

## SHORT COMMUNICATION

# Metal pollutants have additive negative effects on honey bee cognition

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## ABSTRACT

Environmental pollutants can exert sublethal deleterious effects on animals. These include disruption of cognitive functions underlying crucial behaviours. While agrochemicals have been identified as a major threat to pollinators, metal pollutants, which are often found in complex mixtures, have so far been overlooked. Here, we assessed the impact of acute exposure to field-realistic concentrations of three common metal pollutants, lead, copper and arsenic, and their combinations, on honey bee appetitive learning and memory. All treatments involving single metals slowed down learning and disrupted memory retrieval at 24 h. Combinations of these metals had additive negative effects on both processes, suggesting common pathways of toxicity. Our results highlight the need to further assess the risks of metal pollution on invertebrates.

**KEY WORDS:** *Apis mellifera*, PER conditioning, Pollutant interaction, Arsenic, Lead, Copper

## INTRODUCTION

Metal pollution is of increasing concern for both ecosystem and public health (Nriagu and Pacyna, 1988). Over the last century, the widespread use of metals in domestic, industrial and agricultural applications (Bradl, 2005) has considerably elevated their concentrations in water (Mance, 1987) and terrestrial habitats (Krämer, 2010; Su et al., 2014) up to potentially toxic levels.

Pollinators, such as honey bees, are directly exposed to metal pollutants when foraging on contaminated nectar and pollen (Perugini et al., 2011; Xun et al., 2018), and while flying through air containing suspended particles (Thimmegowda et al., 2020). Metals accumulate in the bodies of adults (Giglio et al., 2017) and larvae (Balestra et al., 1992), as well as in hive products (Satta et al., 2012). For instance, bioaccumulation of arsenic (As), copper (Cu) and lead (Pb), resulting from metal production industries (Kabir et al., 2012) and mining (Khaska et al., 2018; Lee et al., 2005), is common in both honey bees (Badiou-Bénéteau et al., 2013; Giglio et al., 2017; Goretti et al., 2020) and their honey (Pisani et al., 2008; Terrab et al., 2005).

The deleterious effects of metals on humans (Tchounwou et al., 2012) and some model animals (mice: Cobbina et al., 2015; flies: Doğanlar et al., 2014) are well known. As, Cu, Pb and other metals have neurotoxic effects that induce neural and neuromuscular alterations, sensory impairment and many other behavioural

dysfunctions (Chen et al., 2016). Deficits in cognition and memory have been reported for As (e.g. humans: Tolins et al., 2014; mice: Tyler et al., 2018; Wu et al., 2006), Pb (e.g. mice: Anderson et al., 2016; humans: Mason et al., 2014) and Cu (e.g. mice: Lamtai et al., 2020; Pal et al., 2013; flies: Zamberlan, 2020). Recent studies showed that low doses of Pb (Monchanin et al., 2021a) and selenium (Se) (Burden et al., 2016) also impair behaviour and cognition in honey bees, suggesting a widespread impact on pollinators. So far, however, very little attention has been given to the potential combined effects of co-exposure to different metals (Monchanin et al., 2021b).

Interactions among stressors are commonly classified as antagonistic (when the effect of one stressor reduces the effect of the other one), additive (when stressors have simple cumulative effects) or synergistic (when stressors together have a greater effect than the sum of their individual effects) (Folt et al., 1999). Additive effects of As, Cu and Pb have been described for humans (Lin et al., 2016), rats (Aktar et al., 2017; Mahaffey et al., 1981; Schmolke et al., 1992) and fishes (Verriopoulos and Dimas, 1988). In rats, for example, co-exposure to Pb and As disrupted brain biogenic amine levels (Agrawal et al., 2015). In humans, it has been hypothesized that combined exposure to Pb and As, or other metal pollutants, has additive or synergistic toxic responses leading to cognitive dysfunction (Karri et al., 2016). To our knowledge, two studies have addressed the impact of metallic cocktails on bee physiology. Honey bees simultaneously exposed to Pb, cadmium (Cd) and Cu accumulated significant levels of these metals in their bodies and had lower brain concentrations of dopamine compared with control honey bees (Nisbet et al., 2018). Cd and Cu exerted a weak synergistic effect on honey bee survival (Di et al., 2020). However, none of these studies investigated potential effects of combined exposure on cognition.

