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1 **Heavy metal pollutants have additive negative effects on honey bee cognition**

2

3 **Running title:** Heavy metal cocktails impair bee cognition

4 **Author list**

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12

13 **Keywords:** *Apis mellifera*, PER conditioning, pollutant interaction, arsenic, lead, copper

14

15 **Summary statement:**

16 Honey bees displayed reduced learning and memory performances following acute exposure to
17 arsenic, copper or lead. Exposure to combinations of these metals induced additive effects.

18 **Abstract**

19

20 Environmental pollutants can exert sublethal deleterious effects on animals. These include
21 disruption of cognitive functions underlying crucial behaviours. While agrochemicals have
22 been identified as a major threat to pollinators, other compounds, such as heavy metals that are
23 often found in complex mixtures, have largely been overlooked. Here, we assessed the impact
24 of acute exposure to field-realistic concentrations of lead, copper, arsenic, and their
25 combinations, on honey bee learning and memory. All treatments involving single metals
26 slowed down appetitive learning and disrupted memory retrieval at 24 h. Importantly,
27 combinations of these metals induced additive negative effects on both processes, suggesting
28 common pathways of toxicity. Our results highlight the need to further assess the risks of heavy
29 metal pollution on invertebrates and to their associated ecosystem services.

30

31 **Introduction**

32

33 Metal pollution is an increasingly important concern for the maintenance of ecosystems and
34 public health (Nriagu and Pacyna, 1988). Over the last century, the widespread use of heavy
35 metals in domestic, industrial and agricultural applications (Bradl, 2005) has considerably
36 elevated their concentrations in water (Mance, 1987) and terrestrial habitats (Krämer, 2010; Su
37 et al., 2014) to potentially toxic levels.

38 Critical pollinators, such as honey bees, are directly exposed to metal pollutants when
39 foraging on contaminated nectar and pollen (Perugini et al., 2011; Xun et al., 2018), and while
40 flying through air containing suspended particles (Thimmegowda et al., 2020). Metals
41 accumulate in the bodies of adult bees (Giglio et al., 2017) and larvae (Balestra et al., 1992) as
42 well as in the hive products (Satta et al., 2012). For instance, concomitant bioaccumulation of
43 arsenic (As), copper (Cu) and lead (Pb), resulting from metal production industries (Kabir et
44 al., 2012) and mining (Khaska et al., 2018; Lee et al., 2005), is common in honey bees (Badiou-
45 Bénéteau et al., 2013; Giglio et al., 2017; Goretti et al., 2020) and their honey (Pisani et al.,
46 2008; Terrab et al., 2005).

47 The deleterious effects of metals on humans are well-known (Tchounwou et al., 2012).
48 Neural and neuromuscular alterations, sensory impairments and many related forms of
49 behavioural dysfunctions highlight the neurotoxic effects of As, Cu, Pb and other metals (Chen
50 et al., 2016). Deficits in cognition and memory have been reported for As (e.g. humans: Tolins
51 et al., 2014; mice: Tyler et al., 2018; Wu et al., 2006), Pb (e.g. mice: Anderson et al., 2016;

52 humans: Mason et al., 2014) and Cu (e.g. mice: Lamtai et al., 2020; Pal et al., 2013; flies:
53 Zamberlan, 2020). Recent studies showed that low doses of Pb (Monchanin et al., 2020a) and
54 selenium (Se) (Burden et al., 2016) also impair behaviour and cognition in honey bees,
55 suggesting a widespread impact on pollinators. However, very little attention has been given to
56 the potential combined effects of co-exposure to different metals (Monchanin et al., 2020b).

