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ORIGINAL RESEARCH ARTICLE

Effect of oxalic acid on the mite *Varroa destructor* and its host the honey bee *Apis mellifera*

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Here, we study the effect of oxalic acid on isolated varroa mites and on varroa mites parasitizing caged honey bees treated with oxalic acid *per os* or topically (by trickling or by sublimation). We also study the effect of oxalic acid (trickling and sublimation) on individual bees, focusing on their lifespan, midgut morphology and function, and Malpighian tubule morphology. Effect on mites: contact of isolated mites with oxalic acid coated surface (Petri dishes treated by sublimation) significantly decreased mite viability. In an experiment on varroa mites parasitizing caged bees treated with oxalic acid, the strongest acaricidal effect was observed following oral application and the lowest when oxalic acid was applied through sublimation. Effect on bees: oxalic acid applied by sublimation did not decrease bee lifespan over the 21 days of observation contrary to trickling, where a nonsignificant lifespan decrease was observed. Topical application of oxalic acid increased the rate of midgut cell apoptosis, with a stronger statistically significant effect seen in the group treated by trickling. However, neither trickling nor sublimation caused epithelial destruction in the midgut and Malpighian tubules or loss of digestive tract function.

Efecto del ácido oxálico en el ácaro *Varroa destructor* y su huésped, la abeja de la miel *Apis mellifera*

Aquí estudiamos el efecto del ácido oxálico sobre ácaros *varroa* aislados y sobre abejas de la miel en cajas parasitadas con ácaros *varroa* mediante el tratamiento con ácido oxálico *per os* o tópico (por dispersión o por sublimación). También estudiamos el efecto del ácido oxálico (por dispersión o en sublimación) en abejas individuales, enfocándonos en su esperanza de vida, la morfología y función del intestino medio, y la morfología y de los túbulos de Malpighi. Efecto sobre los ácaros: el contacto de los ácaros con una capa superficial de ácido oxálico (placas Petri tratadas con sublimación) disminuyó significativamente la viabilidad de los ácaros. En un experimento con abejas de cajas parasitadas por ácaros varroa tratadas con ácido oxálico, el efecto acaricida más fuerte se observó siguiendo la aplicación oral y el más débil cuando el ácido oxálico se aplicó por sublimación. Efecto en las abejas: el ácido oxálico aplicado por sublimación no disminuyó la esperanza de vida de las abejas a lo largo de los 21 días de observación, al contrario que al tratarlas con ácido oxálico dispersado, donde se observó una disminución no significativa de la esperanza de vida. La aplicación tópica de ácido oxálico incrementó el nivel de apoptosis en las células del intestino medio, con un efecto más fuerte y estadísticamente significativo en el grupo tratado con ácido oxálico dispersado. Sin embargo, ni el contacto con el ácido dispersado ni por sublimación, causó la destrucción del epitelio del intestino medio o de los túbulos de Malphigi, ni una pérdida en la función del tracto digestivo.

Keywords: honey bee; *Varroa destructor*; varroosis; acaricide; oxalic acid; sublimation

Introduction

Varroosis, caused by infestation with the ectoparasitic varroa mite *Varroa destructor*, is one of the most destructive diseases of western honey bees *Apis mellifera* (Ellis, Evans, & Pettis, 2010; Le Conte, Ellis, & Ritter, 2010; Potts et al., 2010). Nowadays, most honey bee colonies need regular treatment. If left untreated, the colonies usually collapse within two years after initial infestation (Le Conte et al., 2010). Most control strategies are based on the use of synthetic acaricides as they are economically convenient, their application is simple and application usually does not require any expensive equipment. However, increasing problems with varroa resistance to synthetic acaricides, as well as an increased risk of residue build-up in bee hive substrates and products has raised interest in treatment with non-synthetic substances. These control strategies are mostly based on the use of essential oils or natural organic acids.

Among the organic acids, oxalic acid is one of the most widely used (Nanetti et al., 2003). Its main advantage is the low risk of bee hive contamination and the very low probability of mites developing resistance. The most widespread mode of application is through trickling or by spraying the bees with oxalic acid dissolved in sugar syrup (Charriére & Imdorf, 2002; Nanetti et al., 2003; Nanetti & Stradi, 1997; Toomemaa, Martin, &

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Williams, 2010). It is also possible to apply oxalic acid through sublimation of oxalate crystals using an electrically heated tool (Al Toufailia, Scandian, & Ratnieks, 2015; Radetzki, 2001). In the hive temperatures, oxalic acid vapors promptly change back to a solid phase, as melting point of oxalic acid dihydrate used for the treatments is 101.0 °C (Lipeng et al., 2017).

