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Short communication

Lithium contamination of honeybee products and its accumulation in brood as a consequence of anti-varroa treatment

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ABSTRACT

Varroa destructor, the primary honeybee pathogen, is kept in check by various chemical compounds which may enter the human diet through honeybee products. Lithium is an emerging varroa control substance, and we investigated its accumulation in honey, bee bread, brood and adults along with the mortality of bees. Increased lithium concentrations were detected in workers, fed individually once per os with 10 μL of 25 mM LiCl in sucrose solution (6.50–40.10 mg/kg) or had the same solution available *ad libitum* (39.25–266.00 mg/kg). A three-day treatment of honeybee colonies with 25 mM LiCl in 1L/day sucrose solution increased lithium concentrations in five-day-old larvae, honey, and bee bread: up to 45.0, 1.2, and 47.0 mg/kg, respectively. Lithium concentrations peaked three days post-treatment in both larvae and honey and increased worker mortality was observed. The control colonies exhibited lithium concentrations below the limit of quantification (0.5 mg/kg). Prudence in lithium use is advised.

1. Introduction

Food chains of humans and managed honeybees are coupled via honeybee food stores, specifically honey and stored pollen, known as bee bread. The leading contemporary honeybee pest, the mite *Varroa destructor*, which acts both as an ectoparasite and vector of honeybee viruses (Ramsey et al., 2019; Boecking & Genersch, 2008), is kept in check by use of a variety of chemical substances including synthetic acaricides and organic acids. The residues of these compounds can be found in honeybee products, especially when not used according to good beekeeping practices and/or instructions (Martel et al., 2007). In such a situation, the coupling of the food chain can at least theoretically present a risk for human health (Fang, Chen, Wu, Sung, & Huang, 1995; Proudfoot, 2003). Lithium salts seem to be an emerging varroa control substance (Ziegelmann et al., 2018). As such, lithium needs to be studied from the aspects of human and honeybee diet and well-being.

The varroa population must be effectively reduced several times a year to prevent colony collapse: varroa feeds on the fat body of honeybees, which is vital for both immune response and as a storage organ (Ramsey et al., 2019). Varroa-control methods are roughly divided into technological and chemical approaches, which often complement each other. While the technological approaches are based mostly on brood interruption (Maul, Klepsch, & Assmann-Werthmüller, 1988), the chemical methods are often based on synthetic organic substances that have been shown to have some kind of acaricidal activity. There are only a few approved substances (coumaphos, amitraz, flumethrin, fluvalinate; Rosenkranz, Aumeier, & Ziegelmann, 2010); complementing these are organic acids (oxalic and formic): essential oils, such as monoterpenoid phenol thymol (Jemec Kokalj & Glavan, 2017), can also be used. While both organic acids and essential oils are suitable for organic beekeeping, these are more difficult to handle than synthetic acaricides, and their efficacy can vary greatly among treated colonies (Trouiller & Watkins, 2001). Unfortunately, it seems that varroa has developed resistance to the synthetic acaricidal compounds (e.g., Milani, 1995, Sammataro, Untalan, Guerrero, & Finley, 2005; Rodríguez-Dehaibes, Otero-Colina, Sedas, & Jiménez, 2005). One promising new approach was the use of short RNAi molecules which silence vital varroa genes. This method suggested the use of honeybees as a vector to deliver RNAi molecules to varroa, serving the RNAi in their feed; lithium chloride acts as a solvent of nucleic acids in the process of isolation (Garbian, Maori, Kalev, Shafir, & Sela, 2012). Later, it was demonstrated that lithium ions without RNAi have a similar acaricidal effect, which was unexpected yet potentially advantageous for varroa control. Lithium is not regulated as a veterinary medicine product (VMP), but it is both cost-effective in the form of chloride and reasonably efficient. However, lithium is also a problematic substance, as it

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shortens the lifespan of honeybees (Ziegelmann et al., 2018) and also has an effect on human physiology: lithium directly affects the nervous system in humans (Freeman & Freeman, 2006). Lithium citrate and lithium carbonate are often used to treat bipolar disorders and other mental illnesses (Cade, 1949; Lenox & Watson, 1994) despite side effects such as interference in embryonal development (Schrauzer, 2002).

