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Environmental exposure to metallic pollution impairs honey bee brain development and cognition

Coline Monchanin^{a,b,c}, Erwann Drujont^{a,b}, Gaël Le Roux^d, Philipp D. Lösel^{e,f}, Andrew B. Barron^c, Jean-Marc Devaud^{a,b}, Arnaud Elger^{d,1}, Mathieu Lihoreau^{a,b,*,1}

^a CNRS, University Paul Sabatier, Toulouse III, France

^b Research Center on Animal Cognition (CRCA), Center for Integrative Biology (CBI), University Paul Sabatier, Toulouse III, France

^c Department of Biological Sciences, Macquarie University, NSW, Australia

^d Laboratoire Ecologie Fonctionnelle et Environnement, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France

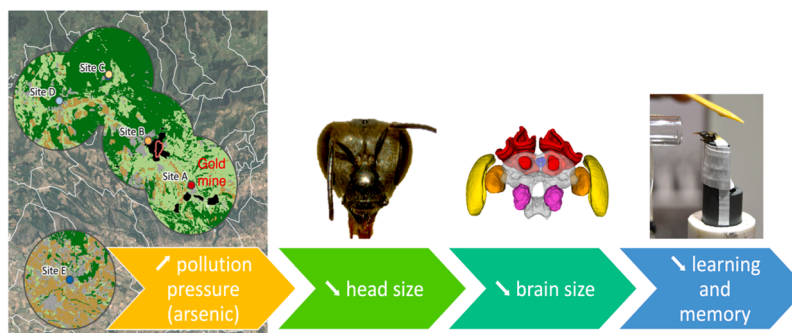
^e Engineering Mathematics and Computing Lab (EMCL), Interdisciplinary Center for Scientific Computing (IWR), Heidelberg University, Germany

^f Department of Materials Physics, Research School of Physics, The Australian National University, ACT, Australia

HIGHLIGHTS

- Effects of metallic pollution on animal behaviour are poorly known.
- We studied honey bees in an historic mining site contaminated with arsenic.
- Live bees closer to the mine had reduced cognitive performance.
- They also developed smaller heads with smaller brains.
- Metallic pollution thus impairs bee behaviour and threatens pollination.

GRAPHICAL ABSTRACT



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ABSTRACT

Laboratory studies show detrimental effects of metallic pollutants on invertebrate behaviour and cognition, even at low levels. Here we report a field study on Western honey bees exposed to metal and metalloid pollution through dusts, food and water at a historic mining site. We analysed more than 1000 bees from five apiaries along a gradient of contamination within 11 km of a former gold mine in Southern France. Bees collected close to the mine exhibited olfactory learning performances lower by 36% and heads smaller by 4%. Three-dimensional scans of bee brains showed that the olfactory centres of insects sampled close to the mine were also 4% smaller, indicating neurodevelopmental issues. Our study raises serious concerns about the health of honey bee populations in areas polluted with potentially harmful elements, particularly with arsenic, and illustrates how standard cognitive tests can be used for risk assessment.

* Correspondence to: Centre de Recherches sur la Cognition Animale (CRCA), Centre de Biologie Intégrative (CBI), Université Paul Sabatier, 118 Route de Narbonne, CEDEX 09, Toulouse 31062, France.

E-mail address: mathieu.lihoreau@univ-tlse3.fr (M. Lihoreau).

¹ Joint last authors

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1. Introduction

Metals and metalloids are naturally found at low levels in the Earth crust and water as a result of volcanic eruptions and soil erosion. Human activities have raised environmental levels far above this baseline [34]. In particular, mining operations [15] and metal smelters have elevated concentrations of toxic pollutants in superficial soils [71] and water [58] across large areas worldwide, exposing people and the wildlife to hazardous concentrations [4]. In humans, metals and metalloids bind to

proteins and enzymes, disrupting their activity and provoking cellular damage. This causes neurotoxicity, oxidative stress, and carcinogenesis [6], leading to sublethal cognitive effects such as sensory impairments, mood disorders or learning and memory deficits [66]. However, the consequences on the wildlife are much less understood.

Bees are key sentinel species, used for risk assessment of environmental pollution on biodiversity loss and exosystemic services [16,63]. Foraging bees are exposed to potentially harmful elements in airborne dusts [61], plant nectar and pollen [43], and drinking water [46]. In

