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Original article

Organic acids and thymol: unsuitable for alternative control of *Aethina tumida* (Coleoptera: Nitidulidae)?

Sven Buchholz[1], Katharina Merkel[2], Sebastian Spiewok[3], Anton Imdorf[1], Jeffery S. Pettis[4], David Westervelt[5], Wolfgang Ritter[6], Michael Duncan[7], Peter Rosenkranz[8], Robert Spooner-Hart[7], Peter Neumann[1,9]

[1]Swiss Bee Research Centre, Agroscope Liebefeld-Posieux ALP, CH-3033 Bern, Switzerland
[2]Institut für Ökologie, AG Populations- und Evolutionsökologie, Universität Bremen, 28359 Bremen, Germany
[3]Institut für Biologie, Martin-Luther-Universität, Halle-Wittenberg, 06099 Halle (Saale), Germany
[4]Bee Research Laboratory, United States Department of Agriculture, Beltsville, MD, USA
[5]Division of Plant Industry, Department of Agriculture and Consumer Services, Gainesville, FL, USA
[6]Chemisches und Veterinäruntersuchungsamt (CVUA), Fachgebiet Bienen, 79123 Freiburg, Germany
[7]Centre for Plant and Food Science, College of Science Technology and Environment, University of Western Sydney, Richmond, Australia
[8]Landesinstitut für Bienenkunde Hohenheim, 14195 Stuttgart, Germany
[9]Department of Zoology and Entomology, Rhodes University, 6140 Grahamstown, South Africa

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Abstract – To explore alternative strategies to synthetic insecticides for control of *Aethina tumida*, the small hive beetle (SHB), treatments already established against two other honeybee pests, *Varroa destructor* and *Galleria mellonella*, were investigated. In the laboratory, eggs, larvae, and adults of SHB were treated with thymol (10, 20, and 50 mg) or with organic acids: 85% formic acid (0.125, 0.25, 0.5, 0.75, 1.0, and 2.0 mL), 15% lactic acid (0.5, 1.0, 1.5, and 2.0 mL), oxalic acid (dihydrate crystals 35 g/L; 0.25, 0.5, 0.75, 1.0, and 2.0 mL), and 65% acetic acid (0.5, 1.0, 1.5, and 2.0 mL). Some of the chosen concentrations of formic and oxalic acid resulted in high mortalities of all SHB life stages. Therefore, they were further evaluated in the field utilising standard methods for control of *V. destructor* in Europe. After exposure to evaporating formic acid (85%, Nassenheider®) and oxalic acid (2 g dehydrate crystals, Varrox®), mortality in all SHB tested stages did not increase significantly. The same was true for trials with 85% (adults) or 60% (eggs and larvae) formic acid, evaporating from sponge tissues in diagnostic trays. In fact, some SHBs used the diagnostic trays to hide or oviposit. Despite treating extracted honey combs with 65% acetic acid, SHBs still reproduced on the combs' pollen cells. In conclusion, none of the tested methods can be recommended to control SHBs.

Apis mellifera **/ formic acid / lactic acid / oxalic acid / pest management / small hive beetle**

1. INTRODUCTION

The most serious threats to European derived honeybees, *Apis mellifera* L., are invasive parasites such as varroa mites (*Varroa destructor* Anderson and Trueman) and tracheal mites (*Acarapis woodi* Rennie) and, more recently, small hive beetles (SHBs), *Aethina tumida* Murray. Adult SHBs feed and reproduce in honeybee colonies in Africa (Lundie 1940; Neumann and Elzen 2004) and cause considerable economic damage to apiculture operations in the USA (Hood 2004) and Australia (Gillespie et al. 2003; Spiewok et al. 2007).

SHB infested colonies in the USA have been treated with the insecticide CheckMite+™ con-

Corresponding author: S. Buchholz,
buchholz.sven.berlin@gmx.de
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taining coumaphos (Elzen et al. 1999; Hood 2000) with varying degrees of success (Mostafa and Williams 2002; Ellis 2005a). In many regions worldwide, varroa populations are already resistant to this agent (Spreafico et al. 2001; Elzen and Westervelt 2002; Pettis 2003). With resistance, pest control requires increasing doses of the active substance to be effective, resulting in another drawback of chemical control—the accumulation of residues in the bee products (Bogdanov et al. 1998; Wallner 1999; Kochansky et al. 2001). Several strategies using alternatives to synthetic insecticides for control of SHB (e.g., Hood 2004; Ellis 2005a, b) have been developed. In-hive traps mounted on frames and filled with liquid compounds (mineral oil, cider vinegar, beer, ethylene glycol, alcohol, and honey) were tested by Hood and Miller (2003) to attract and/or kill adult beetles. Selected entomopathogenic fungi (Ellis et al. 2004; Mürrle et al. 2006) and nematode species (Cabanillas and Elzen 2006; Ellis et al. 2010) were detected, and their potential as biological control organisms was assessed.

Several attempts have also been made to control SHBs and other pests simultaneously. Empty combs have been sprayed with suspensions of spores of *Bacillus thuringiensis* Berliner (B 401®), a method used to control greater wax moth, *Galleria mellonella* L., in an attempt to control SHB reproduction (Buchholz et al. 2006). The application of organic acids or thymol, the essential oil of *Thymus vulgaris* L., can reduce infestation levels by *V. destructor* (Imdorf and Kilchenmann 1990; Rademacher et al. 1999; Imdorf et al. 1999; Mutinelli and Baggio 2004; Rademacher and Harz 2006). Ellis and Delaplane (2006) assessed effects of the varroa treatment Apilife Var™, which includes thymol, on host-seeking behaviour of adult SHBs. Schäfer et al. (2009) evaporated organic acids in nucleus boxes and showed success in reducing numbers of adults with acetic acid and beetle reproduction with formic acid. In storage rooms and honey houses, *G. mellonella* has been successfully controlled with acetic acid (Moosbeckhofer 1993). Lundie (1940) suggested that stored combs could be fumigated with carbon bisulphide to prevent infestation by SHB, and Mostafa and Williams (2002) showed that paradichlorobenzene kept beetles away from stored combs, along with wax moths. As both substances are chemicals, resistance and residue problems will likely arise with increased usage.

