

1 **Chronic exposure to lead impairs honey bee learning**

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3 Coline Monchanin^{1,2#}, Amaury Blanc-Brude¹, Erwann Drujon¹, Mohammed Mustafa
4 Negahi¹, Cristian Pasquaretta¹, Jérôme Silvestre³, David Baqué³, Arnaud Elger³,
5 Andrew B. Barron², Jean-Marc Devaud¹, Mathieu Lihoreau^{1#}

6

7 ¹Research Center on Animal Cognition (CRCA), Center for Integrative Biology (CBI);
8 CNRS, University Paul Sabatier – Toulouse III, France

9 ²Department of Biological Sciences, Macquarie University, NSW, Australia

10 ³EcoLab, Université de Toulouse, CNRS, INPT, UPS, Toulouse, France.

11

12 E-mail address of all authors:

13 coline.monchanin@univ-tlse3.fr, am.bbrude@orange.fr, e.drujon@gmail.com,

14 negahimustafa@gmail.com, cristian.pasquaretta@univ-tlse3.fr, silvestre@ensat.fr,

15 david.baque@univ-tlse3.fr, arnaud.elger@univ-tlse3.fr, andrew.barron@mq.edu.au,

16 jean-marc.devaud@univ-tlse3.fr, mathieu.lihoreau@univ-tlse3.fr

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26 # Corresponding authors:

27 coline.monchanin@univ-tlse3.fr, 0033632423303

28 mathieu.lihoreau@univ-tlse3.fr, 00336470606

29 Centre de Recherche sur la Cognition Animale (CRCA), Centre de Biologie Intégrative
30 de Toulouse (CBI) ; CNRS – Université Paul Sabatier – Toulouse III,

31 118 route de Narbonne, 31052 Toulouse cedex 9, France

32

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36

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38

39 **Abstract**

40 Pollutants can have severe detrimental effects on insects, even at sublethal doses.
41 Agrochemicals have been identified as important causes of pollinator declines, but the
42 impacts of other anthropogenic compounds, such as metallic trace elements
43 contaminating soils and waters, have received considerably less attention. Here, we
44 exposed honey bee colonies to chronic field-realistic concentrations of lead in food and
45 demonstrate that consumption of this single trace element impaired bee cognition and
46 morphological development. Honey bees exposed to the highest lead concentration had
47 reduced olfactory learning performances and developed smaller heads, which may have
48 constrained their cognitive functions. Our results show that lead pollutants can have
49 dramatic effects on honey bee health and may contribute to the widespread decline of
50 pollinators.

51

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

54

55 **Introduction**

56 Metallic trace element (MTE) are naturally present in the environment (Bradl 2005).
57 However, their widespread uses in industrial and domestic applications has elevated
58 their levels in soils (Wuana & Okieimen 2011), water reservoirs, and plant tissues
59 (Hladun *et al.* 2015) far above natural baselines around industrialized and urbanized
60 areas, mining sites and agricultural regions (Zhou *et al.* 2018). Some MTE are well-
61 known neurotoxins (Mason *et al.* 2014) and constitute a direct threat for animals
62 inhabiting contaminated environments, including humans (Chen *et al.* 2016), whose
63 neural development and cognitive functions may be impaired.

64 Pollinators, such as bees, are in front line. These insects have a severely
65 constrained brain size optimised to perform cognitive operations for exploiting
66 scattered plant resources (Giurfa 2013). Bees can encounter airborne MTE particles
67 while flying (Negri *et al.* 2015), and may bring back contaminated water, nectar and
68 pollen to their colony nest (Roman 2007; Formicki *et al.* 2013). MTE bio-accumulate
69 in the bodies of insects (Perugini *et al.* 2011; Lambert *et al.* 2012), because of
70 biochemical and structural similarities to non-toxic molecules (Clarkson 1987) and
71 their incorporation in metabolic pathways (Mertz 1981), but they can also contaminate
72 the nest. In honey bee hives, MTE are found in pollen, honey, wax and propolis (Satta
73 *et al.* 2012; Zhou *et al.* 2018), and can be transferred to the larvae (Balestra *et al.* 1992).

