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Chronic exposure to trace lead impairs honey bee learning

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Data accessibility statement: Raw data are available in Dataset S1 (.xlsx file). Should the manuscript be accepted, the data will be archived in Dryad.

Abstract

Pollutants can have severe detrimental effects on insects, even at sublethal doses, damaging developmental and cognitive processes involved in crucial behaviours. Agrochemicals have been identified as important causes of pollinator declines, but the impacts of other anthropogenic compounds, such as metallic trace elements in soils and waters, have received considerably less attention. Here, we exposed colonies of the western honey bee *Apis mellifera* to chronic field-realistic concentrations of lead in food and demonstrated that consumption of this trace element impaired bee cognition and morphological development. Honey bees exposed to the highest of these low concentrations had reduced olfactory learning performances. In addition, they developed smaller heads, which may have constrained their cognitive functions as we show a general relationship between head size and learning performance. Our results demonstrate that lead pollutants, even at trace levels, can have dramatic effects on honey bee cognitive abilities, potentially altering colony function and pollination service.

Keywords: *Apis mellifera*, heavy metal pollution, PER conditioning, reversal learning, morphometry

1. Introduction

Honey bees and other central-place foraging pollinators rely on their cognitive abilities (learning and memory) to efficiently forage on flowers (Klein et al., 2017; Lihoreau et al., 2011). Yet, these abilities can be easily disrupted by some environmental stressors, even at low exposure levels (e.g. neonicotinoid insecticides: Colin et al., 2019b; Desneux et al., 2007; Henry et al., 2012). In theory, any stressor impairing brain development and/or learning processes may have subtle effects on individual's foraging capacity, with dramatic consequences on colony function, if food supply is compromised (Perry et al., 2015). Here, we focused on the possible sublethal effects of lead (Pb), a metallic trace element (MTE) with well-established neurotoxic properties in vertebrates (Chen et al., 2016; Mason et al., 2014), but whose effects on invertebrates are poorly documented.

MTEs are naturally present in the environment (Bradl, 2005). However, their widespread use in industrial and domestic applications has elevated their levels far above natural baselines in and around urbanised or industrial areas (Hladun et al., 2015; Wuana & Okieimen, 2011). Lead, in particular, is a worldwide pollutant (Cameron, 1992), which can occur at high and persistent concentrations in soils (Han et al., 2002) and in plant nectar between 0.001 and 0.075 $mg.kg^{-1}$ (Gutiérrez et al., 2020). Lead is one among the few MTEs for which international permissible limit values exist (Codex Alimentarius, 2015). However, soil contamination levels are unlikely to decrease in a near future (Marx et al., 2016). These limits defining acceptable levels of lead pollution were defined for humans (Codex Alimentarius, 2015). Pollinators may be particularly exposed to airborne particles while flying (Thimmegowda et al., 2020) and to contaminated water, nectar and pollen when foraging (Formicki et al., 2013). Lead bio-accumulates in the insect body (Mertz, 1981) and it can contaminate pollen, honey and

70 wax in the bee hive (Zhou et al., 2018) and be transferred with food to the larvae
71 (Balestra et al., 1992). Thus, it is likely that pollinators foraging in many urbanised
72 environments are exposed to lead at different life stages.

73 Lead is known to impact the survival (Hladun et al., 2016), physiology
74 (Gauthier et al., 2016; Nikolic et al., 2019), and development of bees (Di et al., 2016),
75 leading to adults with smaller body sizes. While exposure to lead has also been reported
76 to impair some foraging capacities (Sivakoff & Gardiner, 2017; Xun et al., 2018), the
77 impact on cognition has not been assessed. For bees, efficient foraging requires the
78 capacity to associate floral cues (e.g. odorant) with the presence of food (e.g. nectar) in
79 order to develop preferences for profitable resources (Martin Giurfa, 2007). Since the
80 nectar status of flowers changes with time, any such associations must be continually
81 updated with new experience. This demands cognitive flexibility, i.e. the capacity to
82 modify behaviour in response to environmental changes (Scott, 1962). Such flexibility,
83 often assessed with reversal learning paradigms (Izquierdo et al., 2017), is sensitive to
84 many sources of stress and can be impaired in humans exposed to sublethal MTEs levels
85 (Mergler et al., 1994; Rafiee et al., 2020). In honey bee foragers reversal learning
86 performance develops during adulthood and significantly improves at foraging onset,
87 as does the maturation of the underlying brain circuits (Cabirol et al., 2017, 2018). We
88 therefore hypothesised that a chronic exposure to lead could yield alterations in
89 development and learning performances in foraging bees, as it does in mammals
90 (Giordano & Costa, 2012; Grandjean & Landrigan, 2006; Mason et al., 2014).

91 Here, we tested this hypothesis by exposing caged honey bee colonies to field-
92 realistic (low) concentrations of lead for 10 weeks and monitored impacts on the
93 morphology and reversal learning abilities of foraging bees. Given the known impact
94 of lead on morphological development (Di et al., 2016), we also evaluated a potential

basal relationship between body size and cognitive performances in non-contaminated and uncaged bees foraging on natural plant resources.

2. Materials and Methods

2.1. Bee colonies

Experiments on the effects of lead on morphology and cognition were conducted with bees from caged hives from 14/06/2019 (day 1) to 23/08/2019 (day 70) using nine colonies of *Apis mellifera* (Buckfast) maintained in 5 frame hives (Dadant). Each colony was placed in an outside tent (3 m x 3 m) at our experimental apiary (University Paul Sabatier, France) to control the food intake and the foraging experience of bees. Each tent contained two 500 mL feeders. One feeder was filled with sucrose solution (with or without lead, see below) and the other with water. The two feeders were located 1 m apart, 2 m in front of the hive entrance. Caged colonies were given pollen patties (Icko, Bollène, France) once a week directly into the hives.

The experiments on the basal relationship between morphology and cognition were conducted with bees from uncaged hives from 02/2018 to 04/2018, by randomly collecting bees from a pool of 15 colonies (*A. mellifera*, Buckfast) as they foraged on an outside feeder in the same apiary. These non-contaminated bees had free access to natural plant resources.