With the aim of controlling bee pests simultaneously, our objectives were to: (1) screen a wide range of substances against bee pests (organic acids and thymol) in different concentrations in the laboratory to single out their potential for the control of SHB, (2) test the most promising substances in live colonies under field conditions, and (3) test acetic acid as a potential SHB control on stored and extracted honey combs. We expected that the test substances might serve as suitable substitutes for conventional chemical controls of SHBs within bee colonies, storage rooms, or both.

2. MATERIALS AND METHODS

2.1 Laboratory experiments

The experiments were conducted at Rhodes University, Grahamstown, South Africa, in an environmental chamber at 30±1°C in permanent darkness. Beetles used for experiments were obtained from a colony reared in the laboratory (Mürrle and Neumann 2004).

2.1.1 Treatments of SHBs with organic acids and thymol

Three different life stages of SHB were treated using 25 eggs, larvae, or adults per replicate in 600-ml glass beakers. Cohorts of unsexed, 10- to 20-day-old adult SHBs were provided with 5.0 mL H_2O on a wick (pulp paper, Weiland, Dahlwitz Hoppegarten, Germany) and with ~1.0 cm^3 cube of icing sugar mixed with honey (2:1) to prevent dehydration and cannibalism. Wandering larvae were also tested, as they sometimes gather on the bottom board before leaving the hive to pupate (Hood 2004), thereby exposing themselves to treatment agents. The

wandering larvae (aged 10–14 days) were sprinkled with 2.0 mL H_2O per beaker to avoid dehydration (Schmolke 1974). No food was provided because "mature" SHB larvae no longer feed (Lundie 1940). Eggs were collected 1 day prior to commencing the experiment by providing SHB oviposition sites (Hoffmann et al. 2008) in rearing boxes with ~200 adult beetles for a period of 8 h to obtain eggs of known age. A total of 25 eggs were transferred with a single-hair brush onto a 5-mm-thick slice of a grape berry and placed in a glass Petri dish (diameter= 6.0 mm). Each dish was then transferred into a beaker. On the fruit slices, the eggs were unlikely to desiccate, and the hatching larvae were provided with food and moisture (Eischen et al. 1999; Ellis et al. 2002; Buchholz et al. 2008). All beakers were covered with nylon gauze to prevent SHB escape and facilitate gas exchange. One beaker was placed in each corner of a glass box [360×360×360 mm] (Figure 1).

Formic acid, acetic acid, and thymol were applied separately as evaporation treatments (Moosbeckhofer 1993; Rademacher et al. 1999; Imdorf et al. 1999). The portions of 85% formic acid (0.125, 0.25, 0.5, 0.75, 1.0, and 2.0 mL) or 65% acetic acid (0.5, 1.0, 1.5, and 2.0 mL) were each pipetted onto a wick [5× 6 cm] on a glass Petri dish, which was set in the centre of the glass box bottom. Thymol crystals (10, 20, and 50 mg; 99%) were placed on Petri dishes without wicks. One trial with 1.0 mL water on a wick served as control for the evaporation assays.

Solutions of 15% lactic acid (0.5, 1.0, 1.5, and 2.0 mL) and oxalic acid (0.25, 0.5, 0.75, 1.0, and 2.0 mL; made from oxalic acid dihydrate crystals, 35 g/L) were dripped with a syringe directly onto the individuals in the beakers as contact agents. For the respective controls, 1.0 mL water was used per beaker.

The glass boxes were closed tightly with a glass lid. Ventilators (CY206 Sunon®, Elektronica-Online, Schinveld, Netherlands) installed in the centres of each inner lid side facilitated equal distribution of the volatiles within the boxes. For reasons of consistency, the same ventilators were also used in the treatments with contact agents and in all respective controls.

For SHB larvae and adults, six replicate beakers were conducted for each concentration and substance. For eggs, however, eight replicates were used because preliminary investigations showed a high variance in egg mortality, even in control treatments. After 6, 12, 24, and 48 h, the beakers were briefly removed from the glass boxes to record larval and

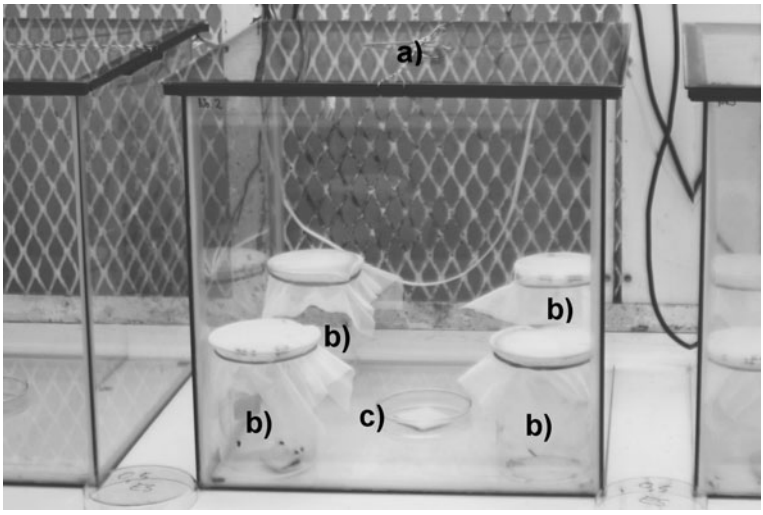

Figure 1. Experimental design to apply organic acids and thymol to different SHB life stages in the laboratory. *a*, ventilator under the lid of the glass box; *b*, beakers containing either eggs, larvae, or adult SHBs covered with nylon gauze; *c*, Petri dish with volatile on pulp paper wick, not applied when contact agents were used.

adult mortality as well as numbers of hatched eggs. None of the dead larvae or adults were removed. In contrast, newly emerged larvae were removed to prevent cannibalism. After 72 h, the experiment was terminated and mortality was assessed for adults and larvae, and the numbers of hatched eggs were counted.