74 Many MTE (e.g. lead, cadmium, selenium, aluminium, manganese) can have
75 detrimental effects on the physiology (Gauthier *et al.* 2016; Nikolić *et al.* 2019),
76 foraging behaviour (Søvik *et al.* 2015; Chicas-Mosier *et al.* 2017; Sivakoff & Gardiner
77 2017) and survival (Di *et al.* 2016; Hladun *et al.* 2016) of bees. However, potential
78 effects on cognition have been largely unexplored. Recent work indicates that acute
79 exposure to some MTE can reduce the sensitivity of bees to sucrose rewards (Hladun

80 *et al.* 2012; Burden *et al.* 2019) as well as elemental forms of learning and memory
81 (Burden *et al.* 2016); while not necessarily being detected by the sensory organs
82 (Burden *et al.* 2019). Just as for agrochemical pollution (Gill *et al.* 2012; Henry *et al.*
83 2012), the potential sublethal effects of MTE on bee cognition due to a chronic exposure
84 may have long-term dramatic consequences on populations. In these central-place
85 foraging insects, any alteration in the learning or memory performances of the foragers
86 may reduce food collection and ultimately impair colony development (Gill *et al.* 2012;
87 Klein *et al.* 2017).

88 Here, we experimentally investigated the effects of chronic lead exposure on
89 honey bee cognition. Lead is of public health significance because of its high degree of
90 toxicity and worldwide distribution (Cameron 1992). Although worldwide lead
91 emissions have been significantly reduced since the phase-out of leaded gasoline
92 (Kierdorf & Kierdorf 2004; Chadwick *et al.* 2011), they are still important in some
93 countries (Li *et al.* 2012) and the concentrations of legacy lead remain high in soils
94 (Han *et al.* 2002; Zhou *et al.* 2018) and plant tissues (Rashed *et al.* 2009). We exposed
95 caged honey bee colonies to field-realistic concentrations of lead for 10 weeks and
96 monitored impacts on the morphology and cognition of individual bees, as well as on
97 colony dynamics. We also evaluated the basal relationship between morphological
98 development and cognitive performances in non-contaminated bees from uncaged
99 colonies foraging on natural plant resources.

100

101 **Materials and Methods**

102

103 *Bee colonies*

104 We ran the experiments with caged hives from 14/06/2019 (day 1) to 23/08/2019 (day
105 70) using nine colonies of *Apis mellifera* (Buckfast) maintained in 5 frame hives
106 (Dadant). Each colony was placed in an outside tent (3m x 3m) at our experimental
107 apiary (University Paul Sabatier, France) to control the food intake and the foraging
108 experience of bees. Each tent contained a feeder with sucrose solution (with or without
109 lead, see below) and a water supply. Both were located 1m apart, 2m in front of the
110 hive entrance. Caged colonies were given pollen patties (Icko, Bollène, France) once a
111 week directly into the hives. We also ran experiments with bees from uncaged hives,
112 by randomly collecting bees from colonies in the same apiary. These non-contaminated
113 bees had free access to natural plant resources.

114

115 *Lead exposure*

116 We assigned the caged colonies to one of three lead treatments (three colonies per
117 treatment): unexposed (hereafter ‘control bees’), exposed to a low (0.075 mg.L⁻¹)
118 concentration of lead (‘L bees’), exposed to a high (0.75 mg.L⁻¹) concentration of lead
119 (‘H bees’). Bees were exposed to lead by ingesting 50% (w/v) sucrose solution from
120 the feeder, to which we added lead (II) chloride (PbCl₂) (Sigma-Aldrich, Lyon, France).
121 The low and high lead concentrations fell within the range of concentrations measured
122 in floral nectar (Eskov *et al.* 2015) and honey (Lambert *et al.* 2012) in contaminated
123 environments. Both concentrations are sublethal to adult honey bees (Di *et al.* 2016).
124 Control hives were fed 50% (w/v) sucrose solution. We maintained the hives in these
125 conditions for 70 days, during which colonies consumed on average 8.5±0.6 (SE) kg of
126 sucrose solution and 616±25 (SE) g of pollen (N=9). We kept track of the foraging
127 experience of the nectar foragers (number of days since the onset of foraging) from
128 each colony by paint-marking bees with a colour code while feeding on the sucrose
129 solution feeder (Posca pen, Tokyo, Japan).