Lithium is delivered to the honeybee colony dissolved in feed (e.g., sucrose syrup), which is transformed to the primary form of carbohydrate storage, the honey, thus linking the honeybee and human food chains. It has often been demonstrated that honeybee feed ends up in extracted honey due to the storage behavior of honeybees (Eyer, Neumann, & Dietemann, 2016), making food chain coupling likely. The present paper addresses some of the concerns above, namely lithium presence in honey, in bee bread, and in honeybee larvae due to feeding with sucrose syrup containing dissolved lithium chloride.

2. Materials and methods

2.1. Honeybee colonies and sampling of larval material

We selected eight colonies balanced in strength (one full-size brood chamber and one full-size honey super, ten frames each) and divided them into control (4) and test (4) groups. Honeybee mortality was followed for 30 days by daily counting dead honeybees in box-like traps installed under hive entrances; baseline mortality was acquired during 18 pre-treatment days. Five days before treatment, we inserted an empty comb for egg-laying in the middle of the nest. On the following day, we labeled areas with new eggs, using the queen bee marker pen. To investigate possible differences in lithium concentration due to different durations of exposure to lithium feed, we repeated the labeling of one-day-old eggs in two-day intervals to obtain four samples of larvae of different age (Table 1), twenty larvae from each hive per sampling. Treatment was initiated by the colonies with test and control solutions on Day 19, five days after first labeling; treatment lasted three consecutive days in which each colony was fed with 1000 mL of solution per day. We dissolved 1.06 g of lithium chloride in 1L of sugar syrup to reach a final solution of 25 mM, shown by Ziegelmann et al. (2018) to be the most appropriate. Samples of larvae were always collected eight days after labeling, just before the brood was covered. We collected 32 samples, homogenized them, and analyzed the homogenate with ICP-MS.

2.2. Sampling of honeybee products

We sampled honey and bee bread from combs that we inserted laterally to the brood nest where the honeybee main food storage is located. For that purpose, we inserted empty combs and labeled their frames. We sampled bee bread on the 4th day post-treatment. Honey was sampled on the 1st, 3rd, and 7th days post-treatment (Table 1).

2.3. Cage experiments

To measure lithium accumulation in adult honeybees, we set up cages with honeybees of equal age. For that purpose, we took a frame with a covered brood just before hatching and set it in an incubator (34.5 °C, 70% RH) over the night. The hatched honeybee workers were then collected into improvised cages, made of perforated inverted 500 mL plastic-cups standing on Petri dishes. On Day 1, we fed individual bees once *per os* either the 10 μL of 1:1 sucrose syrup (control group) or 10 μL of 1:1 sucrose syrup containing 25 mM lithium chloride (Ziegelmann et al., 2018) in case of the test group and placed into the appropriate cage. After feeding, the cages were equipped with simple feeder, made from a syringe, containing 1:1 (v/v) sucrose syrup. Individuals that did not consume the prescribed quantity in *per os* feeding, were placed into *ad libitum* cages equipped with feeders containing 25 mM lithium in 1:1 (v/v) sucrose syrup. The final cage counts were 10 cages in the control group, 21 cages in the *per os* test group and 6 cages in the *ad libitum* test group, each containing 10 workers. Cages were placed into a dark incubator at 28° C, dead individuals were counted daily, and the consumed syrup was replenished. The experiment was concluded on Day 7, when the bees were sacrificed by freezing; heads, thoraxes and abdomens were stored separately for ICP-MS analysis. Samples were pooled due to analytical requirements by treatment (3) and body parts (3), 9 samples in total.