2.2. Lead exposure

Caged colonies were assigned to one of three lead treatments (three colonies per treatment): 1. unexposed (hereafter 'control bees'), 2. exposed to a low (0.075 $mg.L^{-1}$) concentration of lead ('L bees'), 3. exposed to a high (0.75 $mg.L^{-1}$) concentration of

120 lead ('H bees'). Bees were exposed to lead by them ingesting 50% (w/v) sucrose
121 solution from the feeder, to which lead (II) chloride ($PbCl_2$) (Sigma-Aldrich, Lyon,
122 France) was added. The low and high lead concentrations fell within the range of
123 concentrations measured in natural flowers (Eskov et al., 2015; Gutiérrez et al., 2020;
124 Maiyo et al., 2014; Uren et al., 1998) and honey (Ajtony et al., 2007; Naggar et al.,
125 2013; Satta et al., 2012). Both concentrations are sublethal to adult honey bees (LC_{50}:
126 345 $mg.L^{-1}$) (Di et al., 2016). Control hives were fed 50% (w/v) sucrose solution.
127 Feeders were refilled daily so that bees had an *ad libitum* access to food.
128 Caged hives were maintained in these conditions for 70 days. This duration was
129 long enough for colonies to store contaminated food, so that nectar foraging bees
130 sampled for the cognitive assays were likely to have ingested lead during their
131 development. On average, colonies consumed 8.5±0.6 (SE) kg of sucrose solution and
132 616±25 (SE) g of pollen during the experiment (N=9). During this period, we kept track
133 of the foraging experience of all the nectar foragers (number of days since the onset of
134 foraging) by paint-marking bees with a colour code while feeding on the sucrose
135 solution feeder (Posca pen, Tokyo, Japan). Each day was encoded with a new
136 combination of colours. This operation was repeated twice everyday (1 h in the
137 morning, 1 h in the afternoon).
138
139 *2.3. Lead quantification*
140 Lead levels were analysed in samples of the sucrose solution and bees from caged hives
141 using Inductively Coupled Plasma Emission Spectroscopy (ICP-OES, quantification
142 limit: 5-20 $\mu g.kg^{-1}$, precision measure: 1-5%; AMETEK Spectro ARCOS FHX22,
143 Kleve, Germany).

144 Our ability to detect lead was first verified by assaying the lead level in our high
145 lead concentration sucrose solution (0.75 $mg.L^{-1}$). The solution was acidified at 3% of
146 HNO_3 with ultra-pure 69% HNO_3 to avoid precipitation or adsorption in containers. The
147 solution was then diluted with a HNO_3 3% solution to reduce the spectral interference
148 and viscosity effects. With this method, the amount of lead was recovered at 96%
149 (nominal concentration: 0.75 $mg.L^{-1}$, actual concentration: 0.71 $mg.L^{-1}$).
150 The fact that bees exposed to different concentrations of bio-accumulated lead
151 in a dose-dependent manner was then verified. Lead content was assessed in bees
152 collected 30 days after the start of the exposure (i.e. midway through the experiment).
153 For each sample, bees were pooled in batches of 5. Each batch was rinsed with 5 mL
154 HNO_3 at 3% for 30 s. Bees were wet mineralized in 50 mL polypropylene tubes using
155 a Digiprep system (SCP Science, Quebec, Canada) with 5 mL of 69% nitric acid,
156 following a protocol for athropods (Bur et al., 2012; Astolfi et al. 2020). This consisted
157 of a digestion phase carried out at room temperature overnight, followed by a second
158 phase of heating at 80°C for 60 min. The nitric acid was evaporated, and the samples
159 were diluted with 9 mL of 3% HNO_3. Final solutions were at 3% HNO_3 and total
160 dissolved solids below 5%.
161 Certified reference materials (CRMs) were used as quality controls to validate
162 the protocol of mineralization and multi-elementary ICP analysis: waters (SLRS-6,
163 SUPER-05, ION-96.4) and a solid arthropod CRM (PRON-1 river prawn reference
164 material). Recovery coefficients (ratios measured *vs.* certified values) for major and
165 trace elements ranged between 85% and 115%.
166
167 *2.4. Colony dynamics*

168 The effect of lead exposure on colony dynamics was assessed in the caged colonies
169 through continuous measurement of hive parameters in the caged colonies. Hive weight
170 (±0.01 kg) was recorded every hour with an electronic scale (BeeGuard, Labège,
171 France) below each hive. Every two weeks hives were opened and pictures of both sides
172 of each frame were taken with a Panasonic Lumix DMC-FZ200 equipped with a F2.8
173 25-600 mm camera lens. From the pictures, areas of capped brood and food stores were
174 estimated using CombCount (Colin et al., 2018). Each frame was weighted, after gently
175 removing the adult bees, and the total weight of adult bees (total adult bee mass) was
176 determined by subtracting the tare of the hive and the weight of the frames from the
177 weight of the hive.
178
179 *2.5. Learning assays*
180 The cognitive performances of bees from caged and uncaged colonies was assessed
181 using olfactory conditioning of the proboscis extension reflex (PER; Giurfa & Sandoz,
182 2012). Overall, 268 bees from caged colonies were tested (84 control bees, 84 L bees,
183 100 H bees). These bees were exposed to lead for their whole life (foragers exposed
184 from larvae to foraging age, collected between days 46 and 70 from the start of lead
185 treatment) and originated from 8 of the 9 colonies (one control hive showed very low
186 foraging activity). We focused on new foragers (between 24 and 48 h after the onset of
187 foraging) to avoid inter-individual variation in cognitive performance caused by
188 differences in foraging experiences (Cabirol et al., 2018). Another 149 bees from
189 unaceged colonies were tested. Neither the age nor the foraging experience of these
190 bees were controlled.
191 All bees were submitted to a reversal learning task, i.e. a two-stage task
192 assessing the cognitive flexibly of bees in response to changes in flower rewards (Raine

193 & Chittka, 2007). This test mimics the natural situation where one floral species ceases
194 producing nectar before another species starts doing so. Phase 1 is a differential learning
195 phase, in which the bees must learn to differentiate an odour A reinforced with sucrose
196 (50% w/v in water) and an odour B not reinforced (A+ *vs*. B-). Phase 2 is a non-
197 elemental learning phase, in which the bees must learn the opposite contingency (A- *vs*.
198 B+). We used pure limonene and eugenol (Sigma-Aldrich, Lyon, France) as odours A
199 or B alternately on successive days, so that each contingency was used for about half
200 of the bees for each treatment.

201 On the morning of each test, foragers (24-48 h after onset of foraging) were
202 collected on the feeders, cooled on ice and harnessed in restraining holders that allowed
203 free movements of their antennae and mouthparts (Matsumoto et al., 2012; Fig. 1A).
204 Turning of the head was prevented by fixing the back of the head with melted bee wax.
205 All bees were then tested for PER by stimulating their antennae with 50% sucrose
206 solution. Only those that responded for the conditioning phases (77% of all bees tested)
207 were kept for the experiments. These bees were fed 5 µL of sucrose solution and left to
208 rest in a dark incubator for 3 h (temperature: 25±2°C, humidity: 60%).

209 Bees were then trained using an automatic stimulus delivery system (Fig. 1A;
210 Aguiar et al., 2018). Each training phase included five trials with the reinforced odorant
211 and five trials with the non-reinforced odorant in a pseudo-random order with an eight-
212 minute inter-trial interval. Each conditioning trial (37 s in total) started when a bee was
213 placed in front of the stimulus delivery system, which released a continuous flow of
214 clean air (3,300 $mL.min^{-1}$) to the antennae. After 15 s, the odour was introduced to the
215 airflow for 4 s. For rewarded odours, the last second of odour presentation overlapped
216 with sucrose presentation to the antennae using a toothpick soacked in sucrose solution
217 (Fig. 1A) and sucrose feeding by presenting the toothpick to the mouthparts for 4 s. For

218 the unrewarded trials, no sucrose stimulation was applied. The bee remained another
219 15 s under the clean airflow. Bees were kept in the incubator for 1 h between the two
220 learning phases (A+ *vs*. B- and A- *vs*. B+).