The quantification of the volatiles (formic and acetic acid as well as thymol) used in the glass box atmospheres was conducted at the Swiss Bee Research Centre. Air samples were taken (Leichnitz 1985) after 6, 24, 48, and 72 h using an identical experimental setup (Imdorf 2005) but without any SHBs. They were analysed with a gas chromatograph using standard procedures (Imdorf et al. 1995).

2.1.2 Treatments of SHBs with formic and oxalic acid for 8 days

Larval and young adult SHBs were exposed to formic and oxalic acid for 8 days to investigate the agents' potential effects on oviposition and metamorphosis. Two days prior to the experiment, newly emerged adults were sexed according to Schmolke (1974), and genders were kept on a water diet in separate containers to avoid mating. For each replicate, 20 male or female beetles were transferred onto filter paper placed in a glass beaker (600 mL). The beetles were provided with 5 g pollen and 5.0 mL water, and then the beakers were closed with nylon gauze. One hundred young larvae (<48 h old) were collected from rearing boxes and distributed equally into four beakers equipped with pollen and water. To observe possible long-term effects, sublethal doses of 85% formic acid (0.25 mL) and oxalic acid (0.5 mL, 35 g/L) were chosen from the experiment above (Sect. 2.1.1) and applied in the same way. Water and pollen were added after 2, 4, and 6 days, if required. After 8 days, the treatment was terminated, and live individuals were sampled for the second part of the experiment. For both applications and life stages, respective controls with the same amounts of water were performed. After the treatment, female and male beetles were kept in pairs in glass beakers (600 mL). Pollen (3.0 g) and water (5.0 mL on filter paper) were provided, along with oviposition sites. Eight replicate beakers were established per treatment and for their respective controls. After 48 h, the oviposition rate was evaluated by counting eggs.

The live larvae were transferred into 200-mL plastic cups with autoclaved moist soil and kept for pupation in an environmental chamber at 30±1°C. To avoid desiccation and growth of fungi (Lundie 1940; Mürrle and Neumann 2004), the cups were covered with perforated plastic foil. As a result of the formic and oxalic acid treatments, the numbers of live larvae varied between 13 and 25. Therefore, only five replicates could be tested for larvae treated with formic acid, but all eight replicates were tested for those treated with oxalic acid or controls. After 16 days, the numbers of emerged beetles were recorded, since this has been shown previously to be a suitable time to complete development under the given environmental conditions (Buchholz et al. 2006).

2.2 Field experiments

2.2.1 Treatments of adult small hive beetles

The field experiments with adult SHB were performed in an apiary in Umatilla, Florida (USA) during June–September 2004. Two weeks before the experiment, 20 queen-right colonies (predominantly *A. mellifera ligustica*) of similar size (approximately six frames of bees) were set up for each of the three treatments described below. The adult SHBs in the naturally infested colonies were carefully removed from each frame and all other hive parts with an aspirator (Spiewok et al. 2007). They were kept on a honey and water diet at room temperature until they were used for the artificial re-infestation of the bee colonies.

Formic acid (60%) treatment with Nassenheider® evaporator for 13 days Following established European protocols for *V. destructor* treatments (Rademacher et al. 1999), Nassenheider® evaporators (Weiland, Dahlwitz Hoppegarten, Germany) were filled with 180 mL of either 60% formic acid or water (controls). They were affixed to frames and placed in the hive at position 2, next to a honey frame. The liquids were evaporated via a wick (50×60 mm). Tin plates (oven liner, 463×400 mm, Pactif

Co., Illinois, USA), which could be easily removed to assess adult SHB mortality, were placed on the bottom boards. Then, 100 adult SHBs were placed on the frame tops and the hive was quickly closed. The mortality was assessed on a daily basis by counting and removing dead adult SHBs from the tin plates on the bottom board. The reservoirs of the evaporators were refilled as required to ensure continuous evaporation. After 13 days, all remaining adult SHBs were collected from each frame and hive part and then counted.

Formic acid (85%) treatment with sponge tissues for 13 h To obtain a rapid distribution of the volatile agent, the formic acid (85%) was evaporated on a diagnostic tray covered with a grill (white plastic; 250×300×7 mm; Günther Spritzgußtechnik GmbH, Kaufbeuren-Neugablonz, Germany). Sponge tissues (175×195 mm, Wettpex) were placed on the plastic tray and saturated with either 20 mL formic acid or water (control). When each tray was covered with its grill to prevent bee access, it was placed onto the centre of the bottom board. Then, 100 adult beetles were introduced into each colony. As daily temperatures were above 25°C, the experiment was conducted overnight to avoid the rapid evaporation of the acid. All live and dead adult beetles were recaptured and counted after 13 h.

Oxalic acid heat application Directly before treatment, 100 adult SHBs were placed on the frame tops of the colonies (ten replicates per treatment and control) and the hives were quickly closed. To evaporate the oxalic acid, a Varrox® evaporator (Andermatt BioVet AG, Switzerland) was filled with 2 g oxalic acid (dihydrate crystals, 99%) and placed on the bottom board of each hive. The device was powered with a 12-V battery. To provide a continuous distribution of the oxalic acid fumes, the hive entrances were blocked with newspaper during the 2.5 min of heat treatment and for another 15 min after the device was removed. For the controls, the Varrox® evaporator was heated without any substance. After 24 h, all adult SHBs were collected from five treated and five untreated hives. The same procedure was repeated after another 24 h with the remaining ten colonies.

2.2.2 Treatments of SHB eggs and larvae

The field experiments with SHB eggs and larvae were performed in an apiary at Beltsville, Maryland, USA during June–August 2005. The treatments for SHB eggs and larvae, using formic and oxalic acid, were performed in the way as for adult beetles, but with two modifications. Instead of 13 days for the long-term treatment with Nassenheider® evaporators, the SHB life stages were exposed for only 4 days of 60% formic acid fumigation. In addition, to limit non-target effects on bees, we used 60% instead of 85% formic acid for the treatment with sponge tissues on diagnostic trays.

