




# Lithium chloride leads to concentration dependent brood damages in honey bee hives (*Apis mellifera*) during control of the mite *Varroa destructor*

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Received 19 January 2022 – Revised 21 April 2022 – Accepted 27 June 2022

**Abstract** – Lithium chloride (LiCl) has a high efficacy against *Varroa destructor* and a good tolerability for adult bees but the effect of LiCl on the honey bee brood has not been taken into consideration yet. We quantified the mortality of larvae fed with different concentrations of LiCl. For artificially reared larvae already, a concentration of 1 mM had significant toxic effects while under colony conditions, 10 mM was well tolerated. However, a chronic application of the effective concentration of 25 mM elicited brood mortalities between 60 and 90%. Shorter feeding periods of 2 or 4 days reduced the brood damages significantly. Measurements of the lithium concentrations in larvae and pupae during a chronic exposure with 10, 17.5 and 25 mM LiCl revealed respective lithium levels in 5th instar larvae of 7, 13 and 15 mg/kg. No lithium was detectable in 2-day old larvae indicating that pure worker jelly from the hypopharyngeal gland is not contaminated with LiCl. Based on these results, applications of LiCl in colonies with brood should be avoided.

**lithium chloride / varroa destructor / mite control / brood damages**

## 1. INTRODUCTION

The survival of a honey bee colony essentially depends on the success of treatments against the parasitic mite *Varroa destructor*. In combination with virus infections, varroosis is still the crucial factor for winter losses of managed colonies resulting in enormous economic damages for the beekeeping business (Cook et al. 2007; Francis et al. 2013; Gisder and Genersch 2020; Le Conte et al. 2010; Traynor et al. 2020; van Dooremalen et al. 2012). There are numerous products registered for *Varroa* treatment, ranging from organic acids and essential oils to synthetic acaricides (Emmerich 2018;

Rosenkranz et al. 2010; Underwood and Currie 2003). But none of these veterinary products fulfils all requirements of the beekeeper such as a good efficacy independent from environmental conditions (Underwood and Currie 2003), no measurable contaminations of honey bee products (Bogdanov 2006; Thrasivoulou and Pappas 1988), low risk of mite resistances (Milani 1999; Sammataro et al. 2005) and no severe side effects to bees and brood (Rosenkranz et al. 2010). Despite the overall unsatisfactory performance of the currently available products, new acaricidal compounds have not been registered during the past decades. Still, registered products for the treatments of *V. destructor* are nearly exclusively based on a limited number of active substances such as oxalic acid, formic acid, lactic acid, thymol and the synthetic agents tau-fluvalinate, flumethrin, coumaphos and amitraz (Emmerich 2018; Mutinelli 2016). Recently, lithium chloride (LiCl)

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Manuscript editor: Yves Le Conte

has been discovered as a new compound with promising varroacidal properties (Ziegelmann et al. 2018). Lithium chloride is a hydro-soluble and ubiquitous distributed salt and furthermore, a natural component of honey (Tutun et al. 2019). In contrast to the acaricides listed above, LiCl has a systemic mode of action which means that it could easily be applied by adding it to the honey bee food. Ziegelmann et al. (2018) showed that even small concentrations of 25 mM LiCl can lead to mite mortalities of nearly 100% when fed to caged bees. Even under field conditions, a short-term feeding of LiCl killed about 90% of the mites in brood-less colonies, such as artificial swarms (Ziegelmann et al. 2018). Meanwhile, the extraordinary efficacy of LiCl has been confirmed by other working groups in first field experiments during summer (Stanimirovic et al. 2021) or as winter treatment (Kolics et al. 2020b) and it has been shown to be also effective in killing mites by a contact mode of action (Kolics et al. 2020a). Also, first analyses on the residues of lithium in the honey bee products have been performed, revealing a moderate concentration in honey which returns to control level 16 days after treatment and low contaminations in bee bread (Kolics et al. 2021; Prešern et al. 2020). Moreover, no measurable residues were found in bees wax (Kolics et al. 2021). The so far measured lithium levels in honey are in the range of certain commercialized honeys (Kolics et al. 2021) and should, therefore, not be an insuperable barrier for a registration of LiCl as a veterinary product.

An equally important requirement for the suitability of LiCl as a new varroacidal compound is its tolerability for bees and brood. For adult bees, the available data indicate no or only moderate side effects even after overdose treatments of colonies or cage bees with LiCl (Prešern et al. 2020; Stanimirovic et al. 2021; Ziegelmann et al. 2018). In contrast, no published data exist on the effect of LiCl on the development of the honey bee brood. However, first measurements of lithium concentrations in larval tissue after respective treatments revealed similar levels to that in adult bees and might, therefore, pose a risk for the brood (Prešern et al. 2020).

In this study, we quantified the effect of different concentrations of LiCl on the mortality of defined larval stages using both, feeding

of artificially reared larvae and field-realistic applications in free flying colonies. In both approaches, a chronic exposure of the larvae throughout the complete feeding period from the 1st to the 5th instar was simulated and larval mortality was recorded in short-term intervals over the whole period of preimaginal development. We hypothesize that the level of brood damage depends on the duration of the treatment and the applied LiCl concentration. Parallel to this, we analysed the accumulation of lithium in larvae of different age by an ICP-MS method in order to define stage-specific thresholds for larval damages. Here, we expect higher lithium concentrations in particular in older larval instars that receive nectar and pollen in addition to the worker jelly (Böhme et al. 2019; Jung-Hoffmann 1966).

