Research Article

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From consumption to excretion: Lithium concentrations in honey bees (*Apis mellifera*) after lithium chloride application and time-dependent effects on *Varroa destructor*

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Abstract

BACKGROUND: Owing to its systemic mode-of-action and ease of application, lithium chloride (LiCl) is an ideal varroacide for the control of *Varroa destructor* infestations in honey bee colonies. To better understand how LiCl functions within a colony, we screened different parts of honey bee anatomy for lithium accumulation. We wanted to elucidate the time-dependent effects of LiCl on *V. destructor* and its metabolism within honey bees when they were fed continuous LiCl treatments, as well as evaluate potential adverse effects such as accumulation in the hypopharyngeal glands of nurse bees, which could negatively impact queens and larvae.

RESULTS: Cage experiments reveal rapid acaricidal onset, with >95% mite mortality within 48 h of treatment. Bee hemolymph analysis supports these observations, showing a rapid increase in lithium concentration within 12 h of treatment, followed by stabilization at a constant level. Lithium accumulates in the rectum of caged bees (≤475.5 mg kg^{-1} after 7 days of feeding 50 mM LiCl), reflecting the bees' metabolic and excretion process. Despite concerns about potential accumulation in hypopharyngeal glands, low lithium levels of only 0.52 mg kg^{-1} suggest minimal risk to the queen and 1st- and 2nd-instar larvae. Cessation of LiCl treatment results in a rapid decline in mite mortality in the first 5 days, which increases again thereafter, resulting in mite mortality of 77–90% after 10 days.

CONCLUSION: These findings help optimize LiCl application in colonies to achieve high Varroa mortality without unwanted adverse effects and provide important baseline data for future registration.

Keywords: lithium chloride; metabolism; hemolymph; hypopharyngeal gland; *Varroa destructor*

1 INTRODUCTION

Since the mite *Varroa destructor* (Anderson & Trueman) infested the western honey bee *Apis mellifera* (Linneaus), beekeepers have had to treat their colonies with acaricides several times a year to keep them alive. Left untreated, colonies typically die within 1–3 years.[1,2] Unfortunately, all of the currently registered treatment agents have one or more of the following shortcomings. On the one hand, the efficacy of natural products such as organic acids varies substantially with environmental factors such as temperature,[3] the presence of brood and the time of the season. On the other, synthetic acaricides may promote mite resistance[4,5] and entail the risk of residues accumulating in bee products.[6–8] The efficacy of currently available options is often insufficient[9,10] and most beekeepers have to treat multiple times (e.g. Varromed®[11]) or combine mechanical and chemical controls in an integrated pest management (IPM) strategy.[12,13] Both options increase the workload and costs of treatment. Owing to these inherent limitations, there is considerable demand for new treatment methods to combat the Varroa mite.

In 2018, Ziegelmann et al.[14] discovered that lithium chloride (LiCl) mixed into a sugar syrup (Apiinvert®) and fed to caged bees killed >95% of the introduced mites within 1–3 days, depending on the concentration used. The substantial novelty of the LiCl treatment is its systemic mode-of-action (MoA|).[14] Currently all registered varroacides kill mites through a contact MoA, whereby either the mite has to come in direct contact with the compound or the chemical is distributed over the body of the bees to the mite.[15,16] By contrast, feeding LiCl syrup results in a systemic MoA, where the chemical is distributed within the bee´s body and transferred to the mite during feeding. Using the natural food transmission between honey bees called trophallaxis,[17,18] the LiCl food is distributed throughout the whole colony[19] and all of the mites in the dispersal phase feeding on the bees, are easily and

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quickly exposed. Even though several researchers have confirmed the high acaricidal effect of lithium,[14,19–21] adverse effects such as brood loss have also been reported.[19,22] Little is known about how bees metabolize lithium or how quickly and where it accumulates in the host tissues. Understanding these dynamics is crucial to developing LiCl as a viable varroacide with minimal adverse effects on the honey bee brood.

In previous cage experiments, it has been shown that concentrations of 25–50 mM LiCl are well-tolerated by the bees yet highly effective in killing mites[14] and might represent a suitable concentration for Varroa treatment in the field.[19] To understand the rapid onset of efficacy, we recorded the commencement of mite mortality once we started feeding a 50 mM LiCl syrup in cage experiments where individual bees were paired with individual mites. In a second cage experiment, we investigated the duration of mite mortality once LiCl treatment has halted. We also quantified the lithium concentrations in the bee's hemolymph and the rectum after different feeding periods with LiCl sugar syrup that ranged in duration from 6 h to 7 days. Hemolymph serves as a crucial transport medium for nutrients, hormones and waste products throughout the honey bee's body.[23,24] Studying the lithium concentration in the hemolymph provides valuable insights into the movement and distribution from initial uptake by the bee via sugar syrup and how it spreads throughout its body for potential transfer to the mites. In combination with the observed mite mortality, we can define the lithium concentration needed in the hemolymph to kill parasitizing Varroa mites. We also quantified the lithium concentrations in the rectum, the last part of the digestive tract,[25] to understand the metabolism and excretion of lithium by the bees. Following the analysis of hemolymph and rectum, we also investigated whether lithium accumulates in the hypopharyngeal glands of 7-day-old nurse bees reared in LiCl colonies. The accumulation of lithium in the royal jelly secreted by these glands could pose a danger to both the queen and the young larvae, similar to pesticide residues,[26,27] and could be an explanation of the observed brood loss during a LiCl treatment in colonies.[19,22]

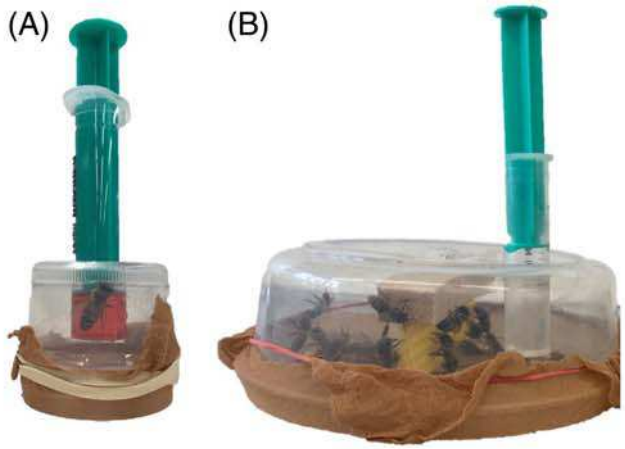

Figure 1. (A) Small cages (40 mL) equipped with a 10-mL feeding syringe containing one bee and one mite, used for the 'onset of active effect' trial. (B) Large cages (600 mL) equipped with wax foundation and a 10-mL feeding syringe, used for the 'duration' and 'build-up rate' trial.