Here, we compared the effects of exposure to single metals or ecologically relevant combinations of these metals on honey bee learning and memory. We hypothesized that combinations of metals may have synergistic negative effects, as has been found with pesticides (Yao et al., 2018; Zhu et al., 2017). We tested individual honey bees in a standard protocol of proboscis extension response (PER) conditioning following acute exposure to As, Pb and Cu alone or in combination. We tested three concentrations of As, considered the most toxic substance (<https://www.atsdr.cdc.gov/cep/index.html>), and added one concentration of Cu or Pb (binary mixtures), or both (tertiary mixture), to reach the molarity of the As solutions.

## MATERIALS AND METHODS

### Metal solutions

Arsenic (NaAsO<sub>2</sub>), lead (PbCl<sub>2</sub>) and copper (CuCl<sub>2</sub>·2H<sub>2</sub>O) were purchased from Sigma-Aldrich Ltd (Lyon, France) and diluted in 50% (w/v) sucrose solution. Control honey bees were fed 50% sucrose solution. Three concentrations of As were used (Table 1): a low concentration (0.13 μmol l<sup>-1</sup>) corresponding to the maximal

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permissible value in drinking water ( $0.01 \text{ mg l}^{-1}$ ) (Codex Alimentarius, 2015), a high concentration ( $0.67 \text{ } \mu\text{mol l}^{-1}$ ) corresponding to half the maximal permissible value in irrigation water ( $0.1 \text{ mg l}^{-1}$ ) (Ayers and Westcot, 1994), and an intermediate concentration ( $0.40 \text{ } \mu\text{mol l}^{-1}$ ). This range of concentrations was reported in water sampled from polluted areas (e.g. mining sites) and in honey (Table S1). For Pb and Cu, we chose  $0.27 \text{ } \mu\text{mol l}^{-1}$  ( $0.055 \text{ mg l}^{-1}$  of Pb and  $0.017 \text{ mg l}^{-1}$  of Cu) so that the binary combinations (As  $0.13 \text{ } \mu\text{mol l}^{-1}$ +Cu  $0.27 \text{ } \mu\text{mol l}^{-1}$  or As  $0.13 \text{ } \mu\text{mol l}^{-1}$ +Pb  $0.27 \text{ } \mu\text{mol l}^{-1}$ ) could be compared with the As intermediate concentration ( $0.40 \text{ } \mu\text{mol l}^{-1}$ ), and the tertiary combination (As  $0.13 \text{ } \mu\text{mol l}^{-1}$ +Pb  $0.27 \text{ } \mu\text{mol l}^{-1}$ +Cu  $0.27 \text{ } \mu\text{mol l}^{-1}$ ) with the As high concentration ( $0.67 \text{ } \mu\text{mol l}^{-1}$ ) (Table 1). These concentrations of Pb and Cu have also been reported in honey samples (Table S1). The mass consumed for As and the concentrations for Cu and Pb fell within sublethal ranges for the honey bee: the  $\text{LD}_{50}$  of elemental As for  $\text{NaAsO}_2$  ranged from  $0.330$  to  $0.540 \text{ } \mu\text{g}$  per bee (Fujii, 1980); the  $\text{LC}_{50}$  of Cu is  $72 \text{ mg l}^{-1}$  (Di et al., 2016) and that of Pb is  $345 \text{ mg l}^{-1}$  (Di et al., 2016).