## 2. MATERIALS AND METHODS

### 2.1. Feeding of lithium chloride to artificially reared larvae

To investigate the impact of LiCl on the development of the honey bee brood, 96 larvae were reared in the laboratory and fed for a period of 6 days with in total 160  $\mu$ l (Table I) of artificial food, containing different concentrations of LiCl (> 99.9%, p.a., ultra-quality, Roth<sup>®</sup>): (1 mM, 1.5 mM and 2 mM). The artificial larval food consists of royal jelly, fructose, glucose, yeast extract, purified water, as described by Aupinel et al. (2005) and the respective amount of LiCl. First, the fructose, glucose and yeast extract were weighed and dissolved in either purified water or LiCl solution and afterwards mixed with the respective amount of royal jelly.

The larvae originated from two colonies of the Apicultural State Institute of Hohenheim, Stuttgart which were equal in colony strength and headed by sister queens. To generate a sufficient number of larvae of the same age, the queen of each hive was caged on one brood frame for at least 24 h in early summer 2017. Two to three days later, the newly hatched 1st instar larvae were transferred to plastic queen cups on top of

**Table I** Amount of food consumed per day for each larva reared in vitro

Rearing day	1	2	3	4	5	6	Total
Consumed food per larva ( $\mu$ l)	10	10	20	30	40	50	160

dental rolls in a 48-well tissue culture plate. Each dental roll was of 5 mm in diameter and wetted with potassium sulphate ( $K_2SO_4$ ) solution to maintain a relative air humidity of about 95%.

For the artificial feeding, we followed the description of Aupinel et al. (2005) and therefore kept the well in an incubator (Memmert IPP500) at 34 °C for 6 days. The larvae were only removed from the incubator for daily artificial feeding and checking on mortality. During the feeding, the well was placed on a heating plate of 35 °C to keep the larvae warm. As soon as the larvae started to defecate and building a cocoon, they were transferred into a new 48-well plate, where the dental rolls were now wetted with sodium chloride (NaCl) to reduce the relative air humidity to 80%, and then placed back in the incubator for another week. All successfully pupated larvae were then kept in small plastic cages, each containing a piece of wax till the time of hatching. Also, a small amount of honey was provided for the newly hatched bees. The mortality of the larvae and pupae was recorded in daily intervals until day 21 of larval development, briefly before adult hatching took place. Dead larvae/pupae were identified by spiracular movements and whether or not they react to a slight touch with a forceps.

After evaluating the first artificial rearing experiment, we conducted a second approach where the larvae were only fed for a period of 3 days with a diet containing 2 mM LiCl to investigate if a shorter feeding period results in lower mortalities and if there is a difference between young and old larvae. For this purpose, the larval phase was divided into two periods which corresponded to young larvae from 1st to 3rd larval instar (L1–L3) and old larvae from the end of the 3rd to the 5th larval instar (L3–L5), respectively (Rembold et al. 1980). The L1–L3 group was fed with LiCl-diet for the first 3 days

of rearing and afterwards replaced by lithium-free food. The L3–L5 group was fed the other way around receiving control diet for the first 3 days followed by a change to LiCl-diet for the rearing day 4 to 6. The survival probability was evaluated the same way as described above.

## 2.2. Assessment of brood development in free flying colonies

Twelve colonies of *A. mellifera* headed by sister queens were established in polystyrene mini-hives (“Mini Plus”) in June 2020 at the campus of the Apicultural State Institute, Hohenheim. Each hive consisted of approx. 7000 worker bees and one sister queen on 18 to 24 small combs (size 16 cm  $\times$  25.2 cm) and only small amounts of food reserves. The colonies were fed with dough made from icing sugar (Südzucker Group) and honey produced from summer nectar of the same region in the ratio 2:1. Four different batches were produced, one untreated control and three batches where different concentrations of lithium chloride (> 99.9%, p.a., ultra-quality, Roth®) were added: 10 mM, 17.5 mM and 25 mM (each  $n=3$ ). The respective concentrations were verified by ICP-MS analysis (see below) of the different batches. The colonies were randomly divided into 4 groups (three colonies each for control, 10, 17.5 and 25 mM LiCl) and at day 0 all colonies received 2 kg of the respective food. Over a period of 8 days, the amount of remaining food was weighted and recorded on daily intervals, in order to make sure that over the entire period of larval development, the nurse bees consumed lithium-contaminated food. After 8 days of feeding and the sealing of the assessed brood cells, the remaining food was weighted and removed.

To quantify the impact of LiCl on the development of individual honey bee larvae within these colonies, we conducted a brood development assessment according to the method described by Schur et al. (2003), starting on the first day of feeding. Briefly, the position of at least 250 eggs per colony in each hive was marked on a transparent sheet (=brood area fixing day (BFD)) and the development of the brood was assessed every 48 h. During each inspection, the labelled cells with viable larvae as well as brood cells that have been cleared out were marked on a new transparent sheet. These data were used to calculate the survival probability for each monitoring day. The monitoring of the brood was terminated on day 16 after BFD before a possible hatching of the eldest brood cells.

