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# 1 **Environmental exposure to metallic pollution** 2 **impairs honey bee brain development and cognition**

3  
4 Running title: Metallic pollution impairs bee cognition

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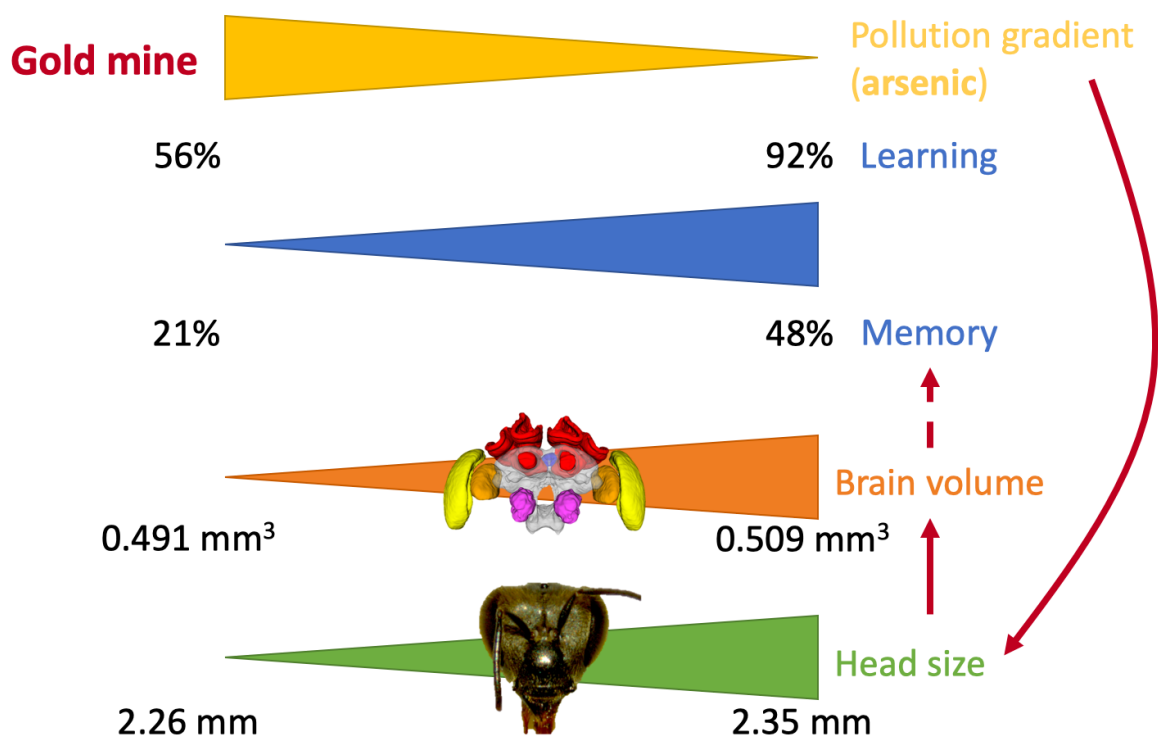
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## 39 **Highlights**

- 40 • We collected honey bees in the vicinity of the former world's largest gold mine, an area  
41 highly polluted with arsenic.
- 42 • Bees closer to the mine had reduced cognitive performance and developed both smaller  
43 heads and smaller brain regions.
- 44 • These sublethal effects of metal pollution on cognition are a major threat for bee  
45 populations and pollination.

46

47 **Graphical abstract**



48

## 49 **Abstract**

50 Laboratory studies show detrimental effects of metal pollutants on invertebrate behaviour and  
51 cognition, even at low levels. Here, we report a field study of Western honey bees sampled from  
52 an historic mining site heavily contaminated with metal and metalloid pollution, particularly  
53 arsenic. We analysed more than 1,000 bees from five apiaries within 11 km of the world's largest  
54 gold mine in Southern France. Bees collected close to the mine exhibited decreased olfactory  
55 learning and memory performances and developed smaller heads. Three-dimensional scans of bee  
56 brains showed that the olfactory centres of bees sampled close to the mine were also smaller,  
57 indicating impairment of brain. Our study raises serious concerns about the health of honey bee  
58 populations in metal-polluted areas and illustrates how standard cognitive tests can be used for  
59 risk assessment.

60

## 61 **1. Introduction**

62

63 Metals and metalloids are naturally found at low levels in the Earth crust and water as a result of  
64 volcanic eruptions and soil erosion. However, over the past century, human activities have raised  
65 environmental levels far above this baseline (Han et al., 2003). In particular, mining operations  
66 (Demková et al., 2017) and metal smelters have elevated concentrations of toxic pollutants such  
67 as arsenic, lead and mercury in superficial soils (Su et al., 2014) and water (Nordstrom, 2002)  
68 across large areas worldwide, exposing people and the wildlife to hazardous concentrations  
69 through inhalation of air and dust, and contaminated food, water, and soil (ATSDR, 2019). In  
70 humans, metals bind to proteins and enzymes, disrupting their activity and provoking cellular  
71 damage. They can cause neurotoxicity, oxidative stress, and carcinogenesis (Azeh Engwa et al.,  
72 2019), leading to sublethal cognitive effects, such as sensory impairments, mood disorders or  
73 learning and memory deficits (Sankhla et al., 2016; Wright and Baccarelli, 2007). However, the  
74 consequences on the wildlife are much less understood.

75 Pollinators are key sentinel species that are exposed to trace metals (Bromenshenk et al.,  
76 1985) in plant nectar and pollen (Krunić et al., 1989) and water (Li et al., 2020). In honey bees,  
77 these elements contaminate honey (Bastías et al., 2013), wax (Tlak Gajger et al., 2016), propolis  
78 (Maragou et al., 2017) and are ultimately ingested by the larvae (Cozmuta et al., 2012). Honey  
79 bees seem unable to detect field-realistic concentrations of metals (Monchanin et al., 2022), and  
80 controlled exposure to metallic pollutants (alone or combined) leads to developmental and  
81 cognitive deficits (Burden et al., 2016; Di et al., 2016; Monchanin et al., 2021a, 2021b). Contact  
82 with high environmental levels of metallic pollutants may thus have severe consequences for  
83 population growth and the associated pollination services.

84 Here, we explored the sublethal effects of environmental exposure of honey bee colonies  
85 to trace metal pollutants in the field. We sampled foragers from apiaries along a pollution gradient  
86 surrounding a former gold mine (Salsigne, France; Fig. 1A-B.) and examined their morphological  
87 development, brain organisation and cognition. During its exploitation (1873-2004) the site  
88 became contaminated mostly with arsenic, but also with lead, cadmium, copper, nickel and zinc.  
89 This contamination has persisted even after the closure and partial remediation of the site (Khaska  
90 et al., 2018, 2019). As the contaminant levels are well above the international permissible limits  
91 (for arsenic: up to 76 times higher in irrigation water (Guerin et al., 2000), 290 times higher in soil  
92 (Koechler et al., 2015)), the Salsigne area is considered as “critically polluted” (Pérez and Valiente,  
93 2005). Hence monitoring human populations and wildlife in this area is of great importance.

