### Title

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### Authors

Di, Ning
Zhang, Kai
Hladun, Kristen R
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### Publication Date

2020-11-01

### DOI

10.1016/j.cbpc.2020.108839

Peer reviewed

Comparative Biochemistry and Physiology, Part C 237 (2020) 108839

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

# Joint effects of cadmium and copper on *Apis mellifera* forgers and larvae

Ning Di[a], Kai Zhang[b], Kristen R. Hladun[c], Michael Rust[d], Ya-Feng Chen[a], Zheng-Yang Zhu[a], Tong-Xian Liu[e], John T. Trumble[d,*]

[a] Institute of Plant and Environment Protection, Beijing Academy of Agriculture and Forestry Sciences, Beijing 100097, PR China
[b] Development Center of Science and Technology, Ministry of Agriculture and Rural Affairs, Beijing 100122, PR China
[c] US FDA Pacific Regional Laboratory, Southwest, Irvine, CA 92612, USA
[d] Department of Entomology, University of California, Riverside, CA 92521, USA
[e] State Key Laboratory of Crop Stress Biology for Arid Areas, Key Laboratory of Northwest Loess Plateau Crop Pest Management of Ministry of Agriculture, Northwest A&F University, Yangling, Shaanxi 712100, PR China

ARTICLE INFO

*Keywords:*
Heavy metals
Synergism
Pollinators
Body burden
Sucrose response threshold

ABSTRACT

Honey bees (*Apis mellifera* L.) are important ecological and agricultural resources. They are among the most widely available pollinators and provide products as well as services. Unfortunately, honey bee populations are susceptible to several environmental threats, including heavy metal exposure. Honey bees can be exposed to heavy metals when foraging on contaminated honey and pollen resources, and in some cases by airborne exposure. We studied the joint acute and chronic effects of cadmium (Cd) and copper (Cu) on *A. mellifera*. A 1:1 solution of the two heavy metals increased larval developmental duration and the mortality of both larvae and foragers in a dose-dependent way, decreased forager feeding, increased body metal burdens, and disrupted the sucrose response behavior of foragers. In combination, Cd and Cu demonstrated a weakly synergistic effect on foragers, but for larvae an initially antagonistic effect at low doses changed to strongly synergistic response at higher concentrations. The sucrose response threshold of foragers decreased significantly when they were dosed with increasing concentrations of the metal mixtures. Overall, the fitness of honey bee larvae and foragers is detrimentally affected when these metals co-occur.

## 1. Introduction

In recent years, metal pollution has become an increasingly important ecological problem around the world (Li et al., 2018; Wan et al., 2019; Zwolak et al., 2019). In agricultural settings soil contamination negatively impacts crops (Hu et al., 2018; Wang et al., 2019; Xiao et al., 2019) and the quality and safety of agricultural products (Lu et al., 2015; Zaanouni et al., 2018). Heavy metals are stable in the environment, which allows potential accumulation to toxic levels in both water supplies and terrestrial habitats (Nriagu and Pacyna, 1988). Heavy metals are difficult to eliminate once present in soil or water, and reportedly cause irreversible damage to survival, feeding, growth, and behavior of organisms, including honey bees (Nicholson et al., 2003; Hongxia et al., 2010; Di et al., 2016). Some reports indicate insect cell ultrastructure and genetic material can be adversely affected by heavy metals, inducing cell apoptosis that can then disrupt cell vigor, cell proliferation, and result in mutation (Stolpe et al., 2017). Heavy metal ions can enter insect bodies during respiration, via air deposition or dislodgeble residues on surfaces, or through ingestion during feeding or grooming (Mogren and Trumble, 2010; Ali et al., 2019; Dabour et al., 2019).

Metal and metalloid pollution in the air, soil and water come from mining activities, industrial production, automobile exhaust, the burning of leaded gasoline, chemical and manure-based fertilizers, some pesticides, geological processes, and plastic films containing metals (Lin et al., 2017; Vu and Wu, 2019). Heavy metals in dust and gases can also enter the soil and water by natural sedimentation or rainfall infiltration when it is released into the air (Sousa et al., 2019).

Contamination by heavy metals such as copper (Cu) and cadmium (Cd) is prevalent in many agricultural systems, often due to superfluous application of fertilizers (Zhou, 2003). In response to fertilizer applications, soil Cd content in farmland from New Zealand increased from 0.39 mg/kg to 0.85 mg/kg in a relatively short time during the 1990s (Taylor, 1997). The soil in large areas of Argentina is reported to be polluted by cadmium, lead, copper and nickel (Carnelo et al., 1997). In farmlands irrigated by sewage sludge and reclaimed water, the mean concentration of Cd increased by over 5-fold (from 0.016 to 0.85 mg/kg) while mean concentrations of Cu increased by 65% (from 26.9 to

* Corresponding author.
*E-mail address:* john.trumble@ucr.edu (J.T. Trumble).

https://doi.org/10.1016/j.cbpc.2020.108839
Received 12 May 2020; Received in revised form 22 June 2020; Accepted 24 June 2020
Available online 26 June 2020

41.17 mg/kg) (Meng et al., 2016). In addition, Cd has been used extensively by the plastics and ceramics industries as colorants, resulting in an increase of soil Cd during degradation (Turner, 2019). Mining is also a major source of Cu and Cd, not just from pollution, but from the use of reclaimed mining water for irrigation (Wang et al., 2006). In the United States, soil concentrations of these metals can range widely (Cd: < 0.01–2 mg/kg, Cu: < 0.06–495 mg/kg (Holmgren et al., 1993).

Both Cu and Cd have also been shown to influence growth and reproduction in insects. For instance, a high concentration of Cd (250 mg/kg) in artificial diet can extend the development period of *Lymantria dispar* larvae (Mirčić et al., 2013). Copper and Ni reduced the survival rate and larval density of *Epirrita autumnata* (Borkhausen) (Switanek et al., 2017). Cadmium and Ni significantly reduced the cocoon length, width, and weight of *Neodiprion sertifer* (Geoffroy) at the concentrations found these insects near a smelter (~6 mg/g Ni and 2 mg/kg Cd) (Heliovaara and Vaisanen, 1989). Cadmium also can reduce the hatch of *Oncopeltus fasciatus* (Dallas) eggs by inhibiting the synthesis of vitellogenin (Cervera et al., 2005).