In temperate climate areas, oxalic acid is usually used as a single-shot treatment performed during the winter broodless period (Charriére & Imdorf, 2002). For trickling, several oxalic acid and sucrose concentrations have been tested in various climatic areas (reviewed by Nanetti et al., 2003). The best results (high efficacy and good tolerability by bees) have been achieved when 4.2% solution was used. However, application of 3.2% oxalic acid has been shown to be an acceptable alternative to 4.2% solution, leading to comparable acaricidal effect (Nanetti et al., 2003). Lower doses than 4.2% were used successfully by several authors – 3.2% solution (Mahmood, Wagchoure, Raja, & Sarwar, 2012), 2.9% solution (Gregorc, Knight, & Adamczyk, 2017; Gregorc & Planinc, 2001; Smodiš Škerl, Nakrst, Žvokejl, & Gregorc, 2010), or 3.4 and 3.7% solution (Gregorc & Planinc, 2001). In products registered in European countries for varroa control, oxalic acid concentration varies between 3.5 and 4.2% (with 4.2% solution being the most widely used).

For sublimation, 2.25 g of oxalic acid is recommended per hive (Al Toufailia et al., 2015). Of the possible modes of application, sublimation has been shown to be the least damaging, being well tolerated by the bees (Al Toufailia et al., 2015; Coffey & Breen, 2016) and allowing the use of lower oxalic acid doses than either trickling or spraying (Al Toufailia et al., 2015).

This work was aimed at evaluation of oxalic acid effect on varroa mites and honey bees. Our first aim was to assess whether oxalic acid applied by sublimation kills varroa mites even after short exposure, as it is possible that mites would tend to avoid the acid after initial contact. Next, we evaluate the effect of oxalic acid on treated bees. Most work concerning oxalic acid to date has been focused on its acaricidal effect (Akyol & Yeninar, 2009; Girişgin & Aydin, 2010; Gregorc & Planinc, 2001, 2004; Rademacher & Harz, 2006), while evaluation of its effect on bees has mostly been limited to monitoring worker mortality, winter queen losses or fitness and survival of experimental colonies (Akyol & Yeninar, 2009; Gregorc & Planinc, 2001; Rademacher & Harz, 2006).

Studies concerning the effect of oxalic acid on individual bees are rather scarce, and there is some controversy between the results obtained. While Aliano, Ellis, and Siegfried (2006) has shown low acute toxicity of oxalic acid for bees, deleterious effects have been described by a number of other authors (Gregorc & Smodiš Škerl, 2007; Martín-Hernández et al., 2007; Toomemaa et al., 2010). In these works, however, oxalic acid was applied at extremely high doses (Martín-Hernández et al., 2007) or in an unusual manner (Gregorc & Smodiš Škerl, 2007; Toomemaa et al., 2010). In our work, we emphasize the use of small doses and modes of application routinely used by beekeepers during field treatments. As part of our studies on the effects of oxalic acid on treated bees, we focused attention on bee lifespan and the integrity of the digestive and excretory systems. For this purpose, food consumption was measured in order to evaluate midgut function, while a histopathological examination of the midgut was performed to analyze midgut morphology. Further, we measured caspase 3 activity in midgut tissue in order to evaluate the apoptosis rate of midgut cells, thereby reflecting potential damage caused by the treatment.

Materials and methods

Treatment of the hive space with sublimated oxalic acid

Sublimated oxalic acid was spread around the bee hive using an electrically heated pan (Varrox; BioVet, Switzerland), according to the manufacturer's recommendations. A pan containing crystalline oxalic acid (oxalic acid dihydrate; Sigma-Aldrich, USA, 1 g per shallow Langstroth hive body) was inserted into the hive through the entrance. The entrance was then closed tightly and the pan connected to a 12 V battery. After 2.5 min, the pan was disconnected and left in the hive for a further 2 min, whereupon the hive was closed for 10 min. This method was used to coat glass Petri dishes used for experiment on isolated varroa mites. The same procedure was used to treat bee hives with bees.

Effect of oxalic acid on isolated mites

Glass Petri dishes were placed vertically into an empty hive consisting of a floor, one shallow hive super and a cover. The hive space was then treated with 1 g of sublimated oxalic acid as described above. The Petri dishes, now coated with fine oxalic acid crystals, were used for the experiments on isolated mites.

Phoretic mites were collected from infested worker bees using powdered sugar (Dietemann et al., 2013). The mites were transferred to Petri dishes with a fine brush within an hour of collection. Four experimental groups were established. In Group 1 (control), mites were kept on clean Petri dishes until the end of the experiment. In Group 2, mites were kept on clean Petri dishes, within which a small dish coated with crystalline oxalic acid was inserted. The small dish was covered with a fine net to prevent direct contact of mites with the oxalic acid crystals. In Group 3, mites were kept on Petri dishes coated with crystalline oxalic acid. After five minutes, they were carefully transferred to clean Petri dishes using a fine brush and kept there until the end of the experiment. In Group 4, mites were kept on Petri dishes coated with crystalline oxalic acid throughout the experiment. Each experimental group consisted of three dishes, each containing 10–12 mites. All Petri dishes were covered and placed into a

thermostat set at 27 °C. Humidity was maintained by placing a piece of wet filter paper inside each dish.