### Bee exposure to metals

We collected honey bees (*Apis mellifera* Linnaeus 1758) returning from foraging trips at the entrance of five different hives in mornings during August 2020. We anaesthetized the bees on ice and harnessed them in plastic tubes, secured with tape and a droplet of wax at the back of the head (Matsumoto et al., 2012). We tested all bees for an intact PER by stimulating their antennae with 50% sucrose. We then fed the responding honey bees  $5 \text{ } \mu\text{l}$  of 50% sucrose solution (see Table 1), making sure they consumed the whole droplet, and left them to rest for 3 h in the incubator (temperature:  $25 \pm 2^\circ\text{C}$ , humidity: 60%). Honey bees that did not respond to the sucrose solution were discarded.

### Absolute learning

Prior to conditioning, we tested all honey bees for the PER by stimulating their antennae with 50% sucrose solution, and kept only those that displayed the reflex. We then performed olfactory absolute conditioning according to a standard protocol using an automatic stimulus delivery system (Aguiar et al., 2018). Honey bees had to learn to respond to an olfactory conditioned stimulus (CS, 1-nonanol; Sigma-Aldrich Ltd) reinforced with the unconditioned stimulus (US, 50% sucrose solution), over five conditioning trials with a ten-minute inter-trial interval. Each trial (37 s in total) began when a bee was placed in front of the stimulus delivery system, which released a continuous flow of clean air ( $3300 \text{ ml min}^{-1}$ ) to the antennae. After 15 s, the odour was introduced into the airflow for 4 s, the last second

of which overlapped with sucrose presentation to the antennae using a toothpick. This was immediately followed by feeding for 4 s by presenting the toothpick to the proboscis. The bee remained for another 15 s under the clean airflow. We recorded the presence or absence (1/0) of a conditioned PER in response to the odour presentation during each conditioning trial. Honey bees spontaneously responding in the first conditioning trial were discarded from the analysis. The sum of conditioned responses over all trials provided an individual acquisition score (between 0 and 4), and honey bees responding at the last trial were categorized as learners.

### Long-term memory

Only honey bees that had learnt the task were kept for the analysis of memory performance. After conditioning, these honey bees were fed  $15 \text{ } \mu\text{l}$  of 50% sucrose solution, left overnight in the incubator, and fed another  $5 \text{ } \mu\text{l}$  of sucrose solution the following morning. Three hours later (24 h post-conditioning), we performed the retention test, consisting of three trials similar to conditioning except that no sucrose reward was presented. In addition to the odour used during the conditioning (CS), we presented two novel odours, in randomized order, to assess the specificity of the memory: nonanal was expected to be perceived by honey bees as similar to 1-nonanol, while 1-hexanol was expected to be perceived differently (Guerrieri et al., 2005). We recorded the presence or absence (1/0) of a conditioned PER to each odourant at each memory retention trial. We classified honey bees according to their response patterns: response to the CS only, response to the CS and the similar odour (low generalization level), response to all odours (high generalization level), no or inconsistent response.

### Statistics

We analysed the data using R Studio v.1.2.5033 (<http://www.rstudio.com/>). Raw data are available from Dryad (Dataset S1, doi:10.5061/dryad.glx3ffbms). We performed binomial generalized linear mixed-effects models (GLMM) (package lme4; Bates et al., 2015), with hive and conditioning date as random factors and treatment as a fixed effect. Using the GLMMs, we evaluated whether molarity or treatment impacted the initial response to antennal stimulation, the spontaneous response in the first conditioning trial, the response in the last trial, the response to each odourant during the memory test, the proportion of honey bees per response pattern in the retention test, and the survival at 24 h. Acquisition scores were standardized and compared with GLMMs using Template Model Builder (Brooks et al., 2017). For all response variables, we compared (1) the treated groups with the control, (2) groups exposed to concentrations of the same molarity (e.g. intermediate [As], [As+Cu] and [As+Pb]), (3) the separate and joint effects of the treatments (e.g. low [As], [Cu] and [As+Cu]) in order to identify interactive effects (antagonistic, additive, synergistic).