The negative effects of heavy metals on honey bees has been widely reported. Although honey bees can acquire toxic levels of metals in many ways, gathering pollen and nectar from contaminated plants is common (Porrini et al., 2014; Xun et al., 2017). Metal accumulation in plants has been demonstrated in common garden experiments. Plants growing in soils treated with aqueous solutions containing 0.3 mg/L Cd or 2 mg/L Cu can accumulate these metals during both the vegetative and reproductive growth periods, and despite very high accumulations in plants, pollinators are still attracted to forage on the flowers (Hladun et al., 2015). Although Cd is not an essential element for plants (unlike Cu), Cd is highly migratory and easily absorbed, accumulated by plants, and readily transferred to honey bees during feeding or foraging (Wang, 2002; Di et al., 2016). Cadmium did not deter feeding by foragers even at the highest levels reported, and copper-containing nectar was readily consumed following proboscis stimulation (Burden et al., 2019). Not surprisingly, honey bees foraging on plants near a garbage incinerator accumulated as high as 17.93 mg/kg Cu (Giglio et al., 2017). As a comparison, honey bees not exposed to Cu had a body burden of < 5 mg/kg (Hladun et al., 2015). In a heavily industrialized district in the USA, the amount of Cu and Cd in foragers was 14–37.68 mg/kg and 0.05–1.75 mg/kg, respectively (Fakhimzadeh and Lodenius, 2000; Veleminsky et al., 1990). When feeding on flowers exposed to motor vehicle pollution, Cd accumulated in bees to 2.87–4.23 mg/kg (Conti and Botre, 2001). In Poland, the foragers were detected to have 20.2–25.5 mg/kg Cu (Roman, 2010). In metal-polluted areas in Italy, foragers accumulated 0.05–0.06 mg/kg Cd (Perugini et al., 2011). When hives were located in industrial areas that were rich in arsenic and Cd, honey bee fecundity declined (Bromenshenk et al., 1985).

Copper and Cd are not only harmful to honey bees, but also pose a threat to the quality and safety of bee products. Honey collected from bee hives near farmland in Nigeria and Turkey contained 25 mg/kg and 0.2 mg/kg Cu, respectively (Achudume and Nwafor, 2010; Yarsan et al., 2007). Honey collected from Egyptian agricultural areas contained Cu and Cd concentrations up to 11 and 0.41 mg/kg, respectively (Rashed et al., 2009). The amount of Cu in honey from Poland's heavily industrialized districts was as high as 23.5 mg/kg (Roman, 2010). Bee waxes from industrial districts in Poland were also reported to accumulate high amounts of Cd (Formicki et al., 2013). Not surprisingly, even after the initial ingestion of contaminated nectar or collection of contaminated pollen by foragers, exposure to these metals continues over time for nest mates and larvae (Hladun et al., 2016). Interestingly, the highest in hive concentrations for some metals (including Cd) were found in the royal jelly, which is fed to all bees (Leita et al., 1996). The independent effects (each metal alone) of Cu and Cd on honey bee foragers and larvae were previously reported (Di et al., 2016), but the effects of simultaneous exposure (joint effects) are unknown. Therefore, our goal was to investigate the joint effects of two co-occurring metals on honey bee larval development, survival and feeding behaviors.

## 2. Materials and methods

### 2.1. Honey bees

*Apis mellifera ligustica* from the apiary at the University of California-Riverside were used in all experiments. In order to standardize the insects tested, the foragers and newly eclosed first instars were from a single colony that had no pesticides applied. The queen was purchased from Allen's Honey & Pollination Co. and was not changed throughout the course of the studies.

### 2.2. General preparation of foragers

Foragers returning to the hive were collected individually in 10 mL clear plastic scintillation vials. Upon returning to the lab, the vials were put on ice just long enough to immobilize the bees. The foragers were harnessed gently with tape in a straw tube holder (0.8 cm in diameter), such that only the head, antennae and proboscis were free to move (after Hladun et al., 2012, Di et al., 2016). The honey bees were fed daily to satiation with a 50% sucrose solution using a micrometer glass syringe (1 μL; Gilmont Instruments, Cole Palmer, Chicago, IL, USA).

### 2.3. Forager acute toxicity assay

After being harnessed into the straw holder, the foragers were fed with 50% sucrose to satiation 24 h before being dosed. Each bee was dosed once with 20 μL of test solution using a glass syringe. The individual concentrations tested for Cd and Cu were 0, 12.5, 20, 25, 50, 100, 150, 200 and 400 mg/L. The joint concentrations of metals in each treatment were 0, 25, 40, 50, 100, 200, 300, 400, and 800 mg/L. Mortality was recorded at 24 h, 48 h and 72 h, and the sucrose solution intake amount was recorded daily. 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. During the experiment, the temperature was maintained at 20.0 ± 0.01 °C and the humidity was maintained at 73.5 ± 0.46%. Except for the feeding process, the assays were conducted in dark conditions. The death rate in the control group was < 15% at 72 h. At the conclusion of this test, foragers were frozen at −60 °C and analyzed for metal content (see Section 2.5).

### 2.4. Forager proboscis extension sucrose response assay

The proboscis extension response threshold assay to sucrose was conducted according to Hladun et al. (2012) and Page et al. (1998). The foragers were collected and harnessed as described previously. Following a 2 h starvation, they were fed with 50% sucrose until satiation. After 24 h, the foragers were randomly assigned to different treatment groups (the same concentrations with the foragers acute toxicity assay). Two hours after dosing, the antenna of each honey bee was tested with 0.1%, 0.3%, 1%, 3%, 10% and 30% sucrose, sequentially. We then recorded if the proboscis extended when the antennae tip was stimulated by the solution. A distilled water test was conducted after each sucrose test to eliminate potential sensitization or passivation effects of repeated sucrose stimulation on the antenna. There were 6 bees in each treatment, each bee was a replicate, and a logistic regression of repeat measurements was used to analyze for difference significances in responses. A non-parametric Chi Square test was used to document significant differences. Because a large number of metal concentrations were tested across a large number of sugar concentrations, we generated a heat map that allows visualization of the responses. Maps were generated using R 3.6.3 (R Core Team, 2020).

### 2.5. Larval development and chronic toxicity tests

To obtain 1 d-old larvae, an excluding cage was placed over a section of comb with sufficient cells to exceed the queens 24 h oviposition capacity, and the queen was inserted. After 24 h, the queen was removed from the frame to prevent further oviposition following the methods of Peng et al. (1992) and Aupinel et al. (2005). Four days later, the frame was brought back to the laboratory and placed in a box (dark, temperature 34.1 ± 0.01 °C, humidity 94.6 ± 0.2%). The approximately 1-d-old larvae were collected within 2 h for experiment and placed onto artificial diet using grafting tools (Sinova, Zhengzhou, China).

The artificial diet consisted of 53% commercial frozen royal jelly, 6% glucose, 6% fructose, 1% yeast extract, and 34% ultra-pure water (Kaftanoglu et al., 2010). The metal compounds were dissolved into the sugar solution portion to reach final target concentrations in the diet. Cadmium and Cu were added to the water as cadmium chloride (Fisher Scientific, Waltham, MA, USA) and copper chloride dihydrate (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 individual concentrations tested for Cd and Cu were: 0, 0.125, 0.5, 2, 6, 10, and 14 mg/L, respectively. Therefore, the total concentrations of metals in each treatment were 0, 0.25, 1, 4, 12, 20 and 28 mg/L. Each individual metal treatment concentration was replicated three times with each replicate containing at least 21 larvae.