For the second brood assessment in the year 2021, we only used the 25 mM LiCl concentration and evaluated the impact of the duration of the feeding on the survival rate of the brood. To realize this, the feeding with LiCl-contaminated food started either in the 1st, 3rd or 5th larval instar resulting in appr. 6, 4 or less than 2 days of exposure to contaminated food. For this purpose, we marked 48–86 individuals of different larval instars (L1, L3 and L5; Rembold et al. 1980) on a brood comb on a transparent sheet. The feeding of the colonies and the inspections every 48 h were performed as above.

### 2.3. Analysis of lithium concentration in food and different larval instars

From the 2020 experiment, samples of the applied food (control, 10 mM, 17.5 mM, 25 mM) were analysed. Samples of 5th instar larvae shortly before brood cell sealing were collected 7 days after the application of the lithium-contaminated food. From each experimental hive ( $n=3$  per group), 20 larvae were pooled, homogenized and then analysed with ICP-MS.

In the 2021 experiments, we analysed the course of lithium concentration over the different developmental larval stages and in relation to the duration of the feeding. For this purpose, we took samples on the 5th, 7th, 10th, 11th, 14th

and 18th day post-treatment, which corresponds to the days after egg-laying. For instance, on the 5th day, we sampled 2-day old larvae considering the 3 days for egg development. This should provide first information on how the lithium accumulates in young larvae and gets metabolized during metamorphosis. Five to 10 larvae/pupae per sample were pooled and each hive ( $n=3$  per group) was sampled 3 times per sampling date.

The content of lithium was determined with the ICP-MS device NexION 300X from Perkin-Elmer. For this purpose, the obtained samples (applied food, larvae) of 0.05 g were completely dissolved with 2 ml of  $\text{HNO}_3$  in a microwave digestion using an Ultra Clave III from MLS (900 watts at 100 bar pressure; heated from 80 to 200 °C in 5 steps) and the digestion solution was then filled up to 10 ml. For the ICP-MS measurement, the sample solution was again diluted tenfold and CertiPUR Rhodium ICP Standard Solution 1000 mg/l Rh, Merck Company was added as an internal standard. The dilutor of a microLAB 600 series was used for dilution. For calibration, a Merck VI standard solution (ICP multi-element standard solution) was diluted to the following concentrations: 0.1 µg/l, 0.2 µg/l, 1 µg/l, 10 µg/l, 20 µg/l. The calibration solutions were prepared with ultrapure  $\text{H}_2\text{O}$  and  $\text{HNO}_3$  (ROTH ROTIPURAN Supra quality 69% from Roth Company) and also diluted with the dilutor. A calibration straight line was prepared on the basis of the solutions. This straight line was the basis for quantifying the lithium content in the digestion solutions ( $\text{LOQ} < 0.025$  mg/kg).

### 2.4. Statistical analysis

Statistical analysis was carried out using IBM SPSS Statistics version 27. The survival probability of the in vitro reared larvae was analysed using a Kaplan–Meier test with log-rank. The survival probabilities of the brood assessments were first tested on normal distribution with Shapiro–Wilk test and consequently analysed using Mann–Whitney- $U$  test for two variables or Kruskal–Wallis test for

more than two variables. Food uptake data were first judged on variance homogeneity followed by ANOVA for 2020 and *t*-test for 2021. The concentration analyses were evaluated using the post hoc Tukey HSD test.