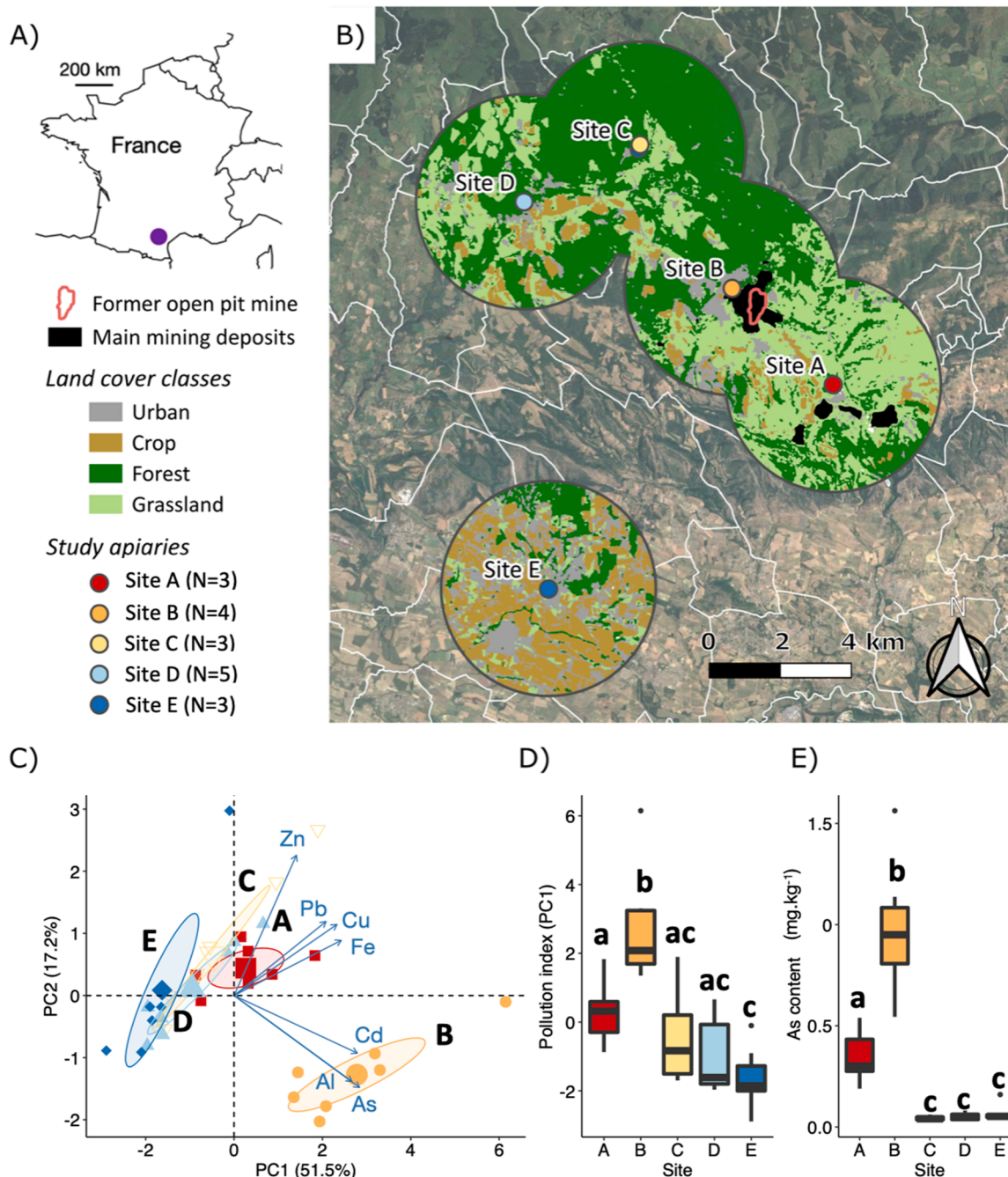


Fig. 1. Study sites and metallic pollution levels. **A)** Former gold mine located in the South of France. N is the number of hives in each apiary. **B)** Location of the five apiaries, with the main land cover classes displayed within a radius of 3 km around each apiary. The major mining deposits in the study area (according to [26]) and the ore processing site are also shown. White lines indicate municipal boundaries. **C)** Principal component analysis (PCA) plot showing the distribution of analysed pools of bees (7 replicates per site) along the two principal components (PC1, PC2) and the relationship among the potentially harmful elements measured. 95% confidence ellipses of the mean are displayed for each site. **D)** Pollution index (score on PC1) at each site. **E)** Arsenic content in the bodies of bees (mg.kg⁻¹ fresh matter) at each site. Boxplots in **D)** and **E)** show median value (intermediate line) and quartiles (upper and lower lines) for each measurement. Different letters (a-c) above boxes indicate significant differences between sites (LMM).

honey bees, these elements also contaminate the nest through honey [7], wax [73] and propolis [49], and are ultimately ingested by all the bees through food or contact with nest materials [14]. Bees seem unable to detect field-realistic concentrations of metals or metalloids [56], and controlled exposure to metallic pollutants (alone or combined) leads to developmental and cognitive deficits [11,17,54,55]. Contact with high environmental levels of metallic pollutants may thus have severe consequences for population growth and the associated pollination services.

Here, we explored the sublethal effects of environmental exposure of honey bee colonies to trace metallic pollutants in the field. We sampled bee foragers from apiaries along a pollution gradient surrounding a former gold mine (Salsigne, France; Fig. 1A-B.) and examined their morphological development, brain organisation and cognition. During its exploitation (1873–2004) the site became contaminated mostly with arsenic, but also lead, cadmium, copper, nickel and zinc. This contamination has persisted even after the closure and partial remediation of the site [39,41]. As the contaminant levels are well above the international permissible limits (for arsenic: up to 76 times higher in irrigation water [32], 290 times higher in soil [42]), the area is considered as “critically polluted” [62]. Hence monitoring human populations and the wildlife in this area is of great importance.

2. Results

2.1. Bees closer to the mine accumulated higher levels of potentially harmful elements

We sampled bees from five apiaries within 11 km of the former gold mine, at different distances along a gradient of exposure to environmental metallic pollution (Fig. 1A-B). First, we verified the gradient of contamination by measuring trace elements (Al, As, Cd, Cu, Fe, Pb, Zn) in the bodies of bees using Inductively Coupled Plasma - Mass Spectrometry [56]. To provide a single measure of overall pollution for each site, we performed a principal component analysis (PCA) using the concentrations of all potentially harmful elements (mg.kg^{-1} fresh matter) in bee bodies (Fig. 1C; Table S1). We used the first principal component (PC1) as a pollution index: high values corresponded to high contamination of bees with all the metals and metalloids (Fig. 1D). Pollution indices differed greatly between sites ($F_{4,30} = 13.42$, $p < 0.001$; Table S1). Bees from site B had the highest content of potentially harmful elements (mean score on PC1 \pm s.e.m: 2.8 ± 0.6), followed by bees from site A (0.3 ± 0.4), while bees from site E had the lowest content (-1.6 ± 0.3) (Fig. 1D). Since the area is known to be heavily contaminated with arsenic, we also compared these values and again found significant variation ($F_{4,30} = 46.33$, $p < 0.001$). Bees from sites A (mean \pm s.e.m: $0.35 \pm 0.05 \text{ mg.kg}^{-1}$) and B ($0.98 \pm 0.12 \text{ mg.kg}^{-1}$) contained higher levels of As than to bees from the three more distant sites (Site C: $0.04 \pm 0.01 \text{ mg.kg}^{-1}$; Site D: $0.05 \pm 0.01 \text{ mg.kg}^{-1}$; Site E: $0.07 \pm 0.02 \text{ mg.kg}^{-1}$) (pairwise comparisons: $p < 0.01$; Fig. 1E). This confirms sites A and B were overall more contaminated than sites C-D, and in particular with arsenic.

2.2. Bees closer to the mine showed lower learning performances

We then conducted learning experiments to test the influence of metallic pollution on bee cognition. We trained bees caught in each of the five apiary to associate an odorant to a sucrose reward using olfactory proboscis extension response (PER) conditioning [51]. Such associative learning is critical for bees to recognise flowers and forage efficiently [27].

Prior to conditioning, we tested all bees for an intact proboscis extension reflex upon antennal stimulation with 50% (w/v) sucrose solution. The proportions of responding bees were similar among sites (GLMM: $p = 0.990$) (Site A: 97.8%, $N = 136$; Site B: 98.5%, $N = 135$; Site C: 97.9%, $N = 140$; Site D: 98.3%, $N = 121$; Site E: 100%, $N = 144$). Therefore, site location did not affect appetitive motivation nor sucrose

perception by bees.

We then trained 673 bees in a five-trial absolute learning task, during which we recorded conditioned responses to an odour. A similar small proportion of bees from each site spontaneously responded at the first odour presentation and was therefore discarded ($N = 42$; GLMM: $p = 0.336$). When considering the 631 remaining bees, learning was observed in all sites (Fig. 2A). However, on the last conditioning trial, a lower proportion of bees from sites A (GLMM: Est = -2.13 ± 0.78 , $p = 0.006$) and C (Est = -1.57 ± 0.78 , $p = 0.044$) had learned the task, compared to those from the farthest site E (A: 56.45%, B: 73.39%, C: 68.94%, D: 78.90%, E: 91.55%). When analysed individually (Fig. 2B), we found that bees from site A had lower acquisition scores (total number of conditioned responses across all conditioning trials) than bees from site B (GLMM: Est = -1.02 ± 0.48 , $p = 0.033$), site D (Est: -1.06 ± 0.49 , $p = 0.031$) and site E (Est = -1.29 ± 0.58 , $p = 0.027$). There was no effect of the odorant used for conditioning (limonene or eugenol) on bees' responses (GLMM: $p = 0.141$). Thus, bees from apiaries closer to the mine had lower learning performances.