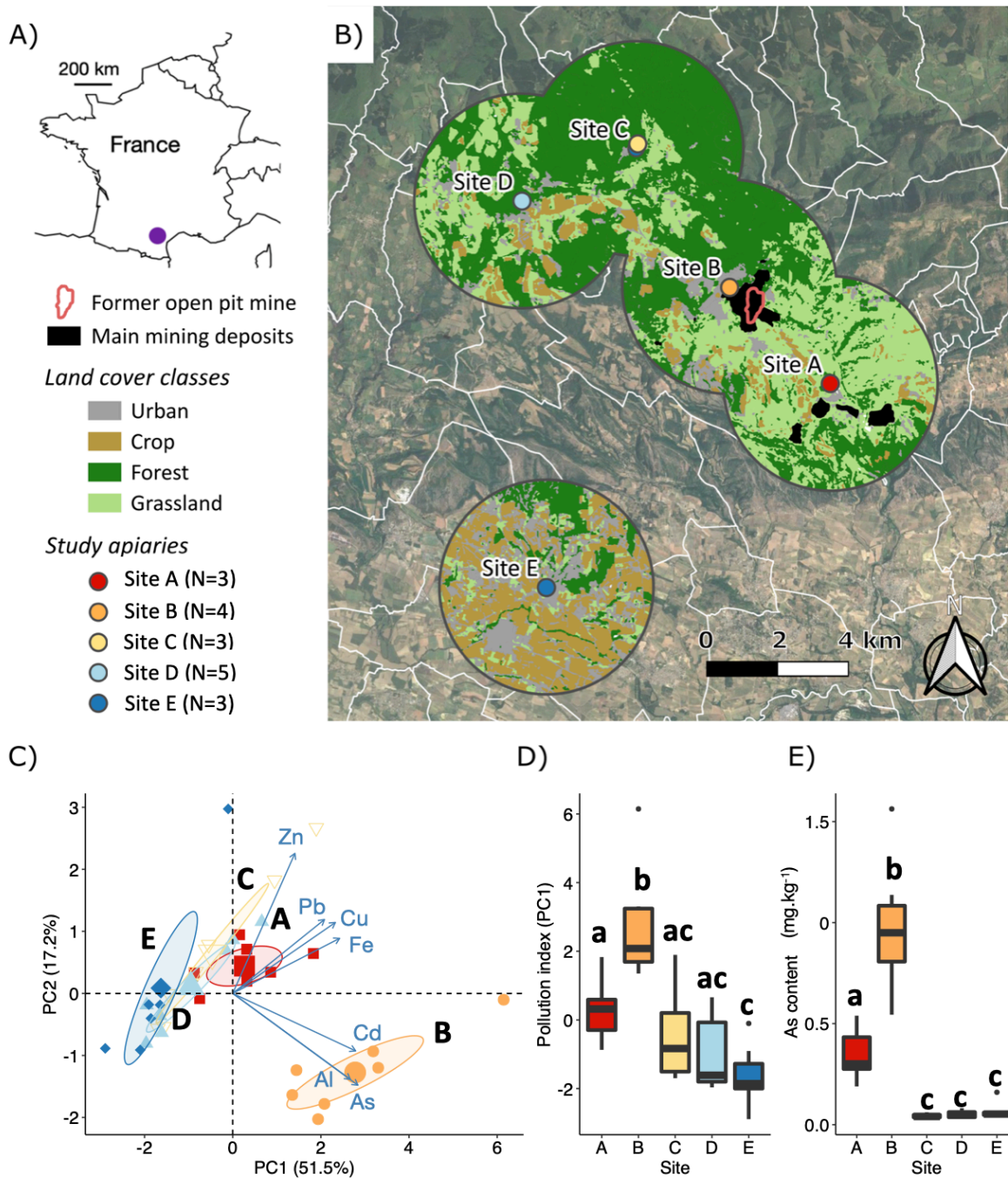
94

## 95 **2. Results**

### 96 *2.1. Bees closer to the mine accumulated higher levels of metals*

97 We sampled bees from five apiaries within 11 km of the former gold mine, at different distances  
98 along an expected gradient of environmental metallic pollution (Fig. 1A-B). First, we verified this  
99 gradient by measuring trace elements (Al, As, Cd, Cu, Fe, Pb, Zn) in the bodies of bees using  
100 Inductively Coupled Plasma - Mass Spectrometry. To provide a single measure of overall pollution

101 for each site, we performed a principal component analysis (PCA) using all metal concentrations  
102 ( $\text{mg}\cdot\text{kg}^{-1}$  fresh matter) in bee bodies (Fig. 1C; Table S1) and used the first principal component  
103 (PC1) as a pollution index: high values corresponded to high contamination of bees with all the  
104 metals (Fig. 1D). Pollution indices greatly different between sites ( $F_{4,30}=13.421$ ,  $p<0.001$ ; Table  
105 S1). Bees from site B had the highest metal content (mean score on PC1  $\pm$  s.e.m:  $2.778 \pm 0.632$ ),  
106 followed by bees from site A ( $0.266 \pm 0.350$ ), while bees from site E had the lowest content ( $-$   
107  $1.630 \pm 0.338$ ) (Fig. 1D). Because the area is known to be highly polluted with arsenic, we also  
108 compared its levels across the sites and again found significant variation ( $F_{4,30}=46.33$ ,  $p<0.001$ ).  
109 Bees from sites A ( $0.350 \pm 0.047 \text{ mg}\cdot\text{kg}^{-1}$ ) and B ( $0.977 \pm 0.122 \text{ mg}\cdot\text{kg}^{-1}$ ) contained higher levels  
110 of As than to bees from the three sites further away (Site C:  $0.043 \pm 0.005 \text{ mg}\cdot\text{kg}^{-1}$ ; Site D:  $0.052$   
111  $\pm 0.007 \text{ mg}\cdot\text{kg}^{-1}$ ; Site E:  $0.067 \pm 0.016 \text{ mg}\cdot\text{kg}^{-1}$ ) (pairwise comparisons:  $p<0.01$ ; Fig. 1E). This  
112 confirms sites A and B were overall more contaminated than sites C-D, and in particular with  
113 arsenic.



114

115 **Figure 1: Study sites and metal pollution levels.** **A)** Former gold mine located in the South of  
 116 France. **B)** Location of the five apiaries (N is the number of hives), with the main land cover classes  
 117 displayed within a radius of 3 km around each apiary. The major mining deposits in the study area  
 118 (according to (GEODERIS, 2012)) and the ore processing site are also shown. White lines indicate  
 119 municipal boundaries. **C)** Principal component analysis (PCA) plot showing the distribution of  
 120 analysed pools of bees (N=7 replicates per site) along the two principal components (PC1, PC2)  
 121 and the relationship among the metals measured. 95% confidence ellipses of the mean are  
 122 displayed for each site. **D)** Pollution index (score on PC1). **E)** Arsenic content in the bodies of



123 bees ( $\text{mg}\cdot\text{kg}^{-1}$  fresh matter). Boxplots show median value (intermediate line) and quartiles (upper  
124 and lower lines) for each measurement. Letters (a-c) indicate significant differences between sites  
125 (LMM).

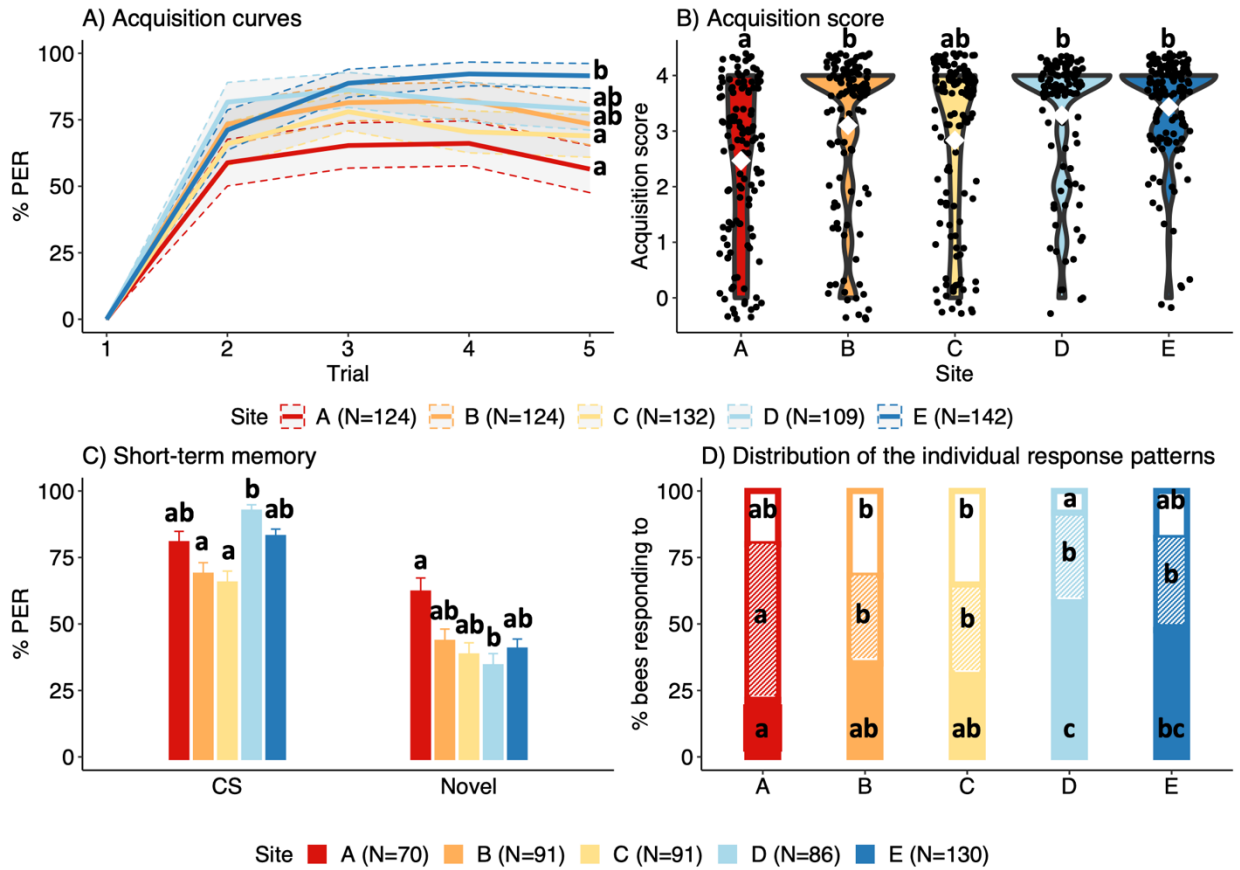
126

## 127 *2.2. Bees closer to the mine showed lower learning performances*

128 Next, we conducted cognitive experiments. We trained bees caught in each of the five apiary to  
129 associate an odorant to a sucrose reward using olfactory proboscis extension response (PER)  
130 conditioning (Matsumoto et al., 2012). Such associative learning is critical for bees to recognise  
131 flowers, specialise on them and forage efficiently (Giurfa, 2013).