The dishes were examined under a dissecting microscope at 2, 4, 6, 12, 24 and 48 h time intervals and the viability of the mites determined. Mites were considered dead when they did not move when stimulated with a fine brush. All mites were examined microscopically at the end of the experiment to evaluate potential cuticular damage caused by oxalic acid crystals.

Effect of oxalic acid on mites parasitizing adult bees

Intact adult bees were sampled from a single colony and kept in a common cage to establish control group, group treated by trickling and group treated *per os*. This colony was then treated with oxalic acid applied by sublimation, as described above. Four hours after treatment, the bees were sub-sampled in order to form the group treated with sublimation.

In Group 1, (intact) bees were left untreated as a control. In Group 2, 2 µl of 3.5% oxalic acid dissolved in a 45% sugar solution (i.e., 70 µg of oxalic acid per bee) was applied topically to the abdomen of each bee. In Group 3, bees were caged individually and allowed to eat 20 µl of 0.35% oxalic acid dissolved in a 45% sugar solution (i.e., 70 µg of oxalic acid per bee). After eating the solution, the bees were placed into experimental cages. In Group 4, bees were treated with oxalic acid applied by sublimation, as described above. Each experimental group consisted of three mite-tight cages containing 15–20 bees with mites in one cage.

Phoretic varroa mites were taken from an infested colony using powdered sugar and gently transferred to all experimental bees (one mite per bee) using a fine brush. Only active, vital mites that were able to attach onto the host immediately were used for the experiment.

Bees with attached mites were observed for five days. Dead bees and mites found on the bottom of the cage were counted daily and removed from the cages. The bees were fed with a 45% sugar solution, with the feed being replaced daily. All mites found on the bottom of the cage were examined microscopically at the end of the experiment.

Effect of oxalic acid on lifespan, food consumption, midgut morphology and apoptosis of midgut cells

Three bee colonies were treated with 3.5% oxalic acid dissolved in 45% sugar solution by trickling the solution over the bees in the hive spaces (5 ml of solution for each space occupied). After four hours, adult worker bees (approximately 60 bees from each treated colony) were sampled and placed into a common cage. Three further colonies were treated with oxalic acid applied by sublimation (1 g of oxalic acid per hive super), as described above. After four hours, adult worker bees (approximately 60 bees from each colony) were sampled and placed into a common cage. Bees from six untreated hives were sampled and placed into a common cage to form a control group.

Bees from each common cage (sublimation, trickling and control) were randomly divided into three smaller cages (50 individuals each). The cages were then placed into a ventilated thermostat and maintained at 27 °C and 60–70% humidity. Bees were fed *ad libitum* with 45% sugar solution, the feeders being cleaned every two days and the sugar solution replaced. All feeders were weighed before cleaning and after changing the sucrose solution and the amount of solution consumed per bee calculated. The caged bees were monitored for 21 days.

Dead bees were counted daily and removed from the cages. At the end of the experiment, all dead bees were examined microscopically to exclude infection with *Nosema* spp. microsporidia, which could negatively affect the lifespan of bees and falsify the results.

Six living bees from each cage (n = 18 for each experimental group) were sampled 72 h after the treatment. The midgut of three bees from each cage (n = 9 for each experimental group) was prepared, fixed in 4% formaldehyde and paraffin - embedded tissue sections were prepared. The sections were then stained with hematoxylin/eosin. The midgut of the remaining three bees from each cage (n = 9 for each experimental group) was prepared and immediately frozen at -80 °C until used for the caspase 3 assay.

At the end of the experiment, the midguts and Malpighian tubules of three bees from each cage (n = 9 for each experimental group) were examined histologically as described above. All hematoxylin/eosin-stained histological sections were examined under polarized light (with crossed polarizers) in order to identify oxalate crystals in the midgut and Malpighian tubules.