2.4. Inductively coupled plasma-mass spectroscopy

Samples were prepared as described elsewhere (Gorjanc et al., 2014). Briefly, all reagents used were of analytical grade or better. For sample dilution and preparation of standards, ultrapure water (MilliQ, Millipore) and ultrapure acid (HNO_3, Merck—Suprapure) were used. The standard was prepared in-house by dilution of certified, traceable, inductively coupled plasma (ICP)-grade single-element standard (Merck CertiPUR). An Agilent Technologies 7500ce ICP-mass spectrometry

Table 1
Sampling protocol. Yellow indicates the treatment interval. The first four columns show brood sampling; the last column indicates the sampling of honey and bee bread. Larvae were sampled in two-day intervals from the same colonies to obtain four samples of larvae of different ages and consequently of different duration of exposure to lithium in the feed.

Day	Larval sample 1	Larval sample 2	Larval sample 3	Larval sample 4	Honey sample /bee bread
15 pre-treatment	egg (labeling)				
16 pre-treatment	egg				
17 pre-treatment	egg	egg (labeling)			
18 pre-treatment	larva	egg			
19 treatment	larva	egg	egg (labeling)		
20 treatment	larva	larva	egg		
21 treatment	larva	larva	egg	egg (labeling)	
22 post-treatment	larva (sampling)	larva	larva	egg	honey
23 post-treatment		larva	larva	egg	
24 post-treatment		larva (sampling)	larva	larva	honey
25 post-treatment			larva	larva	bee bread
26 post-treatment			larva (sampling)	larva	
27 post-treatment				larva	
28 post-treatment				larva (sampling)	honey

(MS) instrument, equipped with a MicroMist glass concentric nebulizer and Peltier-cooled, Scott type spray chamber was used. Prior to ICP-MS analysis, each sample (bee bread and larvae) was weighed (approximately 100 mg) and digested using a microwave-assisted digestion system (CEM MDS-2000) and a solution of 7 mL nitric acid and 1 mL hydrogen peroxide. The digested samples yielded clear solutions, were cooled to room temperature and then diluted with 2% v/v nitric acid until their concentration was within the desired concentration range and analyzed. In the case of honey samples, only dissolution and consequent dilution in 2% v/v nitric acid were needed.

2.5. Statistical analysis

The collected data were processed using a custom-written Python3 script with NumPy and SciPy packages. SciPy implementation of a *t*-test for independent samples was used to compare data between groups. The slope in cumulative mortality analysis was calculated between two data points also using custom-written Python3 script.

3. Results and discussion

3.1. Lithium in honey and bee bread

Honey is the primary form of carbohydrate storage in honeybee colonies, normally located at the sides and above the brood nest in the honeybee colony (Seeley & Morse, 1976). Modern hives are normally divided into a brood chamber and a honey super placed above it to facilitate honey extraction. Only honey stored in the honey super(s) is extracted by beekeepers, while honey in the brood chamber is left for the colony use (Graham, 2015). Our honey samples, collected in the brood chamber on different days, differed in lithium concentrations. In samples collected on the first post-treatment day, we detected between 0.6 and 1.0 mg of lithium/L of honey. The highest concentration was 0.4–1.2 mg of lithium/L of honey, detected in samples collected on the third day post-treatment. The lowest concentrations were detected on Day 7 post-treatment, between 0.05 and 0.9 mg of lithium/L; Fig. 1). Lithium in the control colonies was below the limit of quantification (LOQ < 0.05 mg/L). Good beekeeping practices require that chemical methods of varroa control are applied outside the main nectar flow period, i.e., after honey extraction to prevent contamination of honey (Wallner, 1999; Martel et al., 2007). After the last honey harvest, which in Central Europe is in July at the latest, beekeepers immediately start feeding colonies sucrose syrup to stimulate colony development, to compensate for the extracted honey, and to ensure overwintering of the colonies. The sucrose syrup is transformed by workers into honey and stored in the brood chamber. Leftover food stores are a possible source of contamination: it is well known phenomenon that honeybees transfer honey stores between combs and hive compartments, especially in season during the honey flow when bees transfer stores during the process of nectar conversion to honey and drying (Eyer et al., 2016, Kandolf-Borovšak, 2019). The transfer of honey stores around the hive provides one possible explanation for the measured drop in the lithium concentration in honey, in addition to consumption and replacement with fresh honey. At least hypothetically, this observation presents the possibility that workers inadvertently concentrate lithium in some parts of honey stores. The amount of lithium in honey stores after feeding was in some cases higher than 2.5× of the maximum lithium concentration reported in drinking water from various places in the world (Vita, De Peri, & Sacchetti, 2015).