132 Prior to conditioning, we tested all bees for an intact proboscis extension reflex upon  
133 antennal stimulation with 50% (w/v) sucrose solution. The proportions of responding bees were  
134 similar among sites (GLMM:  $p=0.993$ ) (Site A: 97.8%,  $N=136$ ; Site B: 98.5%,  $N=135$ ; Site C:  
135 97.9%,  $N=140$ ; Site D: 98.3%,  $N=121$ ; Site E: 100%,  $N=144$ ). Therefore, site location did not  
136 affect appetitive motivation nor sucrose perception by bees.

137 We then trained 673 bees in a five-trial absolute learning task, during which we recorded  
138 conditioned responses to an odour. A similar small proportion of bees from each site spontaneously  
139 responded at the first odour presentation and was therefore discarded ( $N=42$ ; GLMM:  $p=0.336$ ).  
140 When considering the 631 remaining bees, learning was observed in all sites (Fig. 2A). However,  
141 by the last conditioning trial, a lower proportion of bees from sites A (GLMM:  $-2.130 \pm 0.777$ ,  
142  $p=0.006$ ) and C ( $-1.572 \pm 0.780$ ,  $p=0.044$ ) had learned the task, as compared to those from the  
143 farthest site E (A: 56.45%, B: 73.39%, C: 68.94%, D: 78.90%, E: 91.55%). When analysed  
144 individually (Fig. 2B), we found that bees from site A had lower acquisition scores (total number  
145 of conditioned responses across all conditioning trials) than bees from site B (GLMM:  $-1.018 \pm$   
146  $0.477$ ,  $p=0.033$ ), site D ( $-1.055 \pm 0.489$ ,  $p=0.031$ ) and site E ( $-1.287 \pm 0.581$ ,  $p=0.027$ ). There was  
147 no effect of the odorant used for conditioning (limonene or eugenol) on bee responses (GLMM:  
148  $p=0.141$ ). Thus, bees from apiaries closer to the former mine had lower learning performance.



149

150 **Figure 2: Learning and short-term memory.** **A)** Acquisition curves show changes in the  
 151 percentages of bees displaying conditioned proboscis extension responses (PER) over the five  
 152 trials. Areas delimited by the dashed lines show 95% confidence intervals. Letters indicate  
 153 significant differences in response proportions at the last trial between sites (GLMM). **B)** Violin  
 154 plots of individual acquisition scores (sum of conditioned responses for each bee, white diamonds  
 155 display mean values). Letters indicate significant differences between sites (GLMM). **C)**  
 156 Percentages of responses to the two odours during the one-hour memory retention test (mean  $\pm$   
 157 s.e.m). Significant differences in response levels for each odour, obtained from GLMM, are  
 158 displayed. **D)** Distribution of bees according to their individual response patterns during the  
 159 memory test: CS-specific responses (coloured), generalized responses to both odours (hatched)  
 160 and inconsistent or absent responses (white). Letters indicate significant differences between sites  
 161 for each response pattern.

162

163 *2.3. Bees closer to the mine showed reduced short-term memory specificity*

164 Using the same bees, we next assessed one-hour memory recall by recording conditioned PER  
165 response to odorants, without sucrose reward. In addition to the odorant used during training  
166 (conditioning stimulus, CS), we tested bees with a novel odorant to evaluate the specificity of  
167 memory (Urlacher et al., 2016). Among bees that had effectively learnt the task (N=468), one-  
168 hour memory recall of the odour-reward association differed between sites (GLMM:  $p=0.002$ ).  
169 Bees from sites B and C responded less to the CS (68.13% and 64.85% respectively) than bees  
170 from site D (GLMM:  $-1.591 \pm 0.504$ ,  $p=0.016$  and  $-1.795 \pm 0.500$ ,  $p=0.003$  respectively).  
171 Additionally, bees from site A responded more to the novel odour than bees from sites D (GLMM:  
172  $1.196 \pm 0.411$ ,  $p=0.036$ ) (Fig. 2C). The individual response patterns (Fig. 2D) showed a clear effect  
173 of site location on the proportion of bees displaying CS-specific memory (GLMM:  $p=0.005$ ) (A:  
174 21.43%, B: 35.16%, C: 30.77%, D: 58.14%, Site E: 47.69%), with significantly fewer specific  
175 responses recorded in bees from site A than from sites D and E (GLMM: resp.  $-1.625 \pm 0.477$ ,  
176  $p<0.001$ ;  $-1.210 \pm 0.477$ ,  $p=0.011$ ); and in bees from site B than from sites C and D (resp.  $-1.162$   
177  $\pm 0.441$ ,  $p=0.008$  and  $-0.898 \pm 0.422$ ,  $p=0.033$ ). Bees from site A also showed higher  
178 generalization of their response to the novel odour as compared to those from all other sites  
179 (GLMM:  $p=0.019$ ; A: 58.57%, B: 32.97%, C: 34.07%, D: 33.72%, E: 34.62%). Thus, the closer  
180 the bees to the mine, the lower their memory specificity.

181

#### 182 *2.4. Bees closer to the mine showed reduced long-term memory*

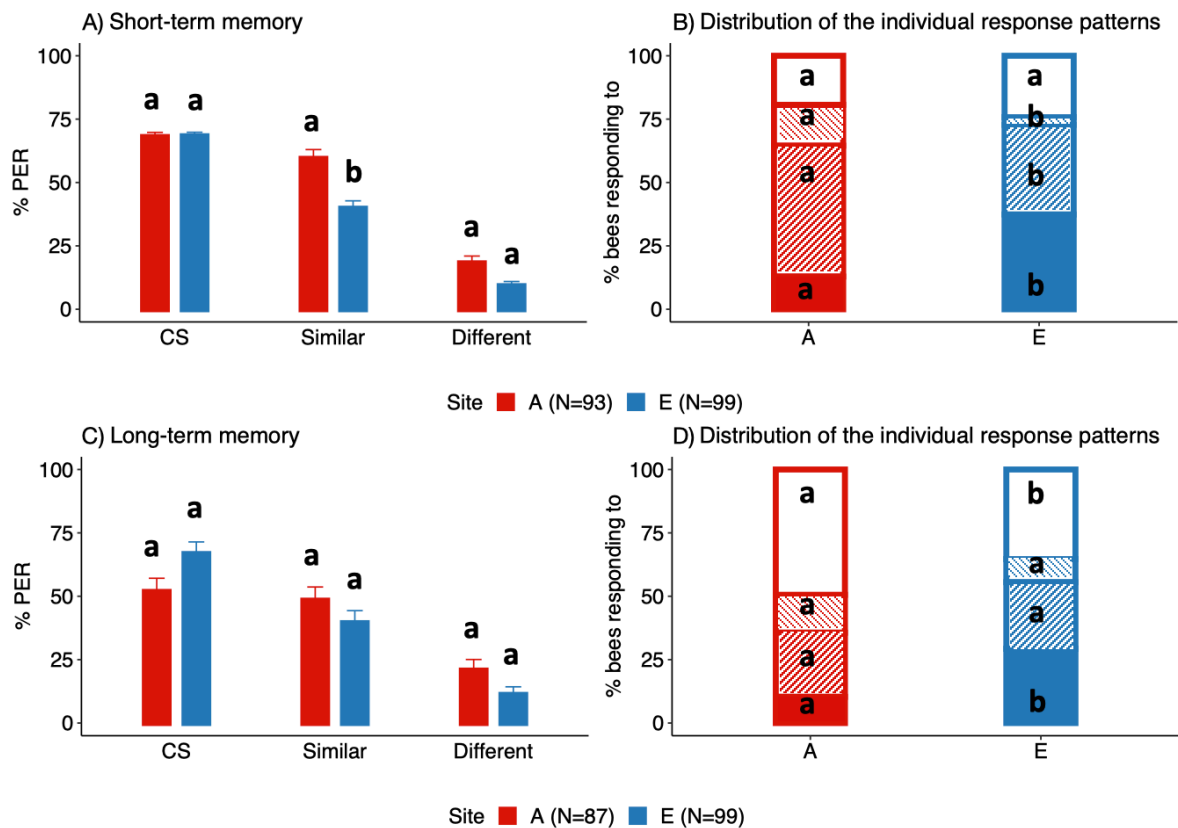
183 To further investigate memory impairments, we conducted a second experiment on bees sampled  
184 in contrasting conditions of metallic pollution: site A (high contamination) and site E (low  
185 contamination). We compared the performances of bees in short-term (1h) and long-term (24h)  
186 memories, the latter guiding foraging decisions and participating in communication between bees  
187 (Farina et al., 2005). To better capture the effect on olfactory generalization, we used two novel  
188 odours in addition to the CS, according to their degree of structural similarity: one perceptually  
189 similar (low generalization level) and one dissimilar (high generalization level) (Urlacher et al.,  
190 2016).