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

Using the same bees, we next assessed one-hour memory recall by recording conditioned PER response to odorants without sucrose reward. In addition to the odorant used during training (conditioning stimulus, CS), we tested bees with a novel odorant to evaluate the specificity of memory [76].

Among bees that had effectively learnt the task ($N = 468$), one-hour memory recall of the odour-reward association differed between sites (GLMM: $p = 0.002$). Bees from sites B and C responded less to CS (68.13% and 64.85% respectively) than bees from site D (GLMM: Est = -1.59 ± 0.50 , $p = 0.016$ and -1.80 ± 0.50 , $p = 0.003$ respectively). Additionally, bees from site A responded more often to the novel odour than bees from sites D (GLMM: Est = 1.20 ± 0.41 , $p = 0.036$) (Fig. 2C). The individual response patterns (Fig. 2D) showed a clear effect of site location on the proportion of bees displaying CS-specific memory (GLMM: $p = 0.005$) (A: 21.43%, B: 35.16%, C: 30.77%, D: 58.14%, Site E: 47.69%), with significantly fewer specific responses recorded in bees from site A than from sites D and E (GLMM: resp. Est = -1.63 ± 0.48 , $p < 0.001$; Est = -1.21 ± 0.48 , $p = 0.011$); and in bees from site B than from sites C and D (resp. Est = -1.16 ± 0.44 , $p = 0.008$; Est = -0.90 ± 0.42 , $p = 0.033$). Bees from site A also showed higher generalisation of their response to the novel odour compared bees from all other sites (GLMM: $p = 0.019$; A: 58.57%, B: 32.97%, C: 34.07%, D: 33.72%, E: 34.62%). Thus, the closer the bees to the mine, the lower their memory specificity.

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

To further investigate memory impairments, we conducted a second experiment on bees sampled in two contrasting conditions of metallic pollution: site A (high contamination) and site E (low contamination). We compared the performances of bees in short-term (1 h) and long-term (24 h) memories, the latter guiding foraging decisions and participating in communication between bees [22]. To better capture the effect on olfactory generalisation, we used two novel odours in addition to CS, according to their degree of structural similarity: one perceptually similar (low generalisation) and one dissimilar (high generalisation) [76].

Like in the first experiment, the proportions of bees initially exhibiting intact PER was similar between both sites (GLMM: $p = 1$; A: 96.17%, E: 100%). After five conditioning trials, a lower proportion of bees from site A had learnt the task compared to site E (GLMM: Est = -2.20 ± 0.87 , $p = 0.011$) (A: 62.00%, E: 92.52%). While they also exhibited a lower mean acquisition score (mean \pm s.e.m: A: 2.27 ± 0.14 , E: 3.22 ± 0.11), the difference was not significant (GLMM: Est = -0.85 ± 0.53 , $p = 0.107$).

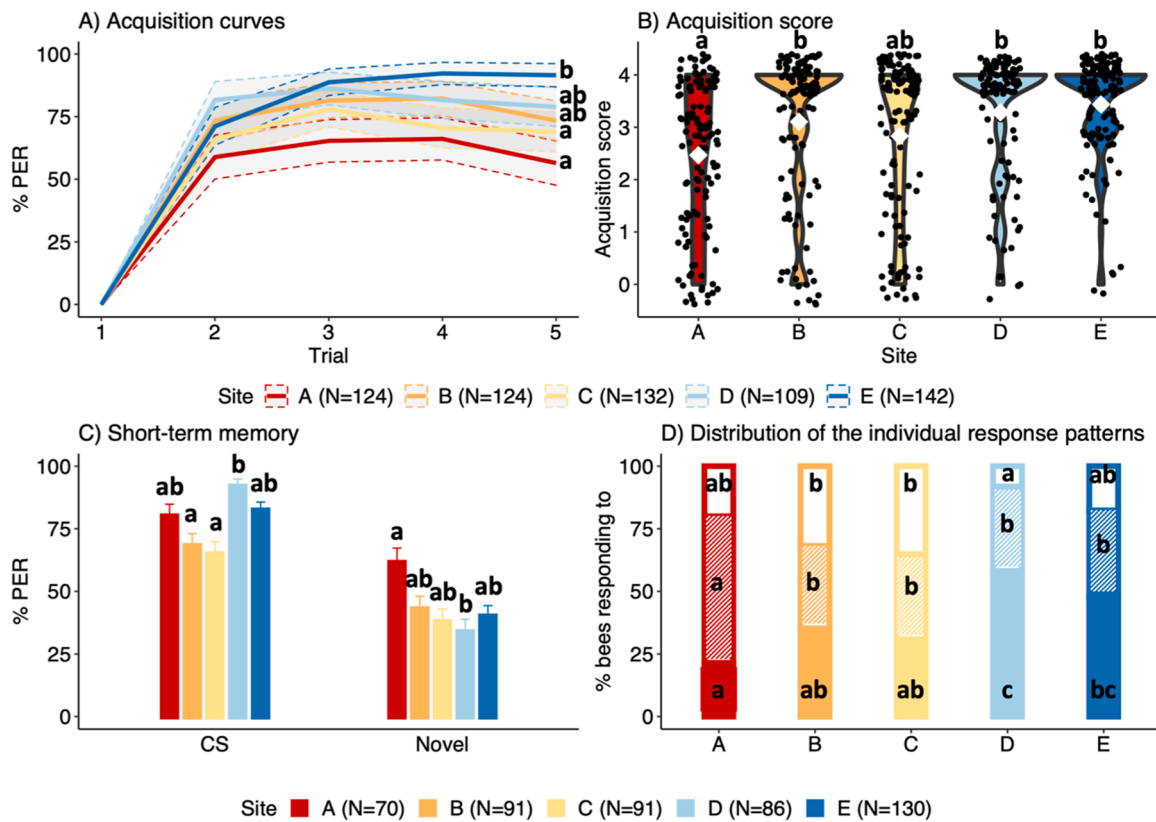


Fig. 2. Learning and short-term memory. **A)** Acquisition curves show changes in the percentages of bees displaying conditioned proboscis extension responses (PER) over the five trials. Areas delimited by the dashed lines show 95% confidence intervals. Different letters next to curves indicate significant differences in response proportions at the last trial between sites (GLMM). **B)** Violin plots of individual acquisition scores (sum of conditioned responses for each bee, white diamonds display mean values). Different letters above plots indicate significant differences between sites (GLMM). **C)** Percentages of responses to the two odours during the non-rewarded one-hour memory retention test (mean \pm s.e.m). Different letters above bars indicate significant differences in response levels for each odour (GLMM). **D)** Distribution of bees according to their individual response patterns during the memory test: CS-specific responses (coloured), generalised responses to both odours (hatched) and inconsistent or absent responses (white). Different letters within bars indicate significant differences between sites for each response pattern (GLMM). A-D) N is the number of bees tested.