To introduce SHB eggs and larvae into the beehive and expose them to the agents, the following method was developed (Figure 2): Comb frames were each equipped with two sections of plastic cells (Permacomb® 12.5×17 cm and 8.5×6 cm) mounted on a non-drawn plastic middle wall. A circular section (8.5 cm in diameter) of the larger Permacomb® piece was filled with 40 g pollen–honey paste (2:1) to provide nutrition. Young SHB larvae (n=25; aged <48 h after hatching) were transferred into the centre of this food section. The combs were sprayed with ~5 mL water to limit larval dehydration. Eggs (aged <20 h; n=152±72) laid on oviposition sites were affixed to the smaller pieces of Permacomb®. Pollen–honey paste (2.5 g) and water (5.0 mL) were each placed into two rows of Permacomb® cells, adjoining the oviposition aid to avoid cannibalism and to enable hatching larvae to reach food and moisture easily. This “observation frame” was covered with metal gauze (mesh width, <2 mm) to avoid removal of SHB eggs and larvae (Neumann and Härtel 2004) and food consumption by the bees. The frame was set on position 5 in the middle of the colony for all experiments. After 4 days, each treatment was terminated and the number of hatched larvae was estimated in the laboratory by evaluating the egg shells. Those appearing clear under microscopic magnification (400×, phase contrast) were classified as “hatched” and those containing white structures (SHB embryos) as “non-hatched”. The larvae from the larger Permacomb® were kept in separate containers in an incubator at 30°C with pollen–honey paste and water ad libitum until they had reached the wandering stage, after which they

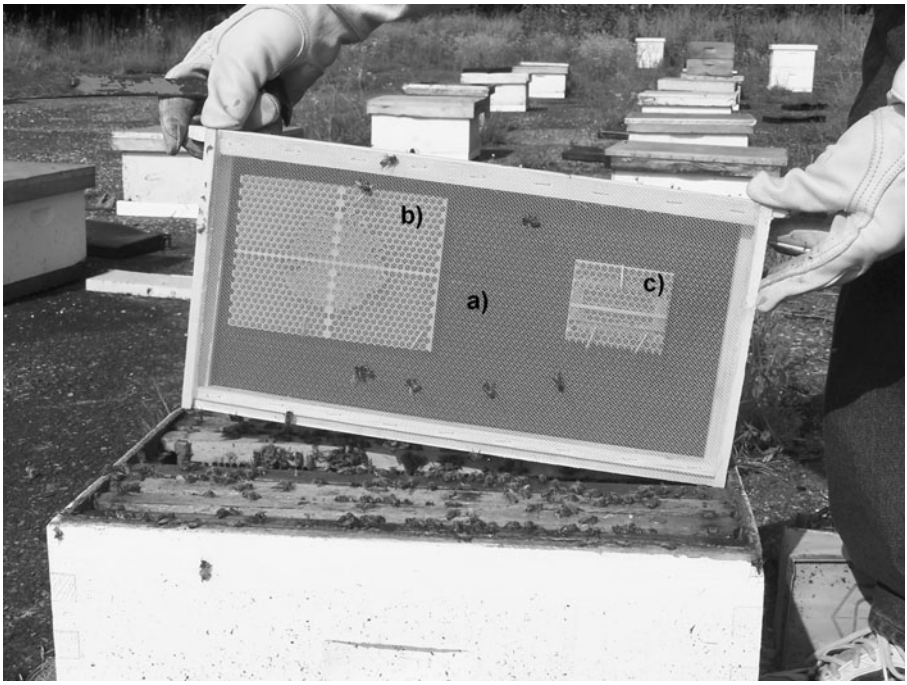

Figure 2. Design of an observation frame used for treating SHBs with organic acids in active beehives. The frame is covered with bee-proof wire mesh and placed in the centre of the beehive. *a*, non-drawn plastic middle wall carrying artificial comb pieces; *b*, Permacomb® section for larvae with circular section (8.5 cm in diameter) of 40 g pollen–honey paste (2:1); *c*, Permacomb® section for eggs on oviposition slides, with 2.5 g pollen–honey paste and 5.0 mL water.

were counted. Ten replicates were performed for both the treatment and the control.

2.2.3 Treatment of SHB on stored combs with acetic acid for 7 days

In Richmond, Australia, an experiment was performed in 2006 to investigate the effects of acetic acid on SHB reproduction in honey houses. After their manual extraction, honey frames were transferred into colonies for 24 h to be cleaned by the bees, which thereby also removed possible SHB eggs or larvae (Neumann and Härtel 2004; Spiewok and Neumann 2006). The cleaned combs were then stored in plastic bags for two days at 4°C to kill any possibly remaining beetles and to avoid re-infection by adult SHBs. The frames were then placed in single Langstroth hive boxes (seven replicates per treatment and control), with upside-down plastic lids in place of bottom boards. The boxes were set up in an extracting room at room temperature (23±3°C). Sponge tissues were also set on the frame tops and saturated with either 20 mL acetic acid (65%) or water as control. Per replicate, 20 unsexed adult beetles (aged approximately 4 weeks) were obtained from rearing containers where they had already laid eggs, and they were transferred into a box that was quickly closed with a lid. The gaps between the lids and boxes were sealed with masking tape to prevent any SHB escape and to maintain a high acid concentration. After 7 days, the boxes were opened to assess mortality of adult SHBs and the presence of SHB eggs and young larvae.

2.3 Data analysis

Data from laboratory short-term treatments were analysed using Spearman rank correlations to detect

dose-dependent mortality within treatment substances and SHB life stages (adults, larvae, and eggs). Kruskal–Wallis tests with multiple comparisons as post-hoc tests were conducted to compare treatments with respective controls (α=0.05). Treatments in laboratory long-term and all field experiments were compared with their respective controls using Mann–Whitney *U* tests (α=0.05). Whenever required, mortalities in the treatments were Abbott-adjusted (Abbott 1925). All analyses were performed using the programme Statistica©.

3. RESULTS

All values are given as means ± SD. If not mentioned in the text, statistical details are shown in the corresponding tables.