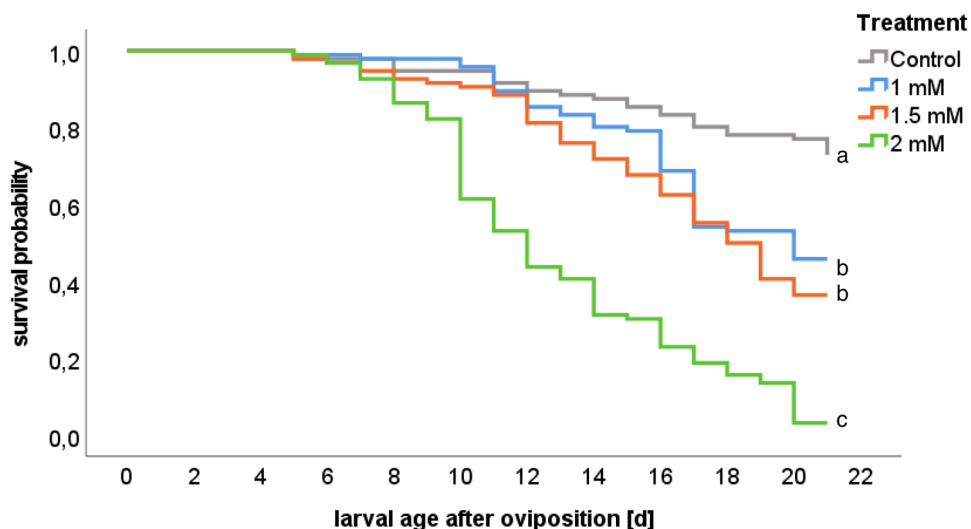
### 3. RESULTS

#### 3.1. Feeding of lithium chloride to artificially reared larvae

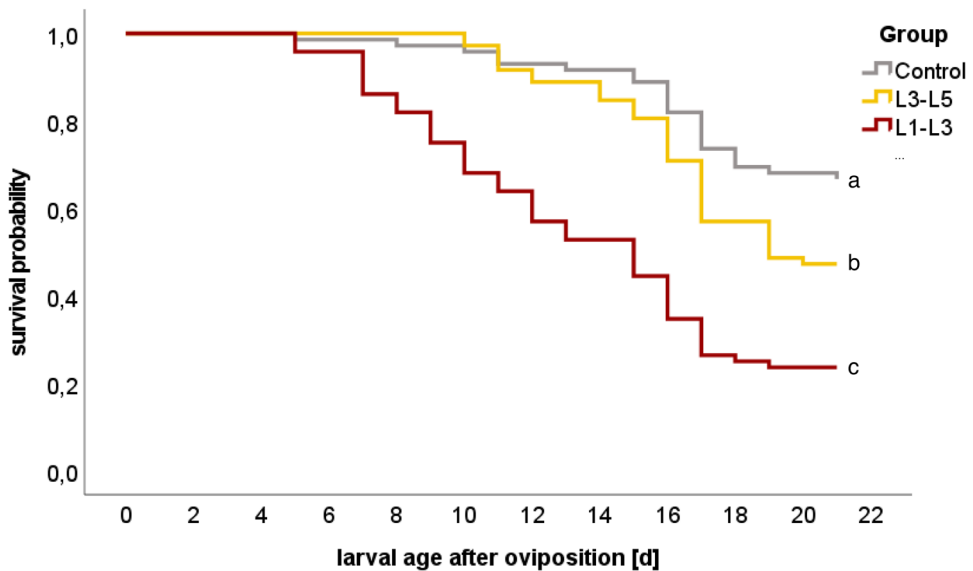
The artificial rearing of larvae allowed an exact application of a defined quantity and concentration of lithium-contaminated food. The survival of the treated larvae was recorded throughout the complete period of the preimaginal development (21 days). The survival of those larvae exposed to lithium-contaminated food was significantly reduced ( $p < 0.001$ ; log-rank test) compared to the control larvae for all concentrations (Figure 1). Higher concentrations of LiCl lead to lower survival

probabilities of the larvae with most of the deaths occurring during the prepupal and pupal stages (after day 10). While there was no significant difference between the 1 mM and 1.5 mM treatment group ( $p = 0.166$ ; log-rank test), the 2 mM treatment revealed a highly significant difference to all other groups ( $p < 0.001$ ; log-rank test). The 2 mM treatment obviously represents an absolute damage threshold for larvae, because only 3% of the larvae survived till day 21.

In the second approach, the larvae were fed only for a period of 3 days with food containing 2 mM LiCl, either from the start of larval development till 3rd larval instar or from the 3rd larval instar till the end of the feeding period in the 5th instar. Relative to the control group, both treated groups showed significant reduced survival rates (Figure 2). However, younger larvae (group L1–L3) were significantly more susceptible to LiCl compared to those larvae treated only in the second phase of larval development (L3–L5) ( $p < 0.001$ , log-rank test, Figure 2).



**Figure 1.** Kaplan–Meier survival curve of artificially reared larvae exposed to different concentrations of LiCl in larval food compared to the control group. 1st instar larvae (day 4 after egg laying) were transferred into artificial cells and fed daily with the corresponding diet ( $n = 4$  well plates/treatment,  $n = 24$  larvae/well plate). Lines not sharing any index letter are statistically different at  $p < 0.001$  (Kaplan–Meier test; log rank (Mantel–Cox)).

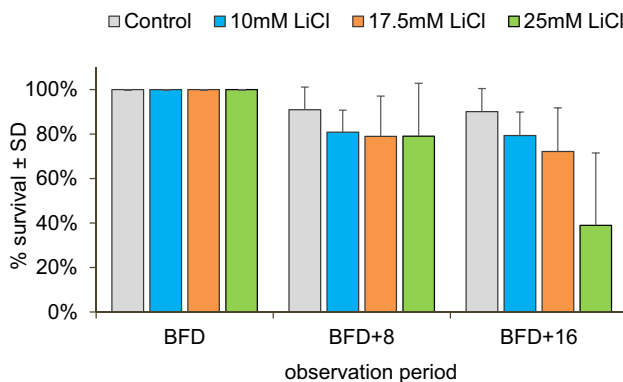


**Figure 2.** Kaplan–Meier survival curve of artificially reared larvae exposed to a diet containing 2 mM of LiCl either from the start of larval development to 3rd larval instar (L1–L3) or from 3rd larval instar to 5th instar (L3–L5) ( $n=3$  well plates/treatment,  $n=24$  larvae/well plate). Lines not sharing any index letter are statistically different at  $p<0.05$  (Kaplan–Meier test; log rank (Mantel–Cox)).

### 3.2. Assessment of brood development in free flying colonies

Our field experiments on the brood assessment clearly showed that LiCl has harmful effects also on larvae in free flying colonies (Figure 3). The

total number of selected eggs for the assessment ranged from 741 to 797 per treatment group. We provided LiCl-contaminated food ad libitum resulting in a continuous food uptake during the entire larval feeding period of about 8 days. There were no differences in food uptake



**Figure 3.** Effect of a chronic feeding of LiCl (10 mM, 17.5 mM and 25 mM) in free flying colonies on the survival of the honey bee brood starting on the BFD (brood area fixing day) till BFD+16 compared to the untreated control ( $n=3$ ). The total number of selected eggs on BFD were 797 (control), 748 (10 mM), 741 (17.5 mM) and 748 (25 mM). The 25 mM treatment elicited high brood mortalities; however, there were no significant differences between the treatment groups ( $p=0.516$ ,  $p=0.123$ , Kruskal–Wallis test).

