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Laboratory bioassays on the impact of cadmium, copper and lead on the development and survival of honeybee (*Apis mellifera* L.) larvae and foragers☆

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HIGHLIGHTS

- Metals such as Cd, Cu and Pb occur in honey bee hives through different routes.
- Lethal and sublethal effects of Cd, Cu and Pb were tested individually.
- Ingestion of metals by larvae caused detrimental effects on growth.
- Both larvae and foragers showed higher body burdens when eating metal-containing nectar.

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ABSTRACT

Honeybees (*Apis mellifera* L.) have been widely distributed around the world to serve as pollinators for agriculture. They can encounter metal pollutants through various routes of exposure, including foraging on contaminated plant resources. Chronic and acute toxicity tests were conducted on larvae using artificial diets and on foragers using solutions of 50% sucrose, which contained cadmium (Cd), copper (Cu) and lead (Pb). We found that mortality increased in both larvae and foragers in a dose-dependent manner. Control larvae had higher relative growth indices (RGI) from day 6 to day 10 compared to all metal treatments, demonstrating substantial negative effects of metals on development. Copper was the least toxic to larvae with an LC_{50} of 6.97 mg L^{-1}. For foragers, Pb had the highest LC_{50}, which was 345 mg L^{-1}. Foragers and larvae accumulated substantial quantities of all metals, and subsequent sucrose consumption decreased after dosing. Overall, honeybee larvae and foragers suffered detrimental effects when they were exposed to ecologically-relevant concentrations of Cd, Cu and Pb.

1. Introduction

Although pollinators can be exposed to metal pollutants via air, water and other mechanisms, a common source of exposure is through ingestion of contaminated pollen and nectar (Celli and Maccagnani, 2003; Hladun et al., 2015a). While many metals can be sequestered and transferred to honeybees, three of the most common are cadmium (Cd), copper (Cu) and lead (Pb) (Hladun et al., 2015a; Satta et al., 2012). For example, once in the soil, Cd is actively absorbed by plant roots, transferred via the vascular system into the nectar and pollen, and subsequently accumulates in pollinators and bee products, including honey, propolis and beeswax, and so on (Bogdanov, 2006; Silici et al., 2013). Cadmium is more reactive and mobile in plants than most of other kinds of metals (Verkleij and Schat, 1990). Not surprisingly, the Cd content in honey was found to be related to that in *Trifolium pratense* L. flowers that the bees foraged upon near a heavily traveled highway (Leita et al., 1996).

Two of the other metals that can accumulate on or in plants and potentially transfer to bees include copper (Cu) and lead (Pb) (Hladun et al., 2015a; Satta et al., 2012). Copper acts as an essential trace element in plants, and is able to accumulate in different plant tissues (Hladun et al., 2015a). Roman (2010) reported that Cu

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content was the highest in honeybee foragers, followed by selenium (Se), lead (Pb), and Cd. As a constituent of bio-active compounds along with other metals such as iron (Fe), zinc (Zn), chromium (Cr), cobalt (Co), molybdenum (Mo) and manganese (Mn), Cu co-acts with several essential proteins to enhance growth and development, but it can also be toxic for plants and honeybees if accumulation exceeds the cellular needs (House, 1961; Perna et al., 2014). Some insects appear to have the capacity to regulate Cu toxicity. For example, locusts feeding on metal contaminated maize were unable to regulate Cd, but had the ability to manage Cu toxicity (Crawford et al., 1996).

Since Pb is not easily translocated within plants (Hladun et al., 2015a), contamination in bees is most likely due to transport through the air and dislodgeable residues resulting from deposition on surfaces contacted by bees. This mechanism is apparently effective as a concentration of up to 2.37 mg kg^{-1} Pb has been reported in honey from contaminated areas (D'Ambrosio and Marchesini, 1982). However, the relatively small amounts of Pb that are translocated within plants can accumulate in flowers, leading to an increase in concentration in honeybees (Hladun et al., 2015a). Honeybee workers fed with nectar containing 0.2 mg kg^{-1} Pb were observed to have 1.4 mg kg^{-1} Pb in their tissues (Pratt and Sikorski, 1982). A study by Bogdanov (2006) suggested that bee hives should be kept at least 3 km away from traffic and incinerators because of the high levels of Pb found in propolis.

Since honeybees have a large foraging area of up to 14 km^2 (Bromenshenk et al., 1985; Ratnieks and Shackleton, 2015), they can be exposed to contaminants continuously (Jones, 1987; Porrini et al., 2003). The fuzzy body of the honeybee is also prone to collecting contaminants (Leita et al., 1996). The levels of metals in forager bodies also vary between regions and by season (Jones, 1987; Velemínský et al., 1990). For instance, the content of Pb and Cd in bee tissues from industrialized regions can be as much as ten times of those from unindustrialized areas (Hoffel, 1985). Cadmium and Pb levels were found to be significantly higher in urban areas than in nonresidential areas (Perugini et al., 2011). In semirural areas and high vehicle emission places in Brazil, Cd and Pb concentrations in pollen were much higher compared to countrysides (Morgano et al., 2010).

Pollutants also accumulate within the hive. A study by Formicki et al. (2013) in Poland showed that Cd accumulated the most in beewax, and Pb content was high in both honey and wax when apiaries are exposed in industrial or agricultural areas. Leita et al. (1996) tested the metal accumulation in bee hives near urban crossroads in Italy and found that the amount of Pb, Zn and Cd on the bodies of honeybee foragers increased over time, and within the hives, royal jelly accumulated the largest concentrations of these metals. Because royal jelly is initially fed to all larvae (not just queens), the presence of metals can potentially affect all members of the colony directly.

Given the presence of Cd, Cu and Pb in diverse areas and within many types of hive products including honey and royal jelly (Leita et al., 1996; Ayestaran et al., 2010), there is a need for baseline information on potential effects of these metals on survival, developmental rates, and body burdens in honeybees. We tested the effect of these metals on both honeybee larvae and foragers to determine the concentrations in food that produce detrimental impacts on the honeybee. Ultimately, these data may be used to determine the acceptability or sustainability of locating pollinator colonies at sites with various levels of metal contamination.