All equipment used to transfer larvae to diets (including grafting tools, cell cups and well plates) was ultraviolet sterilized (Air Clean 600 PCR workstation, ISC Bioexpress) to minimize contamination. Queen cups (Glory Bee Foods, Inc., Eugene, OR) were inserted into holes in 48-well plate (Costar 3526 Cell Culture Plates; Corning). The artificial diet was thoroughly mixed with a pipette. Each cup was provisioned with 250 μL of diet. A single larva was then transferred onto the diet using a grafting tool (Kaftanoglu et al., 2010). Any larvae that died within 24 h of grafting due to handling stress were not included in the subsequent experiments.

Artificial diet (250 μL) was provided to larvae in cell cups (Glory Bee foods, Inc., Eugene, OR). Cell cups were then 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 glycerin with methyl benzethonium chloride was placed at the bottom of the bell jar to prevent contamination and maintain humidity. Mortality was scored daily until pupation. 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). Prepupal larvae and intact pupae were weighed and measured for Cd and Cu content (see Section 2.5). The relative growth indices (RGI) were calculated for *Apis mellifera* larvae exposed to the Cd and Cu combination from day 4 through day 10 using the equations described by Zhang et al. (1993).

### 2.6. Bioassays of metal concentrations 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. Honey bee 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. Then the metals were analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES) (Perkin-Elmer Inc., Shelton CT, USA) (after Hladun et al., 2015). Standard oyster tissue 1566b (freeze-dried, U.S. Department of Commerce National Institute of Standards and Technology, Gaithersburg, MD) was used as a quality control for each run to measure the recovery of metals from a biologically complex sample. Metal recovery was above 90% on all runs. Ultrapure water was also used as a blank control in each run.

### 2.7. Data analyses and calculation of independent and joint effects of Cu and Cd

The sucrose intake amount after metal treatments, the weights of prepupae and pupae, metal accumulation (body burden), as well as the mortality of foragers and larvae were analyzed by one-way ANOVA followed by Tukey's HSD test, SPSS 17.0; SPSS, Chicago, IL, USA) ($P < 0.05$). The Chou-Talalay method in CompuSyn (ComboSyn Inc. Paramus, NJ, 2007) was used to analyze the interaction between Cd and Cu. A repeated measurement logistic regression was conducted on the sucrose response threshold assay of foragers. An ANOVA PROC GLM program (SAS 9.2; SAS Institute, Cary, NC, USA) was used to compare the relative daily average growth index ($P < 0.05$), in which the concentration of heavy metal treatment was taken as an independent variable, the relative daily average growth index was taken as a dependent variable, and the number of days of growth and development was taken as a repeating variable (the first ten days of growth and development were used for data analysis). The larval $LC_{50}$ for metals was calculated using the data of day 8 (before pupation began and control mortality was below 20%) using log-dose response probit analysis (SAS 9.2). For foragers, $LC_{50}$s of the joint effects of metals was calculated using survival at 72 h.

The Chou-Talalay method (Chou and Talalay, 1984; Chou, 2006, 2010) was used to document joint interactions of Cu and Cd. This method has been widely used, especially in the development of anticancer drugs. However, in recent years this has been used to analyze joint effects of in insects (Rust and Hemsarth, 2016). The Chou-Talalay method generates two useful indices. The first is the Combination Index (CI), which is an indicator of the interaction between substances when the proportion of substances in the mixture is constant. For this analysis, CI < 1 represents a synergistic effect, CI = 1 represents an additive effect, and CI > 1 represents an antagonistic effect. The CI is calculated as follows:

$$\frac{(D)_1}{(D_x)_1} + \frac{(D)_2}{(D_x)_2} = CI$$

where $(D_x)_1$ and $(D_x)_2$ represent the concentrations of substances 1 and 2 that cause x% mortality, and $(D)_1$ and $(D)_2$ represent the proportions of substances 1 and 2 that cause x% mortality when combined $(D)_1 + (D)_2$. The second value is the dose response index (DRI), which calculates how much the amount of the combination of heavy metals fed to an insect could be reduced to achieve the same effects seen for individual concentrations. For example, a DRI for Cu of 2.0 would indicate that the amount of the materials in combination could be reduced two-fold and achieve the same result as seen with Cu alone.

## 3. Results

### 3.1. Forager acute toxicity assay

The percent mortality of foragers dosed with joint concentrations of Cd and Cu is shown in Fig. 1. Mortality of foragers increased with the passage of time and as concentration increased. Bee mortality in the low concentration treatment groups (25 mg/L or 40 mg/L) were not not significantly different from that in the control group. However, at when fed 50 mg/L or more, forager mortality increased significantly at 24 h ($F = 6.99$, df = 8, 33, $P < 0.001$), 48 h ($F = 23.805$, df = 8, 33, $P < 0.001$) and 72 h ($F = 26.004$, df = 8, 33, $P < 0.001$) compared

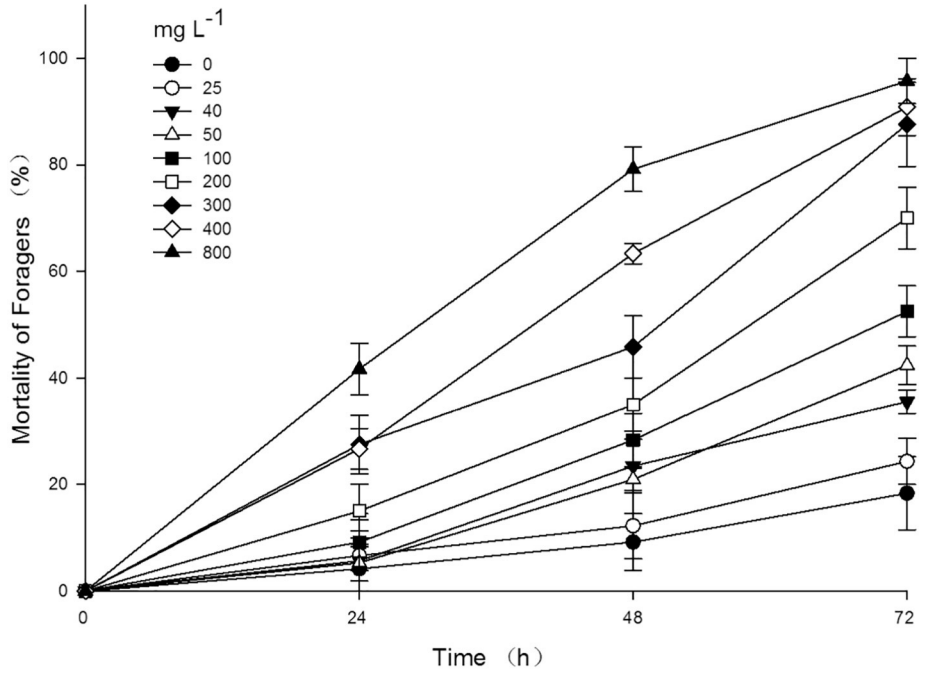


**Fig. 1.** *Apis mellifera* forager mortality over a period of 72 h after joint exposure to Cd and Cu. Bars represent standard errors.

to the controls. Mortality at the highest concentration (800 mg/L) reached 42 ± 4.81% in just 24 h, and peaked at 95.75 ± 4.25% at 72 h. Control mortality at 24 and 72 h was 9.17 ± 5.34% and 18.33 ± 6.87% respectively.