**Table II** Total food uptake in g of dough with different LiCl concentrations during the brood assessments in free flying colonies in the study years 2020 and 2021 ( $n=3/\text{treatment}$ ). There were no significant differences between the treatment groups (2020:  $F(3,8)=0.339$ ,  $p=0.798$ ; 2021:  $t(4)=1.706$ ,  $p=0.163$ )

	Control	LiCl concentration		
		10 mM	17.5 mM	25 mM
2020	1,518 ± 305	1,643 ± 861	1,221 ± 660	1,262 ± 432
2021	1,733 ± 252			1,484 ± 28

between the control and the LiCl-treated colonies ( $F(3,8)=0.339$ ,  $p=0.798$ ); the consumption of food with higher concentrations of LiCl, however, was reduced by approximately 20% (Table II).

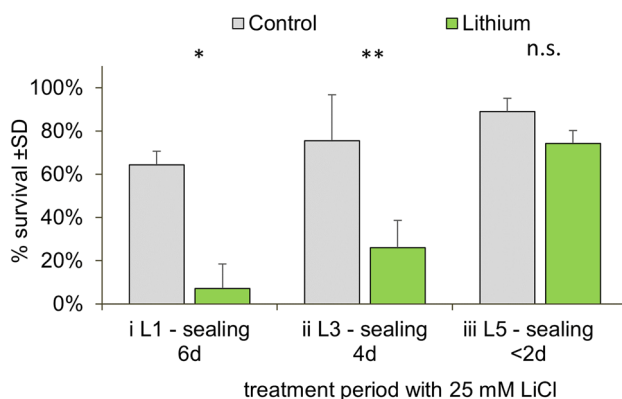
The brood survival rates on BFD + 8, shortly before the sealing of the brood cell, reveal only small differences between the LiCl-treated groups and the untreated control hives, which were not significant different ( $p=0.516$ , Kruskal–Wallis test). However, these differences in brood survival rates increased on BFD + 16, particularly in the highest concentration of 25 mM, where only 39% of the brood survived.

These differences performed during the season of 2020 were not statistically significant due

to a high variation among individual colonies; however, the repetition of the 25 mM application in 2021 clearly revealed a significant effect of this lithium concentration (see Figure 4). We could also confirm the finding from the laboratory experiments (Figure 1), that most of the damages occur during the prepupal or pupal stages when the cells are sealed (BFD + 16) rather than in larval stages (until BFD + 8).

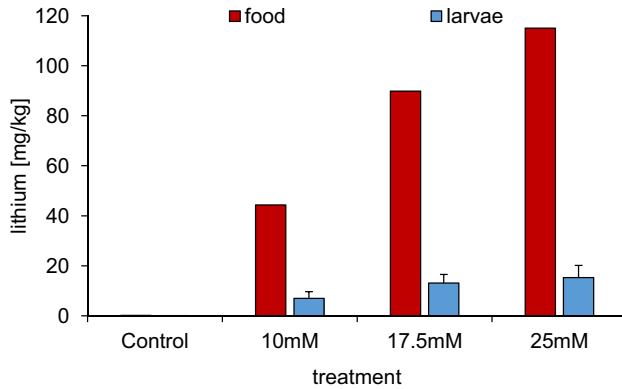
### 3.2.1. Brood assessment with different treatment periods

We can clearly show that the level of brood damages depends on the duration of the application of lithium-contaminated food. A short-term feeding of a period of less than 2 days directly before the sealing of the brood cell had only a slight, but not significant effect on the continued survival of the 5th instar larvae (Figure 4). However, longer feeding periods of 4 days or even over the complete larval development (appr. 6 days) increased the brood mortality rates significantly compared to the respective control group ( $p=0.01$ , Mann–Whitney- $U$  test, Figure 4). In detail, only 26% of the larvae survived a LiCl application over 4 days and only 7% of the larvae survived the 6-day-treatment. The food uptake in



**Figure 4.** Brood survival rate in free flying colonies after feeding of 25 mM LiCl during distinct periods of the larval development: **i** 1st instar larvae till sealing of the brood cell (appr. 6 days), **ii** 3rd instar larvae till sealing of the brood cell (appr. 4 days) and **iii** 5th instar larvae till sealing of the brood cell (<2 days). Data given as mean ± standard deviation of survived brood on day 19 of larval development. The total number of recorded larvae ranged from  $n=272$  to  $n=360$  per group. Compared to the control, there were significant differences in the survival rates of the colonies fed over 6 and 4 days (Mann–Whitney- $U$  test,  $p=0.01$ ,  $p=0.009$ , respectively) but not in the colonies fed for <2 days.





**Figure 5.** Concentration of lithium in the applied food and in the 5th instar larvae 7 days post-treatment with different LiCl concentrations. Data of larvae were each given as mean ± standard deviation ( $n=3$ ). The food was only measured once in the three stock solutions. Control larvae samples were below the limit of quantification ( $LOQ=0.025$  mg/kg).

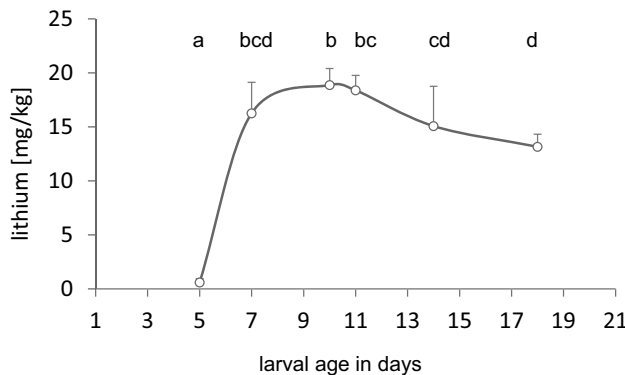
colonies fed with 25 mM LiCl was not significantly different from the food uptake in the control colonies ( $t(4)=1.706, p=0.163$ , Table II).