221 During conditioning, we recorded the presence or absence of a conditioned PER
222 to each odorant at each trial (1 or 0). Each bee was given a learning score for phase 1
223 (1 if the bee responded to A+ and not to B- in the last trial of phase 1, 0 otherwise) and
224 for phase 2 (1 if the bee responded to B+ and not to A- on the last trial, 0 otherwise)
225 (Cabirol et al., 2018). Short-term memory (1 h) was assessed by comparing the
226 responses at the last trial of phase 1 and the first trial of phase 2. Each bee was given a
227 memory score for the two odorants (1 if the bee still responded appropriately to the A+
228 and B- on the first trial of the phase 2, 0 otherwise).

229

230 *2.6. Morphometry*

231 Developmental differences among bees was evaluated by conducting morphometric
232 measures on frozen individuals (-18°C) from caged and uncaged hives.

233 To test the effect of lead exposure on morphology in caged bees, foragers of
234 unknown age were collected on the day before lead exposure (day 0 of the experiment),
235 during lead exposure (day 53 of the experiment) and at the end of the experiment (day
236 70 of the experiment), and their head length and head width were measured (Fig. 2A).
237 Emerging adult bees were also sampled every week from each hive (before exposure,
238 during exposure, and at the end of the exposure period). For each bee, the fresh body
239 weight (±0.001 g) (precision balance ME103T, Mettler-Toledo Gmbh, Greifensee,
240 Switzerland) and eight morphometric parameters were recorded: head length, head
241 width, forewing length, forewing width, femur length, tibia length, basitarsus length,
242 basitarsus width (Fig. 2A; De Souza et al., 2015; Mazeed, 2011).

To test for a relationship between morphology and cognitive performances in the uncaged bees, the head length and head width of the conditioned bees hives were measured after the conditioning experiments. All measurements (±0.01 mm) were taken using a Nikon SMZ 745T dissecting scope (objective x0.67) with a Toupcam camera model U3CMOS coupled to the ToupView software.

2.7. Statistics

All analyses were performed with R Studio v.1.2.5033 (RStudio Team, 2015). Raw data are available in Dataset S1. Lead content of bees was compared using a Kruskal-Wallis test (package FSA; Ogle et al., 2019). The effects of lead exposure on colony parameters were evaluated with a multi-model approach (MMI), with treatment, time since the beginning of the exposure (standardised using rescale function, package arm; Gelman & Su, 2013) and their interaction as fixed effects, and hive identity as random factor. A model selection (package MuMIn; Barton, 2020) was run and conditional model average was applied to evaluate the effects of the different factors on the response variables. A MMI was run followed by a conditional model average to assess the effects of treatment, time of exposure and their interactions on brood area (square-root transformed), food stores area and total adult bee mass.

For learning assays, proportion tests were used, followed by pairwise comparisons with a Bonferroni correction (package RVAideMemoire; Hervé, 2020), to evaluate whether lead exposure changed sucrose responsiveness (i.e. proportions of unresponsive bees across treatments). Generalized linear mixed-effects models (GLMM) (package lme4; Bates et al., 2015) were performed to evaluate the effect of treatment on the behavioural variables (PER responses, learning, reversal and memory scores). Proportions of successful responses during the fifth trial of each learning phase

268 were compared using a binomial GLMM, with odorants, treatments and their
269 interactions as fixed effects, and bee identity nested in the hive identity as random
270 factors. A similar GLMM was run to compare the learning, reversal and memory scores,
271 with hive identity as random factor.

272 For the morphometric analyses on caged bees, LMMs were used for each
273 parameter, considering treatment as a fixed effect, and hive identity as a random factor.
274 To assess the global effect of lead, the nine parameters were collapsed into a principal
275 component analysis (PCA) (package FactoMineR, Lê et al., 2008). Bees were clustered
276 into subgroups based on PCA scores, and clusters were compared with a permutational
277 multivariate analysis of variance (PERMANOVA; package vegan; Oksanen et al.,
278 2019). A LMM was run on individual coordinates from the PCA, with treatment as a
279 fixed effect, and hive identity as a random factor. To assess the effect of head size on
280 the cognitive performances of uncaged bees, head width and head length measures were
281 collapsed into the first component of a PCA and a binomial GLMM was run on learning,
282 memory and reversal scores, with individual coordinates from the PCA as fixed effect,
283 and test day as random factor.

284

285 **3. Results**

286

287 *3.1. Exposure to high lead concentration reduced learning performance*

288 We assessed the effect of lead exposure on cognitive flexibility by conducting reversal
289 learning assays in caged bees. The proportion of bees that responded to the antennal
290 stimulation of sucrose was similar across treatments (control bees: 74% N=113; L bees:
291 69% N=122; H bees: 76% N=132; Chisq=1.423, df=2, p=0.491), indicating that lead
292 exposure did not affect appetitive motivation or sucrose perception.

293 Treatment had no significant effect on learning phase 1, although H bees tended
294 to perform less well (Fig. 1B-C). Upon the last trial of phase 1, bees from all treatments
295 discriminated the two odorants (Binomial GLMM: $p<0.001$ for all treatments), and
296 exhibited similar response levels to odour A (Binomial GLMM: L bees $p=0.877$; H bees
297 $p=0.206$) and B (Binomial GLMM: L bees $p=0.331$; H bees $p=0.459$). The proportions
298 of bees that learned to discriminate the two stimuli (learning score equals to 1) were
299 similar across treatments (Control: 48%; L bees: 43%; H bees: 37%) (Fig. 1C; Table
300 S1). These results were independent of the odours used as stimuli A+ and B- (Binomial
301 GLMM: $F_{1,266}=0.905$, $p=0.526$). The proportion of learners at the end of the first phase
302 was similar across hives, within each treatment group. Therefore, exposure to lead, had
303 no significant effect on performance in the differential conditioning task.

304 Treatment did not significantly affect short-term memory between the two
305 phases neither (Fig. 1D). Bees from all treatments had similar memory scores (Binomial
306 GLMM: L bees $p=0.873$; H bees $p=0.115$). However, H bees had a reduced percentage
307 of correct responses between the two phases (25% compared to 36% for control bees).