2. Materials and methods

2.1. Artificial diet for larval growth during larval bioassays

The artificial diet consisted of 53% (W/W) commercial freshly frozen royal jelly, 6% glucose, 6% fructose, 1% yeast extract, and 34% ultrapure water (Kaftanoglu et al., 2010). The metal compounds were dissolved into the sugar solution portion to reach final target concentrations in the diet. Cadmium, Cu and Pb were added to the water as cadmium chloride (Fisher Scientific, Waltham, MA, USA), copper chloride dihydrate (Acros Organics, Geel, Belgium, purity > 99%) and lead chloride (Acros Organics, Geel, Belgium, purity 99%), respectively. Following a series of pilot experiments to determine the concentration ranges needed to produce between 5 and 95% mortality for each metal, the final concentrations tested for each chemical were: 0, 0.01, 0.04, 0.12, 0.35, 1.05, 3.16, 9.47, and 28.41 mg L^{-1} for Cd; 0, 0.32, 0.64, 1.28, 2.56, 5.12, 10.24, 20.48, and 40.96 mg L^{-1} for Cu; and 0, 0.1, 0.3, 0.9, 2.7, 8.1, and 24.3 mg L^{-1} for Pb. Each metal treatment concentration was replicated three times with each replicate containing at least 22 larvae.

2.2. 1-d-old larvae preparation

Following the methods of Peng et al. (1992) and Aupinel et al. (2005), a queen was put into an excluder cage for 24 h with a frame with empty cells. After 24 h, the queen was removed from the frame to prevent further oviposition. Four days later, the approximately 1-d-old larvae were removed from the wax cells on the frame and placed onto artificial diet using grafting tools (Sinova, Zhengzhou, China).

2.3. Larval chronic toxicity tests

All equipment (including grafting tools, cell cups and well plates) was ultraviolet sterilized (Air Clean 600 PCR workstation, ISC Bioexpress) to minimize contamination. Artificial diet (250 μL) was provided to larvae in the cell cups (Glory Bee foods, Inc., Eugene, OR). Cell cups were then put into 48 well plates (Costar 3526 cell culture plates; Corning), and stored in bell jars (25.1 cm in diameter and 16.8 cm in height) to maintain temperature at 34.1 ± 0.01 °C and 94.6 ± 0.2% humidity in darkness. A dish of glycol with methyl benzethonium chloride (MBC) was placed at the bottom of the bell jar to prevent contamination and maintain humidity. Mortality was scored daily until pupation. Control mortality was below 20%. At the conclusion of the experiment, after the recording process of day 10, dead larvae or prepupae were removed from the well plates and frozen at −60 °C. The prepupae and pupae were then weighed using a microbalance (weighing to 0.0001 g, model HT224, Shinko Denshi Co., Ltd., Tokyo, Japan). A total number of 91, 96, and 118 prepupal larvae were weighed for Cd, Cu and Pb, respectively. The total number of pupae weighed were 111, 56 and 130 for Cd, Cu and Pb, respectively. The relative growth indices (RGI) were calculated for *Apis mellifera* larvae exposed to Cd, Cu and Pb from day 4 through day 10 using the equations described by Zhang et al. (1993).

2.4. Foragers acute toxicity assay

Foragers were collected from the same hive with the same queen maintained at Agricultural Operations at University of California-Riverside. The hive was populated by the western honey bee, subspecies *A. mellifera ligustica*, that was free of parasites. The foragers were collected at the entrance of the hive with scintillation vials, put on ice for a minimum time until immobile, and then harnessed with tape in a straw tube holder (1 cm in diameter). Only

the head, antennae and proboscis were free to move. Then they were fed with 50% sucrose to satiation 24 h before being dosed. Oral toxicity tests were based on standardized procedures recommended by the US Environmental Protection Agency (USEPA, OPPTS 850. 3020. 1996). Sources of the chemicals were the same as reported for the larval tests.

2.5. Foragers LC_{50} assay

For the cadmium chloride and copper chloride dihydrate test groups, each bee was fed with 20 μL corresponding solutions using a micrometer glass syringe (Gilmont Instrument, Cole Palmer, USA). Bees were fed with 30 μL solutions for lead chloride. The treatment concentrations were 0, 26, 52, 104, 208, 416 mg L^{-1} for Cd, 0, 32, 64, 128, 256, 512 mg L^{-1} for Cu, and 0, 267, 295, 330, 365, 400 mg L^{-1} for Pb. The chemicals were dissolved in 50% sucrose solution.

Bees were scored for mortality at 24 h, 48 h, and 72 h after a single dose at 0 h. Mortality for control bees was under 10%. After dosing, the bees that remained alive were fed with 50% sucrose solution at 24 h and 48 h, and the total volumes consumed by each bee were recorded. The temperature was 20.0 ± 0.01 °C during the experiment with a humidity of 73.5 ± 0.46%.

2.6. Bioassays of metal concentration in A. mellifera

Forager and larval tissues were stored at −60 °C after recording mortality. They were then freeze-dried (Labconco Corp freeze dry system, Kansas, Missouri, USA) at −40 °C and −1.758 kg sq. cm^{-1} for 72 h before digestion. Honeybee tissues were weighed with a microbalance and put into 110 mL Teflon vessels. Five mL of concentrated HNO_3 was added to each vessel, after which they were heated in a 570-W microwave oven (CEM Corp) for 20 min. After cooling, the liquid in the vessels was transferred into 50 mL flasks and heated on a hot plate to remove nitrogen oxides. Ultrapure water was then added to filtrate and final volumes were recorded. Standard oyster tissue 1566b (freeze-dried, U.S. Department of Commerce National Institute of Standards and Technology, Gaithersburg, MD) was used for each run as digestion verification, and metal concentration recovery was above 90%. Ultrapure water was used as blank (Hladun et al., 2013b).

2.7. Data analysis

Each metal was analyzed separately. The volume of 50% sucrose consumption after dosing, prepupal and pupal weights, and the mortality of foragers and larvae were analyzed using ANOVA among different treatments (LSD test, SPSS 17.0). Relative growth indices were analyzed using ANOVA (PROC GLM, SAS 9.2; SAS Institute, Cary NC, USA) with repeated measures. The independent variable was metal treatment concentration, RGI was the dependent variable, and day (over the 10 d of development) was the repeated variable. Mean separations were conducted between groups ($\alpha = 0.05$) using a post hoc Tukey's HSD test. Log-dose probit analysis was performed using SAS Version 9.2 (SAS Institute, Cary NC, USA). The LC_{50} of larvae was calculated based on the data from day 8. The foragers LC_{50} was calculated according to the data from 72 h.