## RESULTS AND DISCUSSION

### Exposure to metals does not impact appetitive motivation

The proportion of honey bees that responded to the initial antennal stimulation with sucrose was similar among treatments (GLMM:  $P > 0.05$ ). Therefore, treatment did not affect appetitive motivation or sucrose perception. Consistent with our observations, the ingestion of similar concentrations of Pb and Cu had no effect on responsiveness to increasing concentrations of sucrose (Burden et al., 2019). By contrast, Di et al. (2020) found that honey bees exposed to increasing concentrations of a mixture of Cu and Cd exhibited a decreased ability to distinguish sucrose concentrations,

**Table 1. Concentrations of heavy metals used**

Treatment	Molarity ( $\mu\text{mol l}^{-1}$ )	Concentration ( $\text{mg l}^{-1}$ )			Ingestion of $5 \text{ } \mu\text{l}$ (ng per bee)		
		As	Cu	Pb	As	Cu	Pb
Control	0	0	0	0	0	0	0
Low [As]	0.13	0.01	0	0	0.05	0	0
[Cu]	0.27	0	0.02	0	0	0.09	0
[Pb]	0.27	0	0	0.06	0	0	0.28
Int. [As]	0.40	0.03	0	0	0.15	0	0
[As+Cu]	0.40	0.01	0.02	0	0.05	0.09	0
[As+Pb]	0.40	0.01	0	0.06	0.05	0	0.28
High [As]	0.67	0.05	0	0	0.25	0	0
[As+Cu+Pb]	0.67	0.01	0.02	0.06	0.05	0.09	0.28

Int., intermediate. Combined treatments are shown in grey.

but this may be explained by the much higher (at least 600 times) concentrations used in that study. Thus, in our conditions, any impact on appetitive learning is unlikely to be due to a decrease in motivation for sucrose or sucrose perception.

### Individual and combined exposure to metals reduces learning performance

Two out of the 381 honey bees subjected to the absolute learning task spontaneously responded to the first odour presentation and were therefore discarded. In all groups, the number of honey bees showing the conditioned response increased over trials, thus showing learning (Fig. 1A). However, fewer honey bees exposed to metals learned the task when compared with controls (GLMM:  $P < 0.05$ , except for low [As],  $P = 0.082$ ). Accordingly, the acquisition scores of honey bees from all treatments were lower than those of controls (Fig. 1B). Honey bees exposed to intermediate [As] (GLMM:  $-0.610 \pm 0.246$ ,  $P = 0.013$ ), high [As] (GLMM:  $-0.639 \pm 0.241$ ,  $P = 0.008$ ) and [As+Cu+Pb] (GLMM:  $-0.592 \pm 0.244$ ,  $P = 0.015$ ) had acquisition scores significantly lower than those of controls. Honey bees exposed to [As+Pb] had similar acquisition scores to bees exposed to intermediate [As] (GLMM:  $0.299 \pm 0.234$ ,  $P = 0.201$ ), but honey bees exposed to [As+Cu] performed better (GLMM:  $0.596 \pm 0.241$ ,  $P = 0.013$ ). Honey bees exposed to high [As] and [As+Cu+Pb] exhibited similar acquisition scores (GLMM:  $P = 0.810$ ). We found no difference in the acquisition scores and the proportions of learners between honey bees treated with a single metal and mixed treatments (GLMM:  $P > 0.05$ ), that would have indicated non-additive effects (i.e. antagonistic or synergistic). Thus, exposure to metals significantly reduced learning performance, and combined exposure appeared to exert simple additive deleterious effects.

### Individual and combined exposure to metals reduces long-term memory specificity

To examine possible effects of metal exposure on memory retention, we tested memory 24 h post-training. Only honey bees that had learned the CS-US association at the end of conditioning were tested;