It is noteworthy that the brood survival rate of the control larvae (using 1st instar larvae at the BFD) was considerably lower (Figure 4, left column; 64%) compared to the survival rate in the respective control of the previous year (Figure 3; 90%).

### 3.3. Analysis of lithium concentration in food and different larval instars

First of all, we could confirm the desired concentration of our applied food with 44.3 mg/kg lithium for the 10 mM, 89.9 mg/kg for the

17.5 mM and 115 mg/kg for the 25 mM LiCl concentration. The concentration of lithium in the larvae increased with (i) higher concentrations of LiCl in the food (Figure 5) and (ii) with the time of exposure (Figure 6). In the last instar larvae (L5) from hives treated with 10 mM LiCl, we measured lithium concentrations of 7.0 mg/kg, whereas in the larvae treated with 17.5 mM, the lithium concentration was almost doubled (13.1 mg/kg). The 25 mM treatment leads to the highest concentration of 15.2 mg/kg. Compared to the concentrations of lithium in our applied food, we measured a sixfold lower concentration in the respective larvae (Figure 5).



**Figure 6.** Concentration of lithium in larvae from colonies chronically treated with 25 mM LiCl. Sampling of brood stages was performed on the 5th, 7th, 10th, 11th, 14th and 18th day after egg laying, which corresponds to different developmental stages. Data are given as mean ± standard deviation ( $n=9$ ). Plotted values not sharing any index letter are statistically different at  $p < 0.05$  (post hoc Tukey HSD test).



To analyse the course of the lithium concentration in larvae, we analysed different larval instars during a chronic application of 25 mM LiCl. The lithium concentration differed significantly depending on age of larvae/pupae ( $F(5,42)=71.89$ ,  $p<0.00$ ; Figure 6). In 2-day-old larvae (L2/L3) sampled 5 days after the start of lithium treatment, we only found traces of lithium ( $0.59\pm 0.35$  mg/kg; Figure 6). The concentration of lithium significantly increases already 2 days later (L4) to values of  $16.3\pm 2.9$  mg/kg. In the pre-pupal stage briefly after sealing of the brood cell, the lithium concentration reaches a maximum of  $18.9\pm 1.5$  mg/kg and thereafter decreases slightly towards  $13.2\pm 1.2$  mg/kg during the pupal phase (Figure 6).

During the sampling of larvae and pupae for the residue analysis, we frequently observed that the pupae were malformed with an incomplete development of head, thorax and abdomen while most of the larval instars seemed normally developed. This might indicate that lithium disturbed the metamorphosis of the honey bee larva which is supported by the high mortality and brood removal rates of pre-pupal and pupal stages as shown in Figures 1 and 4.

#### 4. DISCUSSION

Lithium chloride (LiCl) is one of the very few new active compounds with a promising potential to become an effective veterinary product for the control of the mite *Varroa destructor*. This potential is based on the (i) contact and systemic mode of action (“easy to apply”), (ii) high efficacy under broodless conditions, (iii) good tolerability to adult bees and (iv) no identifiable risk for the user and consumer. Despite these advantages of the new compound, a number of details on the mode of action and, in particular, on side-effects on the honey bee brood still need to be clarified. Here, we analysed for the first time the impact of LiCl on the development of honey bee larvae and pupae using both, in vitro reared larvae and larvae reared within the colony.

For the in vitro assay, we used larval diets with very low concentrations of LiCl of 1 to 2 mM which is considerably below the effective treatment

concentration of 25 to 50 mM (Stanimirovic et al. 2021; Ziegelmann et al. 2018) and close to the range of concentrations naturally occurring in certain honeys (Bogdanov et al. 2008). However, even the larval diet with the lowest concentration of 1 mM LiCl led to a survival probability of only 46%. This is significantly lower compared to the untreated control colonies with nearly 80% survived larvae on day 21. Higher LiCl concentrations of 1.5 mM and 2 mM reduced the survival rate even stronger with only three of 96 larvae that survived the 2 mM diet until day 21. This clearly demonstrates the high sensitivity of the honey bee brood to LiCl, particularly when compared to cage experiments with adult worker bees where a continuous feeding of LiCl concentrations of 2 to 25 mM over up to 7 days did hardly reveal any toxic effects (Stanimirovic et al. 2021; Ziegelmann et al. 2018). We could also show that the extent of brood damages depends on the duration of the LiCl application: the survival of larvae receiving the 2 mM diet increased substantially when applied only during the first or during the second phase of larval development. It is not surprising that the younger larvae (L1–L3) are significantly more susceptible to LiCl compared to the older ones (L4, L5). It is noticeable that brood mortality not only occurred during the period when the larvae actively consumed the contaminated food but also even to a higher extent during the non-feeding prepupal and pupal phase. This indicates long-term effects of LiCl on the developmental processes during the metamorphosis.