2 MATERIAL AND METHODS

2.1 Origin of bees and mites

Apis mellifera carnica colonies were located in the local apiary of the State Institute of Bee Research at the University of Hohenheim, Stuttgart, Germany (48° 42' 31.8 " N, 9° 12' 37.5" E), in the vicinity of the botanic gardens. Queenright colonies, headed by healthy sister queens from the local breeding stock managed by the State Institute of Bee Research, University of Hohenheim, Stuttgart, Germany, were housed in polystyrene mini-hives 'MiniPlus' with six frames per box. Each colony consisted of two to three boxes, which held ≈3000–4000 adult bees. According to the needs of the experiments, adult bees were directly sampled from brood combs of untreated donor colonies ($n = 3$) and put in cages to measure the 'onset of efficacy' and the 'build-up rate' in our two trials. For the longer lasting 'duration' trial and the 'hypopharyngeal gland' experiment, we used newly emerged bees, as the age of the bee is important in these experiments. We took frames of emerging brood from untreated donor colonies ($n = 3$) and transferred them to an incubator overnight [, 30 °C, 60% relative humidity (RH); Memmert GmbH, Schwabach, Germany] The next day, the newly emerged bees were either placed directly into cages for the 'duration' trial, or marked with a queen marking pen and released back into the test colonies ($n = 3$ / treatment) for the 'hypopharyngeal gland' trial. All experiments were carried out in 2022. The 'hypopharyngeal gland' trial was repeated in 2023.

The sugar shake method[28] was used to obtain viable female *V. destructor* mites for the cage trials. Highly infested colonies outside of the experimental apiary, which had not received winter treatment against Varroa, were used as mite donors. After the sugar shake, the mites were washed with lukewarm water and placed on a damp cloth. Only mites that showed normal movement upon being touched with a brush were used in the trials and placed on bees in the cages. Upon introduction, all mites immediately attached to the bees and were subsequently observed on different parts of the bees during the daily mortality check, as they are free-moving. The subsequent movements of the mite on the bee were not recorded.

2.2 Cage trials

2.2.1 Onset of efficacy on mites

The onset of the active effect of LiCl on mites was evaluated in cage trials consisting of 40-mL containers [Rotilabo®; Roth, Karlsruhe, Germany; Fig. 1(A)]. A single bee was employed in each cage to assess the direct effect of LiCl feeding on the mite. The containers were sealed with breathable pantyhose that was secured with rubber bands instead of a plastic lid. A hole was drilled into the containers and a 10-mL feeding syringe (Injekt®; Roth) was inserted. The syringes were filled with Apiinvert® sugar syrup (Suedzucker Group, Mannheim, Germany) spiked with 50 mM LiCl (> 99.9%, p.a., ultra-quality; Roth). This concentration has been shown to be effective for treating mites in brood-free colonies.[19] To reach this concentration, we mixed 2.12 g of LiCl-salt in 1 L of syrup. Syringes of control cages were filled with pure Apiinvert® syrup. We placed one hive bee taken directly from a MiniPlus brood frame and one mite into each cage, which was then placed into an incubator (, 28 °C, 60% RH; Memmert GmbH)[29] for the observation period. The mortality of the mites was recorded after 12 h, 24 h and 48 h. After 48 h, the cages were frozen at −20 °C and the bees were checked for mites to ensure that before freezing, the mite was still alive and in the cage at the end of the

experiment. Cages in which the bee died or the mite was not found dead during the trial or was not found on the bee after 48 h were excluded from the analysis.

2.2.2 Duration of mite mortality

In order to assess the duration of the active effect of LiCl on mites following a change in diet from lithium to control sugar syrup, we used 600-mL plastic containers [Lock and Lock®. Framkfurt, Germany; Fig. 1(B)]. We attached a strip of pure beeswax foundation (8 cm × 3.5 cm) in the middle of the container. The cages were sealed with pantyhose and equipped with 10-mL syringes (Injekt®; Roth). Newly emerged bees ($n = 35 \pm 1$) were transferred into cages and placed in an incubator (28 °C, 60% RH; Memmert).[29] Bees were fed for 48 h with 50 mM LiCl syrup (2.12 g LiCl-salt / litre), then the diet was changed to pure sugar syrup (Apiinvert®). Female *V. destructor* mites ($n = 11$) were introduced into the cage at different time intervals after switching from LiCl syrup to sugar syrup (Fig. 2). For each time interval (0 h, 24 h, 48 h, 72 h and 96 h) four cages were used. Bees in the control group were fed with sugar syrup over the whole observation period. Mite mortality was documented daily after mite introduction and dead mites were removed. Additionally, the cages were checked for the presence of any dead bees, which were not counted. Upon removal, the bees were found to be free of mites. The cages were frozen at −20 °C 10 days after mite introduction and the remaining mites on bees were counted.

