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1 **Metal pollutants have additive negative effects on honey bee cognition**

2

3 **Running title:** Metal pollutant 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 performance following acute exposure to
17 arsenic, copper or lead. Exposure to combinations of these metals had 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, metal pollutants, which are often found in
23 complex mixtures, have so far been overlooked. Here we assessed the impact of acute exposure
24 to field-realistic concentrations of three common metal pollutants, lead, copper, arsenic, and
25 their combinations, on honey bee appetitive learning and memory. All treatments involving
26 single metals slowed down learning and disrupted memory retrieval at 24 h. Combinations of
27 these metals induced additive negative effects on both processes, suggesting common pathways
28 of toxicity. Our results highlight the need to further assess the risks of metal pollution on
29 invertebrates.

30

31 **Introduction**

32

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

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

45 The deleterious effects of metals on humans (Tchounwou et al., 2012) and some model
46 animals (mice: Cobbina et al., 2015; flies: Doğanlar et al., 2014) are well-known. As, Cu, Pb
47 and other metals have neurotoxic effects that induce neural and neuromuscular alterations,
48 sensory impairments and many related forms of behavioural dysfunctions (Chen et al., 2016).
49 Deficits in cognition and memory have been reported for As (e.g. humans: Tolins et al., 2014;
50 mice: Tyler et al., 2018; Wu et al., 2006), Pb (e.g. mice: Anderson et al., 2016; humans: Mason
51 et al., 2014) and Cu (e.g. mice: Lamtai et al., 2020; Pal et al., 2013; flies: Zamberlan, 2020).

52 Recent studies showed that low doses of Pb (Monchanin et al., 2021a) and selenium (Se)
53 (Burden et al., 2016) also impair behaviour and cognition in honey bees, suggesting a
54 widespread impact on pollinators. So far, however, very little attention has been given to the
55 potential combined effects of co-exposure to different metals (Monchanin et al., 2021b).

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

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

80

81 **Materials and methods**

82

83 *Metal solutions*

84 Arsenic (NaAsO₂), lead (PbCl₂) and copper (CuCl₂·2H₂O) were purchased from Sigma-Aldrich
85 Ltd (Lyon, France) and diluted in 50% (w/v) sucrose solution. Control honey bees were fed

86 50% sucrose solution. Three concentrations of As were used (Table 1): a low concentration
 87 (0.13 μM) corresponding to the maximal permissible value in drinking water (0.01 mg.L^{-1})
 88 (Codex Alimentarius, 2015), a high concentration (0.67 μM) corresponding to half the maximal
 89 permissible value in irrigation water (0.1 mg.L^{-1}) (Ayers and Westcot, 1994), and an
 90 intermediate concentration (0.40 μM). This range of concentrations was reported in water
 91 sampled from polluted areas (e.g. mining sites) and in honey (Table S1). For Pb and Cu, we
 92 chose 0.27 μM (0.055 mg.L^{-1} of Pb and 0.017 mg.L^{-1} of Cu) so that the binary combinations (As
 93 0.13 μM + Cu 0.27 μM or As 0.13 μM + Pb 0.27 μM) could be compared to the As intermediate
 94 concentration (0.40 μM), and the tertiary combination (As 0.13 μM + Pb 0.27 μM + Cu 0.27
 95 μM) to the As high concentration (0.67 μM) (Table 1). These concentrations of Pb and Cu have
 96 also been reported in honey samples (Table S1). The mass consumed for As and the
 97 concentrations for Cu and Pb fell within sublethal ranges for the honey bee: the LD50 of
 98 elemental As for NaAsO_2 ranged from 0.330 to 0.540 $\mu\text{g/bee}$ (Fujii, 1980), the LC50 of Cu is
 99 72 mg.L^{-1} (Di et al., 2016) and of Pb is 345 mg.L^{-1} (Di et al., 2016).