After initially dosing the bees with metal solutions, the mean amount of 50% sucrose solution consumed at 24 h and 48 h was also recorded (Fig. 2). Bees dosed with heavy metals consumed significantly less sucrose solution compared to controls at 24 h ($F = 13.7$, df = 8, 33, $P < 0.001$) and 48 h ($F = 23.262$, df = 8, 33, $P < 0.001$). Average sucrose solution consumption in the control group was 34.86 ± 1.04 μL and 26.69 ± 1.10 μL at 24 h and 48 h, respectively. In the treatment group dosed with the highest concentration of metal (800 mg/L), sucrose consumption was 12.92 ± 1.31 μL for 24 h and 8.5 ± 1.71 μL for 48 h, respectively, which was significantly lower than that of the control group and all other treatment groups.

### 3.2. Forager proboscis response to antennal stimulation

Foragers in the two highest metal treatment groups showed a reduced tendency for proboscis extension compared to controls when

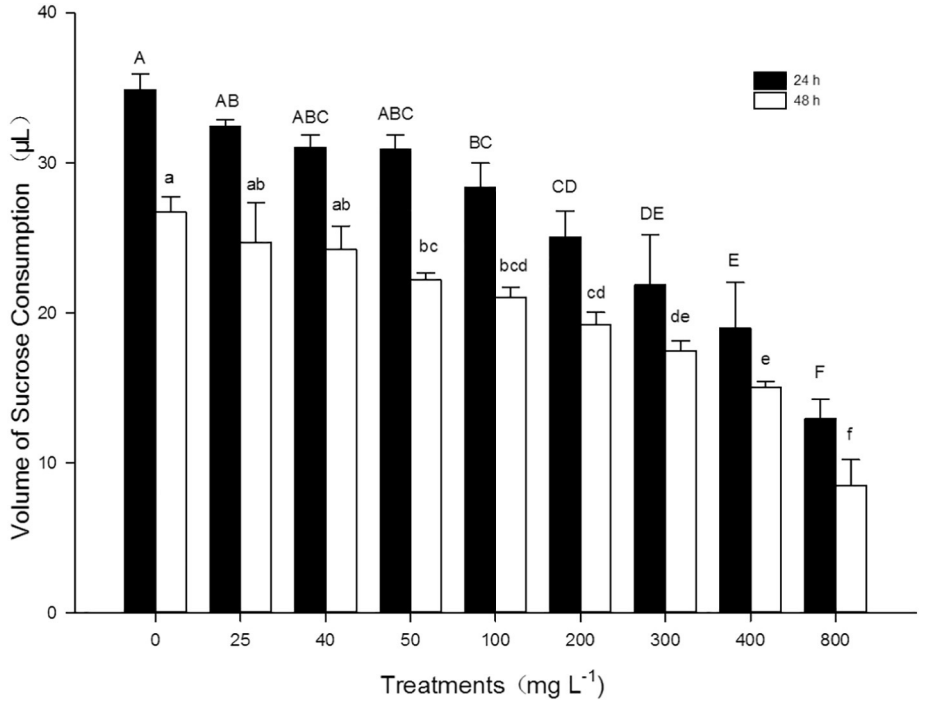


**Fig. 2.** Volume of 50% sucrose consumption by *A. mellifera* foragers at 24 and 48 h following feeding with Cd and Cu. Bars represent standard errors and means within a time interval with the same letter are not significantly different ($P > 0.05$).

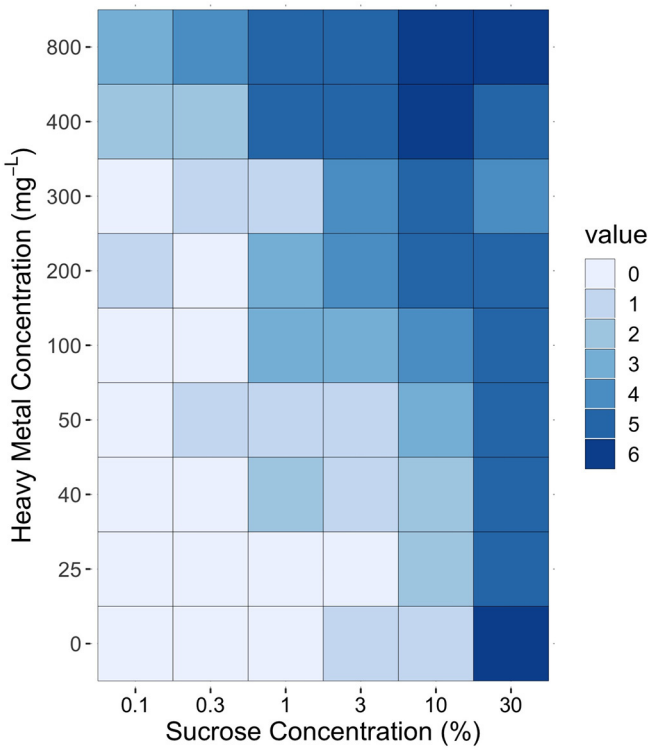


**Fig. 3.** Heat map showing response values for honey bee foragers exposed to a range of both sucrose concentrations and heavy metal concentrations. Lighter colors (lower values) indicate maximum proboscis extensions while the darkest color indicates few bees demonstrated proboscis extension.

their antennae were exposed to solutions with increasing sugar concentrations ($X^2 = 41.23$, $P < 0.001$) (Fig. 3). Across all metal treatments, increasing sucrose concentrations ($X^2 = 42.61$, $P < 0.001$) also affected proboscis extension. Thus, the higher the intake of heavy metals, the worse the ability of the bees to distinguish or respond to sucrose solutions. This is similar to the proboscis extension behavior in response to antennal stimulation reported by Burden et al. (2019) for Cu alone (a decline at higher dosages). This was not evident for Cd. Because Cu is more toxic than Cd (Di et al., 2016; Burden et al., 2019), it appears likely that Cu has the greater effect in joint assays measuring proboscis extension based on antennal stimulation.

### 3.3. Larval development and chronic toxicity tests

In as little as 4 days of feeding on artificial diets containing the highest rates of Cd and Cu, larval survival was significantly reduced compared to the controls (day 4: $F = 26.09$, df = 6, 19, $P < 0.001$) (Fig. 4). From days 4 to 10, the mortality of the larvae in all treatments increased in a dose-dependent fashion. From day 4 to day 6, there was no significant difference between the low concentration treatment groups (0.25 mg/L and 1 mg/L) and the control group (ANOVA: $P > 0.05$). By day 6 all higher rates were significantly different from the controls (d 6: $F = 24.04$, df = 6,19, $P < 0.001$). However, by day 8, there was a significant difference in larval mortality even between the lowest treatment (0.25 mg/L) group and the control group (8 d: $F = 34.506$, df = 6,19, $P < 0.001$). At the conclusion of the test at day 10, larval mortality was significantly different from the control in all treatments (d 10: $F = 45.389$, df = 6, 19, $P < 0.001$).