2.2.3 Build-up rate of lithium in hemolymph and rectum

This experiment aimed to understand the distribution of lithium within the honey bee body and the pathway lithium could take to reach mites feeding on these bees. To assess the rate of digestion and how quickly the lithium is excreted, we analyzed the concentration of lithium in both the hemolymph and the rectum. We exclusively measured the lithium levels in the samples since the chloride anion has no effect on mite mortality.[14]

Adult honey bees ($n = 50 \pm 2$) were kept in 600-mL cages [Fig. 1(B)] in an incubator at 28 °C and fed *ad libitum* with 50 mM LiCl syrup

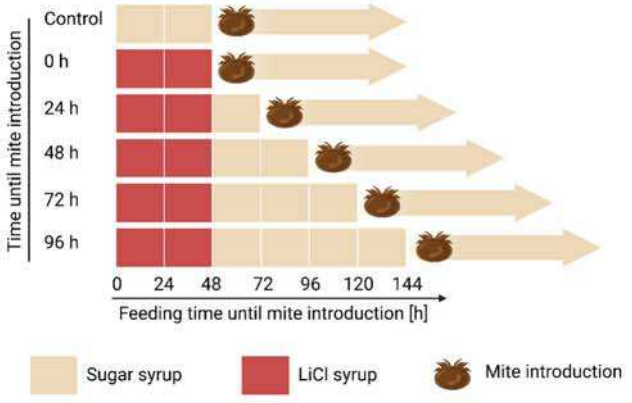

Figure 2. Feeding plan for caged bees and time until mite introduction. Bees ($n = 35 \pm 1$) were fed with 50 mM LiCl for 48 h ($n = 4$ cages / group), then the diet was changed to sugar syrup. *Varroa destructor* mites ($n = 11$ /cage) were introduced into the cages either directly after LiCl feeding ceased (0 h) or at different time intervals during the sugar syrup feeding (24–96 h). The group names indicate the time intervals between the switch to sugar syrup and the introduction of mites. After the mites were introduced, feeding with sugar syrup continued until the end of the 10-day observation period. The bees in the control group were fed with sugar syrup over the whole observation period, before and after mite introduction.

until hemolymph extraction and dissection of the rectum, with samples taken for different feeding periods (Table 1). Eight cages were used for each feeding period, resulting in a total of 104 cages.

At the end of the defined feeding period, the bees were anaesthetized with CO_2 for 5 min. All dead bees were removed beforehand. Wings were removed and a slit was cut with a small scissor into the 3rd abdominal tergite of the bee into the dorsal sinus [Fig. 3(A)]. Then a 10-µL capillary pipette (ringcaps®; Hirschmann, Eberstadt, Germany) was gently pressed against the abdomen [Fig. 3(B)]. Owing to capillary forces, 5–6 µL hemolymph per bee could easily be extracted. The hemolymph from 40 ± 5 bees per cage were pooled to reach a sample volume of 200 µL. For the rectum samples, another 5–10 bees per cage were used to gain a sample volume of 200 µL. The rectum can be easily exposed by pulling the stinger with forceps [Fig. 3(C)]. The concentration of lithium in both hemolymph and rectum was analyzed using inductively coupled plasma-mass spectrometry (ICP-MS).

2.3 Semi-field trials

2.3.1 Lithium in hypopharyngeal glands of nurse bees

In order to determine whether LiCl exposure causes lithium to accumulate in the hypopharyngeal gland (HPG) of nurse bees, we conducted semi-field trials in which young bees were reared in different test colonies for 7 days to develop HPGs in the presence of brood.[30,31]

A frame of newly emerging bees from one of the donor colonies was placed in an incubator overnight (30 °C, 60% RH; Memmert). The next day, the newly emerged bees were marked on the thorax with a queen marking pen (Posca) and then released into test colonies ($n = 3$ / treatment / year). The colonies were either fed with 2 L control sugar syrup (Apiinvert®) or LiCl syrup (Apiinvert® spiked with 25 mM LiCl, which refers to 1.06 g LiCl-salt L^{-1}) which was applied once on the day of bee release into colonies. This lower concentration of 25 mM LiCl has been used in previous experiments evaluating the effects of LiCl on brood development.[22] After 7 days, the marked bees were collected from the colonies and stored in a freezer at −20 °C until dissection of the HPGs.

For dissection, bees were defrosted, decapitated and the head was pinned down in a wax petri dish filled with deionised water. With a scalpel we cut between eyes and forehead and gently pulled with forceps on the exoskeleton to expose the HPG [Fig. 4(A)]. The glands from 10 bees were pooled into a 200-µL tube for later analysis of lithium concentration with ICP-MS. In total we dissected 120 honey bees from control colonies ($n = 6$) and 110 honey bees from LiCl colonies (n = 6) in two consecutive years ($n = 3$ colonies / treatment / year). In the 2nd year, we additionally dissected the honey stomach (crop) from the same bees for lithium analysis, to clarify whether they consumed LiCl-food. To dissect the honey crop, the abdomen was gently pulled with forceps while holding onto the thorax. This results in the crop being exposed [Fig. 4(B)]. Here, in total 80 control bees and 60 LiCl bees were dissected. Again 10 bees were pooled per sample.

2.4 Lithium analysis with ICP-MS

The samples were first weighed and then put into glass tubes to which nitric acid (HNO_3; 2 mL) was added. The tubes were then filled with double-distilled water to reach a final volume of 10 mL. The mixture was homogenized using a vortex. Microwave digestion was performed using an Ultra Clave III (MLS Mikrowellen-Labor-Systeme GmbH, Leutkirch, Germany), with

Table 1. Feeding periods of caged bees ($n = 50 \pm 2$) with 50 mM LiCl syrup until extraction of hemolymph and rectum ($n = 8$ cages / feeding period)

Feeding period with LiCl until extraction											
6 h	12 h	18 h	24 h	30 h	36 h	48 h	60 h	72 h	96 h	120 h	144 h	168 h