100

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

Treatment	Molarity (μM)	Concentration (mg.L^{-1})			Ingestion of 5 μL (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

102

103 *Bee exposure to metals*

104 We collected honey bees (*Apis mellifera*) returning from foraging trips at the entrance of five
 105 different hives in mornings during August 2020. We anaesthetised the bees on ice and harnessed
 106 them in plastic tubes, secured with tape and a droplet of wax at the back of the head (Matsumoto
 107 et al., 2012). We tested all the bees for intact proboscis extension (PER) by stimulating their

108 antennae with 50% sucrose. We then fed the responding honey bees 5 μ L of 50% sucrose
109 solution (see Table 1), making sure they consumed the whole droplet, and left them to rest for
110 3 h in the incubator (temperature: $25\pm 2^\circ\text{C}$, humidity: 60%). Honey bees that did not respond to
111 the sucrose solution were discarded.

112

113 *Absolute learning*

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

130

131 *Long-term memory*

132 Only honey bees that had learnt the task were kept for the analysis of memory performance.
133 After conditioning, these honey bees were fed 15 μ L of 50% sucrose solution, left overnight in
134 the incubator, and fed another 5 μ L of sucrose solution the following morning. Three hours
135 after (24 h post-conditioning), we performed the retention test, consisting of three trials similar
136 to conditioning except that no sucrose reward was presented. In addition to the odour used
137 during the conditioning (CS), we presented two novel odours, in randomized order, to assess
138 the specificity of the memory: nonanal was expected to be perceived by honey bees similarly
139 to 1-nonanol, while 1-hexanol was expected to be perceived differently (Guerrieri et al., 2005).
140 We recorded the presence or absence (1/0) of a conditioned PER to each odorant at each
141 memory retention trial. We classified honey bees according to their response patterns: response

142 to the CS only, response to the CS and the similar odour (low generalization level), response to
143 all odours (high generalization level), no or inconsistent response.

144

145 *Statistics*

146 We analysed the data using R Studio v.1.2.5033 (RStudio Team, 2015). Raw data are available
147 in Dataset S1. We performed binomial generalised linear mixed-effects models (GLMM)
148 (package lme4; Bates et al., 2015), with hive and conditioning date as random factors and
149 treatment as fixed effect. Using the GLMMs, we evaluated whether molarity or treatment
150 impacted the initial response to antennal stimulation, the spontaneous response in the first
151 conditioning trial, the response in the last trial, the response to each odorant during the memory
152 test, the proportion of honey bees per response pattern in the retention test, and the survival at
153 24 h. Acquisition scores were standardised and compared with GLMMs using Template Model
154 Builder (Brooks et al., 2017). For all response variables, we compared (1) the treated groups to
155 the control, (2) groups exposed to concentrations of the same molarity (e.g. Med [As], [As+Cu]
156 and [As+Pb]), (3) the separate and joint effects of the treatments (e.g. Low [As], [Cu] and
157 [As+Cu]) in order to identify interactive effects (antagonistic, additive, synergistic).

158

159 **Results and discussion**

160

161 *Exposure to metals did not impact appetitive motivation*

162 The proportion of honey bees that responded to the initial antennal stimulation with sucrose
163 was similar among treatments (GLMM: $p > 0.05$). All honey bees responded in the other groups.
164 Therefore, treatment did not affect appetitive motivation or sucrose perception. Consistently
165 with our observations, the ingestion of similar concentrations of Pb and Cu had no effect on the
166 responsiveness to increasing concentrations of sucrose (Burden et al., 2019). By contrast, Di et
167 al. (2020) found that honey bees exposed to increasing concentrations of a mixture of Cu and
168 Cd exhibited a decreased ability to distinguish sucrose concentrations, but this may be
169 explained by the much higher (at least 600 times) concentrations used in that study. Thus, in
170 our conditions any impact on appetitive learning is unlikely due to a decreased motivation for
171 sucrose.

