimation tool, and also because the hot tool was inserted into the hive entrance just a few seconds after being loaded with OA. If need be, this could be made certain by only supplying the electricity to the sublimation tool when it has already been loaded with OA and inserted into the hive entrance. However, this would take several minutes extra time per colony to cool down and heat up and would not be practical for a commercial beekeeper treating many colonies. We achieved the same effect by quickly loading the tool with OA at the hive entrance and, within one or two seconds, inserting it before any had sublimated. We did not find it difficult to use the sublimation method.

Our results are very encouraging for beekeepers. They show that a quick and cheap method, sublimation, can kill approximately 97% of the varroa in a broodless honey bee colony. A broodless period is normal in the

honey bee colony's seasonal cycle in many parts of the world, and treatment can be made at this time. Alternatively, colonies with a caged queen could be treated, although this requires considerable additional work by the beekeeper. Swarms and package bees could also be treated within c. eight days of placing into a hive, before any sealed brood is present. Temperature and humidity also need to be taken into account (Aliano & Ellis, 2009; Rademacher & Harz, 2006). One of the goals of varroa control is to develop treatment methods that can be applied at long intervals. Depending on varroa population increase, the level of mite kill from OA sublimation may be sufficient for annual treatment in a winter broodless period without the use of additional control measures in combination with hygienic behavior. This seems to be the case in the study location (Al Toufailia, Amiri, Scandian, Kryger, & Ratnieks, 2014), especially for colonies that are also hygienic. If this is not the case then additional varroa control methods may be necessary.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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