130

131 *Learning assays*

132 We assessed the cognitive performances of bees from caged and uncaged colonies using
133 olfactory conditioning of the proboscis extension reflex (PER) (Giurfa & Sandoz 2012).
134 For caged colonies, we tested bees exposed during their whole life (foragers collected
135 between days 46 and 70 after the start of the exposure) from 8 of the 9 colonies (one
136 control hive showed low foraging activity).

137 All bees were submitted to a reversal learning task, a two-task assessing the
138 cognitive flexibility of bees in response to changes in flower rewards (Raine & Chittka
139 2007). Phase 1 is a differential learning phase, in which the bees must learn to
140 differentiate an odour A reinforced with sucrose (50% w/v in water) and an odour B
141 not reinforced (A+ vs. B-). Phase 2 is a non-elemental learning phase, in which the bees
142 must learn the opposite contingency (A- vs. B+). We used pure limonene and eugenol
143 (Sigma-Aldrich, Lyon, France) as odours A or B alternately on successive days, so that
144 each combination was used for about half of the bees for each treatment.

145 We tested new foragers (between 24 and 48 hours after the onset of foraging) to
146 avoid variations in cognitive performances caused by inter-individual differences in
147 foraging experiences (Cabirol *et al.* 2018). On the morning of each test, we collected
148 foragers from each hive on the feeders (except from one control hive due to a low
149 foraging activity), cooled them on ice and harnessed them in restraining holders, which
150 allowed free movements of their antennae and mouthparts (Matsumoto *et al.* 2012) (Fig.
151 1A). Rotational movements of the head were prevented by fixing the back of the head
152 with melted bee wax. We then tested all bees for PER by stimulating their antennae
153 with 50% sucrose solution, and kept only those that responded for the conditioning

154 (77% of the 543 bees tested in total). These bees were fed 5 μ L of sucrose solution and
155 left to rest in a dark incubator for 3h (temperature: 25 \pm 2 $^{\circ}$ C, humidity: 60%).

156 Bees were trained using an automatic stimulus delivery system (Fig. 1A;
157 (Aguiar *et al.* 2018)). Each training phase included five trials with the reinforced
158 odorant and five trials with the non-reinforced odorant in a pseudo-random order with
159 an eight-minute inter-trial interval. Each conditioning trial (37 s in total) started when
160 a bee was placed in front of the stimulus delivery system, which released a continuous
161 flow of clean air (3,300 mL.min $^{-1}$) to the antennae. After 15 s, the odour was introduced
162 to the airflow for 4 s, the last second of which overlapped with sucrose presentation to
163 the antennae using a toothpick (Fig. 1A) and subsequent feeding for 4 seconds for the
164 rewarded trials. For the unrewarded trials, no sucrose stimulation was applied. The bee
165 remained another 15 s under the clean airflow. Bees were kept in the incubator for 1h
166 between the two learning phases (A+ *vs.* B- and A- *vs.* B+).

167 During conditioning, we recorded the presence or absence of a conditioned PER
168 to each odorant at each trial (1 or 0). Each bee was given a learning score for phase 1
169 (1 if the bee responded to A+ and not to B- in the last trial of phase 1, 0 otherwise) and
170 for phase 2 (1 if the bee responded to B+ and not to A- on the last trial, 0 otherwise)
171 (Cabirol *et al.* 2018). Short-term memory (1 h) was assessed by comparing the
172 responses at the last trial of phase 1 and the first trial of phase 2. Each bee was given a
173 memory score for the two odorants (1 if the bee still responded appropriately to the A+
174 and B- on the first trial of the phase 2, 0 otherwise).

175

176 *Morphometry*

177 We evaluated developmental differences among bees by conducting morphometric
178 measures on frozen individuals (-18 $^{\circ}$ C) from caged and uncaged hives. To test the effect

179 of lead exposure on morphology, we collected foragers from the caged hives the day
180 before exposure (day 0), during exposure (day 53) and at the end of the experiment (day
181 70), and measured their head length and head width (Fig. 2A). We also sampled
182 emerging adult bees every week from each of the caged hives (before exposure, during
183 exposure, and at the end of the exposure period), making sure that no *Varroa sp.* mites
184 were present in the cell. We measured their fresh body weight (± 0.001 g) (precision
185 balance ME103T, Mettler-Toledo GmbH, Greifensee, Switzerland) and eight
186 morphometric parameters: head length, head width, forewing length, forewing width,
187 femur length, tibia length, basitarsus length, basitarsus width (Fig. 2A) (Mazeed 2011;
188 De Souza *et al.* 2015). To test the basal relationship between cognitive performances
189 and morphology, we measured the head length and head width of the non-contaminated
190 bees randomly collected from uncaged hives after the conditioning experiments. We
191 took all measurements (± 0.01 mm) using a Nikon SMZ 745T dissecting scope
192 (objective x0.67) with a Toupcam camera model U3CMOS coupled to the ToupView
193 software.