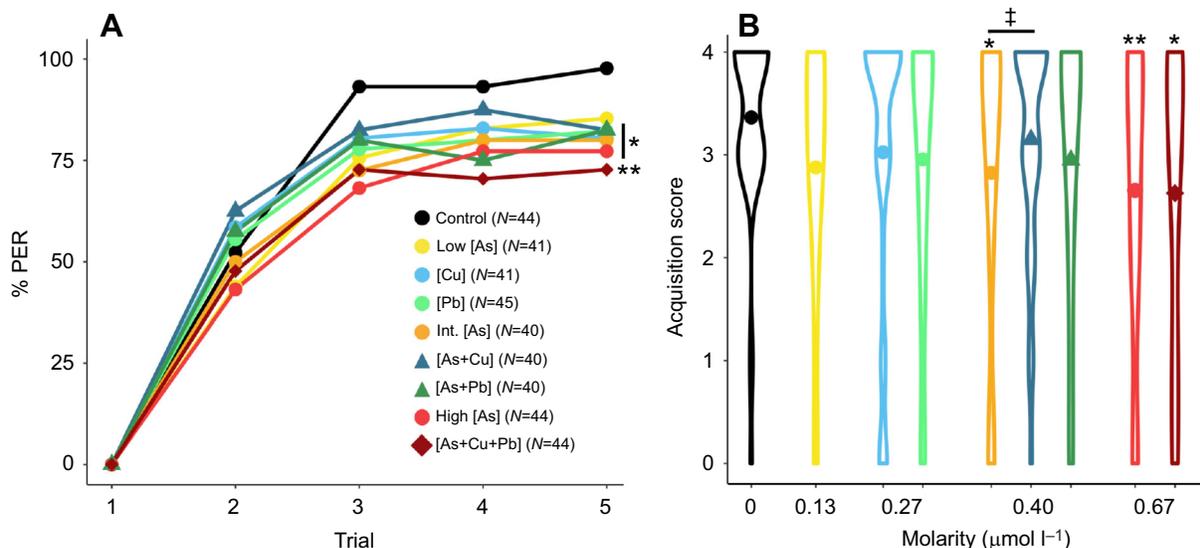
167 out of the 379 honey bees subjected to the absolute learning task did not learn and were therefore not included in the memory test.

We found no effect of treatment on survival at 24 h (GLMM:  $P > 0.05$ ). However, long-term memory was significantly affected (Fig. 2). Overall, treated honey bees responded less to the learned odourant (CS) than did controls, as indicated by a significant effect of exposure to metals on retention levels (GLMM:  $P < 0.05$ ) (Fig. 2A). Yet, this decrease was not significant for honey bees exposed to intermediate [As] (GLMM:  $-0.260 \pm 0.628$ ,  $P = 0.679$ ) and high [As] (GLMM:  $-1.023 \pm 0.570$ ,  $P = 0.073$ ). Finally, there was no clear dose effect on responses to the CS among treated groups (GLMM:  $-0.576 \pm 0.579$ ,  $P = 0.320$ ).