In the short-term memory retrieval test (Fig. 3A-B), bees from both sites, that had effectively learnt the task ($N = 192$), responded similarly to the CS (A: 67.94%; E: 68.25%) (GLMM: Est = 0.12 ± 0.58 , $p = 0.846$). However, bees from site A showed stronger generalisation to novel odours (Fig. 3A), although only significantly to the similar one (GLMM: Est = 1.32 ± 0.31 , $p < 0.001$) (similar: 69.89% vs. 40.40%; dissimilar: 16.13% vs. 3.03%). Patterns of individual responses confirmed the effect of the proximity to the mine on response selectivity (Fig. 3B). Bees from site A showed fewer CS-specific responses (12.90% vs. 37.37%; GLMM: Est = -1.40 ± 0.39 , $p < 0.001$), but rather generalised more to other odours, both similar (51.61% vs. 35.35%; GLMM: Est = 0.67 ± 0.30 , $p = 0.024$) and dissimilar (16.13% vs. 3.03%; GLMM: Est = 1.85 ± 0.80 , $p = 0.020$). Thus, again, bees closer to the mine exhibited higher levels of generalisation and less specific memory.

When tested for long-term memory (Fig. 3C-D), bees from both sites responded in similar proportions to all three odours (Fig. 3C): the CS (A: 51.72%, E: 66.67%; GLMM: Est = -0.63 ± 0.48 , $p = 0.191$), the similar odour (A: 48.28%, E: 39.39%; GLMM: Est = 0.35 ± 0.41 , $p = 0.392$) and the dissimilar one (A: 20.69%, E: 11.11%; GLMM: Est = 0.80 ± 0.63 , $p = 0.199$). However, bees from site A showed fewer CS-specific responses (Fig. 3D; A: 10.34%, E: 29.29%; GLMM: Est = -0.87 ± 0.41 , $p = 0.037$) and more inconsistent or absent responses (A: 49.43%, E: 35.35%; GLMM: Est = 0.85 ± 0.41 , $p = 0.039$). Generalisation responses to the similar odour (A: 25.29%, E: 26.26%; GLMM: Est = -0.22 ± 0.34 , $p = 0.508$) as well as to the dissimilar one (A: 14.94%, E: 9.09%; GLMM: Est = 0.47 ± 0.70 , $p = 0.501$) were equally frequent in bees from both sites. Therefore, a shorter distance to the mine was

associated with lower individual long-term memory specificity, as observed for short-term memory.

2.5. Bees closer to the mine had smaller heads

To assess the potential developmental impact of mine proximity on bee development, we measured five morphological parameters (Fig. 4A) on all the bees collected in the two experiments ($N = 1021$). We assessed the effect of site location on overall morphology with a PCA including all parameters (Fig. 4B; Table S2). Two PCs discriminated overall larger bees vs. smaller bees (PC1) and bees with large and wide heads vs. short wing and femur (PC2) (PERMANOVA: Pseudo- $F=10.92$, $p = 0.001$). Bees from site A were morphologically different from those from all other sites (pairwise PERMANOVA: $p = 0.01$ for all comparisons). Their heads were not longer than those of other bees ($F_{4,12.5} = 1.22$, $p = 0.350$; Fig. 4C), but significantly narrower than at sites D and E (resp. LMM: Est = -0.08 ± 0.03 , $p = 0.037$; Est = -0.08 ± 0.04 , $p = 0.045$; Fig. 4D). Head width and length measures were thus collapsed into the first component of another PCA (explaining 68% of the variance) that we used as a proxy of the head size (Table S3). Bees from the closest site (A) had overall smaller heads than bees from the farthest sites (D and E) (resp. LMM: Est = -0.94 ± 0.41 , $p = 0.044$; Est = -1.01 ± 0.46 , $p = 0.049$; Fig. 4E). However, bees from all sites exhibited similar femur length ($F_{4,12.6} = 0.31$, $p = 0.866$; Fig. 4F), wing length ($F_{4,12.7} = 0.34$, $p = 0.849$; Fig. 4G) and body weight ($F_{4,12.5} = 3.13$, $p = 0.054$; Fig. 4H). This means the bees closest to the mine had smaller heads but no overall smaller bodies.