194

195 *Colony dynamics*

196 We assessed the effect of lead exposure on colony dynamics through continuous
197 measurement of hive parameters in the caged colonies. Colony weight (± 0.01 kg) was
198 recorded every hour with an electronic scale (BeeGuard, Labège, France) below each
199 hive. Every two weeks we opened the hives and took pictures of both sides of each
200 frame with a Panasonic Lumix DMC-FZ200 equipped with a F2.8 25-600 mm camera
201 lens. From the pictures, we estimated the areas of capped brood and food stores using
202 CombCount (Colin *et al.* 2018). We weighted each frame, after gently removing the
203 adult bees, and determined the total weight of adult bees (bee mass) by subtracting the

204 tare of the hive and the weight of the frames from the weight of the hive. Five times
205 during the experiment (day 0, day 24, day 38, day 53, day 70), we estimated *Varroa*
206 loads by collecting ca. 300 bees from uncapped brood frames in a jar and adding 15g
207 of icing sugar. We shook the jar for two minutes, released the bees and counted the
208 number of fallen mites. The number of mites was standardised and expressed as number
209 of *Varroa sp.* phoretic mites per 100 bees (Dietemann *et al.* 2013).

210

211 *Lead quantification*

212 We analysed lead in sucrose solution and bees from caged hives using Inductively
213 Coupled Plasma Emission Spectroscopy (ICP-OES, quantification limit: 5-20 $\mu\text{g.kg}^{-1}$,
214 precision measure: 1-5%; AMETEK Spectro ARCOS FHX22, Kleve, Germany). We
215 assessed lead concentration in sucrose using the high concentration sucrose solution
216 (the low concentration solution fell under the ICP-OES detection thresholds). The
217 solution was acidified at 3% of HNO_3 with ultra-pure 69% HNO_3 to avoid precipitation
218 or adsorption in containers. It was then diluted with a HNO_3 3% solution to reduce the
219 spectral interference and viscosity effects. We assessed lead content in bees collected
220 during the 4th week of exposure. For each sample, we pooled bees in batches of 5. Each
221 batch was rinsed with 5 mL HNO_3 at 3% for 30 s. Bees were wet mineralized in 50mL
222 polypropylene tubes using a Digiprep system (SCP Science, Quebec, Canada) with 5
223 mL of 69% nitric acid. A digestion phase was carried out at room temperature
224 overnight, followed by a second phase of heating at 80°C for 60 min. The nitric acid
225 was evaporated, and the samples were diluted with 9 mL of 3% HNO_3 . Final solutions
226 were at 3% HNO_3 and total dissolved solids below 5%.

227

228 *Statistics*

229 We ran all analyses with R Studio v.1.2.5033 (RStudio Team 2015). We compared lead
230 content of bees using a Kruskal-Wallis test (package FSA; (Ogle *et al.* 2019)). We
231 evaluated the effects of lead exposure on colony parameters with a multi-model
232 approach (MMI), with treatment, exposure duration (standardised using rescale
233 function, package arm; (Gelman & Su 2013)) and their interaction as fixed effects, and
234 hive identity as random factor. We ran a model selection (package MuMIn; (Barton
235 2020)) and applied a conditional model average to evaluate the effects of the different
236 factors on the response variables. To evaluate the effect of *Varroa sp.* load, we used a
237 subset of the colony data for the days when mites were counted. We ran a MMI followed
238 by a conditional model average to assess the effects of treatment, time, *Varroa* counts
239 and their interactions on brood area (square-root transformed), food stores area and bee
240 mass.