172

173 *Individual and joint exposures to metals reduced learning performance*

174 Two out of the 381 honey bees submitted to the absolute learning task spontaneously responded
175 to the first odour presentation and were therefore discarded. In all groups, the number of honey

176 bees showing the conditioned response increased over trials, thus showing learning (Fig. 1A).
177 However, fewer honey bees exposed to metals learned the task when compared to controls
178 (GLMM: $p < 0.05$, except for Low [As], $p = 0.082$). Accordingly, the acquisition scores of honey
179 bees from all treatments were lower than controls (Fig. 1B). Honey bees exposed to Med [As]
180 (GLMM: -0.610 ± 0.246 , $p = 0.013$), High [As] (GLMM: -0.639 ± 0.241 , $p = 0.008$) and
181 [As+Cu+Pb] (GLMM: -0.592 ± 0.244 , $p = 0.015$) had acquisition scores significantly lower than
182 controls. Honey bees exposed to solutions of [As+Pb] had similar acquisition scores to Med
183 [As] (GLMM: 0.299 ± 0.234 , $p = 0.201$), but honey bees exposed to [As+Cu] performed better
184 (GLMM: 0.596 ± 0.241 , $p = 0.013$). Honey bees exposed to High [As] and [As+Cu+Pb] exhibited
185 similar acquisition scores (GLMM: $p = 0.810$). We found no difference in the acquisition scores
186 and the proportions of learners between honey bees treated with a single metal and mixed
187 treatments (GLMM: $p > 0.05$), that would have indicated non-additive effects (i.e. antagonistic
188 or synergistic). Thus, exposure to metals significantly reduced learning performance, and
189 combined exposure appeared to exert simple additive deleterious effects.

190

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

199

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

201 To examine possible effects of metal exposure on memory retention, we tested the capacity for
202 long-term memory retention. Only honey bees that had learned the CS-US association at the
203 end of conditioning were tested, to evaluate retention levels independently of possible biases
204 due to slight variations in learning performance among treatments. 167 out of the 379 honey
205 bees submitted to the absolute learning task did not learn and were therefore discarded. We
206 found no effect of treatment on survival at 24 h (GLMM: $p > 0.05$). However, long-term memory
207 was significantly affected (Fig. 2). Overall, treated honey bees responded less to the learned
208 odorant (CS) than controls, as indicated by a significant effect of exposure to metals on retention
209 levels (GLMM: $p < 0.05$) (Fig. 2A). Yet, this decrease was not significant for honey bees

210 exposed to Med [As] (GLMM: -0.260 ± 0.628 , $p=0.679$) and High [As] (GLMM: -1.023 ± 0.570 ,
211 $p=0.073$). Finally, there was no clear dose effect on responses to the CS among treated groups
212 (GLMM: -0.576 ± 0.579 , $p=0.320$).

213 Yet, individual response patterns (Fig. 2B) revealed a loss of memory specificity. While
214 honey bees from all treatments responded equivalently to the similar odour (GLMM: $p>0.05$),
215 those exposed to higher doses responded more frequently to all odorants, indicating a higher
216 degree of response generalization (GLMM: 1.954 ± 0.775 , $p=0.012$). This was accompanied by
217 a significantly lower proportion of specific (CS-only) responses for honey bees exposed to [Pb]
218 (GLMM: -1.795 ± 0.690 , $p=0.009$), low [As] (GLMM: -1.313 ± 0.589 , $p=0.026$) and
219 [As+Cu+Pb] (GLMM: -1.200 ± 0.588 , $p=0.041$). Exposure also significantly increased the
220 frequency of inconsistent responses as compared to controls (GLMM: $p<0.05$). This was the
221 case for each individual treatment except for Med [As], $p=0.293$). Thus, exposure to metals had
222 a negative impact on memory performance at 24h. The analysis of individual response patterns
223 also revealed additive effects as they neither differ among groups exposed to solutions with the
224 same molarity, nor between single and mixed metal treatments (GLMM: $p>0.05$). Thus, most
225 treatments reduced memory performance at 24h.