57 Interactions among stressors are commonly classified as antagonistic (when the effect
58 of one stressor reduces the effect of the other one), additive (when stressors have cumulative
59 effects) or synergistic (when stressors together have a greater effect than the sum of their
60 individual effects) (Folt et al., 1999). Additive effects of As, Cu and Pb have been described
61 for humans (Lin et al., 2016), rats (Aktar et al., 2017; Mahaffey et al., 1981; Schmolke et al.,
62 1992) and fishes (Verriopoulos and Dimas, 1988). In rats, for instance, co-exposure to Pb and
63 As disrupted brain biogenic amine levels (Agrawal et al., 2015). In humans, it was hypothesized
64 that combined exposure to Pb and As, or other metal pollutants, have additive or synergistic
65 toxic responses leading to cognitive dysfunction (Karri et al., 2016). To our knowledge, two
66 studies have addressed the impact of metallic cocktails on bee physiology. Bees simultaneously
67 exposed to Pb, cadmium (Cd) and Cu accumulated significant levels of those metals and had
68 lower brain concentrations of dopamine compared to unexposed bees (Nisbet et al., 2018). Cd
69 and Cu exerted a weak synergistic effect on bee survival (Di et al., 2020). However, none of
70 these studies investigated potential effects of combined exposure on cognition.

71 Here we explored the effects of an exposure to single or combinations of metals on bee
72 learning and memory. We hypothesised that combinations of metals may have synergistic
73 negative effects, as it has been found with pesticides (Yao et al., 2018; Zhu et al., 2017). We
74 tested individual honey bees in a standard protocol of proboscis extension reflex (PER)
75 conditioning following acute exposure to As, Pb and Cu or a combination of them. We tested
76 three concentrations of As, considered the most toxic substance (ATSDR, 2019), and added
77 one concentration of Cu or Pb (binary mixtures), or both (tertiary mixture), to reach the molarity
78 of the As solutions, allowing us to better assess any combined effects.

79

80 **Materials and methods**

81

82 *Metal solutions*

83 Arsenic (NaAsO₂), lead (PbCl₂) and copper (CuCl₂·2H₂O) were purchased from Sigma-Aldrich
84 Ltd (Lyon, France) and diluted in 50% (w/v) sucrose solution. Control bees were fed 50%
85 sucrose solution. We used three concentrations of As (Table 1): a low concentration (0.13 μM)

86 corresponding to the maximal permissible value in water (0.01 mg.L⁻¹) (Codex Alimentarius,
87 2015), a high concentration (0.67 µM) corresponding to half the maximal permissible value in
88 irrigation water (0.1 mg.L⁻¹) (Ayers and Westcot, 1994), and an intermediate concentration
89 (0.40 µM). This range of concentrations was reported in water sampled from polluted areas
90 (e.g. mining sites) and in honey (Table S1). For Pb and Cu, we chose 0.27 µM (0.055 mg.L⁻¹ of
91 Pb and 0.017 mg.L⁻¹ of Cu) so that the binary combinations (As 0.13 µM + Cu 0.27 µM or As
92 0.13 µM + Pb 0.27 µM) could be compared to the As intermediate concentration (0.40 µM),
93 and the tertiary combination (As 0.13 µM + Pb 0.27 µM + Cu 0.27 µM) to the As high
94 concentration (0.67 µM) (Table 1). These concentrations of Pb and Cu have also been reported
95 in honey samples (Table S1). All concentrations fell within sublethal ranges for the honey bee:
96 the LD50 of elemental As for NaAsO₂ ranged from 0.330 to 0.540 ug/bee (Fujii, 1980), the
97 LD50 of Cu is 72 mg.L⁻¹ (Di et al., 2016) and of Pb is 345 mg.L⁻¹ (Di et al., 2016).

98

99 **Table 1: Concentrations used.** Combined treatments are shown in grey.

Treatment	Molarity (µM)	Concentration (mg.L ⁻¹)			Ingestion of 5µL (ng/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
Med [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

100

101 *Bee exposure to metals*

102 We collected honey bees (*Apis mellifera*) returning from foraging trips at the hive entrance in
103 mornings of August 2020. We then anesthetised the bees on ice and harnessed them in plastic
104 tubes, secured with tape and a droplet of wax at the back of the head (Matsumoto et al., 2012).
105 We fed them 5 µL of 50% sucrose solution (see Table 1) and left them to rest for 3 h in the
106 incubator (temperature: 25±2°C, humidity: 60%).