191 Like in the first experiment, the proportions of bees initially exhibiting intact PER was  
192 similar between both sites (GLMM:  $p=1$ ; A: 96.17%, E: 100%). After five conditioning trials, a  
193 lower proportion of bees from site A had learnt the task compared to site E (GLMM:  $-2.197 \pm$   
194  $0.866$ ,  $p=0.011$ ) (A: 62.00%, E: 92.52%). While they also exhibited a lower mean acquisition score  
195 (mean  $\pm$  s.e.m: A:  $2.27 \pm 0.14$ , E:  $3.22 \pm 0.11$ ), the difference was not significant (GLMM:  $-0.848$   
196  $\pm 0.526$ ,  $p=0.107$ ).

197 In the short-term memory retrieval test (Fig. 3A-B), bees from both sites, that had  
198 effectively learnt the task (N=192), responded similarly to the CS (A: 67.94%; E: 68.25%)  
199 (GLMM:  $0.123 \pm 0.579$ ,  $p=0.846$ ). However, bees from site A had more generalization responses  
200 to novel odours (Fig. 3A), although only significantly to the similar one (GLMM:  $1.3231 \pm 0.305$ ,  
201  $p<0.001$ ) (similar: 69.89% vs. 40.40%; dissimilar: 16.13% vs. 3.03%). Patterns of individual  
202 responses confirmed the effect of the proximity to the mine on the selectivity of responses (Fig.  
203 3B). Bees from site A displayed less CS-specific responses (12.90% vs. 37.37%; GLMM:  $-1.400$   
204  $\pm 0.387$ ,  $p<0.001$ ), generalised more to other odours, both similar (51.61% vs. 35.35%; GLMM:  
205  $0.668 \pm 0.295$ ,  $p=0.024$ ) and dissimilar (16.13% vs. 3.03%; GLMM:  $1.848 \pm 0.795$ ,  $p=0.020$ ).  
206 Thus, here also, bees closer to the mine exhibited higher levels of generalization and less specific  
207 memory.

208 When tested for long-term memory (Fig. 3C-D), bees from both sites responded in similar  
209 proportions to all three odours (Fig. 3C): the CS (A: 51.72%, E: 66.67%; GLMM:  $-0.630 \pm 0.482$ ,  
210  $p=0.191$ ), the similar odour (A: 48.28%, E: 39.39%; GLMM:  $0.352 \pm 0.411$ ,  $p=0.392$ ) and the  
211 dissimilar one (A: 20.69%, E: 11.11%; GLMM:  $0.803 \pm 0.625$ ,  $p=0.199$ ). However, bees from site  
212 A displayed less CS-specific responses (Fig. 3D; A: 10.34%, E: 29.29%; GLMM:  $-0.865 \pm 0.414$ ,  
213  $p=0.037$ ) and more inconsistent or absent responses (A: 49.43%, E: 35.35%; GLMM:  $0.850 \pm$   
214  $0.412$ ,  $p=0.039$ ). Generalization responses were equally frequent in bees from both sites, to the  
215 similar odour (A: 25.29%, E: 26.26%; GLMM:  $-0.224 \pm 0.339$ ,  $p=0.508$ ) as well as to the  
216 dissimilar one (A: 14.94%, E: 9.09%; GLMM:  $0.469 \pm 0.696$ ,  $p=0.501$ ). Therefore, a shorter

217 distance to the mine was associated with lower individual long-term memory specificity, as  
 218 observed for short-term memory.  
 219



220

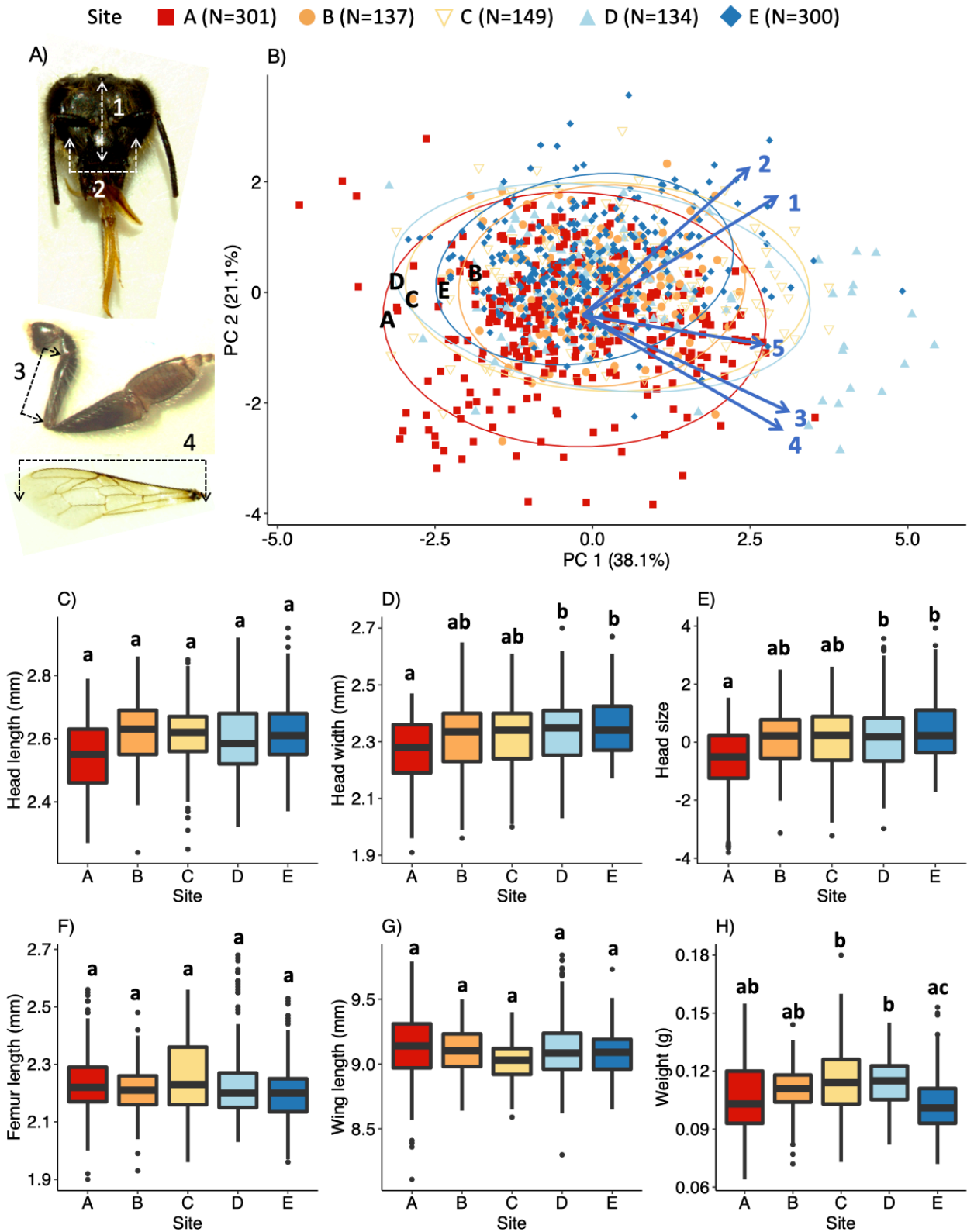
221 **Figure 3: Short-term and long-term memory.** **A)** Percentages of responses to the three odours  
 222 during the one-hour memory retention test (mean  $\pm$  s.e.m). Significant differences in response  
 223 levels for each odour are displayed (GLMM). **B)** Distribution of bees according to their individual  
 224 response patterns during the short-term memory test: CS-specific responses (coloured),  
 225 generalized responses to the similar odour (dense hatches), generalized responses to both similar  
 226 and dissimilar odours (hatched), and inconsistent or no responses (white). Letters indicate  
 227 significant differences between sites for each response pattern. **C)** Percentages of responses to the  
 228 three odours during the 24 h-memory retention test (mean  $\pm$  s.e.m). Significant differences in  
 229 response levels for each odour are displayed (GLMM). **D)** Distribution of bees according to their  
 230 individual responses during the long-term memory test, as in B. Letters indicate significant  
 231 differences between sites for each response pattern.