Table 2
Concentration of lithium in bee bread samples (mg/kg) on Day 4 after completed feeding. Control samples were below limit of quantitative detection (LOQ < 0.5 mg/kg). C: control colonies; Li: test colonies.

	C1	C2	C3	C4	Li1	Li2	Li3	Li4
mg Li/kg bee bread	< 0.5	< 0.5	< 0.5	< 0.5	33	26	47	17

Bee bread represents the colonies' protein storage, important in rearing of the brood. Bee bread samples were collected in test colonies containing different quantities of lithium but all within the same order of magnitude (17–47 mg Li/kg bee bread; Table 2). Lithium in bee bread sampled from control colonies was below the limit of quantification.

The lithium content in honey and bee bread differ in an order of magnitude, yet they are both within recommended daily dietary allowance for an adult human, which was determined at 14.3 μg lithium/kg of body weight (Schrauzer, 2002). Major dietary sources of lithium in the human diet are vegetables and drinking water, depending on the region, while the estimated average daily intake of lithium is 2 mg per adult (Schrauzer, 2002). In the treatment of bipolar disorder in which lithium acts as an active ingredient, the American Psychiatric Associations suggests 0.5–1.2 mmol/L of lithium in serum, which can be achieved by daily intake of 900–1200 mg of lithium (American Psychiatric Association, 2002). While theoretically possible, it is difficult to imagine the consumption of such daily amounts of contaminated honey and/or bee bread to make a notable contribution to the daily consumption of lithium or to lithium concentration in blood plasma.

3.2. Lithium in bees and brood

The second set of consequences that needs to be considered is the effect of lithium on the honeybees themselves. In our experiments, the

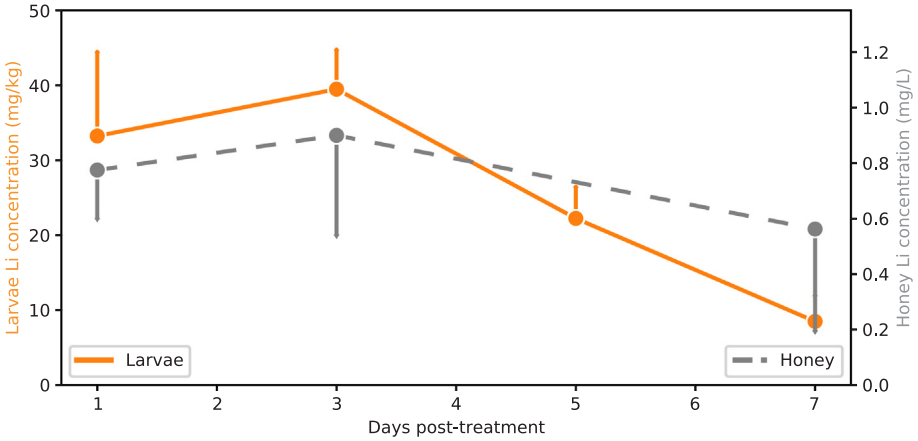

Fig. 1. Concentrations of lithium in larvae (left y-axis) and honey (right y-axis) sampled from test colonies on different days post-treatment. Concentrations of lithium in control samples are below the limit of quantification. The concentration of lithium in larval tissues is more than an order of magnitude higher than in honey.