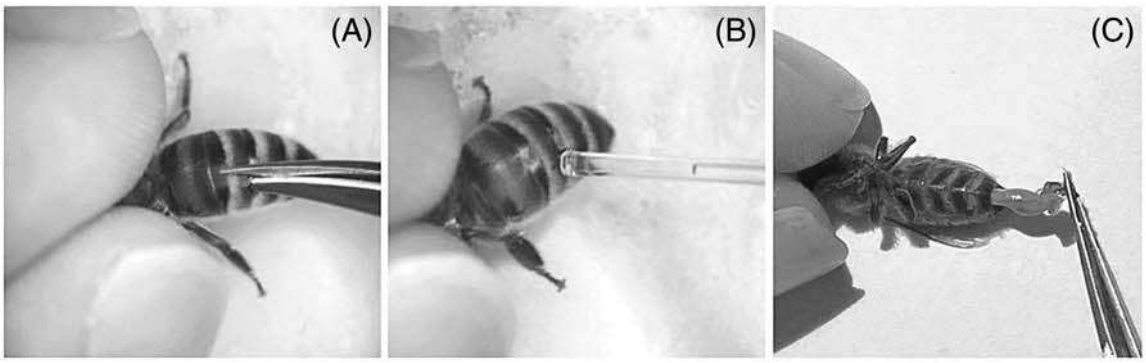

Figure 3. Extraction of hemolymph and rectum: (A) cutting into 3rd tergite of the abdomen, (B) pressing capillary pipette against abdomen to extract hemolymph and (C) exposing rectum by pulling the stinger with forceps.

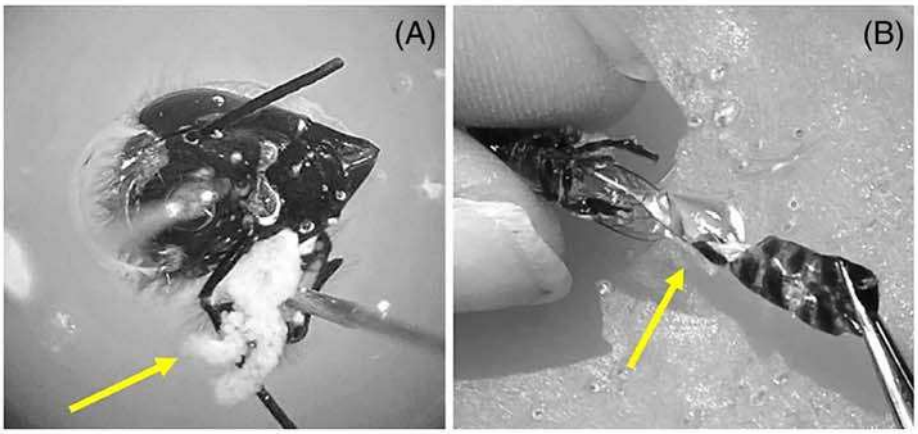

Figure 4. Dissection of the hypopharyngeal gland (A) and honey stomach (crop) (B) of a 7-day-old nurse bee.

the temperature gradually increasing from 80 °C to 200 °C at 900 W and 100 bar. The samples were analyzed using ICP-MS (NexION 300 X; PerkinElmer, Waltham, MA, USA) after being cooled and diluted. Calibration was performed using a multi-element standard solution (Merck KGaA, Darmstadt, Germany) prepared with double-distilled water and HNO_3 at concentrations of 0.1, 0.2, 1, 10 and 20 µg L^{-1}. An internal standard of CertiPUR Rhodium ICP standard solution ($c = 1000$ mg L^{-1} Rh; Merck KGaA, Darmstadt, Germany) was used [limit of quantification (LOQ) < 0.025 mg kg^{-1}].

2.5 Statistics

The collected data were statistically analyzed using the programs JMP Pro17 and IBM SPSS Statistics 27. For the 'onset of efficacy' trial we used a χ^2 test to compare the groups. The survival probability of the mites in the 'duration' trial was analyzed using a Kaplan–Meier test with pairwise log-rank and a Bonferroni correction for multiple comparisons. To analyze the increase in lithium concentration in hemolymph and rectum samples over time, we fitted quadratic polynomial curves with least-squares and tested their slope and curvature. We also compared the mean value per feeding period with the mean value of the following feeding period to analyze significant increases using an ANOVA with pairwise Tukey's honestly significant difference (HSD) test and Bonferroni correction. We tested the lithium concentration in both the honey crop and HPG for normal distribution using the Shapiro–Wilk test. As the data had nonparametric properties, we used the Mann–Whitney *U*-test to compare the groups.

3 RESULTS

3.1 Onset of efficacy on mites

Within 12 h of feeding individual bees with 50 mM LiCl syrup, 38% of the introduced mites were dead on the cage floor (Fig. 5). After an additional 12 h, an additional 40% of the mites were killed. Overall, 95% of the mites died within the first 48 h after commencing the LiCl treatment, significantly higher mortality than in the control group [$\chi^2(3) = 147.72$, $P < 0.001$]. In the control

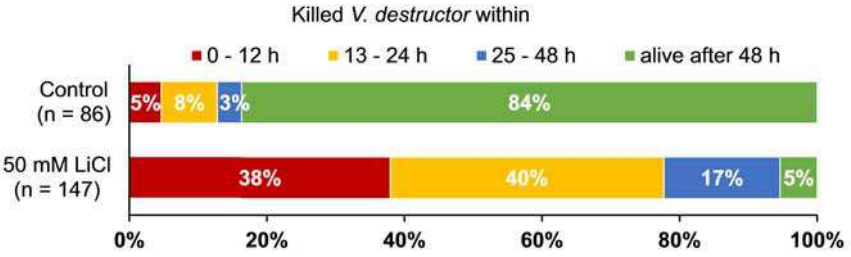

Figure 5. Timing of *Varroa destructor* mortality owing to the active effect of LiCl after application via feeding. Individual cages with one honey bee and one mite were fed with 50 mM LiCl syrup. Bees in the control group were fed with pure sugar syrup. Mite mortality was recorded after 12 h, 24 h and 48 h. Remaining mites on bees after 48 h were identified as 'alive'. Number of mites used per group are given in brackets. The χ^2 test revealed significantly higher mortality in the treatment group compared to control [$\chi^2(3) = 147.72$, $P < 0.001$].

group only 16% of the mites died within the whole observation period while 84% survived till the end of the cage trial. Two bees from the LiCl group were excluded from the analysis as they died during the trial.