232

## 233 2.5. Bees closer to the mine had smaller heads

234 To assess the potential developmental impact of mine proximity on bee development, we measured  
235 five morphological parameters (Fig. 4A) on all the bees collected in the two experiments  
236 (N=1021). We assessed the effect of site location on overall morphology with a PCA including all  
237 parameters (Fig. 4B; Table S2). Two PCs discriminated overall larger bees *vs.* smaller bees (PC1)  
238 and bees with large and wide heads *vs.* short wing and femur (PC2) (PERMANOVA: Pseudo-  
239 F=10.923, p=0.001). Bees from site A were morphologically different from those from all other  
240 sites (pairwise PERMANOVA: p=0.01 for all comparisons). Their heads were not longer than  
241 those of other bees ( $F_{4,12.498}=1.224$ , p=0.354; Fig. 4C), but significantly narrower than in sites D  
242 and E (resp. LMM:  $-0.080 \pm 0.034$ , p=0.037 and  $-0.084 \pm 0.037$ , p=0.045; Fig. 4D). Head width  
243 and length measures were thus collapsed into the first component of another PCA (explaining 68%  
244 of the variance) that we used as a proxy of the head size (Table S3). Bees from the closest site (A)  
245 had overall smaller heads than bees from the farthest sites (D and E) (resp. LMM:  $-0.940 \pm 0.414$ ,  
246 p=0.044 and  $-1.011 \pm 0.455$ , p=0.049; Fig. 4E). However, bees from all sites exhibited similar  
247 femur length ( $F_{4,12.565}=0.311$ , p=0.866; Fig. 4F), wing length ( $F_{4,12.683}=0.336$ , p=0.849; Fig. 4G)  
248 and body weight ( $F_{4,12.498}=3.13$ , p=0.054; Fig. 4H). This means the bees closest to the mine had  
249 smaller heads but no overall smaller bodies.

250



251

252 **Figure 4: Morphometric measurements of bees.** A) Details of the parameters measured: (1)  
 253 head length, (2) head width, (3) femur length, (4) wing length, (5) body weight (not shown). B)  
 254 Principal component analysis (PCA) plot showing the distribution of individuals along the two  
 255 principal components (PC1, PC2) and the relationship among the morphometric measures (same  
 256 number code as in A). 95% confidence ellipses of the mean are displayed for each site. C) Head

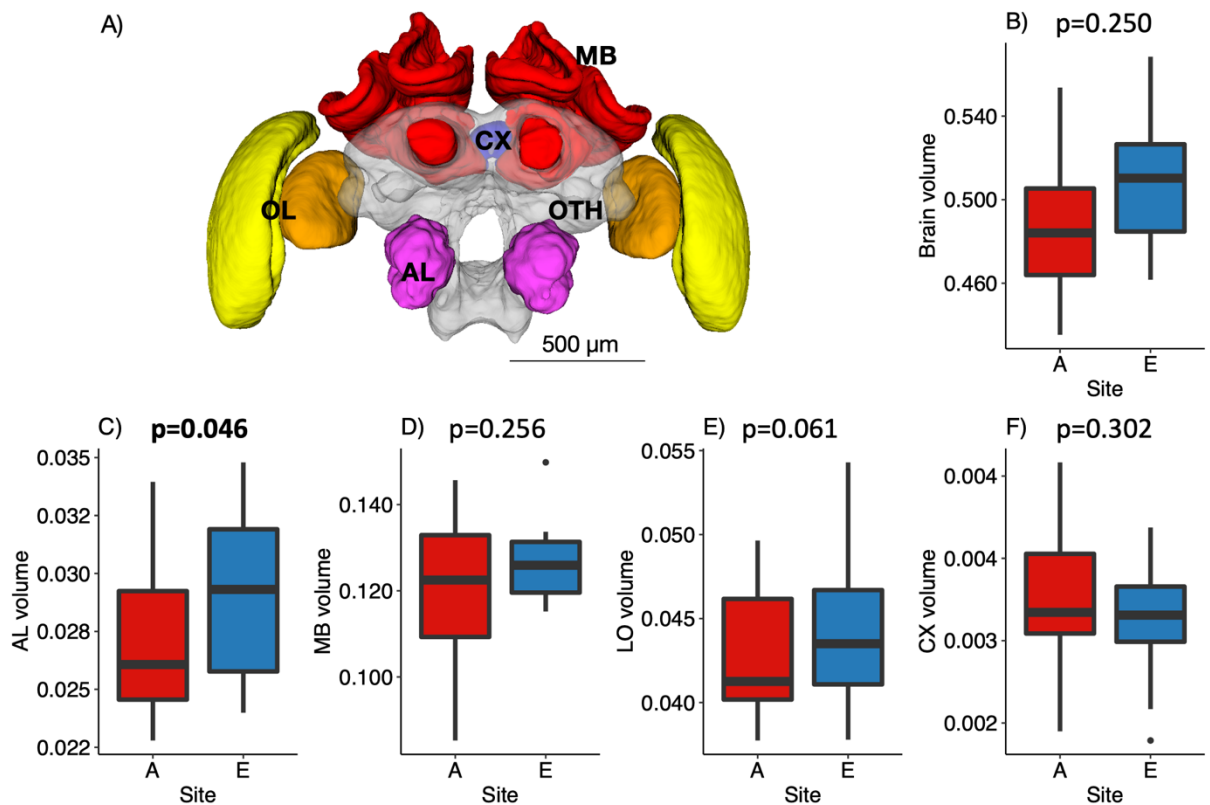
257 length. **D)** Head width. **E)** Head size. **F)** Femur length. **G)** Wing length. **H)** Body weight. Boxplots  
258 show median value (intermediate line) and quartiles (upper and lower lines) for each measurement.  
259 Letters indicate significant differences between sites (LMM).

260

## 261 *2.6. Bees closer to the mine had smaller antennal lobes*

262 Given the observed variations in head size among bees from different sites, we finally assessed  
263 whether proximity to the mine might alter the volume of the brain or of specific neuropils related  
264 to learning and memory deficits (Fig. 5). We quantified volumes from 3D reconstructions of the  
265 brain based on microtomography scanning (Fig. 5A) obtained from a subset of bees from sites A  
266 and E used in the second behavioural experiment (see Fig. 3). Total brain volume was positively  
267 correlated with head size ( $r=0.37$ ,  $p=0.023$ ), but did not differ between sites A and E (Fig 5B).  
268 However, we found differences at the level of individual neuropils. In particular, the antennal lobes  
269 (ALs), i.e. the peripheral brain structures involved for olfactory learning and memory (Giurfa and  
270 Sandoz, 2012), were significantly smaller in bees from site A than in bees from site E (Fig. 5C).  
271 By contrast, none of the other major neuropils of the bee brain (mushroom bodies MBs, optic lobes  
272 OLs, central complex CX) varied between sites (Fig. 5D-G). This suggests the cognitive olfactory  
273 deficits observed in bees sampled near the mine are linked to impaired development of their ALs.  
274 However, we did not find any influence of overall AL volume on the proportion of learners  
275 (GLMM:  $1.116 \pm 0.855$ ,  $p=0.192$ ) nor the acquisition score (GLMM:  $0.239 \pm 0.371$ ,  $p=0.519$ ) in  
276 this small subset of bees (N=38).