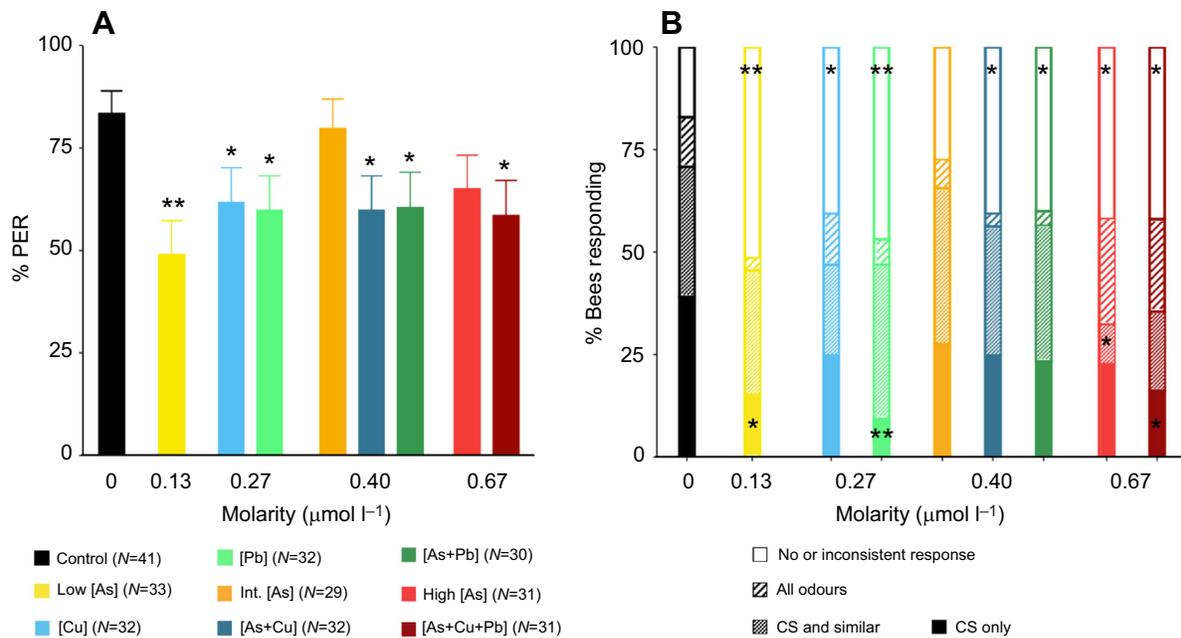
Individual response patterns (Fig. 2B) revealed a loss of memory specificity. While honey bees from all treatments responded similarly to the similar odour (GLMM:  $P > 0.05$ ), those exposed to higher doses responded more frequently to all odourants, indicating a higher degree of response generalization (GLMM:  $1.954 \pm 0.775$ ,  $P = 0.012$ ). This was accompanied by a significantly lower proportion of specific (CS-only) responses for honey bees exposed to [Pb] (GLMM:  $-1.795 \pm 0.690$ ,  $P = 0.009$ ), low [As] (GLMM:  $-1.313 \pm 0.589$ ,  $P = 0.026$ ) and [As+Cu+Pb] (GLMM:  $-1.200 \pm 0.588$ ,  $P = 0.041$ ). Exposure also significantly increased the frequency of inconsistent responses as compared with that in controls (GLMM:  $P < 0.05$ ). This was the case for each individual treatment except for intermediate [As] ( $P = 0.293$ ). Thus, exposure to metals had a negative impact on memory performance at 24 h. The analysis of individual response patterns also revealed additive effects as they did not differ among groups exposed to solutions with the same molarity, nor between single and mixed metal treatments (GLMM:  $P > 0.05$ ). Thus, most treatments reduced memory performance at 24 h.

### The additive effects of metal mixtures may be explained by common pathways of toxicity

Although many mechanisms of metal toxicity have not yet been elucidated, some points of consensus are emerging from the



**Fig. 1. Learning.** (A) Learning curves show changes in the percentage of honey bees displaying the conditioned proboscis extension response (PER) over five training trials. Asterisks indicate significant differences in response rates at the last trial compared with those for control honey bees. (B) Violin plots of acquisition score values (sum of conditioned responses for each honey bee). Symbols (circle: single exposure; triangle: binary mixture; diamond: tertiary mixture) indicate the mean score for each treatment. Significant differences between groups exposed to solutions of the same molarity (‡) or with respect to control honey bees (\*) are indicated (\* $P < 0.05$ , \*\* $P < 0.01$ ; GLMM).



**Fig. 2. Long-term memory.** (A) Percentage of responses to the conditioned stimulus (CS) odour in the 24 h memory retention test (means $\pm$ s.e.m.). (B) Distribution of honey bees according to their individual response pattern during the long-term memory test: response to CS only; response to CS and similar odour; response to all odours; no or inconsistent response. Significant differences from controls are indicated (\* $P$ <0.05, \*\* $P$ <0.01; GLMM).