3.2 Duration of mite mortality

In this experiment, 24 cages containing a total of 820 bees and 254 mites were examined to assess the duration of the active effect of LiCl on the mites after the diet was changed to pure sugar syrup.

Mite mortality in the control group was the lowest at 3–4% per day, resulting in a total survival probability of 77% at the end of the experiment (Fig. 6). Mites placed directly on the bees at the end of LiCl feeding (0 h) had the highest mortality rate with 88% of the mites dying within the first day. In subsequent days, mites continued to die in these cages, and within 5 days the maximum mortality rate of 98% was reached, differing significantly from all other LiCl groups and the control group ($P < 0.001$, log-rank; Fig. 6).

An interval of 24 h from the cessation of LiCl feeding and the introduction of the mites into the cages significantly reduced the miticidal effect of LiCl. In this group only 36% of the mites died during the first 5 days, thus significantly differing from the 0 h group [$P < 0.001$, log-rank until Day (D)5] but not from the control group ($P = 0.852$, log-rank). However, during the following days, the mite mortality of this 24 h group increased, resulting in an overall mortality rate of 86% measured on D10, which was significantly higher than that of the control group ($P < 0.001$, log-rank). A similar course of mite mortality was observed in the groups with longer intervals from diet change to mite introduction of 48 h, 72 h and 96 h. In all of these test groups, no significant difference was observed compared to the control group up to D5, thereafter the mite mortality increased and at the end of the 10-day observation period, all LiCl groups had a significant lower survival probability than the control group ($P < 0.001$, log-rank).

3.3 Build-up rate of lithium in hemolymph and rectum

The concentration of lithium in the extracted hemolymph increased after the start of LiCl feeding, with a significant difference between 6 h (0.5 ± 0.3 mg kg^{-1}) and 12 h (5.2 ± 3.2 mg kg^{-1}) of feeding ($P = 0.034$, Tukey's HSD test; Fig. 7). After 24 h and 48 h the lithium concentration was 7.1 ± 1.7 and 8.4 ± 2.1 mg kg^{-1}, respectively. The lithium concentration at 12 h did not differ significantly from later time periods, indicating that as of this time point there was no significant accumulation of lithium in the hemolymph ($P = 1$, Tukey's HSD test). The highest

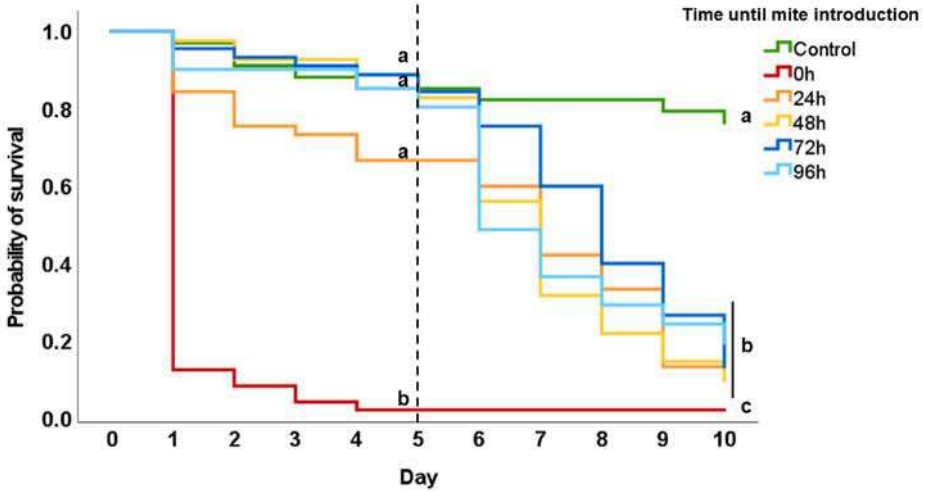

Figure 6. Duration of active effect of LiCl on *Varroa destructor* after diet change (feeding plan in Fig. 2). Observation of mite mortality started after the introduction of the mites on Day 0. Bees ($n = 35 \pm 1$) were fed for 48 h with 50 mM LiCl syrup, then the diet was changed to sugar syrup. Mites ($n = 11$) were introduced into each cage after time intervals of 0 h, 24 h, 48 h, 72 h and 96 h after diet change ($n = 4$ cages / group). Bees in the control group received sugar syrup over the whole observation period. Mortality of mites was documented daily. Kaplan–Meier survival analysis with pairwise comparison showed significant differences between the groups, as indicated by different letters. Log-rank test ($\alpha = 0.05$) with Bonferroni correction was carried out for the first 5 days and over the whole observation period of 10 days.