Caspase 3 assay

Caspase 3 activity was measured using Ac-DEVD-AMC (Sigma-Aldrich, USA) as a substrate, as described by Kremserová et al. (2016). Bee midguts were lysed on ice for 20 min using 80 µl of lysis buffer containing 50 mM HEPES ((4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.2% CHAPS, 5 mM DTT (1,4 dithiothreitol) (all from Sigma-Aldrich, USA) and 0.2 µM aprotinin (Applichem, Germany). The samples were then shaken and centrifuged at 15,000*G* for 15 min at 4 °C. The supernatants and buffers were stored on ice until use. The supernatants (50 µl) were mixed with 150 µl of assay buffer (20 mM HEPES, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA (ethylenediaminetetraacetic acid) [Sigma-Aldrich, USA] and 50 µM caspase 3 [Ac-DEVD-AMC (N-Acetyl-Asp-Glu-Val-Asp-7-amido-4-Methylcoumarin CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate] substrate). Fluorescence was measured using an Infinite 200 fluorimeter (Tecan, Switzerland) with excitation/emission wavelengths of 380/460 nm. After measurement, the frame containing

the samples was placed into an incubator set at 37 °C and the measurement repeated at hourly intervals. The last time point before the fluorescence signal started to decline (4 h) was used for evaluation of caspase 3 activity. The fluorescence values obtained were normalized against protein concentrations in the samples. A commercial Bradford protein assay kit (BioRad, USA) was used for colorimetric measurement of protein concentration.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). The data were analyzed using nonparametric ANOVA (Kruskal–Wallis test). The Dunn's *post hoc* test for multiple comparisons was applied to test statistical significance among particular experimental groups. For data concerning caspase 3 activity, one-way ANOVA was used followed by Tukey HSD test. All tests were performed using GraphPad Prism 5.04 software (GraphPad, Inc., USA). Differences were considered statistically significant if $p < 0.05$.

Results

Effect of oxalic acid on isolated mites

Direct contact with crystalline oxalic acid (Group 4) significantly decreased mite viability - (Figure 1; Table 1). Mite viability was also decreased following short contact with oxalic acid (Group 3) – 63.3% of mites died after 12 h compared to 6.7% in controls (Figure 1). However, mortality was lower than in Group 4 and there was not

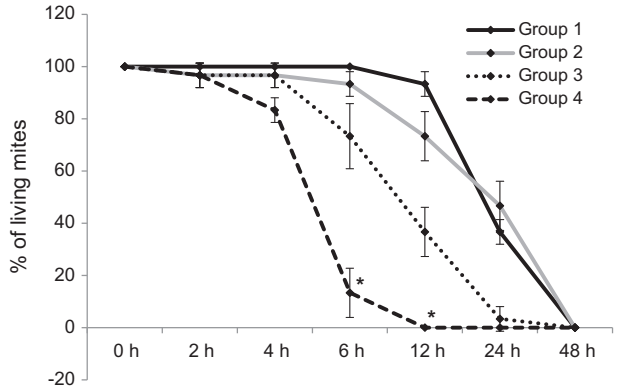

Figure 1. Percentage of viable varroa mites in experimental groups at particular time intervals.
Notes: Group 1: mites kept on clean Petri dishes (control); Group 2: mites on clean Petri dishes with a small oxalic acid-coated dish; Group 3: mites kept on oxalic acid-coated dishes and transferred to clean dishes after five minutes; Group 4: mites kept on oxalic acid-coated dishes throughout the experiment. Data for each experimental group represent the mean ± SEM from three Petri dishes, each containing 10–12 mites. Statistically significant differences from the control are marked with an asterisk.

Table 1. Viability of varroa mites exposed to oxalic acid-coated surface.

	Group 2	Group 3	Group 4
2 h	96.7 ± 2.72	97.2 ± 2.27	97.2 ± 2.72
4 h	96.7 ± 2.72	97.2 ± 2.27	85.1 ± 2.05
6 h	93.3 ± 2.72	85.0 ± 4.08	21.3 ± 2.56**
12 h	78.6 ± 5.83	47.6 ± 4.86	3.2 ± 2.65*
24 h	127.3 ± 14.85	No living mites	No living mites
48 h	No living mites	No living mites	No living mites

Notes: Group 2: mites on clean Petri dishes with a small oxalic acid-coated dish; Group 3: mites kept on oxalic acid-coated dishes and transferred to clean dishes after five minutes; Group 4: mites kept on oxalic acid-coated dishes throughout the experiment. Data represent viability of varroa mites expressed as percentage of viability in controls (Group 1) and are expressed as means ± SEM. Statistical significance is marked with
*$p < 0.05$; **$p < 0.01$.

any statistically significant difference between Group 3 and control. Examination of mites from Groups 3 and 4 under a dissecting microscope revealed oxalic acid crystals attached to their body surface (Figure 2). Attached crystals were also found in mites that were only in contact with oxalic acid for five minutes. Presence of a small dish coated with oxalic acid crystals (Group 2) did not affect mite viability (when compared with the control group). Microscopic evaluation of dead mites at the end of the experiment did not reveal cuticular damage in any group.