3.1 Laboratory experiments

3.1.1 Treatments of SHBs with organic acids and thymol for 72 h

The results from treatments with evaporating substances (formic acid, acetic acid, and thymol) are shown in Table I. All tested life stages showed a strong, positive correlation between the dose of formic acid and the induced mortality. After 72 h, formic acid treatments with concentrations of 2.0 mL for adults, ≥0.75 mL for larvae, and ≥0.5 mL for eggs resulted in significantly higher mortalities in comparison to their respective controls. Compared to their controls, none of the acetic acid dosages resulted in significantly higher numbers of dead adults or eggs. In contrast, all larvae were killed with doses ≥1.5 mL of acetic acid. The mortality of larvae treated with acetic acid indicated a positive dose–mortality relationship. None of the evaluated amounts of thymol increased SHB mortality significantly, regardless of the tested life stages.

The results of the experiments where SHBs had been treated with lactic or oxalic acid are shown in Table II. None of the tested dosages of lactic acid resulted in significantly increased mortality of adults and eggs. However, larvae showed a positive dose–mortality correlation, and dosages of ≥1.0 mL lactic acid resulted in significantly higher mortality than controls. Oxalic acid indicated a positive dose–mortality association for adults and larvae. Compared to the control, significantly more adults died in the highest concentration (2.0 mL) of oxalic acid. All larvae were killed with treatments of ≥0.75 mL oxalic acid. Trials with eggs treated with ≤1.0 mL oxalic acid showed fungal infestation, probably caused by spores on the fruit. In trials with 1.0 mL and 2.0 mL oxalic acid, more than half of the eggs were dead after 72 h.

Reference measurements All concentrations (parts per million) of formic or acetic acid measured from air samples declined after 24 h (Table III). Formic acid concentrations of ≥160 ppm were obtained from dosages of ≥0.5 mL after 6 and 24 h while the highest dosage of 2.0 mL formic acid, resulted in values of ≥680 ppm (6 h) and 960 ppm (24 h). The maximum concentration of acetic acid decreased from 880 ppm after 6 h to 60 ppm after 72 h. In contrast, levels of thymol compounds in the air samples increased with time, resulting in the highest values after 72 h (Table III).

3.1.2 Long-term effects of formic and oxalic acid on larval and adult SHBs

After treatment with 2.0 mL 85% formic acid, adult SHBs laid significantly fewer eggs (8.5±10.3) within 48 h in comparison to untreated adults (59.2±34.2; U=6; P<0.01). In contrast, the numbers of eggs (33.5±22.9) laid by adults treated with 0.5 mL oxalic acid [35 g/L] did not differ significantly from the controls (46.0±55.6; U=32; P>0.95).

The numbers of emerged adults (89.3±10.7) that developed from larvae previously treated with formic acid were not significantly different from those that emerged from the control (78.1±26.4; U=23; P>0.31). The same was true for adults that emerged from larvae previously

Table I. Abbott-adjusted mortalities [percent] of adult SHBs, wandering larvae, and eggs 72 h after application of organic acids and thymol by evaporation in the laboratory (adults and larvae: six replicates each; eggs: eight replicates, with 25 individuals per replicate).

Substance		Mortality		
		Adults	Larvae	Eggs
Formic acid	0.125 mL	0.7±1.6c	1.0±2.6b	4.9±8.3bc
	0.25 mL	1.9±2.1bc	18.0±10.9ab	18.4±13.5abc
	0.5 mL	3.3±6.2bc	69.5±16.8ab	75.0±22.3ab
	0.75 mL	49.4±20.4[a]ac	97±0[a]a	86.5±10.5a
	1 mL	91.3±5.9ab	97±0a	86.5±10.5a
	2 mL	100.0±0.0a	97±0a	86.5±10.5a
H_2O (control)	1 mL	0±0[a]bc	3.1±4.0[a]b	13.5±10.5c
Kruskal–Wallis test		$H_{6,38}$=32.5; P<0.001	$H_{6,36}$=36.4; P<0.001	$H_{6,56}$=43.2; P<0.001
Spearman rank		R=0.89; P<0.001	R=0.94; P<0.001	R=0.74; P<0.001
Acetic acid	0.5 mL	0.7±1.6a	0±0b	3.3±5.2a
	1 mL	0.7±1.6a	17.6±10.7ab	1.8±4.4a
	1.5 mL	0.7±1.6a	97±0a	3.9±5.4a
	2 mL	0.7±1.6a	97±0a	10.6±11.7a
H_2O (control)	1 mL	0±0[a]a	3.1±4.0[a]b	13.5±10.5a
Kruskal–Wallis test		$H_{4,28}$=0.8; P>0.94	$H_{4,28}$=26.7; P<0.001	$H_{4,40}$=8.6; P>0.07
Spearman rank		R=0; P>0.99	R=0.94; P<0.001	R=0.28; P>0.12
Thymol	10 mg	2.7±3.3a	1.0±2.6a	8.6±10.6a
	20 mg	8.6±11.1a	2.1±4.2a	2.6±5.9a
	50 mg	0±0a	0±0a	4.2±8.9a
H_2O (control)	1 mL	0±0[a]a	3.1±4.0[a]a	13.5±10.5a
Kruskal–Wallis test		$H_{3,22}$=8.5; P<0.04	$H_{3,22}$=3.4; P>0.33	$H_{3,32}$=5.6; P>0.13
Spearman rank		R=−0.38; P>0.12	R=−0.18; P> 0.47	R=−0.14; P>0.50

Results are shown as means ± SD with results of Kruskal–Wallis tests and multiple comparisons as post-hoc tests, Spearman's rank correlations with α=0.05. Values followed by different letters indicate significant differences within treatments and life stages

[a] Four replicates

treated with oxalic acid (91.2±6.0) and the respective control (93.3±9.4; U=17; P>0.10).