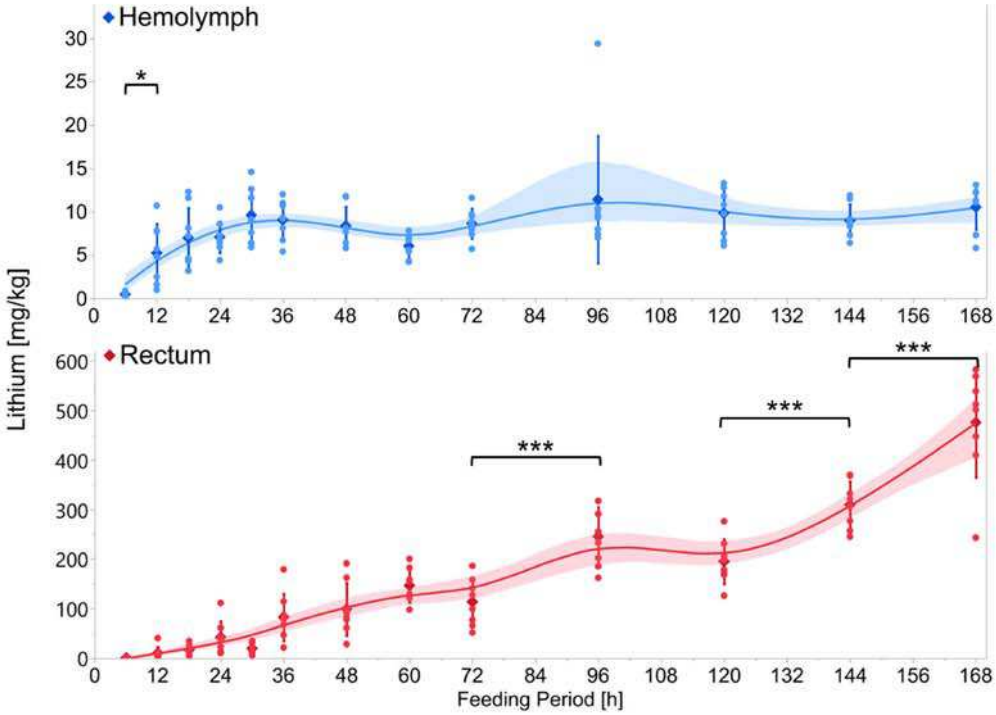

Figure 7. Lithium concentration in hemolymph and rectum extracted after different feeding periods from 6 h to 168 h. Fifty bees were fed with 50 mM LiCl in cages, $n = 8$ cages per feeding period, each given as mean ± SD. Asterisks indicate significant differences between two consecutive feeding periods [*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Tukey's HSD test)]. Polynomial fit revealed a significant effect of the feeding period on lithium concentrations for the rectum samples ($P < 0.001$), which continued to accumulate higher concentrations of lithium throughout the feeding experiment. Hemolymph samples increased significantly at 12 h, but then remained at steady-state. Note that the y-axes are thus of different scales for rectum and hemolymph samples.

lithium concentration was measured after 96 h with 11.4 ± 6.9 mg kg^{-1}. Polynomial fitting, used to look for significant differences over a factor such as time, did not reveal any significant effect of the feeding period on lithium accumulation, indicating a steady state of lithium in the hemolymph throughout all feeding periods.

In comparison, we measured twice as much lithium in the rectum samples within 12 h of commencing the feeding, a concentration of 12.2 ± 11.9 mg kg^{-1}. In the rectums, the lithium accumulated continuously, with a significant effect of the feeding period on the concentration ($P < 0.001$, polynomial fit). There were significant increases in lithium concentrations after 72 h, 120 h and 144 h of feeding ($P < 0.001$, Tukey's HSD test; Fig. 7). The maximum concentration of 475.5 ± 103.4 mg kg^{-1} was reached after 168 h of LiCl feeding.

3.4 Lithium in hypopharyngeal glands of nurse bees within honey bee colonies

In this semi-field experiment, feeding LiCl syrup at a concentration of 25 mM to colonies resulted in an average lithium concentration of 0.52 ± 0.3 mg kg^{-1} in HPG of 7-day-old nurse bees (Table 2). In the honey crop of these bees, we determined an average lithium concentration of 58.1 ± 11.8 mg kg^{-1}, which is ≈100-fold higher. In the control colonies, seven of 12 HPG samples from nurse bees reared without LiCl feeding were below the LOQ (< 0.025 mg kg^{-1}), whereas in the other five samples concentrations of 0.09–0.335 mg kg^{-1} were detected. The average concentration of lithium in samples was 0.09 ± 0.1 mg kg^{-1} and thus significantly lower than the LiCl group ($P < 0.001$, Mann–Whitney *U*-test, Table 2). A similar pattern was seen in the honey crop of control bees, which had an average lithium concentration of 4.07 ± 1.3 mg kg^{-1}, significantly lower compared to LiCl bees ($P = 0.0024$, Mann–Whitney *U*-test).

4 DISCUSSION

The acaricidal effect of lithium salts, specifically lithium chloride, on *V. destructor* has been demonstrated by multiple independent research groups[14,21,32,33] and has been confirmed even under realistic field conditions with different concentrations and applications.[19,20,32] Unlike any other treatment currently available, LiCl has a systemic MoA and can be mixed into sugar syrup and

Table 2. Lithium concentration in hypopharyngeal glands (HPG) and honey crops of 7-day-old nurse bees from colonies fed with 25 mM LiCl syrup or control colonies, fed with sugar syrup

	Lithium (mg kg^{-1})	
Origin of nurse bees	HPG ***	Crop **
LiCl colonies	0.52 ± 0.3 ($n = 11$)	58.1 ± 11.8 ($n = 6$)
Control colonies	0.09 ± 0.1 ($n = 12$)	4.07 ± 1.3 ($n = 8$)

Values are given as mean ± SD, number of analyzed samples are given in brackets. Samples were analyzed with inductively coupled plasma-mass spectrometry (ICP-MS), values below the limit of quantification (<0.025 mg kg^{-1}) were included in the calculation with a value of 0.025 mg kg^{-1}. Significant differences between LiCl and control colonies within the sample group are indicated with asterisks (***$P < 0.001$ and **$P = 0.0024$, respectively; Mann–Whitney *U*-test).

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applied by feeding to bees. This simplifies the possible use as a veterinary medicine and ensures the efficacy is independent from environmental factors such as the weather. Because treatment of *V. destructor* by a systemic MoA is a new approach for varroacides, the details of how quickly it acts when fed as a syrup solution were unknown. Two key questions must be answered to establish an effective treatment plan: (i) How long does it take from commencing LiCl feeding of the bees to the death of the mites? And (ii) How long does the active effect last once the LiCl application is ceased?