241 For learning assays, we tested colony effects by comparing the proportions of
242 bees with learning or reversal scores of 1 between hives in each treatment group. We
243 used proportion tests, followed by pairwise comparisons with a Bonferroni correction
244 (package RVAideMemoire; (Hervé 2020)), to evaluate whether lead exposure changed
245 sucrose responsiveness (i.e. proportions of unresponsive bees across treatments). We
246 extracted individual learning slopes from the raw data (using a linear mixed effect
247 model (LMM, package nlme; (Pinheiro *et al.* 2019)) with individual nested in test day
248 as a random effect and trial as random slope). We then compared learning slopes
249 between treatments, during each learning phase, by performing generalized linear
250 mixed-effects models (GLMM) (package lme4; (Bates *et al.* 2015)), with hive identity
251 as random factor and treatment and standardized duration of exposure as fixed effects.
252 We compared proportions of successful responses during the 5th trial of each learning
253 phase using a binomial GLMM, with odorants, treatments, standardized duration of

254 exposure and their interactions as fixed effects, and bee identity nested in the hive
255 identity as random factors. We ran a similar GLMM to compare the learning, reversal
256 and memory scores. We used a MMI and model average to evaluate the effect of
257 treatment and standardised duration of exposure, and their interactions, on the
258 behavioural variables (PER responses, learning, reversal and memory scores).

259 For the morphometric analyses on caged bees, we used LMMs for each
260 parameter, considering treatment, duration of exposure and their interaction as fixed
261 effects, and hive identity as random factor. To assess the global effect of lead, we
262 collapsed the nine parameters into the first component of a principal component
263 analysis (PCA) (package FactoMineR, (Lê *et al.* 2008)). Bees were clustered into
264 subgroups based on PCA scores, and clusters were compared with a permutational
265 multivariate analysis of variance (PERMANOVA) (package vegan; (Oksanen *et al.*
266 2019)). We ran a LMM on individual coordinates from the PCA, with treatment,
267 exposure duration and their interaction as fixed effects, and hive identity as random
268 factor. To assess the effect of head size on cognitive performance of uncaged bees, we
269 collapsed the head width and length measures into the first component of a PCA and
270 ran a binomial GLMM on learning, memory and reversal scores, with individual
271 coordinates from the PCA as fixed effect, and test day as random factor.

272

273 **Results**

274

275 *Lead bio-accumulated in bees but did not affect colony dynamics.*

276 We quantified lead concentration in sucrose solutions and in the caged bees using ICP-
277 OES. The amount of lead in the high concentration sucrose solution was recovered at
278 96% (nominal concentration: 0.75 mg.L⁻¹, actual concentration: 0.71 mg.L⁻¹). After

279 four weeks of exposure, we found no difference in the lead content of control bees and
280 L bees (control: 0.126 ± 0.031 mg.kg⁻¹ d.m., N=3; L bees: 0.130 ± 0.002 mg.kg⁻¹ d.m.,
281 N=3; Kruskal-Wallis: H=7.636, df=1, p=0.712). However, H bees accumulated
282 significant amounts of lead (H bees: 0.809 ± 0.044 mg.kg⁻¹ d.m., N=5; Kruskal-Wallis:
283 H=7.636, df=1, p=0.039), which validated our method of lead exposure.

284 We tested the effect of lead exposure on colony dynamics using continuous
285 monitoring of hive parameters in caged colonies. Syrup and pollen consumption,
286 dynamics of brood production (Fig. S1A), food stores (Fig. S1B), adult bee mass (Fig.
287 S1C), colony mass (Fig. S1D) and *Varroa sp.* loads (Fig. S1E) were similar in all
288 colonies across treatments (LMM: Treatment effect: p>0.05 for all parameters). We
289 found a strong effect of time since the beginning of the experiment on all these
290 parameters (LMM: brood: $F_{1,51}=75.27$, p<0.001; food stores: $F_{1,51}=4.738$, p=0.035; bee
291 mass: $F_{1,51}=28.38$, p<0.001; colony mass: $F_{1,636}=760.44$, p<0.001; *Varroa sp.*:
292 $F_{1,41}=14.19$, p<0.001). In all colonies, brood production, adult bee mass and colonies
293 mass decreased over time (LMM: p<0.001) while food stores (LMM: p=0.035) and
294 *Varroa sp.* load increased (LMM: p=0.046), irrespective of treatment. Therefore, lead
295 exposure had no influence on colony dynamics and parasite loads. Interestingly,
296 however, colony infestation by *Varroa* interacted with several parameters (Table S1),
297 so that infested colonies exhibited a weaker reduction of brood area (LMM:
298 Time:Varroa effect: p=0.028) and of adult bee population (LMM: Time:Varroa effect:
299 p=0.046), and a weaker increase in food stores (LMM: Time:Varroa: p=0.037),
300 suggesting that infested colonies allocated part of their foraging workforce to a
301 collective response to stress induced by *Varroa*.