Effect of oxalic acid on mites parasitizing adult bees

All three modes of oxalic acid application had an acaricidal effect, with the strongest effect observed following oral application and the lowest when oxalic acid was applied through sublimation (Table 2). Examination of dead mites under a dissecting microscope revealed cutitular damage in a small number of mites. In all, we found just 9.1% (trickling group), 12.2% (feeding group) and 12.5% (sublimation group) of damaged mites in the three oxalic acid-treated groups (without statistically significant differences among the groups). Only two mites suffered potentially fatal injuries (missing legs and split dorsal shield). The rest of the damaged mites displayed only mild compression of the dorsal shield, which was unlikely to affect their viability. No damaged mites were found in the control group; however, few mites were examined in this group due to low mite mortality. Bee mortality was significantly higher only in the group where oxalic acid was orally applied (Table 2).

Effect of oxalic acid on lifespan, food consumption, midgut morphology and apoptosis of midgut cells

Oxalic acid applied by sublimation did not decrease bee lifespan over the 21 days of observation. Higher mortality was observed in bees treated by trickling, but

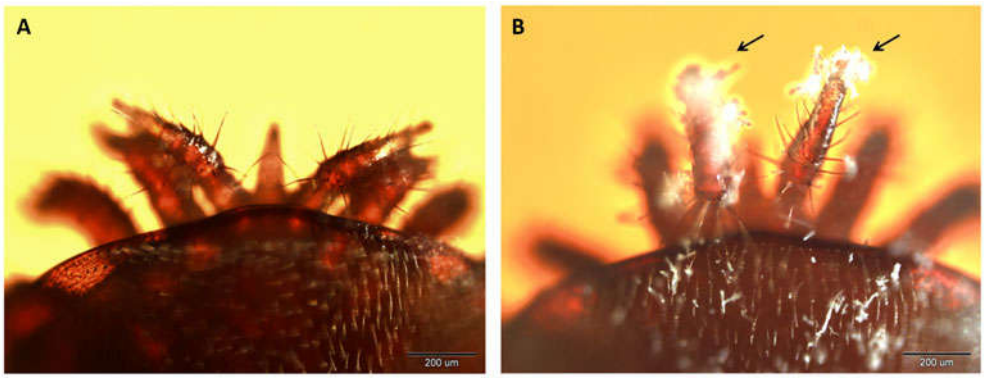

Figure 2. Intact varroa mite (A) and varroa mite from Group 3 (short exposure to oxalic acid) with white crystals of oxalic acid attached to their legs (B).

Table 2. Percentage of living varroa mites and bees after treatment with oxalic acid applied by sublimation, trickling or *per os* (expressed as % of Day 0).

	Living varroa mites (%)					Living bees (%)
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 5
Control	100.00	100.00	97.8 ± 2.22	91.1 ± 4.44	91.1 ± 4.44	95.6 ± 3.63
Sublimation	93.3 ± 3.85	91.1 ± 4.44	80.6 ± 10.56	76.7 ± 8.82	51.1 ± 17.36	86.7 ± 8.32
Trickling	80.0 ± 7.70	71.1 ± 8.89	57.78 ± 8.01*	51.1 ± 11.76	31.1 ± 11.76	86.7 ± 8.32
Feeding	84.7 ± 7.82	76.8 ± 10.08	56.9 ± 8.57*	26.8 ± 8.42*	2.0 ± 2.00**	50.8 ± 3.76*

Notes: Data are expressed as mean ± SEM. Statistical significance is marked with *$p < 0.05$; **$p < 0.01$.

Table 3. Viability of control bees and bees treated with oxalic acid applied by sublimation or trickling.

	Control	Sublimation	Trickling
5 days	97.04 ± 1.48	94.81 ± 1.48	91.85 ± 1.48
10 days	92.59 ± 3.92	93.33 ± 1.28	87.41 ± 3.23
15 days	85.92 ± 3.92	91.11 ± 1.28	72.59 ± 8.73
20 days	72.59 ± 10.29	79.26 ± 1.48	51.11 ± 12.24

Notes: Data for each experimental group represent the mean ± SEM from three cages, each containing 50 bees. Among the experimental groups, no statistically significant differences were found.

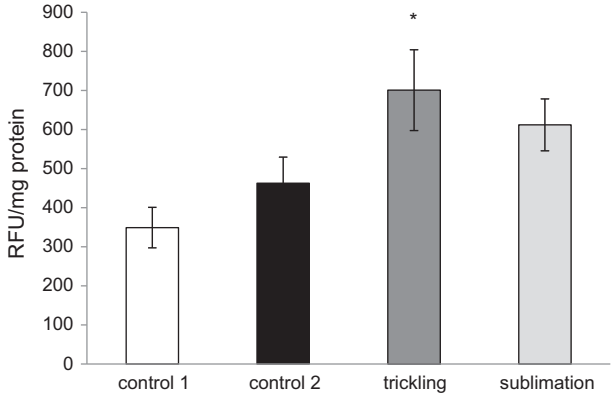

Figure 3. Caspase 3 activity in the midgut of caged bees sampled 48 h after application of oxalic acid.
Notes: Control 1 represents intact bees before caging at the beginning of the experiment. Control 2 represents untreated bees after 48 h of caging. Data are expressed as relative fluorescence units adjusted to protein amount in the samples (RFU/mg protein). Data for each experimental group represent the mean ± SEM of nine bees. Statistical significance from control 1 is marked with an asterisk.