3. Results

3.1. Prepupal and pupal weights after metal treatment

The metal treatments affected prepupal and pupal weights significantly (ANOVA, with P values < 0.017), with the exception of Cd effects on prepupal weight ($F = 1.164$, df = 6.90, $P = 0.334$) (Table 1). Compared to control (0 mg L^{-1}) groups, most treatments decreased the prepupal and pupal weights. For Pb, the weight of pupae grown in 8.1 mg L^{-1} was 0.1386 g, which was significantly lower than the other treatment groups ($F = 4.130$, df = 5,128, $P < 0.01$), and it was 15% lower than the control group. For Cu, both prepupal weights ($F = 8.458$, df = 8.97, $P < 0.001$) and pupal weights ($F = 4.221$, df = 7.55, $P < 0.001$) were significantly reduced. Interestingly, larvae fed with 0.32 mg L^{-1} Cu weighed significantly more at the prepupal stage than the controls or any other treatments. However, the increased weight of these larvae did not result in increased pupal weights.

3.2. Relative growth indices of A. mellifera larvae exposed to Cd, Cu or Pb

For Cd, day ($F = 8.74$, df = 6,108, $P < 0.001$), Cd treatment ($F = 140.54$, df = 8.18, $P < 0.001$), and the interaction of day and Cd treatment ($F = 3.18$, df = 48,108, $P < 0.001$) had overall significant effect on RGI. By day 6, the 1.05, 3.16, 9.47 and 28.41 mg Cd L^{-1} treatments had significantly lower RGIs compared to the control (Fig. 1A).

For Cu, day ($F = 23.43$, df = 6,114, $P < 0.001$), Cu treatment ($F = 17.55$, df = 8.19, $P < 0.001$) and the interaction of day and Cu treatment ($F = 6.79$, df = 48,114, $P < 0.001$) had a significant effect on RGI. On day 6, the 20.48 and 40.96 mg Cu L^{-1} treatments had significantly lower RGIs compared to control. By day 9, all Cu treatments had significantly lower RGIs compared to the control (Fig. 1B).

For Pb (Fig. 1C), day ($F = 9.85$, df = 6.84, $P < 0.001$), Pb treatment ($F = 74.15$, df = 6.14, $P < 0.001$), and the interaction of day and Pb treatment ($F = 9.85$, df = 6.84, $P < 0.001$) had an overall significant effect on RGI. Larvae fed the control (0 mg L^{-1}) had significantly higher RGIs compared to the 0.3, 0.9, 2.7 and 8.1 mg Pb L^{-1} treatments starting on day 8, and the trend continued until d 10 (Tukey HSD test, $P < 0.05$).

Table 1
Prepupal and pupal weights of *A. mellifera* larvae exposed to Cd, Cu or Pb after 10 days. Means within a row for one metal with the same letter are not significantly different (LSD test: $P > 0.05$).

Metal	Treatment Concentration (mg L^{-1})	Mean Prepupa weight (g) (Average ± SE)	Mean Pupa weight (g) (Average ± SE)
Pb	0.00	0.1673 ± 0.0031 a	0.1634 ± 0.0023 a
	0.10	0.1598 ± 0.0048 ab	0.1581 ± 0.0025 a
	0.30	0.1496 ± 0.0055 b	0.1563 ± 0.0052 a
	0.90	0.1665 ± 0.0042 a	0.1700 ± 0.0038 a
	2.70	0.1699 ± 0.0037 a	0.1633 ± 0.0055 a
	8.10	0.1591 ± 0.0049 ab	0.1386 ± 0.0078 b
	24.30	–	–
Cd	0.00	0.1608 ± 0.0063 a	0.1620 ± 0.0047 bc
	0.01	0.1458 ± 0.0078 a	0.1558 ± 0.0052 c
	0.04	0.1602 ± 0.0041 a	0.1608 ± 0.0043 bc
	0.12	0.1573 ± 0.0065 a	0.1740 ± 0.0039 ab
	0.35	0.1651 ± 0.0063 a	0.1675 ± 0.0058 bc
	1.05	0.1632 ± 0.0039 a	0.1618 ± 0.0046 bc
	3.16	0.1607 ± 0.0059 a	0.1860 ± 0.0027 a
	9.47	–	–
	28.41	–	–
Cu	0.00	0.1601 ± 0.0036 a	0.1681 ± 0.0027 ab
	0.32	0.1682 ± 0.0561 b	0.1798 ± 0.0103 a
	0.64	0.1551 ± 0.0047 a	0.1512 ± 0.0074 bc
	1.28	0.1338 ± 0.0070 a	0.1416 ± 0.0131 c
	2.56	0.1371 ± 0.0048 a	0.1426 ± 0.0072 c
	5.12	0.1556 ± 0.0064 a	0.1519 ± 0.0034 bc
	10.24	0.1392 ± 0.0122 a	0.1536 ± 0.0094 bc
	20.48	0.1489 ± 0.0025 a	0.1396 ± 0.0033 c
	40.96	0.1455 ± 0.0037 a	–