308 By contrast, treatment had a clear effect on learning in phase 2 (Fig. 1E-F).
309 Upon the last trial, control and L bees were able to discriminate the two odorants
310 (Binomial GLMM: Control $p<0.001$; L bees $p=0.007$), but not H bees (Binomial
311 GLMM: $p=0.075$). The response level to odours A and B was similar between control
312 and L bees (Binomial GLMM: odour A $p=0.097$; odour B $p=0.513$), but H bees
313 responded less to odour B (Binomial GLMM: $p=0.012$) and more to odour A (Binomial
314 GLMM: $p=0.032$) compared to control. Consequently, H bees exhibited lower reversal
315 scores (13% of learners) than L bees (21%) and controls (33%) (Binomial GLMM: L
316 bees, $p=0.086$; H bees, $p=0.001$) (Table S1, Fig. 1F). There was no effect of the odours
317 used as stimuli A- and B+ (Binomial GLMM: $F_{1,266}=1.300$, $p=0.636$), nor of the hive,

318 on the proportion of learners within treatment groups. Therefore, exposure to a high
319 concentration of lead reduced the performance of bees in the reversal learning task.
320 The dose-dependent effect of lead exposure on bee cognition was correlated
321 with dose-dependent bio-accumulation of lead in bees. Control bees and L bees showed
322 no difference in lead content (controls: 0.126±0.031 $mg.kg^{-1}$ d.m., N=3; L bees:
323 0.130±0.002 $mg.kg^{-1}$ d.m., N=3; Kruskal-Wallis: H=7.636, df=1, p=0.712), whereas H
324 bees accumulated significantly more lead (H bees: 0.809±0.044 $mg.kg^{-1}$ d.m., N=5;
325 Kruskal-Wallis: H=7.636, df=1, p=0.039). This result was also independent from any
326 influence of the state of the colony, since lead treatment had no effect on colony
327 measures (syrup and pollen consumption, dynamics of brood production, size of food
328 stores, total adult bee mass, colony weight; LMM: Treatment effect: p>0.05 for all
329 parameters; for further details see Fig. S1).
330
331 *3.2. Bees exposed to the high lead concentration were shorter with smaller heads*
332 Given the observed effects of chronic exposure on the cognitive flexibility of foragers,
333 we asked whether this might result from compromised development. We measured
334 head size in individuals from the different caged hives. Foragers of unknown age
335 collected on the day before the beginning of treatment (day 0) had similar head
336 measurements irrespective of treatment (LMM: L bees: head length p=0.296, head
337 width p=0.287; H bees: head length p=0.333, head width p=0.394). Foragers collected
338 in the middle (day 53) and at the end (day 70) of the experiment had significantly
339 smaller heads than controls (LMM: L bees: head length p=0.017, head width =0.456;
340 H bees: head length p<0.001, head width p=0.040; Table S2).
341 To better assess this developmental impact of lead exposure, we also collected
342 bees at adult emergence, thereby considering only the preimaginal period. For this

343 analysis, we included different body measures in addition to head length and width (Fig.
344 2A), and used them to perform a PCA (Fig. 2B, Table S3). Two PCs explaining 58%
345 of the variance were sufficient to separate control bees and H bees into two distinct
346 clusters, while L bees were intermediate (PERMANOVA: Pseudo-F=5.575, p=0.002;
347 control bees *vs.* L bees: p=0.975; C bees *vs.* H bees: p=0.003; L bees *vs.* H bees,
348 p=0.189). We focused on PC1 which explained 45.8% of the total variance and was
349 associated with general body size. PC1 was negatively correlated with lead
350 concentration (LMM: p=0.042), so that the H bees tended to be smaller than L bees and
351 control bees (Table S4). H bees displayed a rather homogeneous decrease in most
352 parameter values, resulting in a notable weight loss of ca. 8.33% (Table S4).
353 The fact that emerging and foraging bees exhibited a similar decrease in head
354 size (LMM: age effect p>0.05; Tables S2, S4) suggests that most of the impact of lead
355 exposure on morphology occurred before the adult stage.
356
357 *3.3. Unexposed bees with larger heads showed better learning performance*
358 Because the above data suggests a link between lead-induced learning impairment and
359 alterations of head development in our caged bees, we tested the possibility of a general
360 correlation between performance at adulthood and head size, irrespective of lead
361 treatment. For this, we submitted unexposed adult bees from uncaged hives to a reversal
362 learning task (N=149). We separated bees according to their learning, memory and
363 reversal scores (see Methods), in order to compare the morphometric characteristics of
364 bees with different levels of performance. We ran a PCA on this subset of bees, and
365 used the first component (PC1, 73% of the morphological variance), which collapsed
366 head width and length, as a proxy of overall head size (Fig. 3). In phase 1 of reversal
367 learning, the proportion of learners (79% N=118) increased with head size (Fig. 3A),

368 as did the short-term memory recall (46% N=68) (Fig. 3B). In phase 2, the proportion
369 of learners (18% N=27) also increased with head size (Fig. 3C). Therefore, bees with
370 larger heads showed better learning and memory performances in absence of any cage
371 confinement or lead treatment.
372
373 **4. Discussion**
374
375 Recent studies suggest that MTEs can have sublethal effects on individual bees, with
376 potential detrimental consequences for colonies and the pollination service through
377 altered foraging behaviour (Burden et al., 2016, 2019; Skaldina & Sorvari, 2019; Søvik
378 et al., 2015). Here, we found that honey bees chronically exposed to trace
379 concentrations of lead in food have reduced body sizes and learning abilities. The
380 positive correlation between head size and learning performances in unexposed bees
381 suggests that consumption of lead affects bee development, by reducing head size and
382 cognitive function, and thus constitutes a significant neurocognitive stressor for bees at
383 field realistic levels.
384 Chronic exposure to trace lead led to reduced cognitive performance in an
385 olfactory appetitive condition task. This assay reproduces a foraging situation in which
386 bees need to learn olfactory cues signalling the presence or absence of nectar. Neither
387 differential learning (first learning phase) nor short-term memory were affected.
388 However, we found a decreased performance in reversal learning (second learning
389 phase). Thus, the treatment we used did not induce a general impairment of olfactory
390 discrimination nor a decreased motivation for sucrose. This contrasts with the decreased
391 responsiveness to sucrose exhibited in bees acutely treated with lead at similar
392 concentrations (Burden et al., 2019), suggesting a different impact of chronic lead

393 exposure on bees. The specific impairment of reversal learning indicates a loss of
394 cognitive flexibility, which is crucial for bee foragers to switch preferences for flowers
395 whose value changes over time (Ferguson et al., 2001). Over the long-term, this
396 sublethal impact on individual cognition may compromise the overall foraging
397 efficiency of a colony exploiting changing resources, and thus its survival.

398 Reversal learning has been shown to be more strongly affected by lead exposure
399 than simpler differential learning in rats (Hilson & Strupp, 1997), monkeys (Bushnell
400 & Bowman, 1979) and humans (Evans et al., 1994). These tasks measuring cognitive
401 flexibility are particularly sensitive to the adverse effects of stressful stimuli, or of
402 neurodevelopmental disorders (Dajani & Uddin, 2015). Just like mammals
403 (Schoenbaum et al., 2000), honey bees rely on specific brain regions to perform reversal
404 learning, which are not essential for simple differential conditioning (i.e. phase 1 of the
405 conditioning task in our protocol). These are the mushroom bodies (MBs) (Boitard et
406 al., 2015; Devaud et al., 2007), whose maturation over adulthood relates to the
407 acquisition of the capacity for reversal learning (Cabirol et al., 2017, 2018).
408 Interestingly, adult MB organization is altered following exposure to several forms of
409 stress in bees (Cabirol et al., 2017; Peng & Yang, 2016) and other insects (Jacob et al.,
410 2015; Wang et al., 2007). Thus, the specific reversal impairment of lead-exposed bees
411 might be due to neural circuits being more sensitive to the impact of lead in the MBs
412 than in other brain regions.