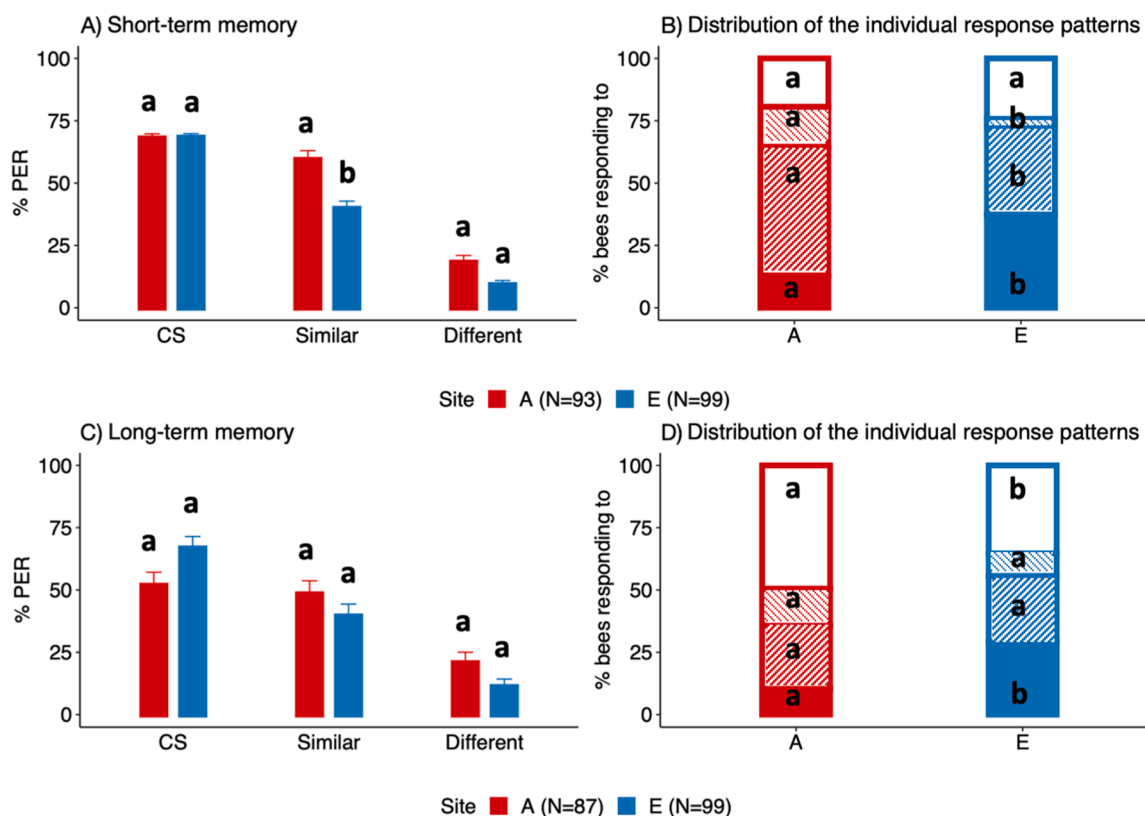


Fig. 3. Short-term and long-term memory. **A)** Percentages of responses to the three odours during the non-rewarded one-hour memory retention test (mean \pm s.e.m.). Different letters above bars show significant differences in response levels for each odour are displayed (GLMM). **B)** Distribution of bees according to their individual response patterns during the short-term memory test: CS-specific responses (coloured), generalised responses to the similar odour (dense hatches), generalised responses to both similar and dissimilar odours (hatched), and inconsistent or no responses (white). Different letters inside bars show significant differences between sites for each response pattern (GLMM). **C)** Percentages of responses to the three odours during the 24 h-memory retention test (mean \pm s.e.m.). Different letters above bars show significant differences in response levels for each odour (GLMM). **D)** Distribution of bees according to their individual responses during the long-term memory test, as in **B)**. Different letters inside bars show significant differences between sites for each response pattern (GLMM). **A-D)** N is the number of bees tested.

2.6. Bees closer to the mine had smaller antennal lobes

We finally assessed whether proximity to the mine might alter the volume of the brain or of specific neuropils related to learning and memory deficits (Fig. 5). We quantified volumes from 3D reconstructions of the brain based on microtomography scanning (Fig. 5A) obtained from a subset of bees from sites A and E used in the second behavioural experiment (see Fig. 3). Total brain volume was positively correlated with head size ($r = 0.37$, $p = 0.023$), but did not differ between sites A and E (Fig. 5B). However, we found differences at the level of individual neuropils. In particular, the antennal lobes (ALs), i.e. the primary brain structures involved in olfactory learning and memory [28], were significantly smaller in bees from site A than in bees from site E (Fig. 5C). By contrast, none of the other main brain neuropils (i.e. mushroom bodies, optic lobes, central complex) varied between sites (Fig. 5D-G). This suggests the cognitive olfactory deficits observed in bees sampled near the mine are related to impaired development of their ALs. However, we did not find any influence of overall AL volume on the proportion of learners (GLMM: Est = 1.12 ± 0.86 , $p = 0.192$) nor the acquisition score (GLMM: Est = 0.24 ± 0.37 , $p = 0.519$) in this small subset of bees ($N = 38$).

3. Discussion

Recent studies show controlled exposure to metallic trace elements impacts honey bee health and cognitive performance suggesting severe consequences for populations in polluted environments [11,54,55].

Here we demonstrated the relationship between environmental metallic contamination and impaired behaviour in the field using cognitive assays on honey bees sampled along a gradient of metallic pollution.

Forager bees from hives closer to the mine, and thus exposed to higher levels of arsenic and other pollutants, developed smaller heads, with smaller antennal lobes, than those from the most distant site. These neurodevelopmental impairments are reminiscent of those observed in mammals following exposure to arsenic [74,75,79]. They are also consistent with reports of longer pupal stages [2], decreased body weight [64], leg deformities [20], smaller heads [31,50] in other insects. The effects of arsenic exposure on brain development are thus likely to contribute to the reduced cognitive abilities observed in our study, since bees exposed to increasingly contaminated environments showed more severe growth defects and deficits in learning and memory. Although we cannot definitely exclude an influence of pesticide exposure on our results [36], the fact that we observed higher cognitive performances in bees from sites dominated by crops (D,E; Fig. 1B) than in sites with fewer crops (A,B; Fig. 1B) indicates metallic pollution was the main cause of cognitive variation in bees. This is further supported by the fact that higher cognitive abilities of bees were consistently observed in the two different end points of the metallic pollution gradient characterised by contrasted land cover (the low contaminated sites D,E; Fig. 1B).

Previous work already pointed out that bees with smaller heads perform less well in olfactory learning tasks [30,54]. Here, the reduced head size, and specifically ALs volume, associated to metal and metalloid exposure might explain the observed olfactory learning impairment, as ALs support olfactory discrimination and learning [53]. We found no