107

108 *Absolute learning*

109 Prior to conditioning, we tested all bees for PER by stimulating their antennae with 50% sucrose
110 solution, and kept only those that displayed the reflex. We then performed olfactory absolute
111 conditioning according to a standard protocol using an automatic stimulus delivery system
112 (Aguilar et al., 2018). Bees had to learn to respond to an olfactory conditioned stimulus (CS, 1-
113 nonanol, Sigma-Aldrich Ltd, Lyon, France) reinforced with 50% sucrose solution, over five
114 conditioning trials with a ten-minute inter-trial interval. Each trial (37 s in total) began when a
115 bee was placed in front of the stimulus delivery system, which released a continuous flow of
116 clean air (3,300 mL.min⁻¹) to the antennae. After 15 s, the odour was introduced into the airflow
117 for 4 s, the last second of which overlapped with sucrose presentation to the antennae using a
118 toothpick with subsequent feeding for 4 seconds by presenting the toothpick to the proboscis.
119 The bee remained another 15 s under the clean airflow. We recorded the presence or absence
120 (1/0) of a conditioned PER in response to the odorant presentation during each conditioning
121 trial. Bees spontaneously responding in the first conditioning trial were discarded from the
122 analysis. The sum of conditioned responses over all trials provided an individual acquisition
123 score (between 0 and 4), and bees responding at the last trial were categorized as learners.

124

125 *Long-term memory*

126 After conditioning, bees were fed 15 µL of 50% sucrose solution, left overnight in the incubator,
127 and fed another 5 µL of sucrose solution the following morning. Three hours after (24 h post-
128 conditioning), we performed the retention test, consisting of three trials similar to conditioning
129 except that no sucrose reward was presented. In addition to the odour used during the
130 conditioning (CS), we presented two novel odours, in randomized order, to assess the
131 specificity of the memory: nonanal was expected to be perceived by bees similarly to 1-nonanol,
132 while 1-hexanol was expected to be perceived differently (Guerrieri et al., 2005). We recorded
133 the presence or absence (1/0) of a conditioned PER to each odorant at each memory retention
134 trial. We classified bees according to their response patterns: response to the CS only, response
135 to the CS and the similar odour (low generalization level), response to all odours (high
136 generalization level), no or inconsistent response.

137

138 *Statistics*

139 We analysed the data using R Studio v.1.2.5033 (RStudio Team, 2015). Raw data are available
140 in Dataset S1. We performed binomial generalised linear mixed-effects models (GLMM)
141 (package lme4; Bates et al., 2015), with hive and conditioning date as random factors and

142 treatment as fixed effect. Using the GLMMs, we evaluated whether treatment impacted the
143 initial response to antennal stimulation, the spontaneous response in the first conditioning trial,
144 the level of response in the last trial, the level of response to each odorant during the memory
145 test, the proportion of bees per response pattern in the retention test, and the survival at 24 h.
146 Note that only bees that had learnt the task were kept for the analysis of memory performance.
147 Acquisition scores were standardised and compared with GLMMs using Template Model
148 Builder (Brooks et al., 2017). For all response variables, we compared (1) the treated groups to
149 the control, (2) groups exposed to concentrations of the same molarity (e.g. Med [As], [As+Cu]
150 and [As+Pb]), (3) the separate and joint effects of the treatments (e.g. Low [As], [Cu] and
151 [As+Cu]) in order to better identify interactive effects (antagonism, additive, synergism).