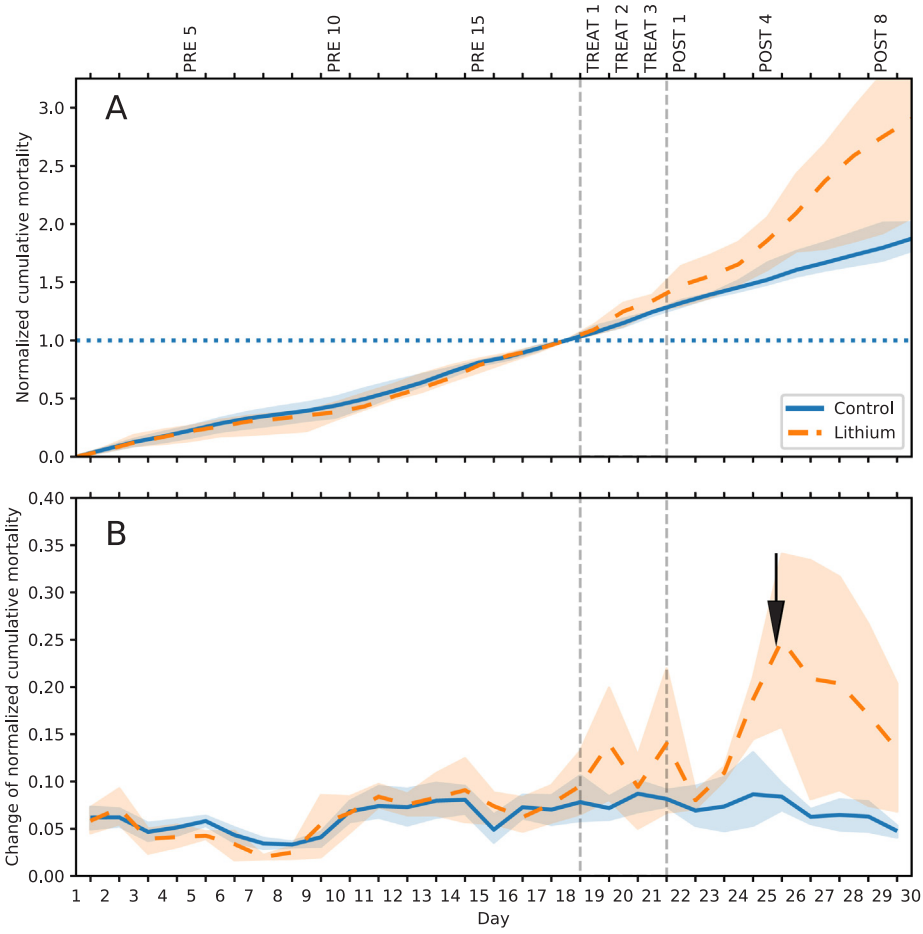

Fig. 2. A: Comparison of the cumulative mortality of adult honeybees between treated and untreated groups. Values were normalized to Day 18 (horizontal dashed line). B: Change of cumulative mortality computed as a slope in datapoints in A. Arrow marks the greatest difference in mortality change between control and lithium groups, approximately 3 days after treatment ended. Dashed lines mark start and end of treatment interval. Values are shown as mean ± SD.

Table 3
Concentrations of lithium in pooled different body parts of adult workers after treatments with sugar syrup containing lithium *per os* and *ad libitum*.

	Head [mg/kg]	Thorax [mg/kg]	Abdomen [mg/kg]
per os control	0.15	0.05	0.15
per os LiCl	6.50	13.10	40.10
ad libitum LiCl	39.25	29.52	266.00