without statistical significance (Table 3). Both modes of oxalic acid application increased the rate of midgut cell apoptosis, with a stronger (and statistically significant) effect observed in the group treated by trickling (Figure 3). None of the treatment methods affected feed consumption per bee (control: 1.24 ± 0.18 ml/bee/21 days, trickling: 1.21 ± 0.20 ml/bee/21 days, sublimation: 1.18 ± 0.17 ml/bee/21 days). Histological examination of hematoxylin/eosin-stained tissue sections did not reveal any sign of midgut tissue destruction at two days after oxalic acid application or at the end of the experiment (Figure 4). Examination of midgut and Malpighian tubules under polarized light revealed no oxalate crystals. Microscopic examination of dead bees found at the bottom of the cages showed no infection by *Nosema* spp. microsporidia in any experimental group.

Discussion

Our data showed that all modes of oxalic acid application exerted an acaricidal effect. When testing the effect of oxalic acid on isolated mites, we used dishes coated with oxalic acid crystals before placement of the mites

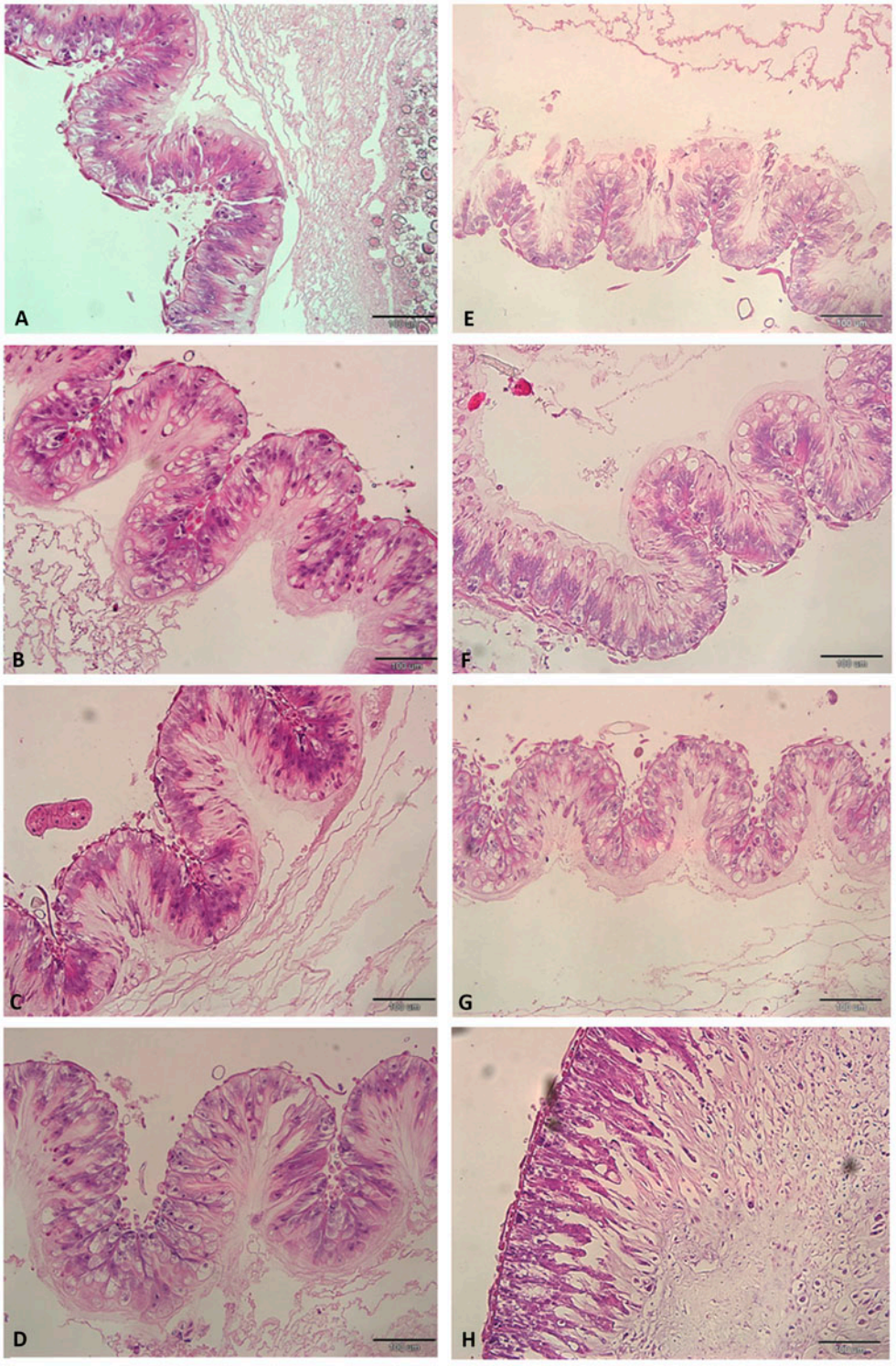

Figure 4. Midguts of experimental bees, longitudinal sections. (A) – control bee before the caging; (B) – control; (C) – trickling; (D) – sublimation, 48 h after the treatment. (E) – control; (F) – trickling; (G) – sublimation, 3 weeks after the treatment. (H) – effect of oxalic acid overdose.
Notes: H (oxalic acid overdose): Epithelial destruction and loss of segmentation in the midgut of bee fed ad libitum with 0.3% oxalic acid in 45% sucrose solution. Hematoxylin/eosin staining, magnification ×200.

to simulate oxalic acid-treated bees and hive surfaces. Within twelve hours, the oxalic acid had killed all mites in permanent contact with the crystals. From a practical point of view, however, it is more likely that mites would reside at sites free of crystals (typically in the space between bee abdominal sternites) after initial contact with the acid. Thus, we included a second experimental group in which the mites were transferred to

oxalic acid-free dishes after short contact with the crystals. We also observed increased mortality in this group, however, with 63.3% of mites dead after 12 h, compared to just 6.7% in the controls. Microscopic examination of the mites after the transfer to clean dishes revealed oxalic acid crystals attached to their body surface. During the experiment, the mites were checked repeatedly with the same result. This probably resulted in a prolongation of the effects of oxalic acid, despite the short initial contact. Given that mites did not ingest bee hemolymph during the experiment, and ingestion of oxalic acid crystals is unlikely, we presume that oxalic acid acts via contact. Contact toxicity of oxalic acid for varroa mites was also described by Aliano et al. (2006). Although the toxic effect of oxalic acid vapor was not excluded in their work, it was presumed to be insignificant due to the low volatility of this compound at laboratory temperatures. Our results are in accordance with this assumption. When direct contact with the acid was prevented, mite mortality did not increase compared to control mites. The mites were also probably unable to detect oxalic acid by olfaction as they made no attempt to avoid the small dish containing oxalic acid crystals; some were even found resting on the net covering it (i.e., 5–10 mm from the acid's surface).