152

153 **Results and discussion**

154

155 *Exposure to metals did not impact appetitive motivation*

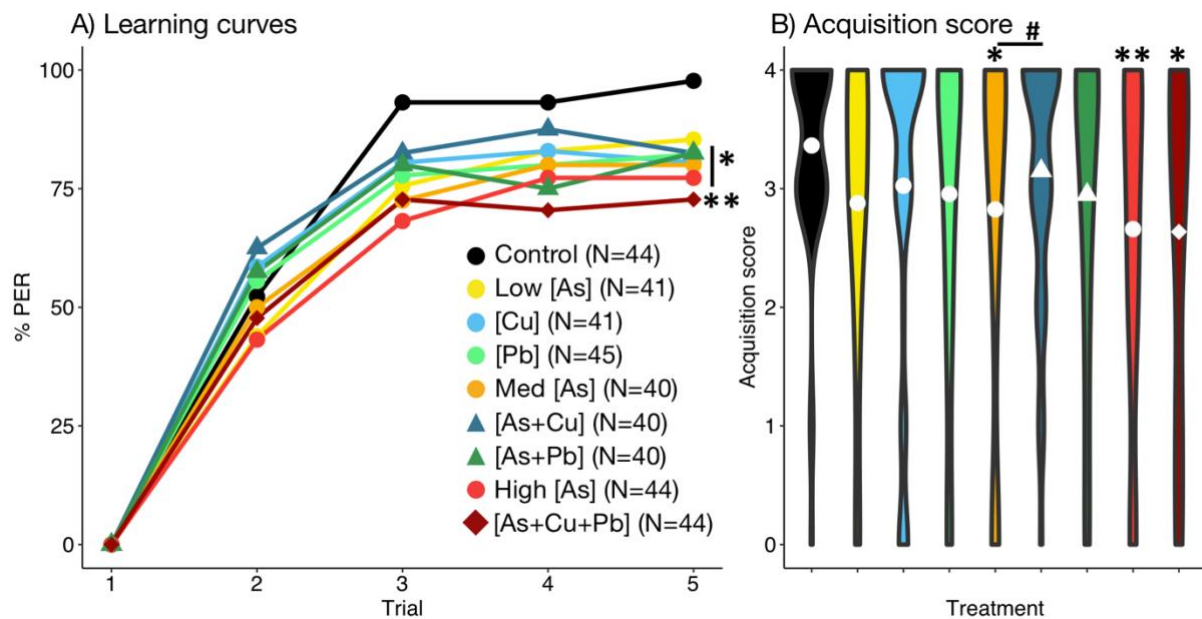
156 The proportion of bees that responded to the initial antennal stimulation with sucrose was
157 similar among treatments (GLMM: $p > 0.05$), with only 2.27% (1 out of 42 bees) of Low [As]
158 and 4.08% (2 out of 46 bees) of [As+Cu+Pb] bees that did not respond. All bees responded in
159 the other groups. Therefore, treatment did not affect appetitive motivation or sucrose
160 perception, as previously reported with similar concentrations of Pb and Cu (Burden et al.,
161 2019).

162

163 *Individual and joint exposures to metals reduced learning performances*

164 Two out of the 381 bees submitted to the absolute learning task spontaneously responded to the
165 first odour presentation and were therefore discarded. In all groups, the number of bees showing
166 the conditioned response increased over trials, thus showing learning (Fig. 1A). However, fewer
167 bees exposed to metals learned the task when compared to controls (GLMM: $p < 0.05$), except
168 for Low [As] (GLMM: -1.920 ± 1.103 , $p = 0.082$). Accordingly, the acquisition scores of bees
169 were significantly affected by treatment (Fig. 1B). Bees exposed to Med [As] (GLMM: $-$
170 0.610 ± 0.246 , $p = 0.013$), High [As] (GLMM: -0.639 ± 0.241 , $p = 0.008$) and [As+Cu+Pb]
171 (GLMM: -0.592 ± 0.244 , $p = 0.015$) had similar acquisition scores, all significantly lower than
172 control bees. Bees exposed to [As+Pb] and Med [As] had similar acquisition scores, but bees
173 exposed to [As+Cu] performed better (GLMM: 0.596 ± 0.241 , $p = 0.013$). Bees exposed to High
174 [As] and [As+Cu+Pb] exhibited similar acquisition scores (GLMM: $p = 0.810$). We found no
175 difference in the acquisition scores and the proportions of learners between individual and

176 mixed treatments (GLMM: $p>0.05$), indicating an additive effect. Thus, exposure to metals
177 significantly reduced learning performance, and combined exposure exerted additive
178 deleterious effects.