Prepupal weights decreased when larvae were fed increasing concentrations of metals (Table 1; $F = 3.067$, df = 4, 58, $P = 0.024$). At 20 or 28 mg/mL, the joint effect of Cu and Cd resulted in no larvae surviving to the prepupal stage. There was no significant difference detected for pupal weight ($F = 0.845$, df = 4, 37, $P = 0.507$) between metal treatments or controls, suggesting that once a minimum weight was achieved, pupation could occur. This is quite similar to the results seen for larvae exposed to the metals individually where prepupal weights needed to exceed 0.13 g before pupation occurred (Di et al., 2016).

The Relative Growth Index (RGI) of larvae fed on Cd- and Cu-contaminated diet varied significantly with time ($F = 12.52$; df = 6, 114; $P < 0.001$), heavy metal treatment ($F = 58.09$; df = 6, 20; $P < 0.001$), and the interaction of time and heavy metal treatment ($F = 8.69$ , df = 36, 114 , $P < 0.001$). The RGI for larvae in the control treatment was about 90% by days 9 and 10, indicating that the population achieved about 90% of the potential maximum growth (Fig. 5). In comparison, the RGI values for larvae fed contaminated diet were significantly lower, and reductions in RGI values were dose dependent. By day 6, all RGI values were significantly less than in the control treatment. Larvae fed with joint concentrations of 1.0 to 12.0 mg/L showed reductions in RGI of up to 60% by day 10, which would greatly slow or potentially stop colony growth. At the highest rates of joint exposure (20 or 28 mg/L), colony death could be expected to occur in a relatively short period of time as RGIs dropped to near zero (Fig. 5).

### 3.4. Bioassays of metal concentrations in A. mellifera foragers and larvae

Foragers accumulated a greater metal body burden in a dose dependent fashion as dosing concentrations increased when bees were fed with Cd or Cu independently (Fig. 6) (Cd; $F = 120.886$, df = 8, 34, $P < 0.001$ and Cu; $F = 120.554$, df = 8, 34, $P < 0.001$). For both metals, once the feeding dose reached 100 mg/L treatment group, the accumulated heavy metal content in the treatment groups increased significantly as compared to controls. Compared with the control group, Cd accumulation in foragers from the 800 mg/L treatment group (163 ± 9.89 μg/g) peaked at over 150 times that of the control group (98.1 ± 7.9 μg/g). Accumulation of Cu for the group fed with 800 mg/L was 204 ± 4.63μg/g, about 10 fold greater than seen in controls.

Larvae feeding on artificial diets spiked with Cd and Cu accumulated different amounts of metals in a dose-dependent manner (Fig. 7). In the control group, the larvae contained only trace amounts of Cd, while the content of Cu in controls was 20.26 μg/g. Metal accumulation in larvae for both Cd and Cu was not significantly different from controls at the two lowest concentrations tested (0.25 mg/L and 1 mg/L). However, both metals accumulated in larvae at significantly greater levels than controls when diets included 4 μg/L or higher concentrations (Fig. 7). In the treatment group exposed to the highest metal concentration (28 mg/L), the accumulations of Cd and Cu in the larvae were 132 μg/g and 143 μg/g, respectively. At this level of contamination, the accumulation of Cu was about seven times that of the control group, while the accumulation of Cu exceeded 100 fold that of the control insects.

### 3.5. Independent and joint effects of Cu and Cd

Log dose probit analyses for insects treated jointly or independently with Cu and Cd indicated that foragers were much less susceptible to either metal than larvae (Table 2). The $LC_{50}$ values for foragers fed Cu were over 10 fold more than for larvae. For Cd, the $LC_{50}$ values for foragers were over 200 fold more than larvae. Thus, bioassays of metal toxicities to honey bees that rely solely on adult bee toxicity will potentially underestimate effects on the colony.

The $LC_{50}$ values for foragers exposed to Cd and Cu jointly or independently were similar and had overlapping 95% confidence intervals, indicating that both metals had similar influences on foragers (Table 2). In contrast, the 95% confidence intervals in the larval assays did not overlap, indicating Cd alone or Cd + Cu had substantially more effect than Cu alone.

The Combination Coefficient (CI) for foragers was generally < 1,

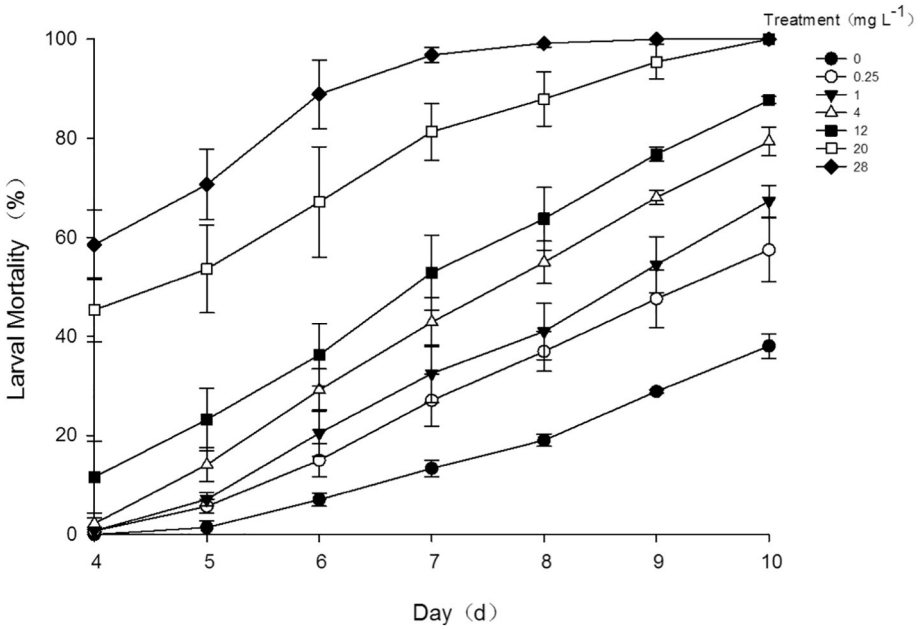


**Fig. 4.** Larval mortality of *A. mellifera* feeding on artificial diets containing a range of concentrations of Cd plus Cu. Bars represent standard errors.

**Table 1**
Prepupal and pupal weights of *A. mellifera* larvae exposed to Cd and Cu for 10 days*.

| Metal | Concentration (mg/L) | Prepupal weight (g) (Mean ± SE) | Pupal weight (g) (Mean ± SE) |
|---|---|---|---|
| Cd*Cu | 0.00 | 0.1652 ± 0.0091 a | 0.1601 ± 0.0081 a |
| | 0.25 | 0.1527 ± 0.0043 ab | 0.1511 ± 0.0064 a |
| | 1.00 | 0.1443 ± 0.0068 ab | 0.1437 ± 0.0207 a |
| | 4.00 | 0.1431 ± 0.0114 ab | 0.1423 ± 0.0118 a |
| | 12.00 | 0.1260 ± 0.0100 b | 0.1303 ± 0.0102 a |
| | 20.00 | – | – |
| | 28.00 | – | – |

* Means within a column with the same letter are not significantly different ($P > 0.05$).

indicating a weakly synergistic effect when the metals co-occurred (Table 3). The DRIs of Cd were consistently above 1, showing that the concentration of Cu (when occurring in combination with Cd) can be reduced by 1.11 to 2.04 times and still achieve the same toxic effect. Similarly, the concentration of Cd (when occurring in combination with Cu) can be reduced by 1.65 to 3.19 times and produce the same mortality (Table 3).