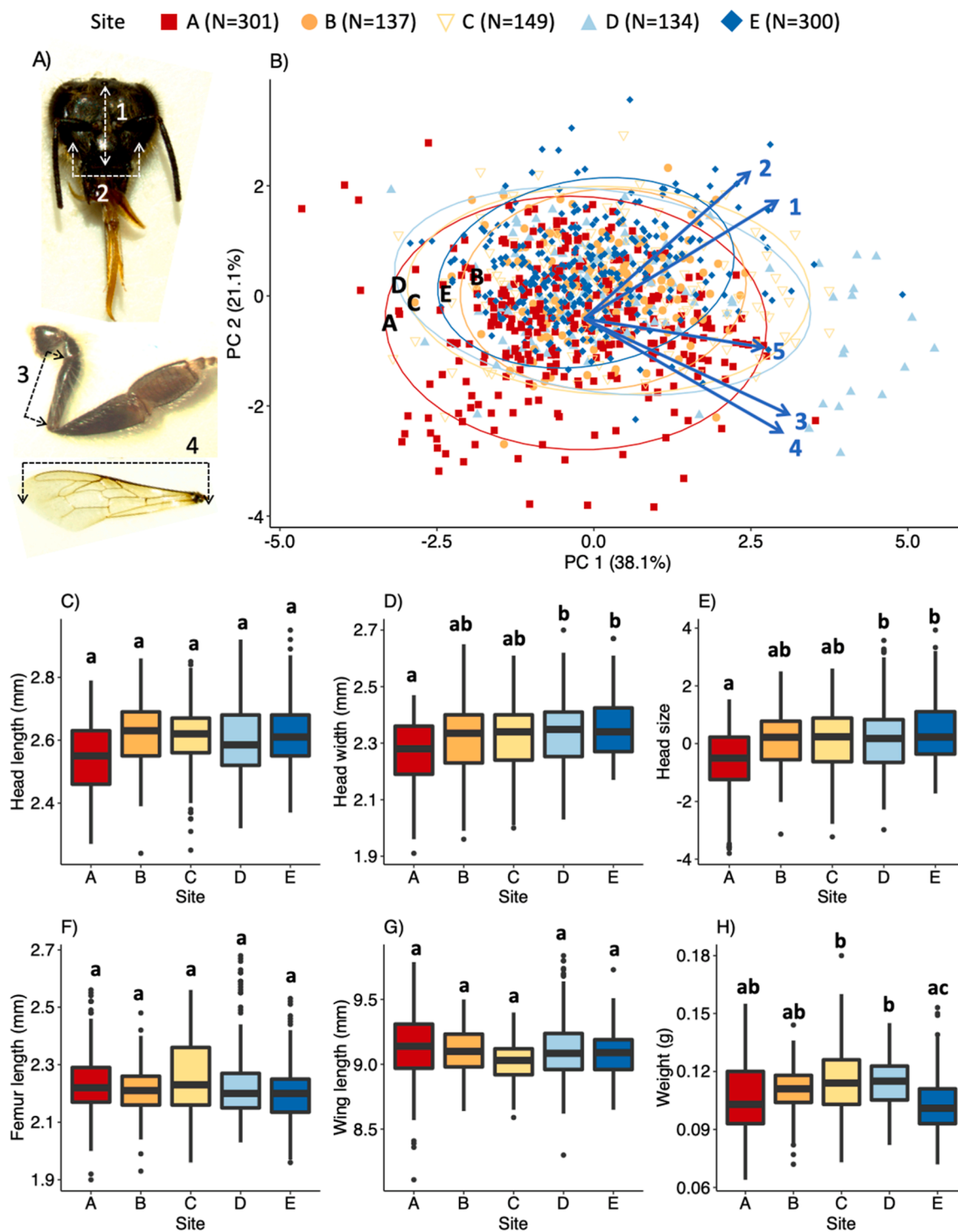


Fig. 4. Morphometric measurements of bees. **A)** Details of the parameters measured: (1) head length, (2) head width, (3) femur length, (4) wing length, (5) body weight (not shown). N is the number of bees measured. **B)** Principal component analysis (PCA) plot showing the distribution of individuals along the two principal components (PC1, PC2) and the relationship among the morphometric measures (same number code as in A). 95% confidence ellipses of the mean are displayed for each site. **C)** Head length. **D)** Head width. **E)** Head size. **F)** Femur length. **G)** Wing length. **H)** Body weight. Boxplots show median value (intermediate line) and quartiles (upper and lower lines) for each measurement. Different letters above boxes indicate significant differences between sites (LMM).

effect on the size of MBs, which are known to support olfactory memory retrieval [52], despite memory impairments correlated with contamination exposure. This suggests that the lower memory specificity in more exposed bees is the consequence of altered coding of odorants in the ALs during learning. Yet, we do not exclude that exposure to higher contamination levels may have a subtler impact on MBs than changes in

overall volume, e.g. by affecting synaptic connectivity [12] with possible consequences on memory specificity [29]. Overall, arsenic exposure is known to affect neural functions, in particular by altering various neurotransmitter levels in mammals [80]: it can lead to neurodevelopmental and cognitive disorders, including learning and memory impairments (reviewed in [74]). Altered GABA signalling might affect

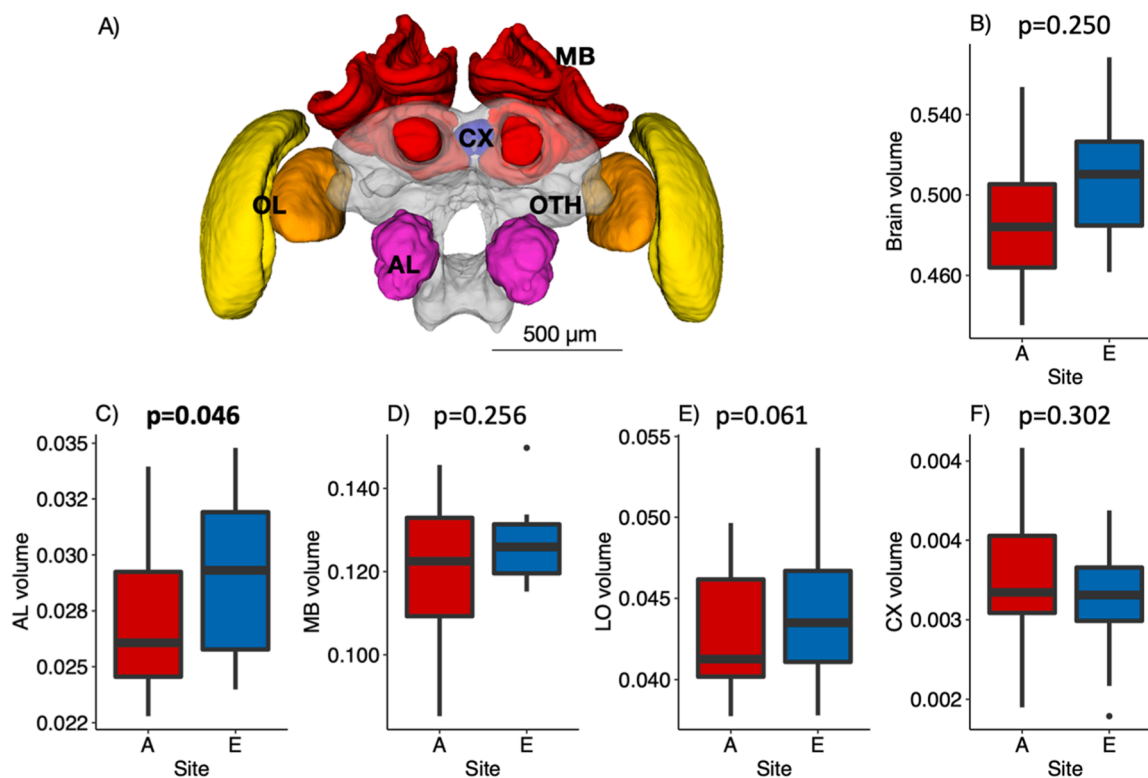


Fig. 5. Brain area volumes (mm^3) of bees from site A ($N = 18$ brains) and E ($N = 20$ brains). **A)** Example of a reconstructed brain (frontal view) showing antennal lobes (AL), mushroom bodies (MB), optic lobes (OL), central complex (CX). **B)** Total brain. **C)** Antennal lobes (primary integration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

odour discrimination [57] and memory specificity [37], and altered amine levels might affect acquisition levels [65]. Bees exposed to high levels of arsenic in this area may therefore have difficulties to find, identify and exploit environmental resources.

Although the impacts of contamination around the former gold mine of Salsigne have been little studied, our conclusions are consistent with epidemiological data obtained in the area, showing an excessive mortality rate among mine workers [67] and arsenic-specific cancer attributed to environmental contamination [18], but also significant bioaccumulation of arsenic in small mammals [19]. A citizen science project carried between 1994 and 1997 found large amounts of arsenic (up to 0.69 mg.kg^{-1}) and lead (up to 0.95 mg.kg^{-1}) in the pollen collected by honey bees [60]. These levels of potentially harmful elements fall within the range of doses that cannot be perceived by bees and are readily ingested in laboratory assays [56]. Hence, bees could be all the more at risk because they cannot avoid such concentrations.