413 Lead exposure is known to impair brain excitation/inhibition balance during
414 development, through multiple effects such as loss of GABAergic interneurons
415 (Stansfield et al., 2015), altered maturation of GABAergic neurons (Wirbisky et al.,
416 2014), decrease in GABA and glutamate release (Xiao et al., 2006) or transport
417 (Struzynska & Sulkowski, 2004), or inhibition of post-synaptic glutamatergic action

418 (Neal & Guilarte, 2010). In insects, although no specific effect of lead on GABAergic
419 signalling has been demonstrated yet, the effects of lead exposure on synaptic
420 development (Morley et al., 2003), presynaptic calcium regulation (He et al., 2009) and
421 acetylcholinesterase activity (Nikolic et al., 2019) are compatible with a disruption of
422 the excitation/inhibition balance. It has been proposed that reaching an optimal value
423 for such balance in MB circuits is what determines efficient reversal learning in mature
424 adults (Cabirol et al., 2017, 2018). If this is somehow disrupted following lead
425 exposure, that would explain the specific impairment observed only during the reversal
426 phase of the task.

427 Importantly, all bees had undergone their larval and pupal stages during the
428 exposure period, providing ample opportunity for the detrimental effects of lead to be
429 caused by larval ingestion of contaminated food brought by foragers. Lead alters larval
430 development in flies and bees (Cohn et al., 1992; Di et al., 2016; Safaee et al., 2014).
431 Further evidence supports the hypothesis of a developmental effect of lead, since bees
432 exposed to the highest concentrations developed lighter bodies, with shorter wings, and
433 smaller heads. In bees, head width is correlated with the volume of the brain (honey bee
434 foragers: Gronenberg & Couvillon, 2010; bumblebees: Riveros & Gronenberg, 2010)
435 and MBs (honey bee foragers: Mares et al., 2005; bumblebees: Smith et al., 2020).
436 Here, we also found that for bees that had not been exposed to lead, those with smaller
437 and shorter heads had a lower learning performance. This suggests there is a general
438 relationship between head size and cognitive performance in a reversal learning task.
439 We did not control for the age of the measured individuals in this part of the study.
440 However, possible age variations among foragers are unlikely to cause any significant
441 head size changes, since this would be expected to stabilise once the adult cuticle is
442 hardened. In addition, reversal learning performance tend to decrease with foraging

443 experience (Cabirol et al, 2018). It is thus unlikely that bees with larger heads in our
444 sample were those that foraged for shorter times. Our results do not necessarily suggest
445 that such a relationship should be expected for all cognitive tasks. Because control bees
446 with larger heads performed better in both phases of the task, and exposed bees with
447 larger heads only performed better in the reversal task , we assume that lead altered
448 brain development in a specific way resulting in a stronger impact on development or
449 performance of MB neural networks.

450 Continuous exposure to environmentally realistic amounts of lead resulted in
451 bioaccumulation of the metal in the bees' bodies. This is likely to have impaired aspects
452 of head and brain development during larval and pupal stages, resulting in adults with
453 deficits in cognitive flexibility in an ecologically relevant cognitive task. Although this
454 mechanistic hypothesis remains to be confirmed, our results clearly indicate a sublethal
455 impact of lead exposure with potential consequences on foraging efficiency.
456 Importantly, the lead contents measured in the bodies of exposed bees in our
457 experiments ranged within the measurements from bees in field conditions (Goretti et
458 al., 2020). The two concentrations of lead in the sucrose solutions used for chronic
459 exposure (0.075 and 0.75 $mg.L^{-1}$) fell below the maximum level authorized in food (3
460 $mg.kg^{-1}$; Codex Alimentarius, 2015) and irrigation water (5 $mg.L^{-1}$; Ayers & Westcot,
461 1994), and the lowest concentration was under the threshold set for honey by the
462 European Union (0.10 $mg.kg^{-1}$; Commission Regulation (EU) 2015/1005, 2015). This
463 indicates that the cognitive and developmental impairments identified in our
464 experimental conditions may be affecting bees foraging on flowers in many
465 contaminated environments.

466 Although our experiment and recent similar approaches (Hladun et al., 2016)
467 did not capture any consequences on colony dynamics, these individual effects

observed over several weeks might ultimately alter colony function, in particular if lead exposure impairs a broader range of behaviours (e.g. communication, feeding, defense). Thus, differences in colony performances could be predicted over longer term (Klein et al., 2017), which might contribute to collapse, as observed for pesticide exposure at sublethal concentrations (Colin et al., 2019a; Meikle et al., 2016). Our results thus call for future studies to better characterize the impact of lead exposure in bee populations, including in combination with other MTEs as such cocktails are often found in contaminated areas (Badiou-Bénéteau et al., 2013; Goretti et al., 2020). More generally, a better assessment of the contribution of heavy metal pollutants to the widespread decline of insects has become an urgent necessity for preserving ecosystem services.