Larvae were much more susceptible to mortality induced by heavy metals than were foragers (Table 4). The CI for larvae indicated the metals were antagonistic at concentrations below 4 mg/L. However, for larvae exposed at 12 mg/L or higher, the CI indicated a moderate to strong synergism. The DRI values followed a similar pattern, with the DRI for Cd and Cu at the two lowest metal concentrations tested ranging from 0.31 to 0.51 for Cd and 0.44 to 0.78 for Cu. However, at concentrations of 4.0 mg/L or higher, the DRIs increased and reached values exceeding 10 at the two highest concentrations. Thus, the synergistic effects of these metals increase sharply as metal concentrations increase.

## 4. Discussion

Because foragers adjust their foraging strategy and initiate recruitment dances according to the sugar content of nectar (Page et al., 1998), changes in ability to distinguish or respond to variable sucrose concentrations could adversely affect forager efficiency. In addition, the average sucrose solution intake amount in the joint metal treatments declined in a dose dependent fashion with over 35% reduction at the higher rates. Reduced nectar intake by foragers could be expected to reduce colony fitness. The observed reduction in sucrose intake may have been due, in part, to a "malaise" that has been reported for some substances (Burden et al., 2019). While foragers generally do not appear able to detect metals and metalloids (Hladun et al., 2012; Di et al., 2016), they have been shown to avoid other toxic substances that they cannot taste by associating the malaise effects with particular compounds (Wright, 2011). Thus, there is a mechanism that allows them able to avoid future exposure. This has been demonstrated for metals such as Cu and nickel, but avoidance was predicated on the availability of alternative, uncontaminated food sources (Burden et al., 2019; Desmedt et al., 2016). Regardless, when foragers have greater mortality and become less efficient, when foraging resources are limited by contamination, and when larval RGI is reduced, negative colony level effects can be expected.

Drawing relationships between honey bee mortality and concentrations of Cd and Cu in pollen and nectar must be done with caution. First, there is very limited information available regarding concentrations of metals in nectar and pollen. Concentrations likely vary between plant species and as flower sources change over time. In one study, Roman, 2007 collected pollen from bees using a pollen collector at the entrance to the hive. This provided a 'snapshot' of whatever flowers were visited, but no information on the plant species visited. When analyzed as mg per kg dry mass of pollen, the amounts of Cd in pollen reached up to 0.660 mg/kg (Roman, 2007). This is well below the 78 mg/L reported as the $LC_{50}$ for foragers, but over twice the $LC_{50}$ reported for larvae (Di et al., 2016). Thus, if only foragers are tested, pollen and nectar concentrations of Cd and Cu would seem environmentally insignificant (in terms of mortality). Second, there are data on the content of Cd and Cu in complete flowers, with reported concentrations in *Raphanus sativus* L. of Cu exceeding 35 μg/g and Cd reaching 15 μg/g. However, for some metalloids like selenium, concentrations in flowers can be much lower than what is found in pollen and nectar, so individual species that are used by foragers would likely influence ingestion rates. Third, an additional concern is that not all Cu or Cd will be accumulated by feeding on nectar and pollen. A substantial amount of metal accumulation can also be from atmospheric deposition followed by ingestion during grooming (Leita et al., 1996).

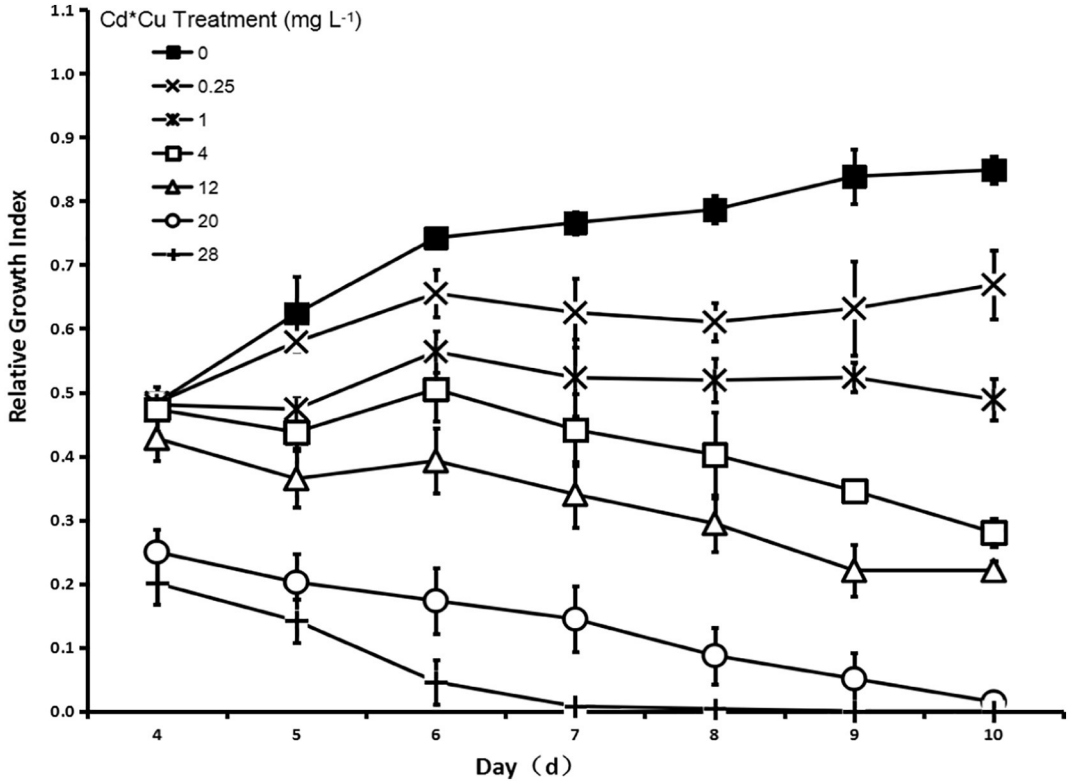


**Fig. 5.** Mean relative growth indexes *of A. mellifera* larvae treated jointly with Cu and Cd over a 10-d period. Bars represent standard errors.