The field experiments confirmed the low tolerability of the honey bee brood to LiCl. However, a substantial increase of larval mortality was only recorded when the colonies received food containing 25 mM LiCl over the entire larval developmental period resulting in a more than 50% lower brood survival rate compared to the control colonies. Lower concentrations of 10 mM and 17.5 mM LiCl elicited only a low reduction in brood survival rates of 20% and 28%, respectively. In the second experiment — performed in the following season — the 25 mM application led to even lower survival probabilities of only 7% compared to 64% in the untreated control (Figure 4). This is surprising because the experiments in the two consecutive

years (2020 and 2021) were performed with the same experimental setup and at the same apiary. This reveals a general problem of the brood assessment as a standard field method for toxicity test of larvae (Medrzycki et al. 2013). Numerous studies have criticized high variations in the brood removal rates even among control colonies or in repetitions with identical treatments (Pistorius et al. 2011). One general reason for these variations is that the brood removal rates in free flying colonies depend on many factors, such as colony strength, the availability of food, the total amount of brood or simply the time of the year (Bigio et al. 2013). Also covert brood diseases and the genotype can lead to different pronounced hygienic behaviour (Panasiuk et al. 2008). At the beginning of our experiments we, therefore, standardized the numbers of bees and brood cells and the amount of stored food as far as possible. However, a particular problem is most likely the fact that we could not quantify which proportion of the applied LiCl has been transferred by the nurse bees to the diet of individual larvae. Though, the food uptake was not significantly different between the LiCl-contaminated food and the LiCl-free control food. Larval food is a mixture of hypopharyngeal gland secretion of the nurse bees with pollen and nectar or honey. The precise composition depends on the age and caste of the larvae that has to be fed (Haydak 1970). So far, it is not clear to what proportion the nurse bees use freshly collected nectar, applied food or stored food for the feeding of the larvae (Babendreier et al. 2004; Brodschneider and Crailsheim 2010; DeGrandi-Hoffman and Hagler 2000). In any case, high foraging activities of our experimental colonies could dilute the concentration of LiCl in the larval diet when freshly collected nectar is preferred over the applied lithium-contaminated dough. An influence of the foraging activity of the bees on the composition of larval food has also been confirmed by Böhme et al. (2019) who identified freshly collected pollen in worker jelly.

Different foraging activities might therefore be responsible for the different brood removal rates in the years 2020 and 2021. Both experimental seasons were rather different concerning

nectar and pollen availability with perfect conditions in the year 2020 in contrast to an unusual cold and rainy summer with an extremely poor nectar flow in the following year. In the 2021 experiments, these unfavourable conditions might have increased (i) the general removal rate of the brood even in the control colonies and (ii) the exposure of larvae to LiCl because the applied LiCl was not diluted with nectar collected from outside. An obvious positive effect of the reduced foraging activity in 2021 is the lower variation within the different treatment groups.

At least, the results of both years show impressively that a long-term application of LiCl in colonies with brood should absolutely been avoided due to high brood damages which are not in accordance with a good beekeeping practice. The application of lower concentrations of 10 mM or less might reduce the brood damages to an acceptable level, but for an effective treatment against *V. destructor*, a concentration of 25 to 50 mM LiCl (Stanimirovic et al. 2021; Ziegelmann et al. 2018) is required.

However, our experiment with different treatment periods demonstrates clearly that not only the concentration of the applied food but also the duration of the LiCl application has a significant impact on the larval development. The survival probability of the brood after a short-term contact with LiCl for a maximum of 2 days was not significantly different to the control group. It has to be analysed in further field experiments whether repeated short-term treatments (“block treatments”) with small amounts of 25 mM LiCl are suitable to minimize the brood damages during a treatment while maintaining a high efficacy. But the therapeutic window for such an approach seems to be small since already a feeding period of 4 days had a clear toxic effect in our experiments. And even in case of no measurable brood damages, the vitality of the bees hatching from treated brood has to be investigated in additional experiments.

So far, we do not know how lithium disturbs the larval and pupal development. Interestingly, it seems that in both, in the in vitro and in the field experiments, most of the damage occurs during prepupal and pupal stage when the metamorphosis

takes place rather than after the moults of the different larval instars. This was supported by yet not quantified observations during the removal of pupae from the brood cells for chemical analysis where most of the pupae showed malformations while most of the larval instars in unsealed brood cells seemed normally developed. Nonetheless, specific susceptibilities of different larval instars under natural conditions and possible long-term effects need to be examined.

There are some evidence that lithium can also alter the development of other organisms than honey bees through inhibition of glycogen synthase kinase-3 $\beta$ , which plays a role in cell fate determination (Klein and Melton 1996; Phiel and Klein 2001). In embryos of the clawed frog *Xenopus laevis*, the exposure to lithium caused an expansion and duplication of dorsal and anterior structures (Kao et al. 1986) and inhibited the morphogenesis of the nervous system (Breckenridge et al. 1987). Among insects, in the genetic model organism *Drosophila melanogaster*, 12 genes were identified with a significant response to lithium exposure (Kasuya et al. 2009). These genes were subdivided into four groups according to their biological function: (i) amino acid transport and metabolism, (ii) detoxication, stress response or self-defense reactions, (iii) psychiatric or neurological disorders, and (iv) others (Kasuya et al. 2009). Especially, the genes of the group (i) could be of interest for a comparative gene expression analysis as an application of lithium seems to influence also the metabolism in honey bee larvae.