226

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

232

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

234 Although many mechanisms of metal toxicity have not yet been elucidated, some points of
235 consensus are emerging from the literature. Firstly, interactions between metals can occur
236 outside the organism (including interactions with the environment (Grobela and Kowalska,
237 2020; Noyes and Lema, 2015)), and during uptake into the organism, leading to potentially
238 toxic processes of speciation, absorption, binding, transport and distribution (Wu et al., 2016).
239 Once metals enter an organism, they can induce, interact or inhibit a range of biological
240 responses and metabolic pathways. By mimicking other essential metals (Bridges and Zalups,
241 2005) or damaging the permeability of biological membranes (Rothschein, 1959), metals allow
242 the uptake or loss of other compounds in/out intracellular compartments (Viarengo, 1994).
243 Metals are known to disrupt signalling and calcium homeostasis (particularly important in

244 neurons) by interfering with the calcium channels (Bridges and Zalups, 2005; Chavez-Crooker
245 et al., 2001; Tamano and Takeda, 2011). This might lead to dysfunction and cytotoxicity due
246 to the disruption of cell signalling and calcium homeostasis. Genotoxicity (Doğanlar et al.,
247 2014) may be achieved through covalent binding to DNA (Brocato and Costa, 2013; Senut et
248 al., 2014). Eventually, oxidative stress and lipid peroxidation of the cell membrane may lead to
249 neuronal death. Additionally, metals in mixture could interact at target sites, but the potential
250 for modulation of that toxic impact is largely unknown (Svendsen et al., 2011). Metal mixtures
251 could change the bioavailability, toxicokinetic and toxicodynamic of each metal, that could
252 directly impact the toxicity towards the organism (Løkke et al., 2013). Based on these shared
253 mechanisms of toxicity that include oxidative stress (Nikolić et al., 2016; Zaman et al., 1995),
254 apoptosis (Raes et al., 2000) and interference with neurotransmitters (Nisbet et al., 2018), the
255 toxic effects of metal pollutants in mixtures may be expected to be additive (von Stackelberg
256 et al., 2013).

257 These effect may affect many aspects of neural activity and brain function in honey
258 bees, as in other species (Karri et al., 2016). Here, we focused on learning and memory of
259 olfactory cues because they play crucial roles in the behavioural ecology of honey bees and
260 other pollinators, for the identification of food resources. Our results in controlled laboratory
261 conditions suggest that exposure to sublethal combinations of toxic elements in the field might
262 alter individual foraging efficiency, and in turn jeopardize survival of pollinator populations.
263 Our findings call for further evaluation of the joint actions of metals (Meyer et al., 2015) to
264 better assess the risk they pose (Nys et al., 2018; Otitoloju, 2003) and better inform regulatory
265 framework (European Commission, 2012). Current risk assessment guidance mainly assesses
266 the effect of individual exposure, that fails to capture potential interactive effects. Hence, the
267 evaluation of metal mixture impacts and their modes of action needs to be developed (Sasso et
268 al., 2010). More generally, the study of the interactions between toxic metals and environmental
269 factors (Naqash et al., 2020) and their impacts on the toxicokinetic of other chemicals (EFSA
270 Scientific Committee et al., 2019) (e.g. pesticides (Sgolastra et al., 2018; Singh et al., 2017),
271 volatile organic compounds (Sasso et al., 2010) etc.) should be implemented in this integrated
272 research framework.

273

274 **Conclusion**

275 In summary, we demonstrated that arsenic, lead, copper or combinations of these metals, at
276 levels found in the environment, slow down appetitive learning and reduce long-term memory
277 specificity in honey bees. These metals show simple additive effects as we found no differences

278 between different solutions of the same molarity suggesting possible non-linear effects
279 (synergism or antagonism). Thus, regarding effects on learning and memory, concentration
280 seem to be more important than identity of any specific metal. Since learning and memory of
281 olfactory cues play crucial roles in the behavioural ecology of honey bees, acute exposure to
282 metal pollutants mixtures could impair fundamental hive function and population growth.

283

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286

287 **Competing interests**

288 The authors declare no competing or financial interests.

289

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297

298 **Data availability**

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

300

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