302

303 *Exposure to high lead concentration reduced learning performance.*

304 We assessed the effect of lead exposure on cognition using an olfactory reversal
305 learning task bees from caged hives. The proportion of bees that responded to the
306 antennal stimulation of sucrose was similar across treatments (control bees: 74%
307 N=113, L bees: 69% N=122, H bees: 76% N= 132; $\chi^2=1.423$, $df=2$, $p=0.491$), indicating
308 that lead exposure did not affect appetitive motivation or sucrose perception.

309 Treatment had no significant effect on phase 1, although H bees tended to
310 perform less well (Fig. 1B). Learning slopes were similar across treatments, as well as
311 the proportions of bees that learned to discriminate the two stimuli (learning score
312 equals to 1) (Tables 1 and S2). These results were independent of the odours used as
313 stimuli A+ and B- (Binomial GLMM: $F_{1,266}=0.905$, $p=0.526$). The proportion of
314 learners at the end of the first phase was similar across hives (Control bees (47%; 48%):
315 $\chi^2<0.001$, $df=2$, $p=1$; L bees (33%; 38%; 55%): $\chi^2=2.993$, $df=2$, $p=0.224$; H bees (41%;
316 18%; 41%): $\chi^2=3.291$, $df=2$, $p=0.193$). Therefore, exposure to lead did not affect the
317 performance of bees in the differential conditioning task.

318 By contrast, treatment had a significant effect on phase 2 (Fig. 1C). Bees from
319 all treatments had similar learning slopes for odour A- (LMM: $p=0.590$ for L bees;
320 $p=0.063$ for H bees). However, H bees started to respond to B+ later than control bees
321 (LMM: $p=0.027$). L bees behaved similarly to controls (LMM: $p=0.830$). By the end of
322 phase 2, L bees and H bees responded less to B+ than controls (Conditional model
323 average: L bees, $p=0.007$; H bees, $p<0.001$) (Tables 1 and S2, Fig. 1C). Treatment also
324 influenced responses to A-, but here L bees responded more ($p=0.033$) than H bees and
325 controls (6% of control bees responded to A+, 10% of H bees, 13% of L bees). Overall,
326 H bees exhibited lower reversal scores (13% of learners) than L bees (21%) and controls
327 (33%) (Conditional average model: L bees, $p=0.088$; H bees, $p=0.001$) (Tables 1 and
328 S2, Fig. 1C). There was no effect of the odours used as stimuli A- and B+ (Binomial

329 GLMM: $F_{1,266}=1.300$, $p=0.636$), nor of the hive on the proportion of learners (control
330 bees: $\chi^2=0.505$, $df=2$, $p=0.477$; L bees: $\chi^2=0.211$, $df=2$, $p=0.9$; H bees: $\chi^2=0.973$, $df=2$,
331 $p=0.615$). Therefore, exposure to a high concentration of lead reduced the performance
332 of bees in the reversal learning task.

333 Comparing the responses of bees between the last trial of phase 1 and the first
334 trial of phase 2, one hour later, gives insight about short-term memory. We only found
335 a tendency for H bees to show a reduced percentage of correct responses between the
336 two phases (Conditional average model: H bees, $p=0.078$) (Tables 1 and S2). The
337 duration of exposure to lead did not affect the response levels of bees, nor their learning
338 scores, at the end of either learning phases (Tables 1 and S2).