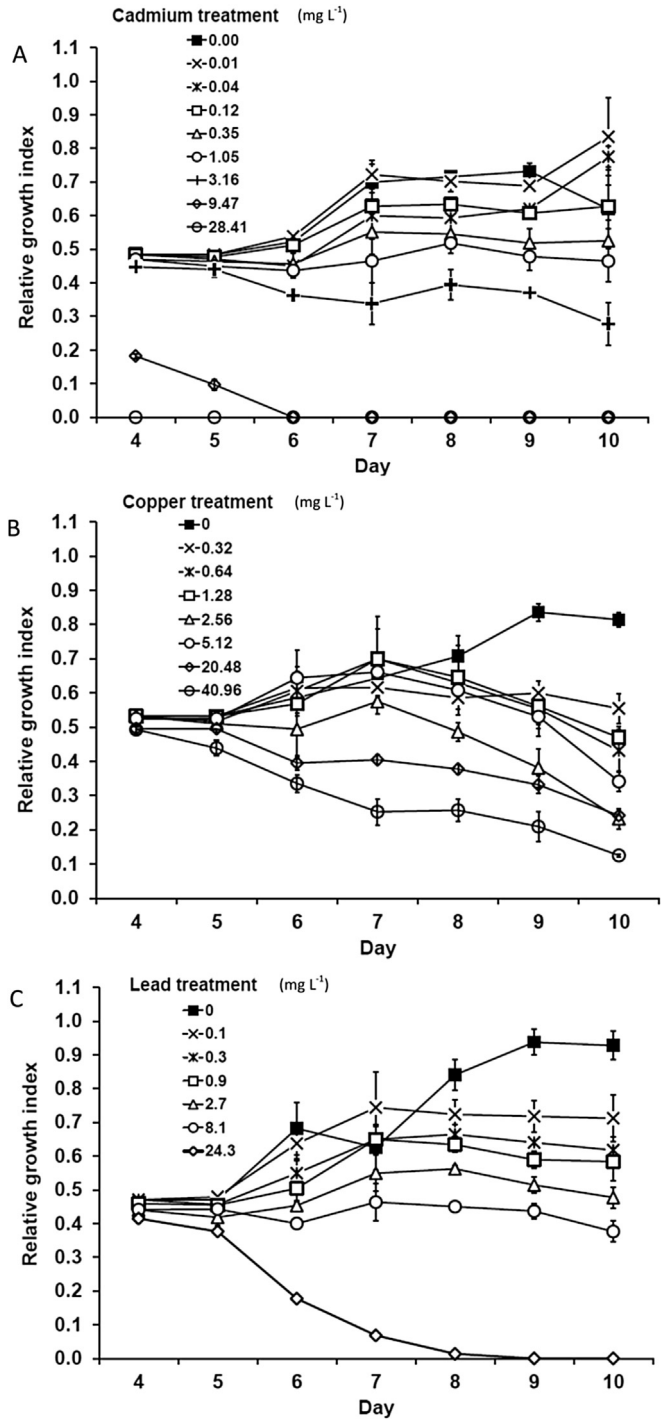

Fig. 1. Mean relative growth indices of *A. mellifera* larvae treated with different metals, Cd (A), Cu (B), and Pb (C), over a 10 days period. Bars represent standard errors.

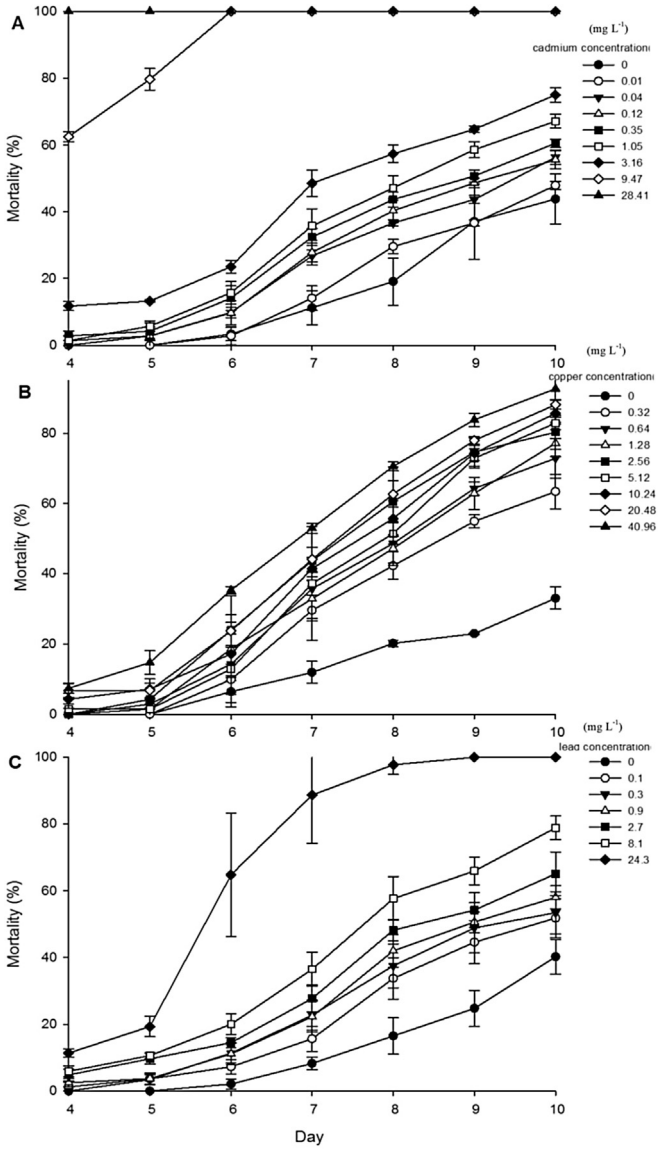

Fig. 2. *Apis mellifera* larval mortality from feeding on artificial diets containing a range of Cd (A), Cu (B) and Pb (C) concentrations during a 10 d period. Bars represent standard errors.

3.3. *Apis mellifera* larval mortality after exposed to Cd, Cu or Pb

Cadmium ($F = 1126.326$, $df = 8.26$, $P < 0.001$) and Pb ($F = 11.505$, $df = 6.20$, $P < 0.001$) significantly affected *A. mellifera* larval mortality starting on day 4 (Fig. 2). The highest concentration of Cd (28.41 mg L^{-1}) killed all the larvae by day 4. For 9.47 mg L^{-1} treatment groups, 79.69 ± 3.24% of the larvae died on day 5, and 100% mortality was reached by day 6.

Copper (Fig. 2B) increased mortality 6 days after exposure ($F = 5.990$, $df = 8.26$, $P < 0.001$). More than half of the treatment groups (5 out of 9) had more than 50% larval mortality by day 8 ($F = 8.680$, $df = 8.26$, $P < 0.001$). From day 4 to day 6 after grafting, the lowest treatment group (0.32 mg L^{-1}) did not cause significant mortality compared to the control, but the mortality rate of that treatment group increased from day 7, and was significantly higher compared to the mortality of control groups. At the end of the recording period (day 10), 92.65% ± 3.10 of larvae had died in the highest dose treatment (40.96 mg L^{-1} Cu).

Similarly for Pb (Fig. 2C), mortality increased from day 4 to day 10 in a dose dependent manner compared to larvae fed control diets. Only the highest treatment group achieved 100% mortality by day 9.

3.4. Metal accumulation in *A. mellifera* larvae

Significant differences in metal accumulation in larvae were observed among different concentrations of Cd ($P < 0.001$), Cu