277

278 **Figure 5: Brain area volumes (mm<sup>3</sup>) of bees from site A (N=18) and E (N=20).** **A)** Example of  
 279 a reconstructed brain (frontal view) showing antennal lobes (AL), mushroom bodies (MB), optic  
 280 lobes (OL), central complex (CX). **B)** Total brain. **C)** Antennal lobes (primary integration centers  
 281 involved in olfactory learning and memory). **D)** Mushroom bodies (secondary integration centers  
 282 involved in multi-sensory learning and memory). **E)** Optic lobes (processing of visual  
 283 information). **F)** Central complex (involved in navigation). Statistical comparisons for the neuropil  
 284 volume between sites were obtained with p-values from LMM. Significant p-values are displayed  
 285 in bold.

286

### 287 **3. Discussion**

288 Recent studies show controlled exposure to heavy metals impacts honey bee health and cognitive  
 289 performance suggesting severe consequences for populations in the field (Burden et al., 2016;  
 290 Monchanin et al., 2021a, 2021b). However no such assessment had yet been made. Here we  
 291 demonstrated the relationship between environmental metal contamination and impaired

292 behaviour in the field using cognitive assays on honey bees sampled along a gradient of metallic  
293 pollution. Bees exposed to higher levels of metals exhibited poorer olfactory learning abilities and  
294 memory specificity. This was associated with decreased head size and reduced volumes of brain  
295 regions supporting olfactory learning. Since functional learning and memory are crucial to the  
296 behavioural and chemical ecology of bees, individuals exposed to metal pollution may be less  
297 efficient at foraging and interacting socially, ultimately jeopardizing the capacity of the colony to  
298 feed, grow and survive.

299 Forager bees closer to the mine, and thus exposed to higher levels of arsenic and other  
300 pollutants, developed smaller heads, with smaller antennal lobes, than those from the most distant  
301 site. These neurodevelopmental impairments are reminiscent of those observed in mammals  
302 following exposure to arsenic (Tolins et al., 2014; Tyler et al., 2018; Wu et al., 2006). They are  
303 also consistent with data from other insects. When feeding on arsenic-contaminated food,  
304 grasshoppers exhibited a decreased body weight (Rathinasabapathi et al., 2007), and moths had  
305 reduced larval survival and a longer pupal stage (Andrahennadi and Pickering, 2008). Oribatid  
306 mites collected along a pollution gradient where arsenic was recorded showed increasing incidence  
307 and severity of leg deformities towards the pollution source (Eeva and Penttinen, 2009). Ants  
308 sampled along a pollution gradient of zinc and cadmium developed smaller heads with increasing  
309 pollution levels (Grześ et al., 2015), and so did midges fed with cadmium or copper (Martinez et  
310 al., 2003). Exposure to stressors during development such as infestation by *Varroa* ectoparasites  
311 (Belaïd et al., 2017) or chronic exposure to lead (Monchanin et al., 2021a) can reduce bee head  
312 growth, and brain growth, e.g. pesticides (Smith et al., 2020). The effects of arsenic exposure on  
313 brain development are thus likely to contribute to the reduced cognitive abilities observed in our  
314 results, since bees exposed to increasing environmental levels show more severe growth defects  
315 as well as with learning and memory deficits. Previous work already pointed out that bees perform  
316 less well in olfactory tasks when they have smaller heads, as a result of natural interindividual size  
317 variability (Gronenberg and Couvillon, 2010; Monchanin et al., 2021a). The reduced head size,  
318 and specifically ALs volume, associated to metal exposure might explain the impaired olfactory

319 learning, as these primary olfactory centres support olfactory discrimination and learning (Menzel  
320 et al., 1996). We found no effect on the size of MBs, known to support olfactory memory retrieval  
321 (Menzel, 2001), despite the observed memory impairments correlating to contamination exposure.  
322 This suggests that the lower memory specificity in more exposed bees might be the consequence  
323 of altered coding of odorants in the ALs during learning. Yet, we do not exclude that exposure to  
324 higher contamination levels may have a subtler impact on MBs, e.g. by affecting synaptic  
325 connectivity (Cabirol et al., 2018) with possible consequences on memory specificity (Groh and  
326 Rössler, 2020), even without changes in overall volume. Overall, arsenic exposure is known to  
327 affect neural functions, in particular by altering various neurotransmitter levels in mammals (Xi et  
328 al., 2010): it can lead to neurodevelopmental and cognitive disorders, including learning and  
329 memory impairments (reviewed in (Tolins et al., 2014)). Altered GABA signalling might affect  
330 odour discrimination (Mwilaria et al., 2008) and memory specificity (Hosler et al., 2000), and  
331 altered amine levels, in particular octopamine, might affect acquisition levels (Rein et al., 2013).  
332 Because learning and memory are critical processes used by honey bees to find, identify and  
333 exploit environmental resources, bees foraging in this area are exposed to high levels of arsenic  
334 that might prove detrimental to their learning and/or memory abilities.

335         Although the impacts of contamination around the former gold mine of Salsigne have been  
336 little studied, our conclusions are consistent with epidemiological data obtained in the area,  
337 showing an excessive mortality rate among mine workers (Simonato et al., 1994) and arsenic-  
338 specific cancer attributed to environmental contamination (Dondon et al., 2005), but also  
339 significant amounts of bioaccumulated arsenic in small mammals (Drouhot et al., 2014). A citizen  
340 science project carried between 1994 and 1997 found a significant accumulation of arsenic (up to  
341  $0.69 \text{ mg.kg}^{-1}$ ) and lead (up to  $0.95 \text{ mg.kg}^{-1}$ ) in the pollen collected by honey bees (Oulès, 1998).  
342 These levels of metals fall within the range of doses that cannot be perceived by honey bees and  
343 are readily ingested in laboratory assays (Monchanin et al., 2022). Hence, bees could be all the  
344 more at risk because they cannot avoid such concentrations. Additionally, here foragers collected  
345 around the mine accumulated amount of arsenic ( $0.350 \text{ mg.kg}^{-1}$ ) below levels reported in other

346 studies, e.g. in Italy (0.367 mg.kg<sup>-1</sup>; (Zarić et al., 2022)), in the US (1.707 mg.kg<sup>-1</sup>; (Fisher, 1984))  
347 or in Serbia (0.996; (Krunić et al., 1989)). Yet, our results indicate a marked effect, and call for  
348 more studies to include simple behavioural and morphometric indicators as a powerful way to  
349 characterize sublethal impacts on pollinators foraging in polluted environments.

350

## 351 **4. Materials and Methods**

### 352 *4.1. Field sites*

353 This study was carried out in 2020 in the vicinity of a former gold mine located in Salsigne, France  
354 (43°18'41''N, 2°22'44''E; Fig. 1A-B). The ores processing, to extract mainly gold and silver, led  
355 to major contamination by arsenic in the surroundings of the industrial plant, in soils (Drouhot et  
356 al., 2014) and water (Guerin et al., 2000), far beyond international maximal permissible limits (for  
357 arsenic: <0.2ppm in food and <0.01ppm in drinking water (Codex Alimentarius, 2015), <0.1ppm  
358 in irrigation water (Ayers and Westcot, 1994), <20ppm in soil (de Vries et al., 2003)). Successive  
359 floods (Gaume et al., 2004; JO Sénat, 2019) contributed to spreading metal pollutants in the valley,  
360 raising public health concerns supported by alarming arsenic levels measured from children in the  
361 area (ARS, 2019). In collaboration with beekeepers, five apiaries were selected within 11 km of  
362 the former gold mine (sites A-E in Fig. 1, Table 1) and have been settled for at least two months  
363 before the beginning of the experiment. Site A was the closest to the former ore processing site. It  
364 was therefore considered as the most polluted due to release of slag, fumes and dust (Drouhot et  
365 al., 2014). Site B was closer to the open mine cast with underground operations, and was also  
366 characterized by high arsenic pollution (Khaska et al., 2015). Both sites were in the vicinity of  
367 main mining deposits (Fig. 1). Sites A to D were located along a north-west transect, following  
368 the predominant wind direction of the area (EVADIES, 2021), potentially involving a higher  
369 exposure to windborne dusts and particles from the mine, while site E was less under wind  
370 influence and would be less impacted, and was therefore considered as less polluted. Land cover  
371 within a 3 km radius of each study apiary was extracted from the Theia OSO 2019 product

372 (Inglada, Jordi et al., 2019), and individual land cover classes were combined into main categories  
 373 (urban, crop, forest and grassland; (Sponsler and Johnson, 2015) see details in Fig. 1 and Fig. S1).  
 374 Site A was surrounded by more grasslands, sites B, C, D by forests and site E by crops and urban  
 375 area (Fig. S1).