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Figures

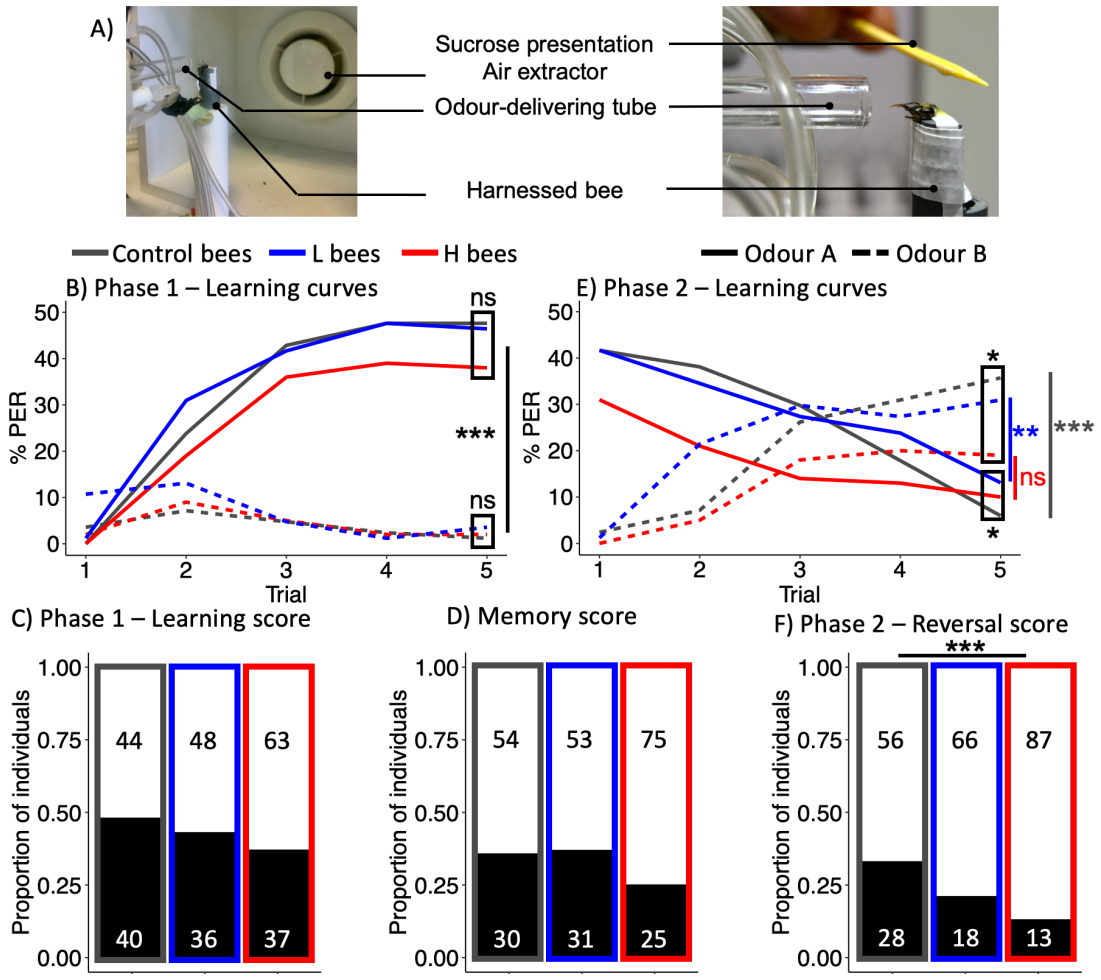

Figure 1: Learning and memory performances of bees from caged hives exposed to lead treatments. A) Picture of a harnessed bee in the conditioning set-up. **B), E)** Line plots show the percentage of proboscis extension responses (PER) elicited by odour A (solid line) and odour B (dashed line) during phase 1 **(B)** and phase 2 **(E)** of reversal learning. Control bees (N=84, dark grey), bees exposed to a low concentration of lead (L bees: 0.075 mg.L^{-1}; N=84, blue) or a high concentration of lead (H bees: 0.75

829 mg.L^{-1}; N=100, red). Statistical comparisons of the response level at the last trial were
830 obtained with p-values from the binomial GLMM (see details in Table S1). **C), D), F)**
831 Bar plots show the proportions of learners (black) and non-learners (white) in the last
832 trial of phase 1 **(C)** and phase 2 **(F)**, with sample size displayed. **D)** Bar plots show the
833 proportions of bees remembering (black) or not (white) during the 1 h memory recall,
834 with sample size displayed. Statistical comparisons were obtained with p-values from
835 the binomial GLMM (Table S1) (ns: non-significant, $p>0.05$; *$p<0.05$; **$p<0.01$;
836 ***$p<0.001$).

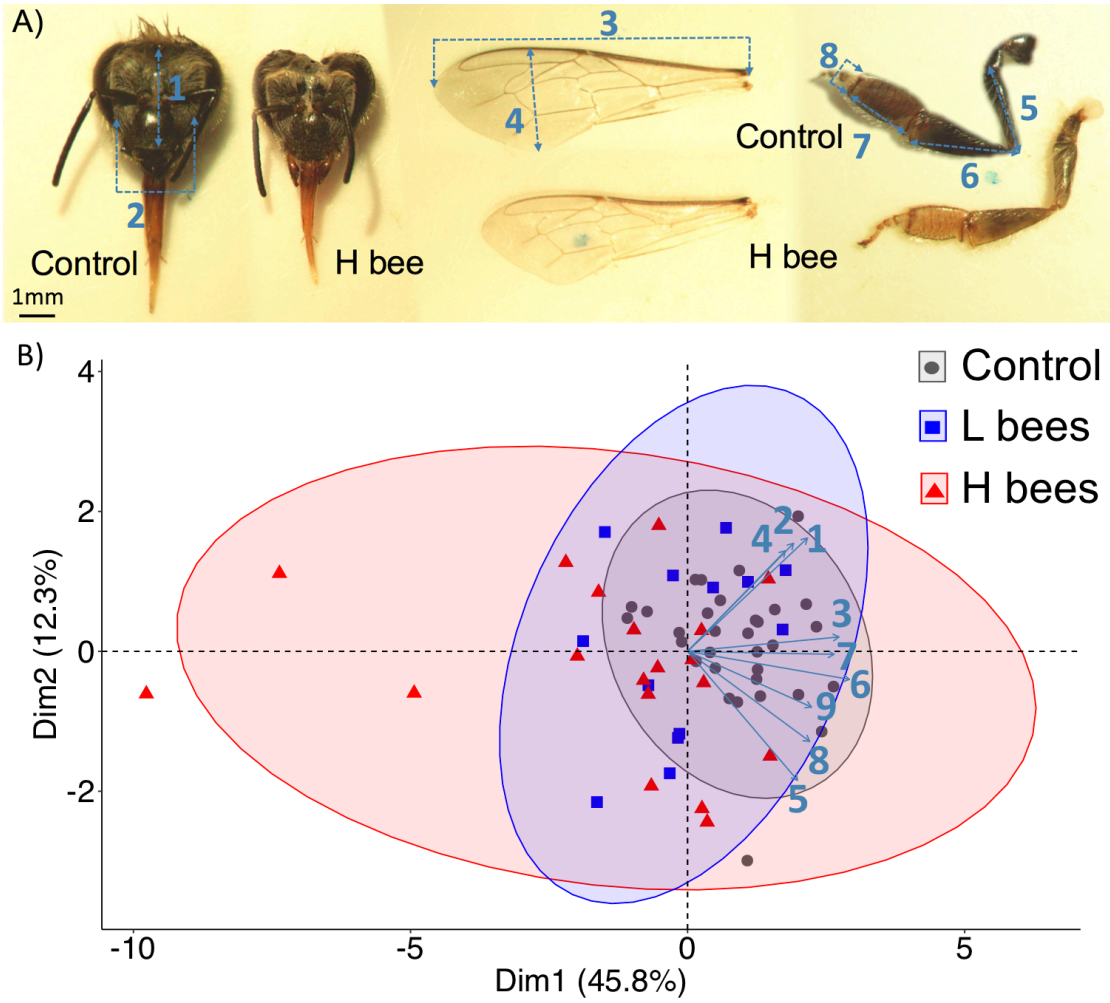

Figure 2: Morphometric analysis of bees from caged hives exposed to lead treatments. A) Details of the parameters measured. This example shows morphological differences in emerging bees. (1) Head length, (2) Head width, (3) Wing length, (4) Wing width, (5) Femur length, (6) Tibia length, (7) Basitarsus length, (8) Basitarsus width, (9) Bee weight (not shown). **B)** Principal component analysis (PCA) map shows the relationship among the morphometric measures (same number code as in A). 95% confidence ellipses of the mean are displayed for each treatment. Controls: bees unexposed to lead (N=32); L bees: bees exposed to the low concentration of lead (0.075 $mg.L^{-1}$) (N=13); H bees: bees exposed to the high concentration of lead (0.75 $mg.L^{-1}$) (N=19).