In mites parasitizing living bees, an acaricidal effect was observed for all three modes of application, i.e., oral application, application by sublimation and application by trickling. In order to maintain the relevant dose in the group treated by sublimation, the whole colony was treated and treated bees were used to establish the group. A dose of 70 μg per bee was used during topical application by trickling, which is approximately equal to the average oxalic acid dose per bee used during field treatment by trickling (Charriére & Imdorf, 2002). This dose is far below the 48 h LD_{10} (176.68 μg per bee) referred to by Aliano et al. (2006). The same dose of 70 μg per bee was used when applying oxalic acid orally, though the solution was first diluted tenfold to facilitate ingestion by the bees and to prevent precocious bee death due to damage to the digestive tract caused by too concentrated acid. In this group, mites did die despite not coming into outer contact with oxalic acid. Nozal, Bernal, Gómez, Higes, and Meana (2003), when describing oxalic acid concentration dynamics in bee tissue from different modes of application (oral versus topical), noted that, in both cases, oxalic acid was still present in hemolymph after some time. As oxalic acid concentration in bee or mite tissues was not measured in our experiment, it is not clear whether the death of mites was the direct consequence of oxalic acid presence in the bee hemolymph. There is a further possibility that oxalic acid did not act directly in the orally-treated group, but caused metabolic disturbances and changes in hemolymph composition in treated bees, which finally led to the death of parasites tightly adapted to the host. Oral application of oxalic acid could not be recommended (at least in concentrations used in our work). Some authors report that this mode of application causes digestive tract damage of treated bees (Gregorc & Smodiš Škerl, 2007) and leads to high bee mortality (Ebert, Kevan, Bishop, Kevan, & Downer, 2007).

We also considered the possibility that oxalic acid-treated bees were more effective in damaging parasitic mites and removing them from the body, since Schneider, Eisenhardt, and Rademacher (2012) described increased grooming behavior after oxalic acid application. While grooming events were not monitored in our experiment, all mites found at the bottom of the cages were examined microscopically. The low number of damaged mites and the minor extent of damage suggest that grooming behavior probably did not play a crucial role in mite mortality. Given that some of the mites were still alive, it may be that oxalic acid affects the mite's ability to hold onto the host's body and/or its ability to re-invade the host. Intact mites caged together with bees were able to attach onto the host's body quickly without falling off.