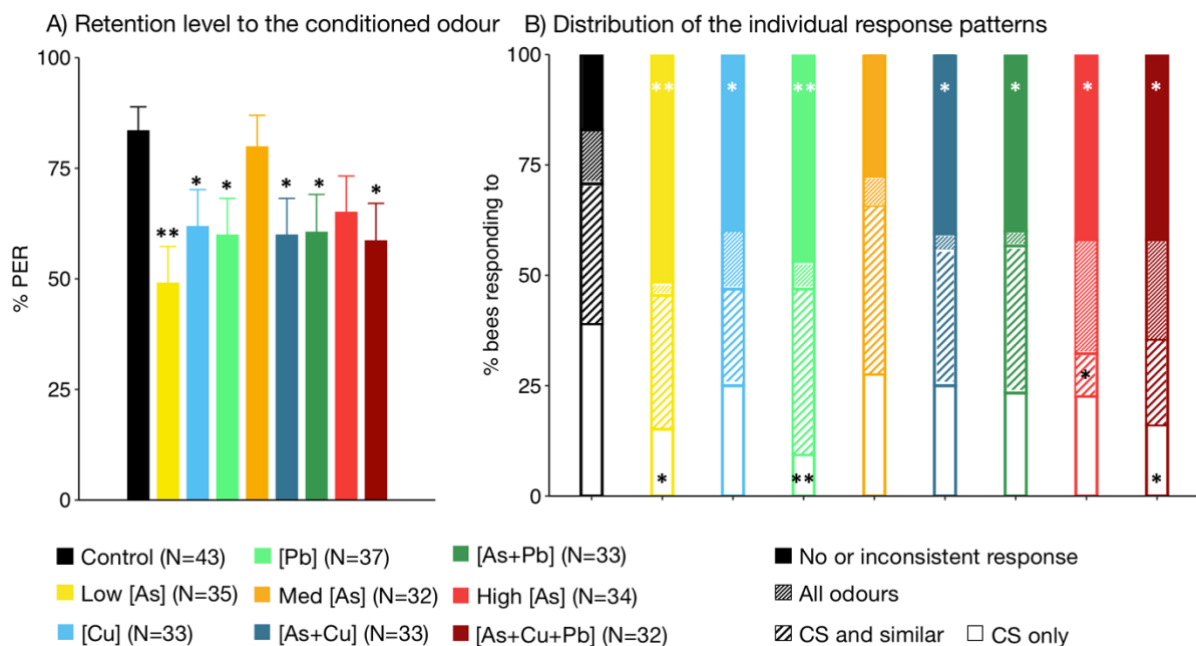
179
180 **Figure 1: Learning.** **A)** Learning curves show changes in the percentages of bees displaying
181 the conditioned proboscis extension response (PER) over five training trials. Asterisks indicate
182 significant differences in responses at the last trial compared to control bees. **B)** Violin plots of
183 acquisition score values (sums of conditioned responses for each bee). Symbols (*circle*: single
184 exposure; *triangle*: binary mixture; *diamond*: tertiary mixture) indicate the mean score for each
185 treatment. Significant differences between groups exposed to the same molarity solutions (#)
186 or with respect to control bees (*) are indicated (#/* $p<0.05$, ** $p<0.01$; GLMM).