In our first experiment, we determined the duration required to achieve a lethal LiCl concentration for mites in the honey bee. We specifically used individual bees paired with a single mite to determine the onset of efficacy, as larger groups of individuals could influence the dynamic of food distribution and transmission. These cage experiments showed a strikingly fast onset of LiCl induced mortality on *V. destructor* mites via honey bee feeding. Almost 40% of the mites were killed within 12 h, this mortality reached 80% by 24 h and within 48 h over 95% of the mites were dead. Both Ziegelmann et al.[14] and Stanimirovic et al.[21] showed that in bigger cages with 50 bees and 25 mites it took >4 days to reach a mite morality of >95%, however they used half the LiCl concentration of our experiment.

Our hemolymph extracted from bees fed a 50 mM LiCl syrup clearly demonstrates that the lithium concentration increases rapidly once feeding starts and then plateaus after 12 h to a steady state. Despite continuous feeding of LiCl syrup, an equilibrium is reached within the bee between the uptake and catabolism of lithium in the hemolymph. Other studies with different substances have demonstrated that the metabolism of bees could be even faster. For example, the toxins amygdalin and quinine were detected in bee hemolymph extracted just 1 h after feeding it to the bees.[34]

We measured the lithium concentration in the hemolymph of the host bees, although Ramsey et al.[35] described the fat body as the primary nutrient from their hosts for dispersing *V. destructor* mites. We chose hemolymph for several reasons. (i) The extent to which hemolymph or fat body provides the crucial nutrition for the parasitizing mites remains controversial. Recently, Piou et al.[36] succeeded in rearing Varroa mites for long periods of time under *in vitro* hemolymph feeding conditions and mentioned that 'we do not know whether the necessary nutrients come mostly from lysed hemocytes or fat body cells or directly from molecules circulating in the hemolymph'. The authors suggested that Varroa mites consume both hemolymph and fat body cells. Meanwhile, Han et al.[37] showed through biostaining and proteomic analysis that the life history stage affects the proportion of hemolymph and fat body in the Varroa mite's diet. (ii) As mentioned in the introduction, the hemolymph is the transport medium in the honey bee's body and the intention of our experiments was to follow the path from the uptake of lithium by the bee in the gut to its transfer to the mites that feed on these bees. The fat body is also bathed in hemolymph, which is important for the secretion and absorption of molecules between the tissues and the circulating fluid.[38] (iii) We have established a method for separating the hemolymph and fat body of adult bees (Grünke M, unpublished), but this method is extremely time-consuming and not suitable for serial analysis and sampling in the quantities required for the ICP-MS method. However, a first comparison of the lithium concentration in fat body and hemolymph after 3 days of feeding 50 mM LiCl to adult bees showed an almost identical lithium concentration of ≈11 mg kg^{-1}. Irrespective of the proportion of hemolymph and fat body consumed by the Varroa mite, the hemolymph data should be suitable to reflect the miticidal threshold of lithium concentration in the bee body.

Owing to the rapid effect of the consumed lithium on the parasitizing mites – 38% mortality after 12 h and 95% after 48 h – we could here for the first time quantify the threshold needed to induce mortality. A concentration of 5–8 mg kg^{-1} lithium in the hemolymph is sufficient to kill mites that parasitize these bees. This is an important parameter for the development of lithium applications in full-sized colonies under field conditions. To achieve a 95% acaricidal efficacy, the concentration, dosage and composition of lithiated food should be designed so that the majority of bees in a colony achieve a hemolymph concentration of >5 mg kg^{-1} for ≈48 h.

Our bee rectum samples show that the concentration of lithium in the rectum rises with the duration of feeding. In contrast to the hemolymph, there is a continuous increase without a steady state. After just 12 h, the concentration of lithium in the rectum at 12.2 mg kg^{-1} is twice that in the hemolymph, and peaks at 475 mg kg^{-1} at the end of the experiment on D7 of feeding. As caged bees do not defecate, waste products, including lithium, accumulate in the rectum over time. We cannot exclude that the absence of defecation in cage trials may have slightly influenced the lithium build-up in the hemolymph due to the absorption of some water and salts through the rectal pads.[39] However, the continuous increase in lithium concentration over the week suggests that there is no particular absorption of LiCl from the rectum to the hemolymph. More important, the accumulation of lithium in the rectum is a useful result, as it means that the lithium is continuously metabolised and then excreted outside the colony by the bees, ensuring that it does not accumulate when applied to free-flying colonies. A similar course was shown by Du Rand,[40] who fed nicotine nectar to caged worker bees and measured the concentration of nicotine and its metabolites in the hemolymph, digestive tract and rectum over time. Nicotine concentration decreased in the crop, remained constant in the hemolymph and increased in the rectum,[40] mimicking what we saw with lithium in our study.

Our knowledge of the lithium metabolism in insects is generally poor. We can only speculate which physiological factors contribute to the here observed dynamic of uptake, degradation and excretion of lithium. First, food is ingested and temporarily stored in the honey stomach (crop). It then enters the midgut through the proventriculus.[41] It is likely that the epithelial cells located in the midgut absorb lithium and carry it to the hemolymph, while the Malpighian tubules clean the hemolymph by removing waste metabolites and toxins.[42] A similar working hypothesis of lithium homeostasis has been described by Jans et al.[43] in the model organism *Drosophila melanogaster*. In honey bees, the detoxifying system consists of enzymes such as cytochrome P450 monooxygenases, which are responsible for the metabolism of toxins such as drugs, pesticides or chemicals,[44,45] and other xenobiotics such as lithium. The constant exchange of the hemolymph with the surrounding tissues, together with this enzymatic detoxification activity might allow for the steady state of the lithium concentration in the hemolymph, but also the accumulation in the rectum.