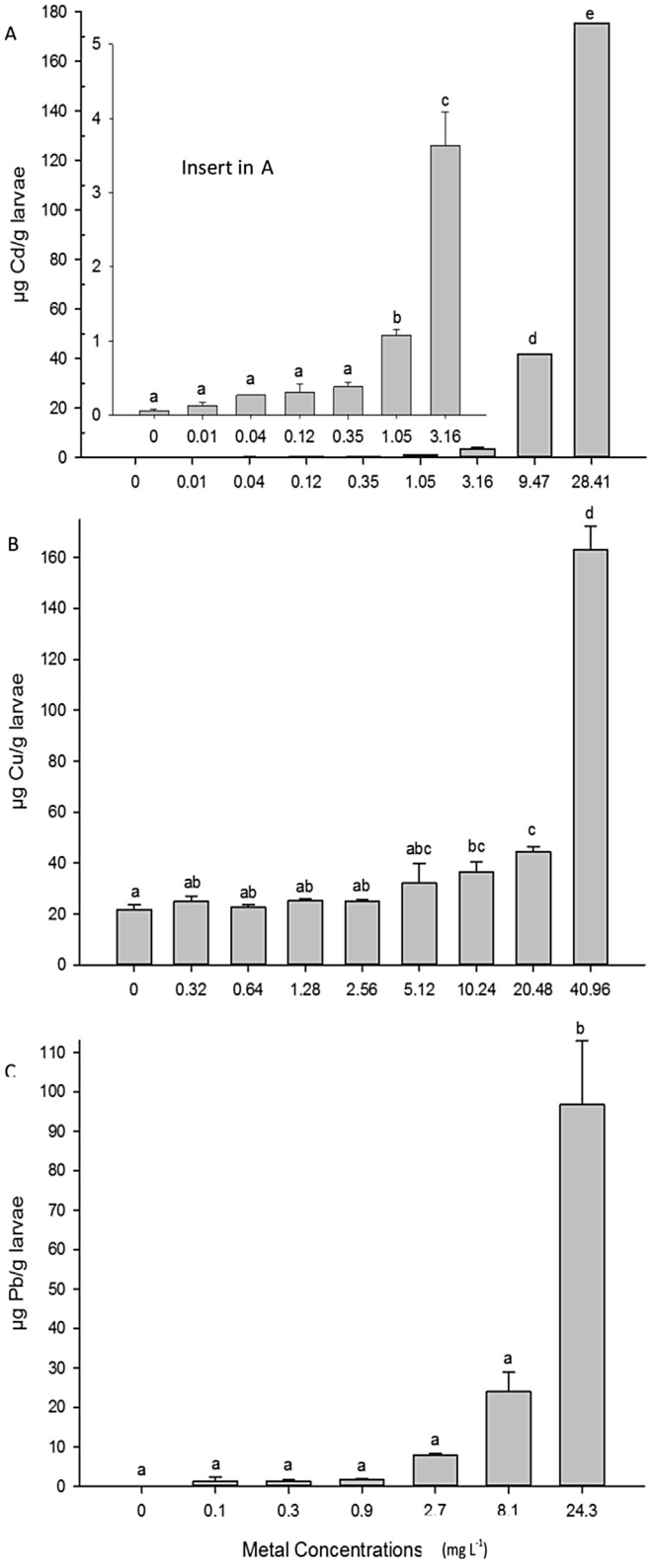

Fig. 3. Metal accumulation in *A. mellifera* larvae after being dosed with gradient Cd (A), Cu (B) and Pb (C) solutions. Bars represent standard errors and means with the same letter are not significantly different (LSD test: $P > 0.05$). There were no error bars for 9.47 mg L^{-1} and 28.43 mg L^{-1} Cd treatments because larvae died in early stage and ended up with quite small body size, so only one sample was tested for each of the two treatments. The insert in A is an expanded view of data from 0 mg L^{-1} to 3.16 mg L^{-1}.

($P < 0.001$) and Pb ($P < 0.01$) treatments, with higher doses causing more metals to accumulate (Fig. 3). All treatment groups had higher metal concentrations than the control groups (Fig. 3). For Cd and Pb, control groups only showed trace amounts of those metals, i.e., 0.060 μg Cd g^{-1} larvae and 0.047 μg Pb g^{-1} larvae. The Cu control group had 21.79 μg Cu g^{-1} larvae, which is not unexpected since, unlike Cd and Pb, Cu is a required metal for insects (Peng et al., 2014). As indicated in Fig. 3, for the highest two concentrations for Cd, Cu and Pb, the larvae body metal accumulation increased substantially, compared to the lower concentrations.

3.5. LC_{50} of *A. mellifera* larvae and foragers treated with metals

Log-dose probit analysis of larval survival found that there was no overlap among 95% confidence intervals for Cd, Cu and Pb, indicating that Cd was the most toxic metal among the three metals tested with an LC_{50} of 0.275 mg L^{-1} (Table 2). Copper showed the least toxicity with an LC_{50} of 6.97 mg L^{-1}. For foragers, there was an overlap among the 95% confidence intervals for Cd, Cu and Pb, suggesting the metals have similar toxicities in terms of adult mortality.

3.6. Forager mortality of *A. mellifera* after dosing with Cd, Cu or Pb

The mortality of foragers generally increased both over time and with an increase of metal concentrations (Fig. 4). For Cd, significant differences were observed at 24 h ($F = 4.387$, $df = 5.35$, $P < 0.01$), 48 h ($F = 4.420$, $df = 5.35$, $P < 0.01$), as well as 72 h ($F = 17.590$, $df = 5.27$, $P < 0.001$) after dosing. The higher the metal concentration was, the more rapidly the mortality increased. Also, there were significant differences at 24 h ($F = 5.453$, $df = 5.35$, $P < 0.01$), 48 h ($F = 13.622$, $df = 5.35$, $P < 0.001$) and 72 h ($F = 26.145$, $df = 5.35$, $P < 0.001$) for Cu. Forager mortality reached approximately 50% for the 512 mg L^{-1} Cu treatment group at 24 h, and the mortality for the same concentration at 72 h was 93.75%. No significant difference occurred at 24 h ($F = 1.353$, $df = 5.41$, $P = 0.265$) after the foragers were dosed with Pb. However at 48 h ($F = 4.636$, $df = 5.41$, $P < 0.01$) and 72 h ($F = 10.966$, $df = 5.41$, $P < 0.001$) timepoints, there were significant differences between control groups and treatment groups for Pb.

3.7. Sucrose consumption by *A. mellifera* foragers after Cd, Cu or Pb treatments

Compared to controls, foragers from metal treatments consumed less of the supplemental 50% sucrose provided after feeding on metal solutions (Fig. 5). At 24 h after dosing, foragers fed Cd ($F = 6.097$, $df = 5.29$, $P < 0.001$), Cu ($F = 6.142$, $df = 5.29$, $P < 0.001$), and Pb ($F = 4.168$, $df = 5.41$, $P < 0.01$) showed a significant reduction in sucrose consumption compared to controls. After 48 h, foragers did not recover their appetite. Bees fed Cd

Table 2
Mean lethal concentrations (LC_{50}) for *A. mellifera* (Hymenoptera: Apidae) exposed to Cd, Cu or Pb.