literature. Firstly, interactions between metals can occur in the environment of the organism (Grobelak and Kowalska, 2020; Noyes and Lema, 2015), and during uptake into the organism, leading to potentially toxic processes of speciation, absorption, binding, transport and distribution (Wu et al., 2016). Once metals enter an organism, they can induce, alter or inhibit a range of biological responses and metabolic pathways. For example, by mimicking other essential metals (Bridges and Zalups, 2005) or damaging the permeability of biological membranes (Rothschein, 1959), metals enable the uptake or loss of other compounds from intracellular compartments (Viarengo, 1994). Metals are also known to disrupt signalling and calcium homeostasis (particularly important in neurons) by interfering with calcium channels (Bridges and Zalups, 2005; Chavez-Crooker et al., 2001; Tamano and Takeda, 2011). This might lead to dysfunction and cytotoxicity as a result of the disruption of cell signalling and calcium homeostasis. Genotoxicity (Doğanlar et al., 2014) may be achieved through covalent binding to DNA (Brocato and Costa, 2013; Senut et al., 2014). Eventually, oxidative stress and lipid peroxidation of the cell membrane may lead to neuronal death. Additionally, metals in mixtures could interact at target sites, but the effect on toxicity of that interaction is largely unknown (Svendsen et al., 2011). Metal mixtures could change the bioavailability (Gong et al., 2020), toxicokinetics and toxicodynamics (Gao et al., 2016) of each metal, which could impact the toxicity for the organism (Løkke et al., 2013). Based on these shared mechanisms of toxicity that include oxidative stress (Nikolić et al., 2016; Zaman et al., 1995), apoptosis (Raes et al., 2000) and interference with neurotransmitters (Nisbet et al., 2018), the toxic effects of metal pollutants in mixtures is expected to be additive (von Stackelberg et al., 2013). Of note, these conclusions emerge from studies mostly conducted on vertebrates, thus possibly leaving aside specificities of meta actions in invertebrate organisms.

Mixtures of metals may affect many aspects of neural activity and brain function in honey bees, as in other species (Karri et al., 2016).

Here, we focused on learning and memory of olfactory cues because they play crucial roles in the behavioural ecology of honey bees and other pollinators, for the identification of food resources. Our results in controlled laboratory conditions suggest that exposure to sublethal combinations of toxic elements in the field might alter individual foraging efficiency, and in turn jeopardize survival of pollinator populations. While we could not identify interactive effects in such conditions, this will need to be confirmed in field experiments where exposure conditions will differ, and we should study a broader range of behavioural responses (flight, activity, navigation). Our approach aims to fill a gap in the evaluation of combined actions of metals (Meyer et al., 2015), which appears necessary to better assess the risks they represent (Nys et al., 2018; Otitoloju, 2003) and better inform regulatory frameworks (European Commission, 2012). Current risk assessment guidance mainly assesses the effect of exposure to individual metals, which fails to capture potential interactive effects. This is of particular importance for honey bees and many other species, where contaminated food is transferred and shared among individuals. Hence, evaluation of the impact of metal mixtures and their modes of action needs to be developed (Sasso et al., 2010). Additionally, interactions between toxic metals and environmental factors (Naqash et al., 2020) as well as with other chemicals (EFSA Scientific Committee et al., 2019) (e.g. pesticides: Sgolastra et al., 2018; Singh et al., 2017; volatile organic compounds: Sasso et al., 2010, etc.) should be implemented in an integrated research framework.

### Conclusion

In summary, we have demonstrated that As, Pb, Cu or combinations of these metals, at levels found in the environment, slow down appetitive learning and reduce long-term memory specificity in honey bees. These metals show simple additive effects as we found no difference in effects between different solutions of the same molarity, suggestive of possible non-linear effects (synergism or antagonism). Thus, regarding effects on learning and memory,

concentration seems to be more important than the identity of any specific metal. Given that learning and memory of olfactory cues play crucial roles in the behavioural ecology of honey bees, acute exposure to mixtures of metal pollutants could impair fundamental hive function and population growth.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: C.M., J.D., M.L., A.B.B.; Methodology: C.M., J.D., M.L., A.B.B.; Investigation: C.M., E.D.; Data curation: C.M.; Writing - original draft: C.M.; Writing - review & editing: C.M., J.D., M.L., A.B.B.; Visualization: C.M.; Supervision: J.D., M.L., A.B.B.; Funding acquisition: J.D., M.L., A.B.B.

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#### Data availability

Raw data are available from the Dryad digital repository (Monchanin, 2021): doi:10.5061/dryad.ghx3fbbms

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