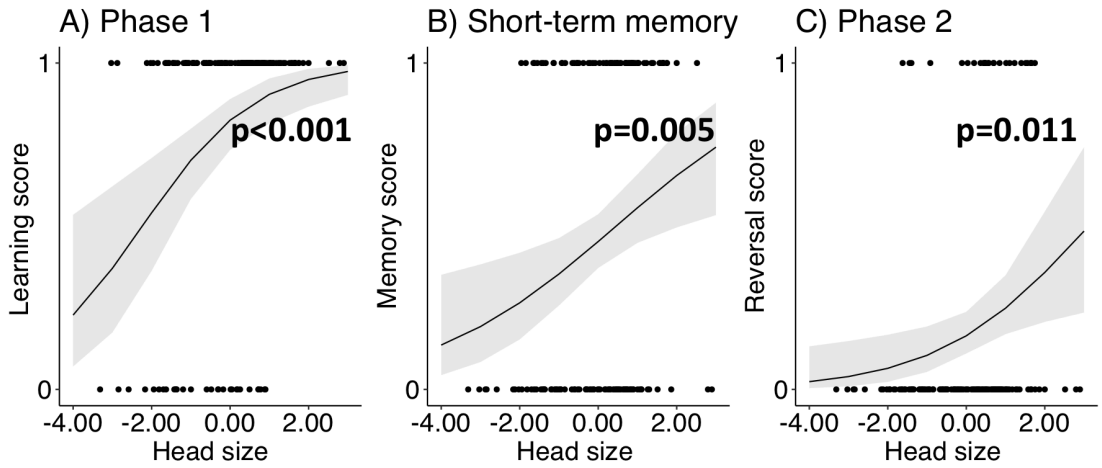

Figure 3: Relationship between head size and cognitive performance in bees from uncaged hives unexposed to lead treatments. Data points represent the individual data for learners (learning score=1) and non-learners (learning score=0). Fitted lines of head size effect are displayed in black with 95% confidence intervals in grey. N = 149 bees. **A)** Learning score at the end of phase 1. **B)** Short-term memory score. **C)** Reversal score at the end of phase 2. Statistical comparisons were obtained with p-values from the binomial GLMM testing bees coordinates in PC1 on cognitive scores, significant values (<0.05) are shown in bold. Increasing head size significantly enhanced the learning performances in phase 1 (Binomial GLMM: estimate±SE, 0.693±0.188, p<0.001) and phase 2 (0.523±0.205, p=0.011), as well as short-term memory recall (0.415±0.149, p=0.005).

861 **Supporting materials**

862

863 **Table S1: Parameter estimates from the binomial GLMM for response levels at**
864 **the end of both learning phases, and for learning, reversal and memory score**
865 **models in bees from caged hives exposed to lead treatments.** Significant p-values
866 (<0.05) are shown in bold. SE=conditional standard errors.

Conditional average	Estimate	SE	p-value
PER response at the end of Phase 1			
Intercept	-0.1002	0.2303	0.664
Low concentration	-0.0505	0.3266	0.877
High concentration	-0.4146	0.3277	0.206
Odour B-	-4.4110	1.0472	**<0.001**
Low concentration:Odour B-	1.1762	1.2091	0.331
High concentration:Odour B-	0.9418	1.2733	0.459
PER response at the end of Phase 2			
Intercept	-2.7600	0.4611	**<0.001**
Low concentration	-0.2146	0.3280	0.513
High concentration	-0.8622	0.3418	**0.012**
Odour A-	-2.1722	0.5143	**<0.001**
Low concentration:Odour A-	1.0820	0.6518	0.097
High concentration:Odour A-	1.4250	0.6638	**0.032**
Learning score			
Intercept	-0.0953	0.2185	0.663
Low concentration	-0.1924	0.3104	0.535
High concentration	-0.4369	0.3010	0.147
Memory score			
Intercept	-0.5878	0.2277	**0.010**
Low concentration	0.0515	0.3209	0.873
High concentration	-0.5108	0.3243	0.115
Reversal score			
Intercept	-0.6931	0.2315	**0.003**
Low concentration	-0.6061	0.3525	0.085
High concentration	-1.2078	0.3768	**0.001**

867

868 **Table S2: Analysis of the morphological parameters of forager bees from caged**
869 **hives exposed to lead treatments.** Median, minimum and maximal values of each
870 morphological parameter of forager bees from caged hives, per treatment and
871 percentage of variation between medians compared to control bees. Estimated
872 regression parameters, standard errors (SE) and p-values of the linear mixed effects
873 models. Significant differences with control group ($p<0.05$) are shown in bold.

Morphological parameters	Treatment	Median (min-max)	Variation compared to control	Estimate±SE	p-value
Head length (mm)	Control	2.88 (2.55-3.07)			
	Low concentration	2.78 (2.33-2.99)	-3.60%	-0.1054±0.0432	**0.017**
	High concentration	2.69 (2.42-2.87)	-7.06%	-0.1877±0.0395	**<0.001**
Head width (mm)	Control	2.42 (2.27-2.62)			
	Low concentration	2.41 (2.16-2.52)	-0.41%	-0.0294±0.0354	0.456
	High concentration	2.30 (2.18-2.48)	-4.99%	-0.0990±0.0324	**0.040**

874

875 **Table S3: Principal component analysis (PCA) on the morphometry of emerging**
876 **bees from caged hives exposed to lead treatments.** Correlation coefficients >0.4 in
877 absolute value are shown in bold.
878

Variable	PC1	PC2
Bee weight	**0.654**	-0.233
Head length	**0.633**	**0.474**
Head width	**0.560**	**0.452**
Wing length	**0.799**	0.060
Wing width	**0.516**	**0.421**
Femur length	**0.580**	**-0.539**
Tibia length	**0.854**	0.117
Basitarsus length	**0.773**	-0.012
Basitarsus width	**0.644**	-0.376
% Total variance	45.84	12.32
Cumulative proportion of total variance	45.84	58.17

879

880 **Table S4: Analysis of the morphological parameters of emerging bees from caged**
881 **hives exposed to lead treatments.** Median, minimum and maximal values of each
882 morphological parameter of emerging bees from caged hives, per treatment and
883 percentage of variation between medians compared to control bees. Estimated
884 regression parameters, standard errors (SE) and p-values of the linear mixed effects
885 models. Significant differences with control group ($p<0.05$) are shown in bold.