Metal	Number of insects tested	LC_{50} (mg L^{-1})	95% confidence limits
Foragers			
Cadmium	172	78	44–122
Copper	177	72	36–114
Lead	221	345	93–429
Larvae			
Cadmium	629	0.275	0.13–0.54
Copper	658	6.970	3.09–22.21
Lead	605	1.120	0.46–2.46

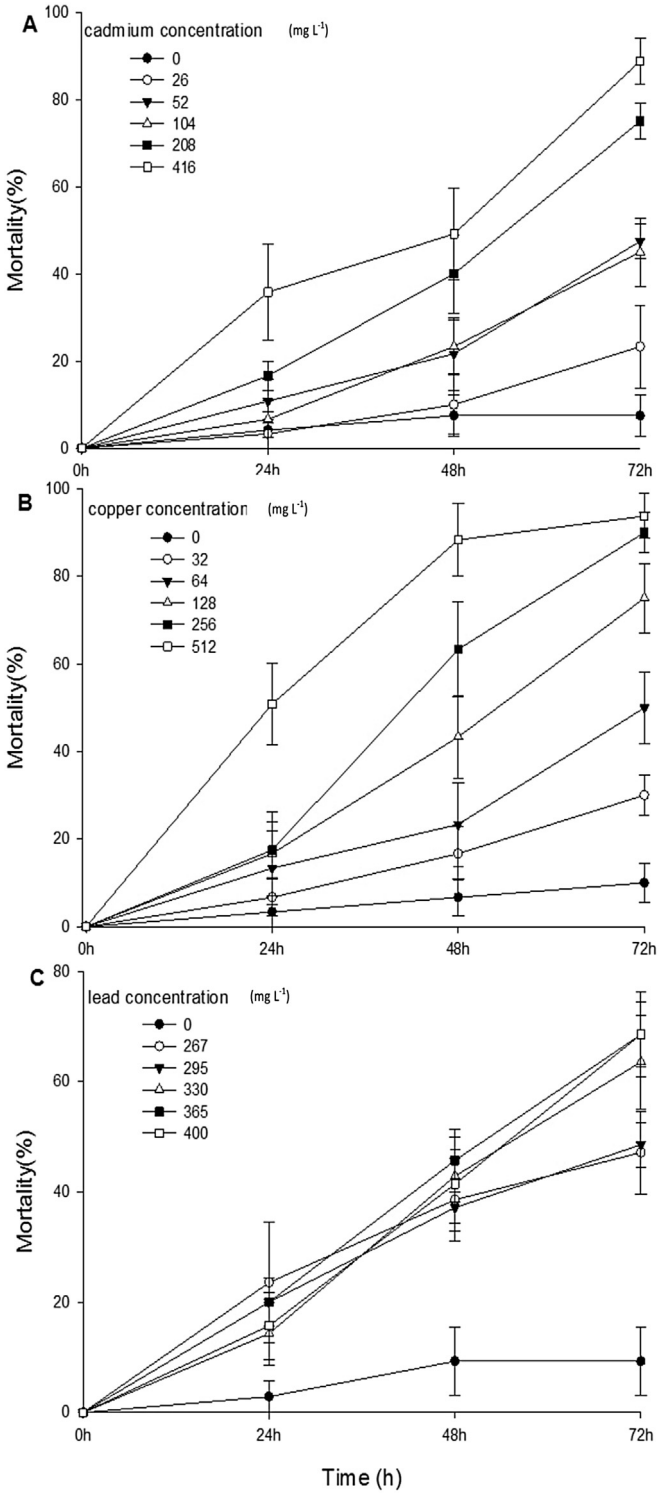

Fig. 4. *Apis mellifera* forager mortality after dosing with Cd solutions (A), Cu solutions (B), and Pb solutions (C), over a period of 72 h. Bars represent standard errors.

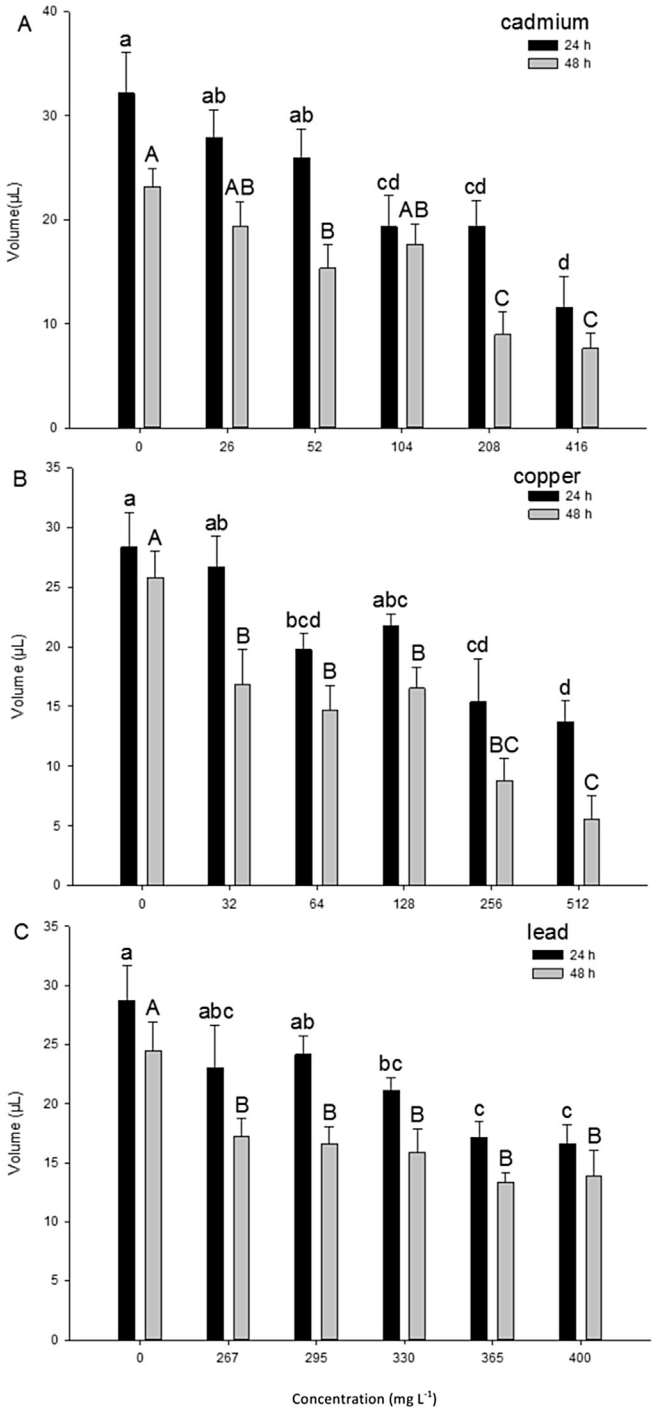

Fig. 5. Volume of 50% sucrose consumption by *A. mellifera* foragers after dosing for 24 h and 48 h for Cd (A), Cu (B) and Pb (C). Bars represent standard errors and means with the same letter are not significantly different (LSD test: $P > 0.05$).