Thus, the few samples that have been reported from pollen may not provide a complete picture of total exposure. Finally, there is considerable evidence that metal accumulation or biomagnification occurs following repeated feeding Silici et al. (2013). The primary causal factors are not known, but when Cd concentrations in honey can reach 1.9 μg/g and royal jelly has 2.9 μg/g, negative effects on larvae could be expected. When whole colonies were fed a sugar syrup containing 0.024 mg/kg of Cd or 0.25 μg/g of Cu (in addition to water and pollen) for 60 days, forager body burdens exceeded 4.5 μg/g for Cd and 600 μg/g for Cu (Hladun et al., 2016). Thus, using body burdens is currently the most useful comparative measure to document potential mortality effects. However, using body burdens alone probably underestimates the

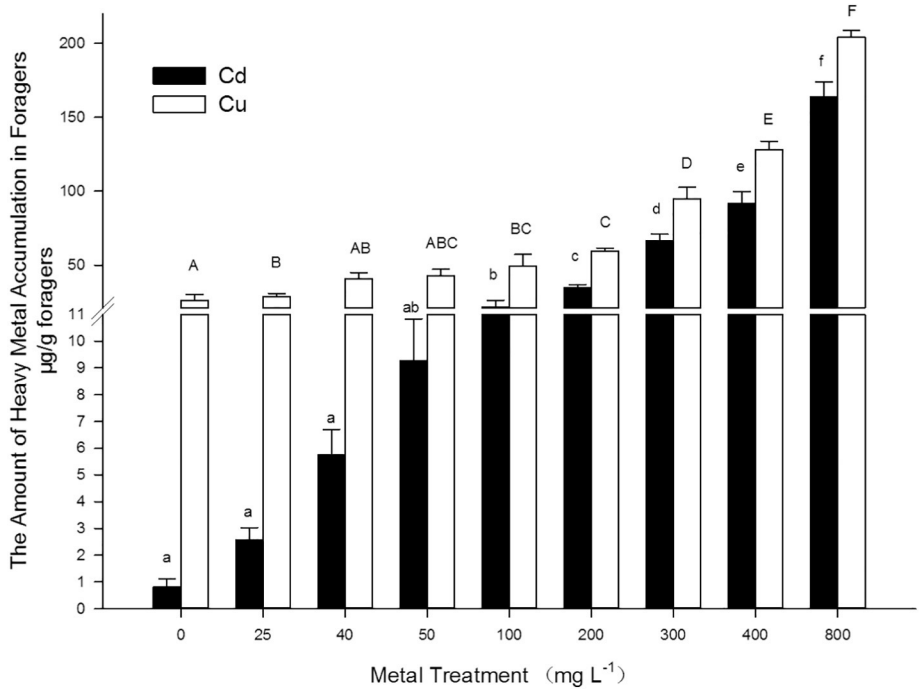


**Fig. 6.** Metal accumulation in *A. mellifera* foragers fed solutions containing either Cd or Cu. Bars represent standard errors. Means within Cd treatments with the same lowercase letters are not significantly different ($P > 0.05$). Means within Cu treatments with the same uppercase letters are not significantly different ($P > 0.05$).

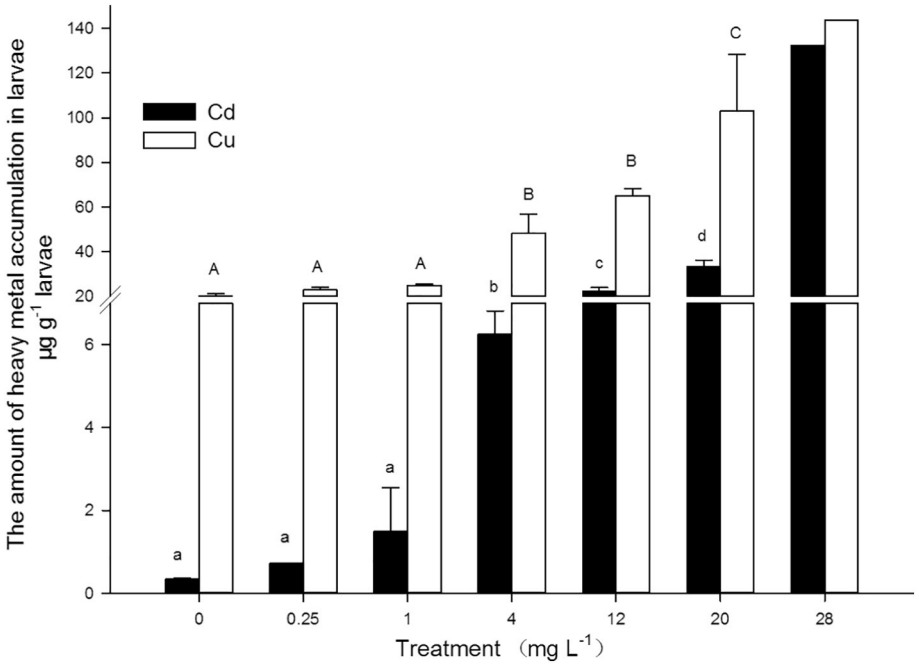


**Fig. 7.** Metal accumulation in *A. mellifera* larvae fed diets containing increasing concentrations of Cu and Cd. Bars represent standard errors. Mean values of Cu with different capital letters are significantly different as are mean values of Cd with different lower case letters (LSD test: $P < 0.05$). There were no error bars for 28 mg/L treatment group because most larvae died prematurely (in a younger stage) and only a small number larvae reached a late instar comparable to other treatments.

**Table 2**
Mean lethal concentrations ($LC_{50}$) of joint and independent exposure to Cd and Cu for *A. mellifera* foragers and larvae.

| Metal | Number Tested | $LC_{50}$(mg/L) | 95% Confidence Interval |
|---|---|---|---|
| Foragers | | | |
| Cd | 172 | 78 | 44–122 (Di et al., 2016) |
| Cu | 177 | 72 | 36-114 (Di et al., 2016) |
| Cd*Cu | 186 | 72.3 | 50–98 |
| Larvae | | | |
| Cd | 629 | 0.275 | 0.13–0.54 (Di et al., 2016) |
| Cu | 658 | 6.970 | 3.09–22.21 (Di et al., 2016) |
| Cd*Cu | 631 | 1.575 | 0.97–5.25 |

**Table 3**
The Chou-Talalay analysis[a] results for *A. mellifera* foragers jointly treated with Cd and Cu.

| Total metal concentration (mg/L) | Fa | CI | DRI Cd | DRI Cu |
|---|---|---|---|---|
| 25 | 0.243 | 0.94 | 2.22 | 2.04 |
| 40 | 0.356 | 0.97 | 2.26 | 1.91 |
| 50 | 0.424 | 0.96 | 2.34 | 1.89 |
| 100 | 0.53 | 1.35 | 1.71 | 1.3 |
| 200 | 0.7 | 1.5 | 1.65 | 1.11 |
| 300 | 0.876 | 0.93 | 2.96 | 1.69 |
| 400 | 0.908 | 0.95 | 3 | 1.63 |
| 800 | 0.958 | 0.97 | 3.19 | 1.52 |

[a] Fa indicates the effect coefficient, i.e. the mortality caused by each treatment. CI (Combination Index): CI > 1, antagonism; CI = 1, additive effect; CI < 1, synergism. DRI (Dose Reduction Index) provides an estimate of the dose reduction of metals occurring jointly that will generate the same effect as compared independent Cd or Cu exposure. For example, at 25 mg/L, the DRI for Cd indicates that concentrations of the joint treatment can be reduced 2.22 times and still achieve the same mortality as Cd alone.

full effects of a metal because this measure does not include any sublethal effects such as reduced feeding, malaise, or forager recruitment problems associated with metal ingestion (Burden et al., 2019).