mortality of adult bees differed between the colonies. The cumulative mortality values were normalized to Day 18 (last pre-treatment day) when the treatment started; changes in the slope of cumulative mortality was used to detect lithium effects (Blejec, 2005). Increased mortality in the lithium-treated colonies was recorded with the start of the treatment (Fig. 2A), demonstrated as a peak change in normalized cumulative mortality on Day 4 post-treatment (Fig. 2B). We have compared the differences in slope change between the control group (0.083 ± 0.02, mean ± SD) and the lithium group (0.25 ± 0.1, mean ± SD) on that day and found a statistically significant difference (*t*-test, $t = 3.01$, $p = 0.024$). The fact that we detected lithium in pollen stores should raise concerns at least for the embryonic growth of honeybee larvae supported by increased concentrations of lithium in larval tissues. Larvae were sampled in even intervals after treatment ended. The concentration of lithium was expectedly high on Day 1 post-treatment, between 20 and 45 mg Li/kg of larval tissue, but the highest concentration was detected on Day 3 post-treatment (35–46 mg Li/kg) and dropped to range from 4 to 8 mg Li/kg of larval tissue on Day 7 post-treatment (Fig. 1). At the same time, lithium in larvae sampled in control colonies was below the level of quantification (LOQ < 0.5 mg/kg). In rat whole-embryo cultures, for example, a reduction of embryonic growth was detected at 50 mg Li/kg (Klug, Collins, Nagao, Merker, & Neubert, 1992). In invertebrates, the literature is scarcer, yet it is known that in the classic invertebrate model (i.e., the worm *Caenorhabditis elegans*) 750 µmol decreases growth and development (Inokuchi et al., 2015). Matching concentrations of lithium in larvae and bee bread shows that lithium may have an impact on the embryonic development of honeybees. It is true, however, that an increase in the mortality of workers could be ascribed to acute poisoning, not developmental problems. In cage experiments, different concentrations of lithium were detected in the samples of 7-day old workers, depending on the feeding mode and the body part analyzed. The sample containing thoraxes from the control group had the least amount of lithium (0.052 mg/kg), while the abdomens of the *ad libitum* sample contained 266 mg/kg. The control group contained the lowest concentrations of lithium, the and *ad libitum* samples always contained the highest concentrations of lithium, regardless of the body part, which was according to expectations (Table 3). Abdominal content could be higher in comparison because the gut was not removed before the analysis.

Finally, insects are part of the diet in many different cultures around the world. While there is no insect-eating tradition in the so-called “western-hemisphere”, several recent studies have been conducted in that field. For example, in a hedonic evaluation of edible insect species, the honeybee larvae were ranked first (Bednářová, Borkovcová, Mlček, Rop, & Zeman, 2013); another study focused on the nutritional and odor characterization of honeybee larvae and pupae (Haber, Mishyna, Martinez, & Benjamin, 2019); a third study defined research protocols and recommendation on the evaluation and harvesting honeybee brood (Jensen et al., 2016). Our results show a more than tenfold lithium concentration in larvae compared to honey, which is a noteworthy point, especially through the prism of the increasing acceptance of insects in the Western diet.

4. Conclusions

We have demonstrated the transfer of lithium in honeybee feed to the next generation of honeybees and the possibility of lithium contamination of human food through honeybee food stores. Lithium chloride is affordable, and its acaricidal efficacy seems to be reasonably high to prompt many beekeepers to try lithium treatment in their colonies without consideration about the possible undesired effects. Lithium chloride is not authorized as a veterinary medicine product (VMP) for use in beekeeping, and no maximum residue levels (MRL) have been determined for lithium. In comparison, the existing synthetic acaricides authorized as VMPs, have their MRL determined for honey (amitraz, 0.2 mg/kg; coumaphos, 0.1 mg/kg; τ-fluvalinate 0.05 mg/kg; EU Pesticide Database). It seems that concentrations of lithium in honey and pollen stores do not reach alarming levels regarding human well-being, but we would urge caution: there is certainly an undesired detrimental effect on the honeybee well-being. Some of the "classic" acaricides are also known to have a negative impact on bees, evident as altered behavior, development, and higher mortality (e.g., Haarmann, Spivak, Weaver, Weaver, & Glenn, 2002, Pettis, Collins, Wilbanks, & Feldlaufer, 2004, Bevk, Kralj, & Čokl, 2011, Dai, Jack, Mortensen, Bustamante, & Ellis, 2018). "Classic" substances could also put beekeepers (e.g., Radakovic et al., 2013) and consumers at risk (Martel et al., 2007). For these reasons, we would suggest a careful comparison between lithium chloride and other substances already in use before the rejection or acceptance of lithium salts in *Varroa* treatment.

CRediT authorship contribution statement

JP, JB, UK and MŠ did the conceptualization. JP and MŠ designed methodology. UK, JB and MŠ did investigation. JP supervised the work. UK and JP did the formal analysis. JP, UK and MŠ did the writing – original draft. JP, JB and MŠ did the writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127334.

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