4. Conclusion

Honey bees sampled close to the mine showed decreased olfactory learning and memory performances and developed smaller heads with smaller brain olfactory centres. Since functional learning and memory is crucial to the behavioural and chemical ecology of bees, individuals exposed to metallic pollution may be less efficient at foraging and interacting socially, ultimately jeopardizing the capacity of the colony to feed and grow. The identification of sublethal effects of metallic pollution in a sentinel species raises serious concerns about invertebrate populations and their associated ecosystem services in contaminated areas. Importantly, the bees collected in our study area contained amounts of arsenic (0.35 mg.kg^{-1} on average) below those reported in other studies, e.g. in Italy (0.37 mg.kg^{-1} ; [81]), in the US (1.71 mg.kg^{-1} ;

[23]) or in Serbia (0.99 ; [43]), thus raising the urgent need to investigate the effects of metallic pollution on pollinators more broadly. Further development of behavioural and morphometric indicators for field assessment of these sublethal effects hold considerable promises for guiding effective management of metallic pollutions.

5. Methods and protocols

5.1. Field sites

This study was carried out in 2020 in the vicinity of a former gold mine located in Salsigne, France ($43^{\circ}18'41''\text{N}$, $2^{\circ}22'44''\text{E}$; Fig. 1A-B). The ores processing, to extract mainly gold and silver, led to major contamination by arsenic in the surroundings of the industrial plant, in soils [19] and water [32], far beyond international maximal permissible limits (for arsenic: $<0.2 \text{ ppm}$ in food and $<0.01 \text{ ppm}$ in drinking water [13], $<0.1 \text{ ppm}$ in irrigation water [5], $<20 \text{ ppm}$ in soil [78]). Successive floods [25] contributed to spreading metallic pollutants in the valley, raising public health concerns supported by alarming arsenic levels measured from children in the area [3]. Five apiaries were selected within 11 km of the former mine (sites A-E in Fig. 1, Table 1) and have been settled for at least two months before the beginning of the experiment. Site A was the closest to the former ore processing site. It was therefore considered as the most polluted due to release of slag, fumes and dust [19]. Site B was closer to the open mine cast with underground operations, and was also characterized by high arsenic pollution [40]. Both sites were in the vicinity of main mining deposits (Fig. 1). Sites A to D were located along a north-west transect, following the predominant wind direction of the area [21], potentially involving a higher exposure to windborne dusts and particles from the mine, while site E was less under wind influence and would be less impacted, and

Table 1
Location of the study sites and details on the hives and the number of bees collected for each experiment.

Site	Geographical coordinates	Distance from the ore processing site (km)	Number of hives	Hive history	Total number of bees collected per hive and used for morphological measurements	Number of bees tested per hive during absolute learning (short-term memory in bracket)	Number of bees tested per hive during long-term memory (short-term memory in bracket)	Number of bees used for micro CT
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	Hive A1: 45 (28) Hive A2: 48 (20) Hive A3: 31 (22)	Hive A1: 45 (39/36) Hive A2: 50 (16/15) Hive A3: 55 (38/36)	Hive A1: 8 Hive A2: 9 Hive A3: 8
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	Hive B1: 41 (21) Hive B2: 41 (31) Hive B3: 21 (19) Hive B4: 21 (20)	NA	NA
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	Hive C1: 47 (25) Hive C2: 44 (38) Hive C3: 41 (28)	NA	NA
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	Hive D1: 20 (15) Hive D2: 16 (12) Hive D3: 20 (7) Hive D4: 33 (32) Hive D5: 20 (20)	NA	NA
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	Hive E1: 46 (41) Hive E2: 49 (45) Hive E3: 47 (44)	Hive E1: 22 (22/22) Hive E2: 40 (35/35) Hive E3: 45 (42/42)	Hive E1: 0 Hive E2: 18 Hive E3: 4

was therefore considered as less polluted. Land cover within a 3 km radius of each study apiary was extracted from the Theia OSO 2019 product [38], and individual land cover classes were combined into main categories (urban, crop, forest and grassland; [70] see details in Fig. 1 and Fig. S1). Site A was surrounded by more grasslands, sites B, C, D by forests and site E by crops and urban area (Fig. S1). Records of pesticide use are not available for the study area.

5.2. Bees

Between July and August 2020, we collected returning forager honey bees (*Apis mellifera*, Buckfast strain) at the hive entrance (Table 1) on the day before the behavioural experiments. We housed bees in plastic boxes containing groups of 20 individuals with access to 400 μL of 50% (w/v) sucrose solution (thus ca. 20 μL per bee following trophallaxis) and kept the plastic boxes overnight in an incubator (28 ± 1 °C, 70% humidity) [77]. In the morning of the following day, we cooled bees on ice and harnessed them in plastic tubes, secured with tape and a droplet of wax at the back of the head. We then manually fed individual bees with 5 μL of 50% sucrose solution and left them to rest for 3 h in the incubator [51].

5.3. Conditioning

We first tested the proboscis extension reflex (PER) of all bees by stimulating their antennae with 50% sucrose solution, and only kept those that responded for the conditioning. We performed olfactory absolute conditioning, in which bees must learn to associate an odour (conditioned stimulus, CS) delivered by an automatic stimulus delivery system [1] with a 50% sucrose reinforcement [51]. In the first experiment (Fig. 2), we used pure limonene and eugenol as the CS (Sigma-Aldrich Ltd, Lyon, France) alternately on successive days, so that each combination was used for about half of the bees. In the second experiment (Fig. 3), we used 1-nonanol (Sigma-Aldrich Ltd, Lyon, France). The conditioning included five trials with a ten-minute inter-trial interval. Each conditioning trial (37 s in total) started when a bee was placed in front of the stimulus delivery system, which released a continuous flow of clean air ($3.3 \text{ ML} \cdot \text{min}^{-1}$) to the antennae. After 15 s, the odour was introduced to the airflow for 4 s, the last second of which overlapped with sucrose presentation to the antennae using a toothpick and subsequent feeding for 4 s. The bee remained another 15 s under the clean airflow. We recorded the presence or absence of a conditioned PER to each odour at each conditioning trial (1 or 0), and the sum of conditioned responses was used to calculate an individual acquisition score (we did not consider trial 1 because untrained bees were expected to respond only after the paired odour-sucrose association). Acquisition scores ranged between 0 (the bee never responded to the odour) and 4 (the bee responded at every trial). After the last conditioning trial, we moved the bees back into the incubator for 1 h, before submitting them to memory tests.