Pollutants in nature are rarely isolated, and there are frequently multiple heavy metal pollutants in a contaminated site with multiple valence states and complex components. However, the majority of

**Table 4**
The Chou-Talalay analysis[a] results for *A. mellifera* larvae jointly fed with Cd and Cu.

| Total metal concentration (mg/L) | Fa | CI | DRI Cd | DRI Cu |
|---|---|---|---|---|
| 0.25 | 0.37 | 3.25 | 0.51 | 0.78 |
| 1 | 0.41 | 5.47 | 0.31 | 0.44 |
| 4 | 0.55 | 1.21 | 1.54 | 1.78 |
| 12 | 0.638 | 0.56 | 3.57 | 3.6 |
| 20 | 0.879 | 0 | > 10 | > 10 |
| 28 | 0.992 | 0 | > 10 | > 10 |

[a] Fa indicates the effect coefficient, i.e. the mortality caused by each treatment. CI (Combination Index): CI > 1, antagonism; CI = 1, additive effect; CI < 1, synergism. DRI (Dose Reduction Index) provides an estimate of the dose reduction of metals occurring jointly that will generate the same effect as compared independent Cd or Cu exposure. For example, at 12 mg/L, the DRI for Cd indicates that concentrations of the joint treatment can be reduced 3.57 times and still achieve the same mortality as Cd alone.

studies report only the impact of individual contaminants (Yang, 1994; Jensen et al., 2006). Among 17 studies describing the interaction effects of $Cu^{2+}$ with other substances, 5 reported a synergistic effect, 11 indicated antagonistic effects, and 1 showed an additive effect (Cedergreen, 2014). From the perspective of toxicokinetics, the interaction of various metals can affect the absorption, metabolism, secretion, and distribution of metal ions. For example, Cd and Hg have similar physical and chemical properties, and they interact with each other due to the sharing of some transporters in the transport process in vivo (Sakar, 2012). Until recently, studies on the joint effects of various pollutants have been largely focused on aquatic organisms, but there are an increasing number of publications for terrestrial insects (Mogren and Trumble, 2010). Until the 1990s, > 95% of honey bee toxicology studies focused on the effects of a single toxic substance at a time (Lin et al., 1998). Our study is one of a very limited number of reports that documents joint effects of two heavy metals on multiple stages of honey bees. Because of the limited number of published reports detailing interactions of metals on honey bees, predicting joint effects at this time is difficult.

Our research found that when Cd and Cu were mixed 1:1 and fed to *A. mellifera* foragers, there was a dose-dependent mortality. However, the joint effects of Cu and Cd were much greater on larvae as compared

to foragers. At the concentrations we tested, differences in mortality between larvae in any treatment group and the control larvae did not become significant until 4 d of feeding on the contaminated diet. The US Environmental Protection Agency guidelines for acute oral toxicity testing of honey bees (US EPA, 1996) calls for evaluation at 24 h to 48 h. Concentrations necessary to cause significant larval mortality in 48 or 72 h would have to be much higher than we tested, and would likely indicate that ecologically relevant concentrations of Cd and Cu would have little effect. Thus, current EPA toxicity procedures substantially underestimate potential toxic effects by not considering the effects over the lifespan of the forager or the possible impact on the immature stages.

Synergism between the metals was impacted by the life stage tested. The Chou-Talalay analysis showed that when the two heavy metals co-occurred, Cu could be reduced by 1.11 to 2.04 times and Cd by 1.64 to 3.19 times to achieve the same effect on foragers when compared to one metal alone. In the case of larvae, the synergism was much stronger, and Cu concentrations could be reduced by up to 22 times and Cd by up to 38 times to achieve the same effect on larvae when compared to one metal alone. Thus, in areas where both metals are present, the concentrations needed to cause any given level of mortality are substantially lower than when Cu or Cd are independently available.

One benefit of measuring interactions using the Chou-Talalay analysis is that this technique generates synergism/antagonism evaluations for each dosage tested (see Tables 3 and 4). This is different from the typical approach to measuring synergism that uses $LC_{50}$ values to calculate such interactions. Using the $LC_{50}$-based methods, a single value is generated that is an assessment of the interactions over the entire range of tested concentrations (Tabashnik, 1992). While this procedure can be conducted repeatedly to allow statistical comparisons of expected versus observed values at different proportions of the joint $LC_{50}$ (for example at 10% or 25% of the joint $LC_{50}$, see Jensen et al. (2006) for an example), the advantage to creating synergism/antagonism evaluations with the Chou-Talalay method is that results are automatically generated for each dose tested. This allows rapid assessment of possible changes in synergism/antagonism with concentration and is also economical in the number of replicates.

There are few studies that have measured changes in synergism across a range of doses. Rust and Hemsarth (2016) showed that when the ratio of methoprene to pyriproxyfen was higher than 20:1, the two insect growth regulators (IGRs) began to show an antagonistic effect on fleas. Their results showed that ratios between 20:1 to 1:1 were synergistic and provided the maximum effect and economic benefit when two IGRs are used to prevent cat flea development. In our study, the combination of the two metals was antagonistic for honey bee larvae at low concentrations and strongly synergistic at concentrations at or above 12 mg/L of diet. For foragers, the joint effects were weakly synergistic across 6 of the 8 concentrations tested. Thus, the importance and frequency of changes in interactions with dose can be important for insects, but the frequency with which this occurs and any patterns that may be present will require additional studies.

## 5. Conclusions

This study provides baseline data on the antagonistic and synergistic effects of joint exposure to Cd and Cu on *A. mellifera* foragers and larvae. The 1:1 mixture solutions of the two heavy metals increased larval development duration and the mortality of both larvae and foragers, decreased the foragers sucrose intake, increased body metal burdens and disrupted the sucrose response behavior of foragers. Our study reveals that the co-existence of Cd and Cu act in a weakly synergistic fashion on honey bee foragers, but synergism was stronger for larvae. There also was evidence that joint effects on larvae changed from antagonistic to synergistic with increasing doses. Additional studies will be needed to verify if the observed variability in joint effects with increasing concentrations is typical for most metals or insects, or if this effect was specific to combinations of Cu and Cd on honey bee larvae.

## Declaration of competing interest

The authors declare they have no conflicts-of-interest.

## Acknowledgments

The authors thank S. Prager for helping with the data analysis. We also thank D. De La Riva, M. Jones, G. Kund and M. Pennington for giving advice during the assay. P. K. Visscher provided access to the hive dedicated for this study. This work was funded by the United States Department of Agriculture National Institute of Food and Agriculture grant (2012-67013-19446), the Beijing Excellent Person Program (2018000020060G181), the National Key Research and Development Program of China (2018YFD02004), the Youth scientific research fund of Beijing Academy of Agricultural and Forestry Sciences (QNJJ201917), and the Youth Program of the National Natural Science Foundation of China (1901945).

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