339

340 *Bees exposed to the high lead concentration were shorter with smaller heads.*

341 We assessed the effect of lead exposure on bee development by measuring
342 morphological parameters on foragers and emerging adults from the caged hives.
343 Foragers collected on the day before the beginning of lead exposure (day 0), had similar
344 head measurements irrespective of treatment (LMM: Treatment effect: $p=0.529$ for
345 head width, $p=0.509$ for head length). However, foragers collected on the 8th or 10th
346 week of lead exposure had significantly smaller heads than controls (L bees: head
347 length $p=0.017$; H bees: head width $p=0.040$, head length $p<0.001$; Table S3). This
348 effect was independent of the duration of exposure (LMM: Treatment:Time effect:
349 $p=0.711$ for head width, $p=0.663$ for head length).

350 We further explored the effects of lead treatment on the morphology of adults
351 (Fig. 2A) using a PCA on the nine morphometric parameters measured on emerging
352 bees (Fig. 2B, Table S4). Two PCs explaining 57% of the variance were sufficient to
353 separate control bees and H bees into two distinct clusters, while L bees remained in

354 between (PERMANOVA: Pseudo-F=3.313, p=0.004; control bees vs. L bees: p=1; C
355 bees vs. H bees: p=0.021; L bees vs. H bees, p=0.177). PC1 explained variations in
356 general body size while PC2 explained variations in wing and basitarsus width, and
357 femur length. PC1 was negatively correlated with the interactive effect between
358 concentration and duration of exposure (LMM: Treatment:Time effect: p<0.001).

359 Overall, H bees displayed a decrease in the same order of magnitude for most
360 of the parameters, except for weight (-12.5%) and head width (-2.99%) (Table S5). This
361 indicates that bees exposed to the high concentration of lead during larval development
362 had a lighter body with shorter legs and wings, as well as smaller heads, compared to
363 unexposed bees (Table S6).

364

365 *Bees with bigger heads showed better learning performance.*

366 We explored the basal relationship between head size and learning performance by
367 testing bees unexposed to lead from uncaged hives to a reversal learning task (N=149).
368 The first component (PC1) of the PCA collapsing head width and length explained
369 73.6% of the variance and represented the global head size. In phase 1, the proportion
370 of learners (79% N=118) increased with head size (Fig. 3A), while short-term memory
371 recall was unaffected (Fig. 3B). In phase 2, the proportion of learners (16% N=23) was
372 also affected by head size (Fig. 3C). Therefore, bees with bigger heads showed better
373 learning performances in absence of any cage confinement or lead treatment.

374

375 **Discussion**

376 Recent studies suggest that MTE can have sublethal effects on individual pollinators,
377 with potential detrimental consequences for populations (Søvik *et al.* 2015; Burden *et*
378 *al.* 2016, 2019; Skaldina & Sorvari 2019). Here, we demonstrate that honey bees

379 chronically exposed to field-realistic concentrations of lead in food have reduced body
380 sizes and learning abilities. The positive correlation between head size and learning
381 performances in unexposed bees suggests that consumption of this single trace element
382 affects bee development, constraining brain size and cognitive function, thus
383 constituting a major source of environmental stress for bees.

384 The impact of lead was independent of the duration of exposure. While our
385 experimental setup allowed contamination through foraging over several weeks, similar
386 effects were observed in adults exposed for varying durations. Yet, all bees had
387 undergone their larval stage during the exposure period, suggesting that most of the
388 detrimental effects of lead is caused by larval ingestion of contaminated food brought
389 by foragers. Lead alters larval development in flies and bees (Cohn *et al.* 1992; Safaee
390 *et al.* 2014; Di *et al.* 2016) and, in mammals, alterations in neural development are
391 correlated with cognitive impairments, including learning and memory (Grandjean &
392 Landrigan 2006; Giordano & Costa 2012; Mason *et al.* 2014).

393 In our experiments, lead did not generally affect olfactory or gustatory
394 perception or appetitive motivation, as previously observed in more systematic sucrose
395 sensitivity tests (Burden *et al.* 2019), nor differential learning (first learning phase) or
396 short term memory. Rather, we found a specifically decreased ability in reversal
397 learning (second learning phase), a task involving cognitive plasticity that allows bees
398 to update their memories about relevant associations between flower cues and food
399 (Ferguson *et al.* 2001). A higher sensitivity to lead in reversal learning has been
400 documented in rats (Hilson & Strupp 1997; Garavan *et al.* 2000), monkeys (Bushnell
401 & Bowman 1979) and humans (Evans *et al.* 1994; Finkelstein 1998). Most probably,
402 this reflects a stronger impact of lead intoxication on the development and/or function
403 of brain circuits that are more specifically involved in a non-elemental task, such as

404 reversal (e.g. orbitofrontal cortex in mammals (Schoenbaum *et al.* 2000)), than in
405 simpler forms of learning like differential learning that do not involve the resolution of
406 ambiguities (Giurfa 2013).