376

377 **Table 1: Location of the study sites and details on the hives.**

Site	Geographical coordinates	Distance from the ore processing site (km)	Number of hives	Hive history	Number of bees collected per hive
Site A (Lastours)	43°19'12''N, 2°22'57''E	1	3	Queens from 2019. Installed in May 2020	Hive A1: 95 Hive A2: 104 Hive A3: 102
Site B (Villanière)	43°20'40''N, 2°20'49'' E	4.5	4	Queens from 2020. Installed in April 2020	Hive B1: 44 Hive B2: 45 Hive B3: 25 Hive B4: 23
Site C (Caudebronde)	43°22'46''N, 2°18'50''E	9.2	3	Queens from 2019. Installed in May 2020	Hive C1: 52 Hive C2: 52 Hive C3: 45
Site D (Cuxac)	43°21'57''N, 2°16'26''E	10.4	5	Queens from 2020. Installed in May 2020	Hive D1: 22 Hive D2: 19 Hive D3: 32 Hive D4: 36 Hive D5: 25
Site E (Ventenac)	43°16'3''N, 2°17'2''E	9.6	3	Queens from 2019. Installed in 2019	Hive E1: 92 Hive E2: 112 Hive E3: 96

378

#### 379 4.2. Bees

380 Between July and August 2020, we collected returning forager honey bees (*Apis mellifera*,  
 381 Buckfast strain) at the hive entrance (Table 1) on the day before the behavioural experiments. We  
 382 housed bees in plastic boxes containing groups of 20 individuals with access to 400  $\mu$ L of 50%  
 383 (w/v) sucrose solution (thus ca. 20  $\mu$ L per bee following trophallaxis) and kept the plastic boxes  
 384 overnight in an incubator ( $28 \pm 1$  °C, 70% humidity) (Villar et al., 2020). In the morning of the  
 385 following day, we cooled bees on ice and harnessed them in plastic tubes, secured with tape and a  
 386 droplet of wax at the back of the head (Matsumoto et al., 2012). We then fed them 5  $\mu$ L of 50%  
 387 sucrose solution and left them to rest for 3 h in the incubator.

### 389 *4.3. Conditioning*

390 We first tested the proboscis extension reflex (PER) of all bees by stimulating their antennae with  
391 50% sucrose solution, and only kept those that responded for the conditioning. We performed  
392 olfactory absolute conditioning, in which bees must learn to associate an odour (conditioned  
393 stimulus, CS) delivered by an automatic stimulus delivery system (Aguiar et al., 2018) with a 50%  
394 sucrose reinforcement (Matsumoto et al., 2012). In the first experiment (Fig. 2), we used pure  
395 limonene and eugenol as the CS (Sigma-Aldrich Ltd, Lyon, France) alternately on successive days,  
396 so that each combination was used for about half of the bees. In the second experiment (Fig. 3),  
397 we used 1-nonanol (Sigma-Aldrich Ltd, Lyon, France). The conditioning included five trials with  
398 a ten-minute inter-trial interval. Each conditioning trial (37 s in total) started when a bee was  
399 placed in front of the stimulus delivery system, which released a continuous flow of clean air  
400 ( $3,300 \text{ mL}\cdot\text{min}^{-1}$ ) to the antennae. After 15 s, the odour was introduced to the airflow for 4 s, the  
401 last second of which overlapped with sucrose presentation to the antennae using a toothpick and  
402 subsequent feeding for 4 seconds. The bee remained another 15 s under the clean airflow. We  
403 recorded the presence or absence of a conditioned PER to each odour at each conditioning trial (1  
404 or 0), and the sum of conditioned responses was used to calculate an individual acquisition score  
405 ranging between 0 and 4. After the last conditioning trial, we put the bees back into the incubator  
406 for 1 h, before submitting them to memory tests.

407

### 408 *4.4. Memory tests*

409 Retention tests were performed at 1 h (both experiments) and 24 h (second experiment only) post-  
410 conditioning. For long-term memory assays, bees were fed 15  $\mu\text{L}$  of 50% sucrose solution after  
411 the short-term memory test, left overnight in the incubator, and fed the following morning with 5  
412  $\mu\text{L}$  of sucrose to ensure their survival. This second test was performed using the same procedure  
413 as for short-term retrieval, 24h after the end of conditioning.

414 In addition to the odour used during the conditioning (CS), novel odours were presented,  
415 following the same dynamics of the conditioning trial but with no sucrose reward. For novel  
416 odours, in the first experiment we used alternatively limonene and eugenol (depending on which  
417 was the CS). In the second one, we used alternatively 1-nonanol and nonanal for odours perceived  
418 as similar to the CS (1-nonanol), and 1-hexanol for a dissimilar odour, based on different levels of  
419 generalization (Guerrieri et al., 2005). We recorded the presence or absence of a conditioned PER  
420 to each odour at each trial (1 or 0).

421 We classified bees according to their response during the memory test. In the first  
422 experiment, the response patterns were: response to CS only, response to both odours, no or  
423 inconsistent (response to novel odour only) response. In the second experiment (Fig. 3), we  
424 classified bees as: responding to the CS only, showing generalization toward the similar odorant  
425 (i.e. responding to the CS and the similar odour, low generalization level), showing generalization  
426 to both the similar and dissimilar odorants (i.e. responding to all odours, high generalization level),  
427 no or inconsistent response. Only bees that learnt the task were kept for the analysis of the memory  
428 performances.

429

#### 430 *4.5. Morphometry*

431 All bees were frozen after the behavioural assays and stored at -18°C. An experimenter blind to  
432 bee identity measured their fresh body weight ( $\pm 0.001$ g) (precision balance ME103T, Mettler-  
433 Toledo GmbH, Greifensee, Switzerland) and took measures ( $\pm 0.01$  mm) under a Nikon SMZ 745T  
434 dissecting microscope (objective x0.67) with a Toupcam camera model U3CMOS coupled to the  
435 ToupView software. We measured the head length, head width, wing length and femur length  
436 (Fig. 4A) (Monchanin et al., 2021a).

437

#### 438 *4.6. Brain scanning and volume measurements*

439 We performed micro-computed tomography (micro CT) scanning of 47 foragers from the second  
440 experiment (Site A: N= 25; Site E: N=22). We removed the front part of the head (Smith et al.,

441 2016) and fully submerged the heads in phosphotungstic acid solution ( $5 \text{ mg.L}^{-1}$  in a 70/30%  
442 ethanol/water solution) for 15 days. Each head was scanned with a resolution of  $5 \text{ }\mu\text{m}$  using a  
443 micro CT station EasyTom 150/RX Solutions (Montpellier Ressources Imagerie, Montpellier,  
444 France). Raw data for each brain scan was reconstructed using X-Act software (RX Solutions,  
445 Chavanod, France). We then re-oriented the reconstructed scan to the same plane-of-view, and  
446 each brain was re-sliced into a new series of two-dimensional images. Based on the staining and  
447 segmentation quality, we kept 38 brains (Site A: N= 18; Site E: N=20). Using a trained neural  
448 network to predict the segmentation of every brain (Lösel et al., 2022), we then segmented the  
449 main neuropils of the brain (Brandt et al., 2005). The antennal lobes (AL), the mushroom bodies  
450 (MB) (comprising medial and lateral calyx, peduncle and lobe), the central complex (CX)  
451 (comprising the central body, the paired noduli and the protocerebral bridge), the optic lobe (OL)  
452 (comprising the medulla and lobula). Neuropil absolute volume was calculated using the voxel  
453 count function of AVIZO 2019.1 (Thermo Fisher Scientific, Waltham, USA).