3.2 Field experiments

3.2.1 Treatments of adult SHBs

The results of field experiments with infested beehives treated with formic and oxalic acid are shown in Table IV. The application of 60% formic acid with Nassenheider® evaporators did not result in significant differences compared to the control after 13 days of treatment, neither in numbers of recaptured adults nor in missing adults or adult mortality. The same was true for the application of 85% formic acid on sponge tissues in diagnostic trays from which the acid evaporated for an overnight period of 13 h. The heat application (Varrox®) of oxalic acid on SHB infested bee colonies showed no significant differences in adult mortality or in numbers of living

Table II. Abbott-adjusted mortalities [percent] of adult SHBs, wandering larvae, and eggs 72 h after contact application of organic acids in the laboratory (adults and larvae: six replicates each; eggs: eight replicates; with 25 individuals per replicate).

Substance	[mL]	Mortality		
		Adults	Larvae	Eggs
Lactic acid	0.5	0±0a	13.8±24.3bc	19.9±32.5ab
	0.75	0±0a	81.3±8.2ab	12.4±12.7a
	1	0±0a	77.8±22.4ac	9.2±12.4ab
	2	0.1±0.2a	97.3±2.1a	11.8±16.3ab
H_2O (control)	1	1.3±2.0a	0.7±1.6b	11.5±6.6b
Kruskal–Wallis test		$H_{4,30}$=4.0; P>0.40	$H_{4,30}$=24.7; P<0.001	$H_{4;40}$=11.03; P>0.03
Spearman rank		R=0.28; P>0.18	R=0.84; P<0.001	R=−0.07; P>0.69
Oxalic acid	0.25	0±0b	83.3±23.1ab	–
	0.5	1.4±2.0ab	88.7±16.9ab	–
	0.75	0±0b	99.3±1.6a	–
	1	1.4±2.0ab	99.3±1.6a	53.5±11.9[a]a
	2	28.4±17.6a	99.3±1.6a	58.0±15.2a
H_2O (control)	1	1.3±2.0b	0.7±1.6b	11.5±6.6b
Kruskal–Wallis test		$H_{5,36}$=24.6; P<0.001	$H_{5,36}$=22.0; P<0.001	$H_{2,20}$=14.9; P<0.001
Spearman rank		R=0.64; P<0.001	R=0.44; P<0.015	–

Values are shown as means ± SD and the results of Kruskal–Wallis tests and Spearman rank correlations (α=0.05). Different letters indicate significant differences within treatments and life stages (multiple comparisons as post-hoc tests)

[a] Four replicates

Table III. Concentrations of formic and acetic acid [parts per million] and thymol [microgrammes per litre] after time [hours] in air samples of experimental design for laboratory SHB treatment.

Substance	Dosage	6 h	24 h	48 h	72 h
Formic acid [ppm]	0.125 mL	28	22	12	8
	0.25 mL	80	24	12	10
	0.5 mL	**160**	**160**	50	20
	0.75 mL	**240**	**240**	70	24
	1.0 mL	**320**	**400**	140	80
	2.0 mL	**(680)**	**(960)**	200	100
Acetic acid [ppm]	0.5 mL	130	36	12	6
	1.0 mL	320	220	80	36
	1.5 mL	560	560	120	100
	2.0 mL	880	480	100	60
Thymol [µg/L]	10 mg	0.6	1.8	1.4	1.9
	20 mg	1.1	2.7	3.0	2.9
	50 mg	3.3	3.5	4.7	**6.4**

Values effective against *Varroa destructor* mites (Charrière et al. 1992; Bolli et al. 1993) are in bold. Values in parentheses are toxic to bees (Charrière et al. 1992; Bolli et al. 1993) and therefore cannot be recommended for application in hives

Table IV. All recaptured, dead recaptured, and missing adult *A. tumida* (Abbott-adjusted mortality) after treatments with organic acids (controls for formic acid: water; for oxalic acid: heat without substance) in bee colonies (ten replicate hives per trial with 100 introduced adults per hive).

Agent	Formic acid (FA)				Oxalic acid dihydrate (OAD)			
Application	Nassenheider®, 13 days		Diagnostic trays, 13 h		Heat evaporation, 24 h[a]		Heat evaporation, 48 h[a]	
	Treatment (FA 60%)	Control (water)	Treatment (FA 85%)	Control (water)	Treatment (2 g OAD)	Control (heat only)	Treatment (2 g OAD)	Control (heat only)
All recaptured adults [%]	27.3± 12.4	28.6± 15.4	87.2± 31.2	92.7± 17.8	42.4± 15.2	45.0± 10.8	38.4±4.0	33.8± 10.1
MWU	U=50; P>0.96		U=46; P>0.73		U=11; P>0.75		U=8; P>0.29	
Dead recaptured adults [%]	0±0	37.8± 21.3	0±0	0.4±1.1	10.1± 21.2	0±0	0±0	1.5±3.4
MWU	U=50; P>0.99		U=40; P>0.45		U=8; P>0.29		U=10; P>0.60	
Missing adults [%]	72.7± 12.4	71.4± 15.4	12.8± 31.2	7.3±17.8	57.6± 15.2	55.0± 10.8	61.6± 4.00	66.2± 10.1
MWU	U=50; P>0.96		U=46; P>0.73		U=11; P>0.75		U=8; P>0.29	

Means ± SD and comparisons between treatments and respective controls are shown

MWU Mann–Whitney *U* tests, α=0.05

[a] Five replications

recaptured beetles compared to the control, regardless of whether adults were sampled after 24 or 48 h.

For both formic acid and oxalic acid treatments, we observed that with increasing duration of the experiments, the numbers of missing adult SHBs increased. This was true for both the treatments and the controls.

3.2.2 Treatments of SHB eggs and larvae

In comparison to the controls, the application of 60% formic acid for 4 days with Nassenheider® evaporators or diagnostic trays did not result in significant differences, either in numbers of larvae that hatched from introduced eggs or in the development of young to wandering larvae (Table V). The same was true for the treatments with oxalic acid (Varrox®).

3.2.3 Treatment of SHB on stored combs with acetic acid for 7 days

There was no significant difference in mortality of adult SHBs between treatment (23.2± 18.9%) and control (14.4±13.4%; U=17; P> 0.30) after 7 days of use of acetic acid. In six out of seven treated hive bodies, young larvae were found on extracted honey frames, which did not differ significantly from control trials, where all hive bodies were infested with young larvae (U=21; P>0.65).