Morphological parameters	Treatment	Median (min-max)	Variation compared to control	Estimate±SE	p-value
Bee weight (g)	Control	0.12 (0.10-0.14)			
	Low concentration	0.11 (0.06-0.12)	-9.35%	-0.0108±0.0064	0.142
	High concentration	0.11 (0.06-0.013)	-8.33%	-0.0173±0.0058	**0.029**
Head length (mm)	Control	2.89 (2.67-3.03)			
	Low concentration	2.90 (2.47-2.97)	0.34%	-0.0712±0.0679	0.365
	High concentration	2.65 (2.15-3.01)	-9.06%	-0.2021±0.0615	**0.050**
Head width (mm)	Control	2.42 (2.24-2.71)			
	Low concentration	2.41 (2.23-2.58)	-0.21%	-0.0339±0.0501	0.530

	High concentration	2.32 (2.02-2.47)	-4.09%	-0.1624±0.0452	**0.022**
Wing length (mm)	Control	8.79 (8.42-9.08)			
	Low concentration	8.84 (8.39-9.03)	0.62%	-0.0030±0.1199	0.981
	High concentration	8.75 (7.57-8.96)	-0.40%	-0.2846±0.1086	**0.048**
Wing width (mm)	Control	3.12 (2.71-3.35)			
	Low concentration	3.10 (2.72-3.34)	-0.81%	-0.0547±0.0506	0.285
	High concentration	3.13 (2.64-3.38)	0.16%	-0.0331±0.0447	0.462
Femur length (mm)	Control	2.30 (2.15-2.53)			
	Low concentration	2.26 (2.08-2.40)	-1.77%	-0.0680±0.0491	0.227
	High concentration	2.27 (1.90-2.46)	-1.32%	-0.0718±0.0442	0.178
Tibia length (mm)	Control	3.04 (2.90-3.18)			
	Low concentration	3.04 (2.81-3.15)	0%	-0.0532±0.0608	0.430
	High	3.04 (2.60-3.14)	0%	-0.0916±0.0560	0.189

	concentration				
Basitarsus length (mm)	Control	2.06 (1.94-2.24)			
	Low concentration	2.05 (1.95-2.15)	-0.73%	-0.0247±0.0537	0.665
	High concentration	2.04 (1.63-2.21)	-1.23%	-0.0634±0.0492	0.264
Basitarsus width (mm)	Control	1.16 (1.05-1.40)			
	Low concentration	1.16 (1.04-1.25)	0%	-0.0066±0.0371	0.868
	High concentration	1.10 (0.95-1.27)	-5.45%	-0.0690±0.0337	0.118
Bees coordinates in PC1	Control	1.01 (-1.09-2.63)			
	Low concentration	-0.17 (-1.89-1.77)		-0.9940±0.9397	0.346
	High concentration	-0.65 (-9.77-1.49)		-2.6526±0.8607	**0.042**

886

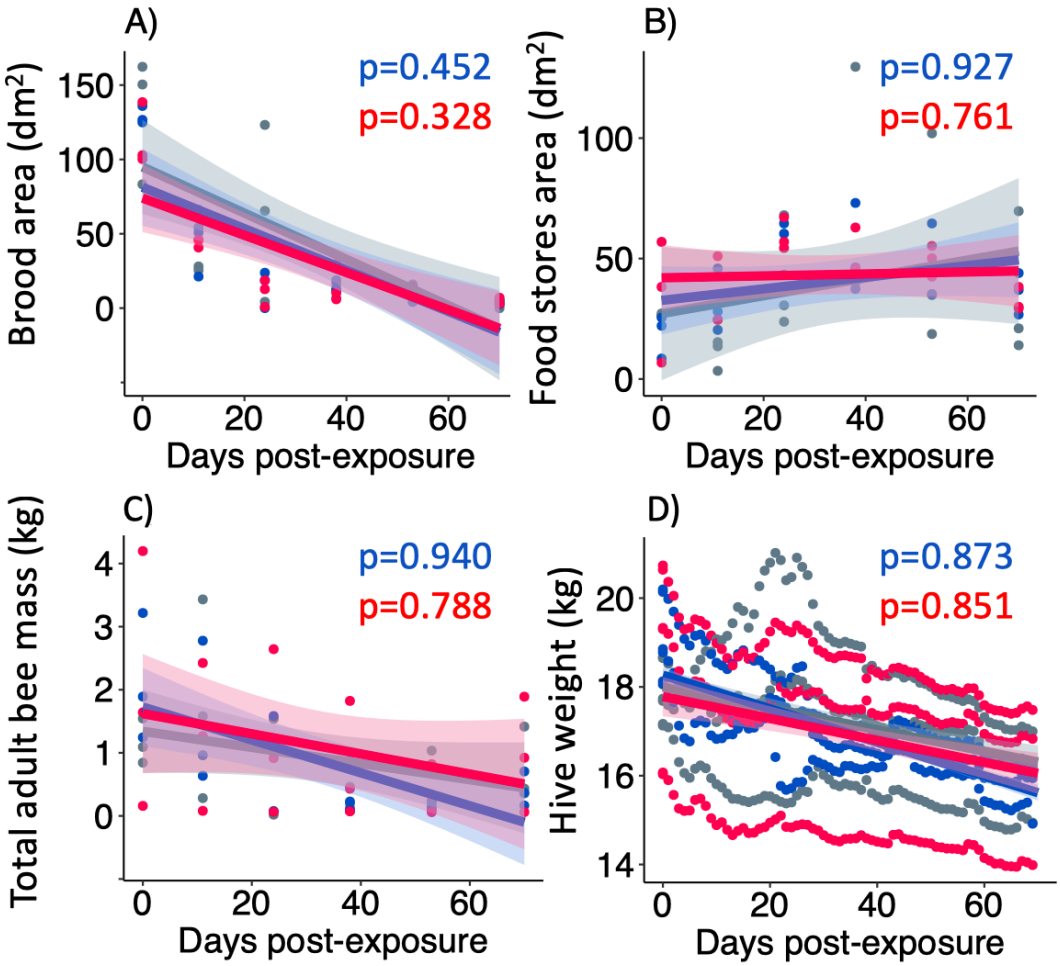

Figure S1: Amount of brood, food stores, total bees mass, hive weight for caged hives exposed to lead treatments throughout the experiment. Control colonies (N=3, grey), colonies exposed to a low concentration (0.075 mg.L^{-1}; N=3, blue) or a high concentration (0.75 mg.L^{-1}; N=3, red) of lead. Evaluations for brood, food stores and bees were conducted every 15 days for all hives. Total adult bee mass was recorded every hour and averaged on a daily basis. **A)** Area of capped brood cells. **B)** Area of food (honey and pollen) stores. **C)** Total adult bee mass. **D)** Hive weight. Estimate trends are displayed in solid lines. 95% confidence level interval are displayed in the same colour code as treatment. P-values were obtained from LMMs and are displayed for the treatment effect.

Dataset S1: Raw data (.xlsx file). 'Colony monitoring': data on the caged colonies parameters. Brood area (cm2), honey store (cm2), total adult bee mass (kg) were assessed during visits to the colonies. The amount of syrup (g) and pollen (g) given to the colony on a daily or weekly basis. The weight of the hive was obtained by averaging the hourly data per 24hr. **'PER caged'**: proboscis extension reflex (1/0) recorded for each trial of the differential and reversal phases. Learning, reversal and memory scores are given for each bee (see 'Learning assays' in the main text for details on the calculation)**. 'Morphometry emerging'**: Morphometric parameters measured on emerging bees: weight (g), head length (mm) and width (mm), wing length (mm) and width (mm), femur length (mm), tibia length (mm), basitarsus length (mm) and width (mm). **'Morphometry foragers'**: Morphometric parameters measured on forager bees: weight (g), head length (mm) and width (mm). **'PER uncaged'**: proboscis extension reflex (1/0) recorded for each trial of the differential and reversal phases. Learning, reversal and memory scores are given for each bee (see 'Learnings assays' in main text for details on the calculation). **'Pb content in bees' bodies'**: analysis of Pb content in the bodies of bees using ICP-OES.