454

#### 455 *4.7. Metal analysis*

456 Metal contents in bee bodies were analysed using Inductively Coupled Plasma - Mass  
457 Spectrometry (Monchanin et al., 2022). Individuals and pools of three bees were digested with  
458 ultrapure nitric acid (Optima grade for ultratrace elements, 65% w/w; ThermoFisher Scientific) in  
459 a class 10000 clean room in pre-washed 15 ml Digtubes (SCP Sciences, Villebon sur Yvettes,  
460 France) at  $100^{\circ}\text{C}$  overnight on a hot plate. They were then diluted to 2%  $\text{HNO}_3$  and metal  
461 concentrations were measured using inductively Coupled Plasma - Mass Spectrometry at  
462 Observatoire Midi-Pyrenees ICP-MS platform on a Thermo ICAP T-Q-ICP-MS (Bremen  
463 Germany) (ICP-MS, quantification limit:  $<0.01 \mu\text{g.kg}^{-1}$ , precision measure: 5%). The accuracy of  
464 the analytical method was controlled using certified reference materials: NIST-1515 (Apple  
465 Leaves, National Institute of Standards and Technology, Gaithersburg, Maryland, USA) ; IAEA-  
466 336 (Lichen, International Atomic Energy Agency, Vienne, Austria) ; DORM-4 (Fish protein,  
467 Conseil National de Recherches du Canada, Ottawa, Ontario, Canada).



468

#### 469 *4.8. Statistics*

470 We analysed the data using R Studio v.1.2.5033 (RStudio Team, 2019). All data are available in  
471 Dataset S1.

472 For the metal analysis, we conducted a PCA (package FactoMineR; (Lê et al., 2008)) on  
473 the levels of the seven selected metals (Szentgyörgyi et al., 2017). Linear mixed-effects models  
474 (LMM) (package lmerTest; (Kuznetsova et al., 2017)) were run on the pollution index (PC1) and  
475 arsenic content considering site as fixed effect, and hive as random factors.

476 For the conditioning trials, we performed generalized linear mixed-effects models  
477 (GLMM) (package lme4; (Bates et al., 2015)), fitted with binomial family, with hive and  
478 conditioning date as random factors and site as fixed effect. Using GLMM, we evaluated whether  
479 site location would impact the percentage of initial responses to antennal stimulation, spontaneous  
480 responses at the first conditioning trial, conditioned responses at the last conditioning trial and  
481 responses to each odour during memory test, as well as the proportion of individual response  
482 patterns during retrieval. GLMMs were followed by ANOVAs to test the overall significance of  
483 fixed categorical variables using the Anova function (package car; (Fox and Weisberg, 2019)).  
484 Acquisition scores were transformed as in (Smithson and Verkuilen, 2006), using the formula  $((x$   
485  $* (n - 1) + 0.5) / n$ ), to avoid zeros and ones in the normalized variables. They were then compared  
486 with GLMM (package Template Model Builder; (Brooks et al., 2017)), and fixed categorical  
487 variables significance was tested using Anova.glmTMB function of that package.

488 For the morphometric analyses, we conducted a PCA on the five parameters measured, and  
489 clusters were compared with permutational multivariate analysis of variance (PERMANOVA)  
490 (package Vegan; (Oksanen et al., 2019)). In addition, head width and length measures were  
491 collapsed into the first component of a PCA, which was used as a proxy of the head size. Linear  
492 mixed-effects models (LMM) were run for each morphological parameter, considering site as  
493 fixed effect, and hive and date as random factors. LMM were followed by F-tests to test the  
494 significance of fixed categorical variables.

495 For the brain analyses, we conducted, for each neuropil, a LMM with hive as random factor  
496 and site as fixed effect.

497

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516

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757

758 **Supporting materials**

759

760 **Table S1: Metal contents (mean  $\pm$  s.e.m, mg.kg<sup>-1</sup> fresh matter) and pollution index (PC1 –**  
 761 **outcome of the PCA representing the metal pollution in the study area) of bees collected (N=7**  
 762 **replicates per site) from the five sites and their contribution in the principal component**  
 763 **analysis (PCA). Correlation coefficients  $>0.4$  in absolute value are shown in bold.**

<b>Metal content</b>	<b>Site A</b>	<b>Site B</b>	<b>Site C</b>	<b>Site D</b>	<b>Site E</b>	<b>PC1 correlation</b>	<b>PC2 correlation</b>
Al	5.040 $\pm$ 0.753	46.283 $\pm$ 5.737	4.409 $\pm$ 0.712	11.204 $\pm$ 3.248	3.841 $\pm$ 0.458	<b>0.80</b>	<b>-0.41</b>
As	0.350 $\pm$ 0.047	0.977 $\pm$ 0.122	0.043 $\pm$ 0.005	0.052 $\pm$ 0.007	0.067 $\pm$ 0.016	<b>0.85</b>	<b>-0.44</b>
Cd	0.081 $\pm$ 0.015	0.156 $\pm$ 0.011	0.079 $\pm$ 0.003	0.058 $\pm$ 0.007	0.050 $\pm$ 0.010	<b>0.83</b>	-0.28
Cu	9.038 $\pm$ 0.486	8.515 $\pm$ 0.693	6.666 $\pm$ 1.016	7.377 $\pm$ 0.850	6.405 $\pm$ 0.607	<b>0.69</b>	0.34
Fe	102.442 $\pm$ 6.30	108.26 $\pm$ 9.258	104.819 $\pm$ 8.401	82.119 $\pm$ 9.340	54.285 $\pm$ 3.166	<b>0.72</b>	0.26
Pb	0.823 $\pm$ 0.221	0.870 $\pm$ 0.573	0.652 $\pm$ 0.448	0.173 $\pm$ 0.040	0.313 $\pm$ 0.109	<b>0.62</b>	0.35
Zn	34.843 $\pm$ 1.302	40.823 $\pm$ 2.840	41.377 $\pm$ 5.367	35.250 $\pm$ 4.606	38.775 $\pm$ 11.766	<b>0.42</b>	<b>0.67</b>
Pollution index (PC1)	0.266 $\pm$ 0.350	2.778 $\pm$ 0.632	-0.464 $\pm$ 0.521	-0.952 $\pm$ 0.411	-1.630 $\pm$ 0.338	/	/

764



765 **Table S2: Principal component analysis (PCA) on the morphometry of bees collected from**  
 766 **the five sites.** Pearson correlation coefficients between the variables and the two first principal  
 767 components. Coefficients >0.4 in absolute value are shown in bold.

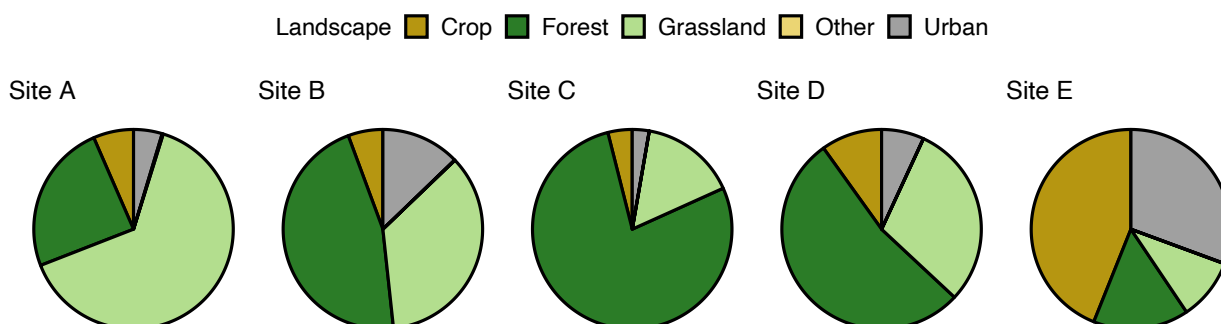
Variable	PC1	PC2
Body weight	<b>0.59</b>	-0.12
Head length	<b>0.63</b>	<b>0.50</b>
Head width	<b>0.55</b>	<b>0.62</b>
Wing length	<b>0.64</b>	<b>-0.49</b>
Femur length	<b>0.67</b>	<b>-0.40</b>
% Total variance	38.14	21.06
Cumulative proportion of total variance	38.14	59.20

774

775 **Table S3: Principal component analysis (PCA) on the head morphometry of bees collected**  
 776 **from the five sites.** Pearson correlation coefficients between the variables and the two first  
 777 principal components. Coefficients >0.4 in absolute value are shown in bold.

Variable	PC1	PC2
Head length	<b>0.83</b>	<b>-0.57</b>
Head width	<b>0.83</b>	<b>0.57</b>
% Total variance	67.61	32.39
Cumulative proportion of total variance	67.61	100

782



783

784 **Figure S1: Landscape composition of study sites at 3 km radius around each apiary.** To  
 785 simplify the landscape composition, we condensed the cover classes into five major aggregate  
 786 classes: crop (cultivated crops; army green), forest (deciduous and evergreen; dark green),

787 grassland (pasture/hay; light green), and urban (grey). Remaining land cover (yellow) consisted  
788 of barren land and open water. Surface expressed as percentage.