($F = 9.275$, $df = 5.35$, $P < 0.001$), Cu ($F = 7.279$, $df = 5.30$, $P < 0.001$), and Pb ($F = 4.957$, $df = 5.41$, $P < 0.001$) continued to consume a significantly lower volume compared to controls. This response was concentration dependent. For Cd, Cu and Pb, the most significant differences were seen between controls and the highest treatment groups ($p < 0.001$ at both 24 h and 48 h).

3.8. Metal accumulation in *A. mellifera* foragers after Cd, Cu or Pb treatments

Foragers fed Cd ($F = 21.585$, $df = 5.17$, $P < 0.001$), Cu ($F = 41.666$, $df = 5.15$, $P < 0.001$), and Pb ($F = 44.201$, $df = 5.14$, $P < 0.001$) accumulated significant quantities of individual metals in a dose dependent manner (Fig. 6). Foragers accumulated significantly more metal than controls when they were dosed with at least 104 mg L^{-1} of Cd in the sucrose solution. Those fed 416 mg L^{-1} Cd

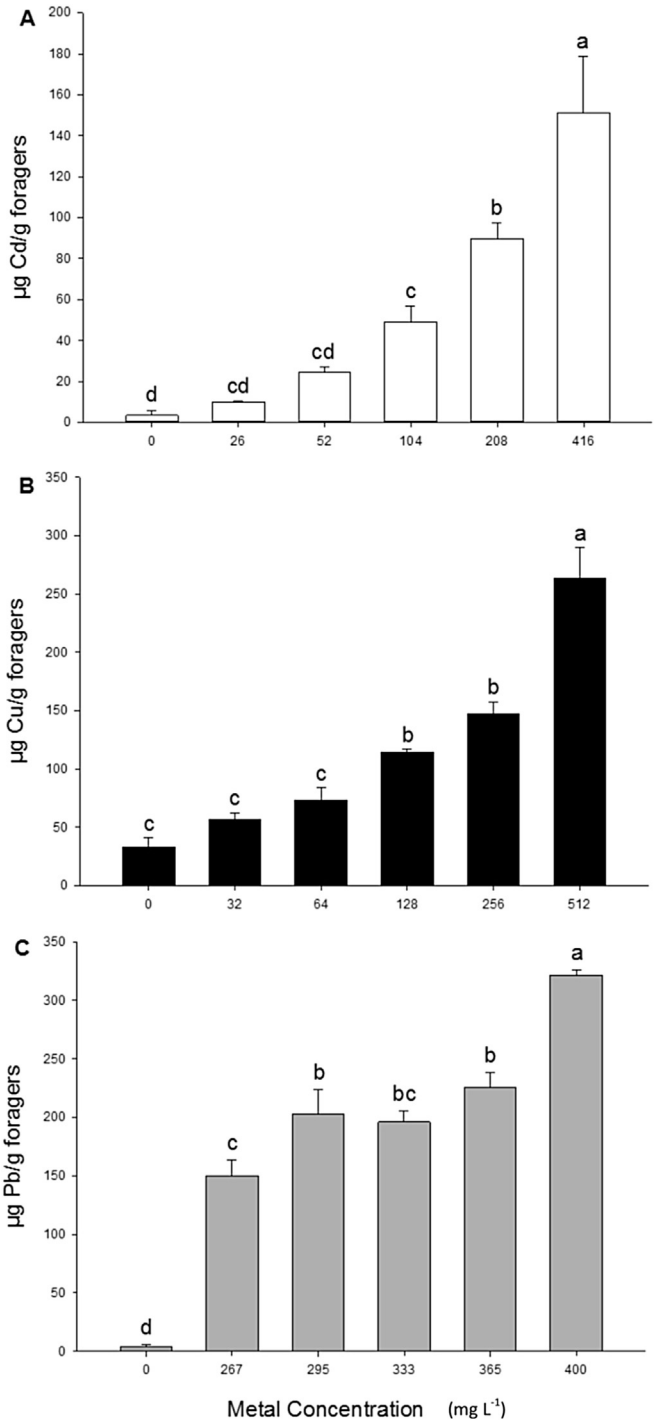

Fig. 6. Metal accumulation in *A. mellifera* foragers body after being dosed with solutions containing Cd (A), Cu (B) and Pb (C) solutions. Bars represent standard errors and means with the same letter are not significantly different (LSD test: $P > 0.05$).

accumulated the highest amounts of this metal, about 50 times more as compared to control bees (Fig. 6A). The control groups contained only trace amounts of Cd. Foragers fed lower concentrations of Cu (32 mg L^{-1} and 64 mg L^{-1}) did not significantly accumulate the metal compared to control groups. *Apis mellifera* foragers dosed with higher concentrations (128, 256, and 512 mg L^{-1}) significantly increased the body burdens of Cu (Fig. 6B). Compared to controls, foragers fed with Pb also accumulated significantly more Pb. The 400 mg L^{-1} treatment groups had approximately 100 times more Pb compared to controls (Fig. 6C).

4. Discussion

Apis mellifera is an essential pollinator for agriculture worldwide, and has been widely researched as a biomonitor of pollutants in the environment (Velemínský et al., 1990; Van der Steen et al., 2015). Silici et al. (2013) studied the trace element concentrations in honey and honeybee bodies near thermal power plants in Turkey and found that mean values for Cu, Cd and Pb were much higher in honeybee bodies than in honey, suggesting bioaccumulation was occurring for these metals. However, data on metal effects on *A. mellifera* survival and development are quite limited. The most extensive reports for honeybees are available on the effects of selenium, a metalloid element found at toxic concentrations in soil and plants in the western United States (Lemly, 1997). Several studies by Hladun et al. (2012, 2013a, 2013b) have documented significant effects of selenium on honeybee survival, development, behavior, and pollination ecology. Bromenshenk et al. (1985) found reduced hive productivity for colonies placed near industrialized regions where high concentrations of arsenic and Cd co-occurred. However, to our knowledge, ours is the first report detailing baseline data on honeybee forager and larval survival and development following exposure to Cd, Cu, and Pb, individually. All three metals showed developmental consequences for the larvae and important sublethal and lethal effects on foragers. Our results compliment the whole colony effects of these metals reported by Hladun et al. (2015b).