5.4. Memory tests

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

In addition to the odour used during the conditioning (CS), novel odours were presented, following the same dynamics of the conditioning trial but with no sucrose reward. In the first experiment, we alternatively used limonene and eugenol as novel odours (depending on which was the CS). In the second experiment, we used 1-nonanol and nonanal for odours perceived as similar to CS (1-nonanol), and 1-hexanol for a

dissimilar odour based on different levels of generalisation [33]. We recorded the presence or absence of a conditioned PER to each odour on each trial (1 or 0).

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

5.5. Morphometry

Bees were frozen after the behavioural assays and stored at $-18\text{ }^{\circ}\text{C}$ for two weeks. An experimenter blind to bee identity measured their fresh body weight ($\pm 0.001\text{ g}$) (precision balance ME103T, Mettler-Toledo GmbH, Greifensee, Switzerland) immediately after removal from freezer in order to avoid water evaporation. The experimenter then took measures ($\pm 0.01\text{ mm}$) under a Nikon SMZ 745 T dissecting microscope (objective x0.67) with a Toupcam camera model U3CMOS coupled to the ToupView software. We measured the head length, head width, wing length and femur length (Fig. 4A) [54].

5.6. Brain scanning and volume measurements

We performed micro-computed tomography (micro-CT) scanning of 47 foragers from the second experiment (Site A: $N = 25$; Site E: $N = 22$). We removed the front part of the head [68] and fully submerged the heads in phosphotungstic acid solution ($5\text{ mg}\cdot\text{L}^{-1}$ in a 70/30% ethanol/water solution) for 15 days. Each head was scanned with a resolution of $5\text{ }\mu\text{m}$ using a micro-CT station EasyTom 150/RX Solutions (Montpellier Ressources Imagerie, Montpellier, France). Raw data for each brain scan was reconstructed using X-Act software (RX Solutions, Chavanod, France). We re-oriented the reconstructed scan to the same plane-of-view, and each brain was re-sliced into a new series of two-dimensional images. Based on the staining and segmentation quality, we kept 38 brains (Site A: $N = 18$; Site E: $N = 20$). Using a trained neural network to predict the segmentation of every brain [47, 48], we then segmented the main neuropils of the brain [9]. The antennal lobes (AL), the mushroom bodies (MB) (comprising medial and lateral calyx, peduncle and lobe), the central complex (CX) (comprising the central body, the paired noduli and the protocerebral bridge), the optic lobe (OL) (comprising the medulla and lobula). Neuropil absolute volume was calculated using the voxel count function of AVIZO 2019.1 (Thermo Fisher Scientific, Waltham, USA).

5.7. Elemental analysis

Metal and metalloid contents in bee bodies were analysed using Inductively Coupled Plasma - Mass Spectrometry [56]. We used a routine analysis for trace elements in organic materials [35]. We digested randomly selected individual bees and pools of three bees with ultrapure nitric acid (Optima grade for ultratrace elements, 65% w/w; ThermoFisher Scientific) in a class 10,000 clean room in pre-washed 15 ML Digitubes (SCP Sciences, Villebon sur Yvettes, France) at $100\text{ }^{\circ}\text{C}$ overnight on a hot plate. They were then diluted to 2% HNO_3 and elemental concentrations were measured using inductively Coupled Plasma - Mass Spectrometry at Observatoire Midi-Pyrenees ICP-MS platform on a Thermo ICAP T-Q-ICP-MS (Bremen Germany) (ICP-MS, quantification limit: $<0.01\text{ }\mu\text{g}\cdot\text{kg}^{-1}$, precision measure: 5%). This approach incorporates a technology that combines a collision cell with a reaction cell. The accuracy of the analytical method was controlled using certified reference materials: NIST-1515 (Apple Leaves, National

Institute of Standards and Technology, Gaithersburg, Maryland, USA); IAEA-336 (Lichen, International Atomic Energy Agency, Vienne, Austria); DORM-4 (Fish protein, Conseil National de Recherches du Canada, Ottawa, Ontario, Canada).

5.8. Statistics

We analysed the data using R Studio v.1.2.5033. All data are available in Dataset S1.

For the analysis of potentially harmful elements, we conducted a PCA (package FactoMineR; [45]) on the levels of the seven selected elements [72]. Linear mixed-effects models (LMM) (package lmerTest; [44]) were run on the pollution index (PCI) and arsenic content considering site as fixed effect, and hive as random factors.

For the conditioning trials, we performed generalised linear mixed-effects models (GLMM) (package lme4; [8]), fitted with binomial family, with hive and conditioning date as random factors and site as fixed effect. Using GLMM, we evaluated whether site location would impact the percentage of initial responses to antennal stimulation, spontaneous responses at the first conditioning trial, conditioned responses at the last conditioning trial and responses to each odour during memory test, as well as the proportion of individual response patterns during retrieval. GLMMs were followed by ANOVAs to test the overall significance of fixed categorical variables using the Anova function (package car; [24]). Acquisition scores were transformed as in [69], using the formula $((x * (n - 1) + 0.5) / n)$, to avoid zeros and ones in the normalized variables. They were then compared with GLMM (package Template Model Builder; [10]), and fixed categorical variables significance was tested using Anova.glmTMB function of that package.

For the morphometric analyses, we conducted a PCA on the five parameters measured, and clusters were compared with permutational multivariate analysis of variance (PERMANOVA) (package Vegan; [59]). In addition, head width and length measures were collapsed into the first component of a PCA, which was used as a proxy of the head size. Linear mixed-effects models (LMM) were run for each morphological parameter, considering site as fixed effect, and hive and date as random factors. LMM were followed by F-tests to test the significance of fixed categorical variables.

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

Environmental Implication

We believe our study is environmentally relevant for the following reasons: We study a historic mining site contaminated with metal and metalloid pollutants (particularly arsenic, but also with lead, cadmium, copper, nickel and zinc). These are known to be highly toxic "hazardous materials" for plants and animals (including humans). We assess the effects of environmental contamination by these elements of a sentinel species: the Western honey bee. Our innovative approach, focusing on sublethal effects, enables a precise analysis of risk assessment for pollinators and the broader biodiversity exposed to these cocktails of pollutants.

CRedit authorship contribution statement

CM, ABB, JMD, AE and ML designed the study. CM and ED collected the behavioural and chemical data. CM and ML collected the brain data. CM analysed the behavioural data. GLR analysed the chemical data. PDL analysed the brain data. CM wrote the first draft. All authors revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data Availability

Data will be made available on request.

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Author Statement

CM, ABB, JMD, AE and ML designed the study. CM, ED, GLR collected the data. CM and PDL analysed the data. CM wrote the first draft of the manuscript. ABB, JMD, AE and ML contributed substantially to revisions.

Data Accessibility Statement

Should the manuscript be accepted, the data supporting the results will be archived in Dryad public repository.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2023.133218](https://doi.org/10.1016/j.jhazmat.2023.133218).

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