Such experiments are a prerequisite to better understand the negative effects of lithium on the physiological processes during larval development. Until then, our ICP-MS analyses of the lithium concentration in differently treated larvae provide a first hint on the threshold concentration that is still tolerable for a successful preimaginal development. As honey bee larvae do not defecate before the end of 5th larval instar, we can exactly determine the concentration of lithium that has accumulated in the in vitro reared larvae by calculating the concentration of lithium in the consumed food. During the artificial rearing, we fed a total of 160  $\mu$ l of LiCl contaminated food to each larva. For the 1 mM application,

this corresponds to a consumption of 6.8  $\mu$ g LiCl or 1.1  $\mu$ g pure lithium per larva. Considering a body weight of the larvae at the end of the feeding phase (5th larval instar before defecation) of about 160 mg (Rembold et al. 1980), we can calculate a lithium concentration in these larvae of 6.9 mg/kg reared on the 1 mM diet. This value is exactly equal to the concentration of lithium measured in larvae from hives treated with a ten-fold higher concentration (10 mM) of LiCl during the complete period of larval development (7.0 mg/kg). It should be considered that during the larval feeding phase, lithium might enter the larval body not only by feeding but also through the cuticle. It has been proven that LiCl is effective on mites by topical application (Kolics et al. 2020a). The honey bee larvae have continuously contact to the liquid food which is applied in frequent intervals. Whether a trans-cuticular uptake is relevant for the observed side effects has to be proven in additional approaches.

After the feeding of higher LiCl concentrations within the colony, we found a corresponding increase of lithium levels in the respective larvae. In detail, the 2.5-fold increase of LiCl in the applied food (10 to 25 mM) resulted in a 2.2-fold increase of lithium within the larvae (7 to 15.3 mg/kg). It is remarkable that the concentration of lithium in the larvae is always considerably lower compared to the concentration in the applied food (Figure 5) and confirms the above-mentioned statement that under natural brood rearing conditions, only a small part of the lithium from the applied food ends up in the larval diet. The reasons for these differences are most likely (i) dilution of the LiCl food by nectar, (ii) metabolization of LiCl including defaecation by the hive bees and (iii) no or little accumulation of LiCl in the hypopharyngeal glands of the nurse bees and, therefore, in the worker jelly. The latter assumption is supported by the extremely low lithium concentrations in the 5-day-old larvae (egg laying as starting point) which according to Rembold et al. (1980) corresponds to the beginning of the 3rd larval instar. Up to this age, the larvae exclusively receive worker jelly from the hypopharyngeal gland (Babendreier et al. 2004; Haydak 1970) which obviously was largely uncontaminated by lithium.

Albeit our *in vitro* experiment with stage-specific applications of LiCl revealed a significant higher susceptibility of the younger larvae compared to the older ones, these first larval instars are likely not exposed to lithium-contaminated food under natural conditions. This means that an application of LiCl will not contaminate the worker jelly neither the royal jelly. This can be regarded as positive because a treatment with LiCl is neither a risk for worker larvae of the 1st and 2nd instar nor the queen, which is fed with royal jelly throughout the lifetime (Jung-Hoffmann 1966). The latter is supported by Kolics et al. (2021) who did not detect any lithium residues in queens 28 days after applying 1 l of 25 mM LiCl syrup within the hive.

From days 5 to 7 of larval development, the concentration of lithium increases rapidly to the maximum value. This concentration remains at the same level during metamorphosis and the beginning of the pupal followed by an only slight reduction during the pupal phase. Finally, the low standard deviations of the different residue analyses, each based on 9 brood samples, clearly indicate an even distribution of the lithium within the entire brood nest of the treated hives.

## 5. CONCLUSION

Lithium is not only highly effective in the treatment of bipolar disorders (Schou 2001) but has also become a promising substance to combat the parasitic mite *Varroa destructor*. Here, we clearly show that a concentration of 25 mM LiCl, which is required to effectively kill the mites on adult bees, leads to severe brood damages when applied over the whole larval developmental period. But our results also indicate that lithium is not or only in traces incorporated in the worker or royal jelly synthesized in the hypopharyngeal glands of the nurse bees. Although this should yet be confirmed by direct measurements of the lithium concentration in hypopharyngeal glands and in larval food, young worker larvae and queen larvae in general seem to be less endangered by a LiCl application. The preliminary consequence for a possible application is that a LiCl treatment should be performed exclusively in brood-free

colonies, at least in colonies without elder brood stages, whether a repeated short-term application of LiCl could be a conceivable solution for colonies with brood has to be analysed in additional field experiments.

## ACKNOWLEDGEMENTS

We would like to explicitly thank Barbara Horn and Dr. Monika Bach (core facility, University of Hohenheim) for the chemical analyses of the lithium content in larval samples.

## AUTHOR CONTRIBUTION

CR and PR developed the design of the study. CR conducted the field experiments, analysed the data and drafted the manuscript. MM performed the *in vitro* experiments. JR conducted the brood assessment studies in 2021. PR supervised the experiments and finalized the manuscript. All authors read and approved the final manuscript.

## FUNDING

Open Access funding enabled and organized by Projekt DEAL. The project (281C301A19) is supported by funds of the Federal Ministry of Food and Agriculture (BMEL) based on a decision of the Parliament of the Federal Republic of Germany via the Federal Office for Agriculture and Food (BLE) under the innovation support programme.

## AVAILABILITY OF DATA AND MATERIAL

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

## CODE AVAILABILITY

Not applicable.

## DECLARATIONS

**Ethics approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Conflict of interest** The authors declare no competing interests.

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