Our second cage experiment on the duration of the active effect of LiCl on mites is consistent with this assumption of lithium metabolism, showing that the active effect decreases rapidly when LiCl feeding is halted. A 24-h interval between the cessation of LiCl feeding and introduction of mites into the cages significantly reduced and delayed mite mortality, whereas most of the

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mites placed directly on bees after the diet change (0 h group) died within a day. This can be explained by (i) the dilution of lithium in the hemolymph once bees can feed on pure sugar syrup and (ii) the fast metabolism of the bees, as explained above. The hemolymph data from the build-up trial illustrates that within 12–18 h the concentration of lithium remains widely stable with *ad libitum* feeding. Nevertheless, switching to a lithium-free diet results in a clear and rapid stop of mite mortality, at least for the first 5 days after ceasing the lithium feeding. With this change in diet, the concentration of lithium in the honey bee seems to be reduced below the threshold of 5–8 mg kg^{-1}, as otherwise we would see high levels of mite mortality (see above). However, there must still be a small amount of lithium circulating in the bee's body, sufficient to cause a kind of chronic effect on the mites that feed on these bees for >5 days, as mite mortality increases with longer observations. The caged bees did not have a chance to defecate inside the cages, and therefore could not excrete the accumulated lithium. To complement our build-up analysis, it would be helpful to have clearance rates for lithium. This would answer the question of how long lithium remains in crops, hemolymph and rectums once LiCl syrup feeding is halted.

Our data demonstrate that to function as a rapidly working varroacide, LiCl needs to circulate through full-sized and free-flying colonies for ≥48 h with the aim of establishing a steady-state concentration of >5 mg kg^{-1} in the hemolymph, which should result in 95% mortality of mites. However, it is important to remember that this only applies to broodless colonies where only mites in the dispersal phase are present, and that in colonies, unlike cage trials, there are options for food storage and additional external food sources, which are likely to dilute the LiCl food applied. Further field experiments should therefore focus on how to distribute LiCl food quickly and effectively in a large colony of >10 000 bees. The social exchange of food, called trophallaxis, is crucial for the rapid spread within a colony.[17,18]

From a prior field experiment, we know that during a 5-day treatment with 50 mM LiCl candy, lithium was detected in the honey crop of pooled bees, sampled from the center of the colony, as early as 1 day postapplication.[19] However, this 5-day treatment with 50 mM LiCl candy was only able to kill 77.5% of the mites, whereas a prolonged treatment of 9 days with the same concentration killed >98%.[19] It is likely that under field conditions a major part of the crop content will be stored in food combs and only a small part will be consumed and digested by the bees. To clarify this, field experiments on LiCl application should be combined with hemolymph analysis from individual bees engaged in different tasks and collected from different parts of the hive over numerous time points during a LiCl treatment trial. Such approaches would provide more insight into how widespread LiCl treatments are shared within a colony, and allow adaptations of treatment intervals to achieve a faster and better distribution of lithium.

Another possible way to reach higher lithium concentrations in a shorter timeframe in the treated bees is to increase the applied LiCl concentration. Kolics et al.[32] found that by trickling 40 mL of a 500 mM LiCl solution in brood-free colonies, mite mortality reached 79.7% within 3 days; this increased to 97.3% with repeated trickling. Although their colonies were broodless at the time of treatment, subsequent brood viability was not assessed. Rein et al.[19] demonstrated that a 50 mM LiCl concentration resulted in high removal of honey bee brood, probably because LiCl food is temporarily stored in combs, then mixed with worker and royal jelly and used for larval feeding. Therefore, it is questionable whether a 10-fold greater concentration, such as 500 mM, is safe for the bee brood. It is also unclear if the amount of LiCl used during a trickling treatment is sufficiently low to prevent accumulation in the bees or hives, which could potentially harm the bee brood.

This study aimed to investigate whether lithium accumulates in the HPG of nurse bees reared in colonies with LiCl treatment. The accumulation of Lithium in HPG could pose a risk to young larvae and the queen if the worker and royal jelly becomes contaminated. Our ICP-MS analyses showed that the HPG had the lowest lithium concentration of all bee organs and fluids which we investigated, with 0.09 mg kg^{-1} in bees from control colonies and 0.52 mg kg^{-1} in bees from LiCl-treated colonies. Analysis of the crop samples from the same bees confirmed their LiCl consumption. As we did not perform a wash of the extracted HPG before analysis, the slightly higher lithium concentrations in the samples could be a consequence of slight contamination with lithium from the hemolymph or other parts of the head, where for example, concentrations of ≤80 mg kg^{-1} lithium have been found.[46] Still, the lithium concentration in the HPG is very low and only 5% of that found in the hemolymph. Additionally, we found low concentrations of lithium in the crop of bees from the control colonies, which could be attributed to robbing, as the colonies were located next to each other in the same apiary. Nevertheless, when considering the data from Rein et al.[22] which shows only traces of lithium in 2-day-old larvae, and Kolics et al.[46] which detected no lithium in the queens, it can be assumed that a LiCl treatment does not contaminate the worker or royal jelly to an extent that could cause damage to either queens or young larvae which are exclusively fed with jelly from the HPG. The use of stored LiCl food incorporated into the brood food fed to older larvae could result in brood loss during treatment in free-flying colonies,[19] should be prevented. Without strategies to minimize brood exposure, the use of lithium in brood-rearing colonies cannot be recommended.

5 CONCLUSION

LiCl has excellent potential as a new treatment for honey bee colonies infested with *V. destructor*. Our findings indicate that LiCl is effectively consumed by the bees, reaching a miticidal concentration in the hemolymph within 12–24 h of commencing to feed the treatment and resulting in the death of >95% of the mites within 48 h. Low concentrations were detected in the analyzed HPG, making such treatments presumably safe for the queen and young larval stages, which are fed exclusively on the secreted jelly. In addition, the metabolism of bees results in a rapid depletion of lithium in their bodies, which minimises any potential long-term effects.

AUTHOR CONTRIBUTION

CR: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing – Review & Editing, Visualization, Project administration, Funding acquisition. **MG**: Conceptualization, Methodology, Investigation. **KT**: Writing – Review & Editing, Supervision. **PR**: Conceptualization, Methodology, Validation, Writing – Review & Editing, Supervision, Project administration, Funding acquisition.

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DATA AVAILABILITY STATEMENT

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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