Most studies concerning oxalic acid as a varroacide have focused on its acaricidal effect, with just a few studies examining its effect on individual bees. In the vast majority of cases, treatment by trickling or spraying was used as it is easier to accomplish and does not require any expensive equipment (reviewed by Rademacher & Harz, 2006). These studies have all shown oxalic acid to have low acute toxicity for bees (Aliano et al., 2006). The possibility of a delayed toxic effect cannot be excluded, however, and should be considered as long-term survival of long-aged winter bees until their natural replacement by new generations is crucial for successful overwintering and fitness of the colony (van Dooremalen et al., 2012). We have found that treatment by sublimation did not lead to increased mortality during three weeks of experiment. In trickling group, bee mortality was markedly (but non-significantly) higher than in the control group. Reduced lifespan of bees treated by trickling has also been described by Schneider et al. (2012). Al Toufailia et al. (2015), recommends to use sublimation over trickling or spraying as sublimated oxalic acid is better tolerated by bees and a lower dose is sufficient to assure a high acaricidal effect. Also Coffey and Breen (2016) report higher tolerability of sublimated oxalic acid over oxalic acid applied by trickling.

The detrimental effect of oxalic acid on bees has been described by a number of authors. Martín-Hernández et al. (2007) demonstrated severe irreversible damage to the bee's midgut, rectum and Malpighian tubules at 24, 48 and 72 h after topical application of oxalic acid. Note, however, that these authors used a much higher oxalic acid concentration (1,320 μg per bee) than in our study. Gregorc and Smodiš Škerl (2007) refer to severe midgut epithelial cell necrosis after administering oxalic acid orally. This mode of application is not used in field treatments, however, as it leads to high bee mortality (Ebert et al., 2007). Toomemaa et al. (2010) described a

1.5% – 2% oxalic acid solution as showing high toxicity to bees. In their work, however, oxalic acid was applied by submersing the whole bee into the solution. As a result, the dose per bee was probably much higher than that used during field treatment by trickling as a larger body area came into contact with the solution. In our work, we intentionally used the dose and form of administration commonly used by practical beekeepers in order to evaluate the impact of such treatment on bees. For trickling, we followed the advice of Charriére and Imdorf (2002), who suggest using a 3.5% oxalic acid solution in 50% sugar solution for trickling in typical Central European climatic conditions in order to assure both sufficient acaricidal effect and successful overwintering and fitness of the treated colonies. For treatment by sublimation, we used 1 g of oxalic acid per hive super, which approximates to the dose of 2.25 g per whole hive recommended by Al Toufailia et al. (2015). Both Carrasco-Letelier, Mendoza, and Ramallo (2012) and Al Toufailia et al. (2015), however, have shown that even higher doses are well tolerated by bees. Also other authors report good oxalic acid tolerability at colony level even when relatively high doses are used (Nanetti et al., 2003; Coffey & Breen, 2016). In our work, histological examination of the midgut and Malpighian tubules did not reveal any epithelial destruction and we excluded the possibility of oxalate crystal formation, which could also result in tissue damage and dysfunction. Tissue sections were examined under polarized light as described by Chen et al. (2012) and no oxalate crystals were observed in the midgut or Malpighian tubules. We also monitored bee feed consumption in order to evaluate midgut functionality. Food intake did not differ between the experimental groups, suggesting undisturbed function of the midgut epithelium, i.e., no malabsorbtion or maldigestion developed that could lead to energy stress, stimulating the bees to consume higher amounts of food. We did find an increase in caspase 3 activity, however, an effector caspase frequently used as a marker of apoptosis intensity. Physiologically, apoptosis is part of natural tissue turnover. In general, physiological midgut epithelial cell turnover ranges between 5 and 10% in both adult bees and larvae (Gregorc & Bowen, 1997; Gregorc, Pogacnik, & Bowen, 2004; Gregorc & Smodiš Škerl, 2007). Any increase in apoptosis rate is indicative of cell damage. Although midgut morphology and function remained unaffected in our oxalic acid-treated bees, it should be noted that both apoptosis and replacement of apoptotic cells is an energetically costly processes (Elmore, 2007). Should winter supplies in the beehive be inadequate, therefore, it cannot be excluded that an increase in apoptosis rate could cause precocious exhaustion of long-aged winter bees, which could ultimately lead to colony weakening or death.

It can be concluded that oxalic acid applied at rates typically used by practical beekeepers for routine treatment had an acaricidal effect and did not cause destruction or loss of function in the digestive tract of treated bees. However, trickling with oxalic acid significantly increased midgut cell apoptosis rate and non-significantly decreased lifespan of treated bees. Therefore, treatment by sublimation should be preferred during field treatment, despite the higher expense and labor input needed. In the future, wider use of oxalic acid for varroosis control could help prevent development of resistant varroa strains and preserve the efficacy of synthetic acaricides.

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