Of the many ways that honeybees can be exposed to these metals, ingesting plant nectar and pollen is probably the most common route of exposure (Celli and Maccagnani, 2003). For example, radishes in the genus *Raphanus* are frequently planted as a nectar and pollen source for maintaining and supporting honeybee colonies in California for up to several months in the dry season (Hladun et al., 2013a; Califlora, 2015). Cadmium, Cu and Pb can accumulate to substantial amounts in the flowers of *Raphanus sativus* and honeybees foraging on these flowers have been shown to accumulate significant amounts of Cd, Cu and Pb (Hladun et al., 2015a). Copper and Pb were reported to biotransfer from *Aster tripolium* L. to honey (Ernst and Bast-Cramer, 1980). No reports are currently available on the potential detection or avoidance of these metals by bees. Even lethal concentrations of Se in flowers did not deter duration or frequency of *A. mellifera* visitation to *R. sativus* (Hladun et al., 2013a). The foraging behavior of bumblebees was not influenced by the presence of aluminum (Al), but was suppressed (although not eliminated) by the presence of nickel in *Streptanthus polygaloides*, a nickel hyperaccumulating plant that sequesters over 1000 µg g^{-1} of nickel in plant tissues (Meindl and Ashman, 2013, 2014).

Copper was the most abundant metal found in honeybee bodies in our study. Although the forager LC_{50} (72 mg L^{-1}) is higher than the concentrations found in flowers of *R. sativus* exposed to ecologically-relevant amounts of soil contaminated with Cu (flowers = 30+ µg g^{-1} dry weight; Hladun et al., 2015a), the concentration of Cu in the pollen or nectar might vary from that of the total flower. Nonetheless, if accumulation or biomagnification of the metals occurs following feeding, as suggested by the report of Silici et al. (2013), the impact could be severe. Larvae had an LC_{50} of only about 7 mg L^{-1}, along with the increased body metal burden and mortality when exposed to high concentrations, severe reduction in whole colony health could be expected. If the reductions in pupal weight (Table 1) from even relatively low concentrations translate into reduced weight of the foragers, then the colony level effects would likely be substantial. Concentrations of Cu reported in honey vary by region, but the highest levels found in

Brazil exceeded 33 μg g^{-1} (Santos et al., 2008). At this concentration, our data suggest substantial reductions in larval growth and survival. The concentrations reported here for bees were not unusual. The Cu concentration in Hemiptera caught near a refinery in England was reportedly 265 mg kg^{-1}, while levels reached 731 mg kg^{-1} in ants, 421 mg kg^{-1} in Curculionidae, and 160 mg kg^{-1} in Lepidoptera larvae (Hunter et al., 1987).

There was a substantial variation in toxicity for Cd between foragers and larvae. The accumulation in *R. sativus* flowers was only 15 μg g^{-1} dry weight (Hladun et al., 2015a), which provided some tolerance compared to the LC_{50} levels for foragers of nearly 80 μg g^{-1} dry weight in our study. However, there has not been any information published on the levels of Cd in larvae. From our results, the LC_{50} for Cd was less than 0.3 mg L^{-1}, suggesting larval survival could be rather substantially reduced. The contents of Cd in honey and royal jelly found in bee products from a hive near a high vehicle traffic area was 1.9 and 2.9 μg g^{-1} fw, respectively (Leita et al., 1996). At these concentrations, our data suggest that the survival of larvae in these areas is at risk.

Lead was the least toxic compound we evaluated. For foragers the LC_{50} was nearly 350 mg L^{-1}, but the concentrations found in flowers of *R. sativus* were below 1 μg g^{-1}, probably because most of the limited accumulation of Pb was sequestered largely in the roots (Hladun et al., 2015a). This is not surprising given that Pb in contaminated soil is not readily bioavailable to plants (Davies et al., 2003). Lead appeared to be the least palatable metal for foragers in our test, because they refused to consume the same volume of contaminated sucrose solution as the control group when the Pb concentration was above 400 mg L^{-1}. Notably, they were able to regurgitate some solution if the Pb concentration was too high. However, the highest concentration treatment groups still accumulated a body burden of more than 100 times that found in the controls. The mortality of foragers fed Pb at 72 h after dosing was only approximately 69%. Maximum levels of Cd, Pb and Cu in honeybees in Finland ranged from 1.8 mg kg^{-1}, 18 mg kg^{-1} and 41 mg kg^{-1}, respectively, and those were 133, 36 and 108 times the concentrations detected from honey (Fakhimzadeh and Lodenius, 2000). This again suggested that bioaccumulation was occurring in foragers. As with the other metals we tested, the larvae were more susceptible to Pb than foragers. Interestingly, Leita et al. (1996) found Pb levels in honey and royal jelly ranging from 1.8 to 13.1 μg g^{-1}. Because larvae in our study had an LC_{50} of approximately 1 μg g^{-1}, which was lower than Cu, a reduction in larval survival and the growth of the whole colony could be expected.

There are two important caveats with this analysis. First, if the ingestion of these metals causes a 'malaise-effect' in workers, such as seen with other toxins (Ayestaran et al., 2010; Hurst et al., 2014), then the dose dependent reduction in sucrose ingestion seen in our study (Fig. 3) could reduce forager energy levels. From our results, larvae treated with higher concentrations of metal had lower RGIs from day 6, and some treatment groups never reached the pupal stage. This 'malaise-effect' would result in negative hive-level effects at lower concentrations than the levels needed to impact forager survival. While our study provides baseline effects of Cd, Cu, and Pb on foragers and larvae, additional research will be necessary to elucidate behavioral effects.

Second, exposure to mixtures of metals is likely in complex, polluted environments such as near smelters, coal-burning power plants, in industrialized regions, or in runoff waters from mining operations. Our study provides baseline information on toxicity of individual metals, but combinations of toxicants can have unexpected effects (Jensen et al., 2007). To fully understand the effects of combinations of metals, additional research will be needed to determine possible synergism or antagonism in environments with multiple metals.

5. Conclusions

Our study provides baseline information on the detrimental effects of Cd, Cu and Pb on *A. mellifera* larvae and foragers. Each metal individually slowed larval development, caused reduction in prepupal and pupal weights, decreased the survival rate of both larvae and foragers in a dose dependent manner, reduced the foragers' consumption of 50% sucrose, and increased body metal burdens in both larvae and foragers. Collectively, these effects will likely have a negative impact on whole colony health. However, to fully understand the effects of metal exposure in honeybees, studies are needed to document the effects of mixtures of metals, which may cause unexpected potentiation or antagonism, and to determine the behavioral effects of metals on behavior and pollination ecology of honeybees.

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