4. DISCUSSION

4.1 Laboratory experiments

The main aim of our study was to kill the adult beetles with the alternative agents before

Table V. Hatched SHB larvae [percent] from introduced eggs and numbers of wandering larvae developing from young ones during treatments with organic acids in bee colonies.

Agent	Formic acid (60%)				Oxalic acid dihydrate	
Application	Nassenheider® evaporator		Diagnostic trays		Heat evaporation (2 g crystals 99%)	
	Treatment	Control	Treatment	Control	Treatment	Control
Hatched larvae	65.5±14.7	63.4±17.7	54.3±13.4	56.10±8.0	67.4±14.1	73.2±15.2[a]
MWU	U=49; P>0.93		U=46; P>0.76		U=29; P>0.11	
Wandering larvae	34.8±26.1	29.2±27.5	80.4±13.8	81.2±11.0	78.7±14.7[a]	82.7±11.7[a]
MWU	U=40; P>0.44		U=48; P>0.87		U=46; P>0.76	

Per treatment and control: ten replicate hives each with 152±72 eggs (defined as n=100%) and 25 wandering larvae. Means ± SD and comparisons between treatments and respective controls are shown

MWU, Mann–Whitney U test, α=0.05

[a] Nine replicates

they were able to reproduce. However, the wandering larvae turned out to be the most susceptible stage to the tested organic acids. At the commencement of our studies, we expected that eggs would be most susceptible, as they are more exposed to the substances due to their small size and disadvantageous volume/surface area ratio compared to the much larger wandering larvae and adults. Although the transfer of the eggs might have had a negative effect on their survival, the hatching rate in the controls was >85%. For future laboratory experiments, it may be better to leave the fragile eggs in their oviposition sites so they can be easily counted and possible injuries to the eggs during transfer would be avoided. In addition, eggs in between two slides simulate their natural situation inside the capped brood cells or in tight crevices. Therefore, in our field experiment, we left the eggs on the slides.

None of the concentrations of thymol showed any effect on SHB mortality of the tested life stages. According to previous laboratory experiments (Imdorf et al. 1995), the thymol concentration for an effective control of *V. destructor* should be between 5 and 15 μg per litre of air. In our laboratory experiments, this amount was achieved with the highest dosage of 50 mg after 72 h. In *V. destructor* treatments, the essential oil is usually applied in long-term treatments of 2 to 4 weeks (Imdorf et al. 1999; Floris et al. 2004). However, a bee colony can either be heavily infested with or even be killed by SHBs within 4 weeks (Elzen et al. 1999; Neumann and Elzen 2004). Furthermore, Ellis and Delaplane (2006) showed that the thymol-based product Apilife Var™ had no effect on the host-finding ability of adult SHBs. Therefore, thymol was ruled out as an agent worthy of being further tested in the field.

For lactic and acetic acid, positive dosage–mortality correlations were recorded for the larval stage only. All the air concentrations of acetic acid that were measured in the glass boxes were lower than those that would be reached in a regular treatment against wax moths (Imdorf 2005).

The mortalities of adults, wandering larvae, and eggs of SHB were correlated with increasing concentrations of formic acid and oxalic acid. The measured formic acid concentrations in the atmospheres of the laboratory trials of ≥160 ppm would have been effective against varroa mites within beehives (Imdorf 2005). Values of ≥680 ppm would cause severe impacts on honeybee colonies, such as disorientation of bees (Przewozny et al. 2003), queen loss, and/or dead brood (Gregorc et al. 2004).

Such concentrations should therefore not be applied in a colony. Levels of 320–400 ppm were measured after 6 and 24 h when 1.0 mL formic acid evaporated. These data would be comparable to 24-h values of a short-term treatment with 60% formic acid (Charrière et al. 1992).

We were surprised that fungal presence occurred only in the egg trial with oxalic acid. We therefore assume that the growth of the unidentified fungus was enhanced by small amounts (0.25, 0.5, and 0.75 mL) of oxalic acid and inhibited by higher concentrations (1.0 and 2.0 mL) of the agent. This is supported by observations that some fungal species produce small amounts of oxalic acids themselves (Lapeyrie 1988).

We observed long-term effects of formic acid and oxalic acid on larvae and adults, but these would not be sufficient for effective control. Although formic acid reduced the magnitude of reproduction significantly, the remaining offspring could still cause considerable damage to the beehive. As the effect of oxalic acid on oviposition was less than for formic acid, the damage that future larvae would cause in this scenario is likely to be more severe. Neither formic nor oxalic acid significantly decreased the rate of successful pupation, so the hatching adult SHBs would remain a severe threat to other colonies.

4.2 Field experiments

The promising effects in the laboratory trials could not be achieved in the field experiments, regardless of the tested developmental stage of SHB (adult, eggs, or young larvae). This might be due to different factors. For example, in the laboratory, the beetles were exposed to the agents without any hiding places. In contrast, eggs laid in crevices or in capped brood cells (Ellis et al. 2003; Neumann and Elzen 2004; Spiewok et al. 2007), as well as larvae that mine within combs (Lundie 1940; Schmolke 1974), are likely to be less exposed to the treatments. A high portion (>50%) of the introduced adult SHBs could not be relocated in the colonies neither in longer-term trials (e.g., 13 days in the formic acid investigation) nor in shorter trials (e.g., 48 h in the oxalic acid investigation). However, this could not be attributed to the application of the treatments, as the same result was recorded in the controls. We therefore assume that one or more of the following factors may have been responsible for these results. Neumann and Hoffmann (2008) showed that not all beetles will be collected in a survey using an aspirator on all frames and hive parts. After a 5-day treatment, they killed the colony and dissected all hive parts, and they found 14.06±10.53% additional adults. However, this number is much lower than the proportion of missing adults in our study. Another explanation might be the high mobility of adult SHB (Spiewok et al. 2008). SHB adults may have left the apiary during the experiment since they were not recollected in any of the test colonies. Alternatively, dead beetles may have been removed from the unscreened tin foil pans and carried outside of the hive by worker bees. This might be prevented by using screen bottom boards in future experiments. Furthermore, we observed considerable numbers of live adults under the sponge tissues, probably using the devices as a hiding place despite the presence of formic acid. In two out of ten treated hives and in four out of ten controls, female SHBs even laid eggs on the sponge tissues used to evaporate the fluids inside the trays. These observations have led to further investigations of diagnostic trays as in-hive traps for catching SHBs (Buchholz et al. 2009). Finally, we showed that SHBs reproduce on extracted honey combs with pollen cells despite a 7-day treatment with acetic acid that would have been effective against *G. mellonella*. This indicates the requirement of a stringent sanitary policy in honey houses, as already stated by Lundie (1940). In contrast to our study, Schäfer et al. (2009) showed a significant increase of adult SHB mortality in nucleus boxes without bees with a successive 70% acetic acid treatment (20 mL for 10 days in the field and another 20 mL for 5 days at room temperature of 27°C). We assume that the smaller volume of nucleus