187

188 *Individual and joint exposures to metals reduced long-term memory specificity*

189 We found no effect of treatment on survival at 24 h (GLMM: $p>0.05$). However, long-term
190 memory was significantly affected (Fig. 2). Bees from all treatments, except Med [As] and
191 High [As], responded less to the learnt odour (CS) than control bees (GLMM: $p<0.05$) (Fig.
192 2A). Bees from all treatments responded similarly to the similar odour (GLMM: $p>0.05$). For
193 the different odour, only bees from High [As] responded more than controls (GLMM:
194 1.242 ± 0.625 , $p=0.047$), indicating a higher degree of response generalization. We found no
195 difference in the responses to the three different odours when comparing the groups exposed to
196 metal solutions with the same molarity, nor between individual and mixed treatments (GLMM:
197 $p>0.05$), thus indicating an additive effect.

198 Individual response patterns (Fig. 2B) revealed a loss of memory specificity in bees
 199 exposed to [Pb] (GLMM: -1.795 ± 0.690 , $p=0.009$), Low [As] (GLMM: -1.313 ± 0.589 , $p=0.026$)
 200 and [As+Cu+Pb] (GLMM: -1.200 ± 0.588 , $p=0.041$). The distributions of individual response
 201 patterns also revealed additive effects as they did not differ among groups exposed to solutions
 202 with the same molarity, nor between single and mixed metal treatments (GLMM: $p>0.05$).
 203 Importantly, bees from all treatment groups but one (Med [As]) failed to respond consistently
 204 more often than controls (GLMM: $p<0.05$). Thus, most treatments reduced memory
 205 performance at 24h.
 206



207
 208 **Figure 2: Long-term memory.** A) Percentages of responses to the CS odour in the 24 h-
 209 memory retention test (mean \pm s.e.m). B) Distribution of bees according to their individual
 210 response pattern during the long-term memory test: response to CS only; response to CS and
 211 similar; response to all odours; no or inconsistent response. Significant differences with controls
 212 are indicated (* $p<0.05$, ** $p<0.01$; GLMM).
 213

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

215 Although many mechanisms of metal toxicity have not yet been elucidated, some general
 216 common points already emerge from the literature. By mimicking other ions (Bridges and
 217 Zalups, 2005), metals can disrupt signalling and calcium homeostasis (particularly important in
 218 neurons) by interfering with the calcium channels (Bridges and Zalups, 2005; Chavez-Crooker
 219 et al., 2001; Tamano and Takeda, 2011). This might lead to dysfunction and cytotoxicity due

220 to the disruption of cell signalling and calcium homeostasis. Genotoxicity (Doğanlar et al.,
221 2014) may be achieved through covalent binding to DNA (Brocato and Costa, 2013; Senut et
222 al., 2014). Eventually, oxidative stress and lipid peroxidation of the cell membrane may lead to
223 neuronal death. Based on their shared mechanisms of toxicity, such as oxidative stress (Nikolić
224 et al., 2016; Zaman et al., 1995), apoptosis (Raes et al., 2000) and interference with
225 neurotransmitters (Nisbet et al., 2018), the toxic effects of heavy metals mixtures may be
226 expected to be additive.

227

228 **Conclusion**

229 We demonstrate that arsenic, lead, copper or combinations of these metals, at levels found in
230 the environment, slow down appetitive learning and reduce long-term memory specificity.
231 Importantly, these metals show additive effects as we found no differences between different
232 solutions of the same molarity, suggesting that concentration was more important than identity
233 of any specific metal for the toxicity. Learning and memory of olfactory cues play crucial roles
234 in the behavioural ecology of bees, for the identification of profitable resources, social
235 interactions and the recruitment of nestmates (Farina et al., 2005; Grüter et al., 2006).
236 Therefore, ultimately, acute exposure to metal pollutants mixtures could impair fundamental
237 hive function and population growth, a snowball effect that may be even more critical in many
238 solitary bees that cannot rely on new cohorts of workers to replace contaminated individuals
239 and engage in food provisioning (Klein et al., 2017).

240

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243

244 **Competing interests**

245 The authors declare no competing or financial interests.

246

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254 **Data availability**

255 Raw data will be available on Dryad repository upon publication.

256

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