boxes (compared to the regular single Langstroth box in the current study) might have resulted in higher aerial acetic acid concentration. In addition, Schäfer et al. (2009) exposed the adult beetles to more and higher-concentrated acid for a longer period compared to our study, which might have also resulted in the increased SHB mortality.

In conclusion, our data suggest that thymol and most of the organic acids applied are unsuitable controls of SHB. However, we recommend conducting further experiments with acetic acid using high concentrations, long exposure times, and combs in stacked Langstroth boxes.

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Acides organiques et thymol: inappropriés comme moyens de lutte alternatifs contre *Aethina tumida* (Coleoptera: Nitidulidae)?

***Apis mellifera* / acide formique / acide lactique / acide oxalique / lutte intégrée / petit coléoptère des ruches**

Zusammenfassung – Organische Säuren und Thymol: Ungeeignet als alternative Kontrolle von *Aethina tumida* (Coleoptera: Nitidulidae)? Der Kleine Beutenkäfer, *Aethina tumida*, ist ein ernstzunehmender Schädling der westlichen Honigbiene, *Apis mellifera*, dessen Bekämpfung sich im Bienenstock als schwierig herausgestellt hat. Im Labor testeten wir verschiedene Konzentrationen organischer Substanzen (Ameisen-, Milch- und Oxalsäure sowie Thymol), die in Europa zur Kontrolle der parasitären Milbe, *Varroa destructor*, verwendet werden. Zudem verwendeten wir Essigsäure, ein Wirkstoff, der den Befall gelagerter Honigwaben mit der großen Wachsmotte, *Galleria mellonella*, abwehrt. Im Vergleich zu den Kontrollen resultierten die Ameisensäure-Konzentrationen von 0,5 mL (Eier), 0,75 mL (Wanderlarven) und 2,0 mL (erwachsene Käfer) in signifikant höheren Mortalitäten (Table I). Gleiche Ergebnisse erzielten wir für Käfer und Wanderlarven bei der Verwendung von Oxalsäure (Table II). Daher wurden diese beiden Substanzen für weitere Feldversuche ausgewählt. Milchsäure (Table I) und Thymol (Table II) wurden aufgrund ihrer geringen Wirkung gegen den Käfer nicht weiter untersucht.

In Experimenten an Bienenstöcken mit einem kontrollierten Befall mit dem Kleinen Beutenkäfer (Figure 2), wurde der Effekt von Ameisen- und Oxalsäure auf drei Lebensstadien des Schädlings (Eier, junge Larven und erwachsene Käfer) getestet. Zur Applikation der Substanzen verwendeten wir Methoden, die in Europa zur Bekämpfung von *V. destructor* eingesetzt werden. Weder das Verdunsten von Ameisensäure mit der Nassenheider® Apparatur über 13 Tage, noch eine Übernacht-Behandlung mit Schwammtüchern auf Diagnoserahmen (Schwammtuchmethode) ergab einen signifikanten Anstieg der Mortalitäten des Schädlings. Gleiche Ergebnisse erzielten wir 24 und 48 h nach einer Oxalsäure Behandlung mit dem Varrox®-Verdampfer (mit 2,5 min Hitze und 15 min Einwirkungszeit).

Wir vermuten, dass die erwachsenen Käfer der Behandlung ausgewichen sind, indem sie sich an Orte im Bienenstock zurückgezogen haben, an denen die Konzentration der Wirkstoffe niedrig war. Oder sie haben den Bienenstock einfach verlassen. Letzteres bestätigt unsere Beobachtung, dass weniger Käfer wiedergefunden wurden, je länger die Dauer des Experiments währte (Table IV). Außerdem könnten tote erwachsene Käfer durch Arbeitsbienen aus dem Stock entfernt worden sein. Ein weiterer Grund für die niedrigen Schädlingsmortalitäten könnte eine zu geringe Konzentration von Ameisen- und Oxalsäure gewesen sein. Allerdings würden höhere Dosen wahrscheinlich schädliche Effekte auf das Bienenvolk hervorrufen (z.B. den Verlust der Königin oder Brutschäden).

Nachdem erwachsene Beutenkäfer einer siebentägigen Essigsäurebehandlung auf geschleuderten Honigwaben ausgesetzt waren, konnten keine signifikanten Unterschiede in der Mortalität zwischen Behandlung und Kontrolle festgestellt werden.

Zusammengefasst kann keine der untersuchten Methoden für die Kontrolle auch nur eines Lebensstadiums des Kleinen Beutenkäfers empfohlen werden,

unabhängig ihrer Effektivität gegen *V. destructor*. Basierend auf unseren Ergebnissen empfehlen wir weitere Experimente mit Essigsäure. Dabei sollten höhere Konzentrationen und längere Behandlungszeiträume untersucht werden, um die Effizienz der Säure als Schutz vor einem Befall gelagerter Waben mit dem Kleinen Beutenkäfer zu evaluieren

Ameisensäure / *Apis mellifera* / Kleiner Beutenkäfer / Milchsäure / Oxalsäure / Schädlingsbekämpfung

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