407 In honey bees, effective reversal requires the normal function of the mushroom
408 bodies (MBs), that are brain centers otherwise dispensable for differential conditioning
409 (Boitard *et al.* 2015). The specific reversal impairment of lead-exposed bees might thus
410 be understood if neural circuits would be more affected in the MBs than in other brain
411 regions. Since reversal learning requires GABAergic transmission in the MBs (Boitard
412 *et al.* 2015), alterations in GABA release observed in mammals exposed to lead (Lasley
413 *et al.* 1999) might contribute to the specific impairment of reversal learning in exposed
414 bees. More generally, lead exposure is known to alter the maturation of the brain
415 excitation/inhibition balance during development, through multiple effects such as loss
416 of GABAergic interneurons (Stansfield *et al.* 2015), altered maturation of GABAergic
417 neurons (Wirbisky *et al.* 2014; Neuwirth *et al.* 2018), decrease in GABA and glutamate
418 release (Lasley *et al.* 1999; Xiao *et al.* 2006) or transport (Strużyńska & Sulkowski
419 2004), inhibition of post-synaptic glutamatergic action (Neal & Guilarte 2010). In
420 insects, although no specific effect on GABAergic signalling has been demonstrated
421 yet, the known effects of lead exposure on synaptic development (Morley *et al.* 2003),
422 presynaptic calcium regulation (He *et al.* 2009) and acetylcholinesterase activity
423 (Nikolić *et al.* 2019) are compatible with a disruption of the excitation/inhibition
424 balance. We proposed earlier that reaching an optimal value for such balance in MB
425 circuits is what determines efficient reversal learning in mature adults, and particularly
426 in the so-called calyces of the MBs, where a sparse coding of odorants is believed
427 crucial for efficient reversal (Cabirol *et al.* 2017, 2018). Developmental lead exposure
428 could jeopardize this balance in many ways, like in mammals, and result in impairment

429 of the reversal learning phase only. Indeed, early environmental conditions shape MB
430 circuits and reversal learning performances of adult bees (Cabirol *et al.* 2017).

431 Further evidence supports the hypothesis of a developmental effect of lead,
432 since bees exposed to the highest concentrations developed lighter bodies, with shorter
433 legs and wings and smaller heads. Interestingly, unexposed bees with smaller and
434 shorter heads had a lower learning performance (albeit not specifically in the reversal
435 phase). In bees, head width tend to be correlated with the volume of the brain (Honey
436 bees: Gronenberg & Couvillon 2010; Bumblebees : Riveros & Gronenberg 2010), the
437 MBs and the MB calyces (Honey bees: Mares *et al.* 2005; Bumblebees: Smith *et al.*
438 2020). Lead exposure during larval development is thus likely to have impaired aspects
439 of head and brain development, that affect preferentially MB-dependent cognitive tasks
440 such as reversal learning. In our experimental conditions, continuous exposure to
441 environmentally realistic amounts of lead resulted in bioaccumulation of the metal in
442 the bees' bodies, which may explain the dose-dependent effects observed on cognition
443 and morphology.

444 Irrespective of the detailed mechanisms by which lead impairs bee learning, our
445 results on an ecologically relevant cognitive task raise the concern of pollinators
446 exposed to environmental metallic pollution. Importantly, lead contents measured in
447 the bodies of exposed bees in our experiments ranged within the measurements from
448 bees in natural conditions (Goretti *et al.* 2020). The two concentrations of lead in the
449 sucrose solutions used for chronic exposure (0.075 and 0.75 mg.L⁻¹) fell below the
450 maximum level authorized in food (3 mg.kg⁻¹; Codex Alimentarius 2015) and irrigation
451 water (5 mg.L⁻¹; Ayers & Westcot 1994), and the lowest concentration was under the
452 threshold set for honey by the European Union (0.10 mg.kg⁻¹; Commission Regulation
453 2015). This suggests that bees foraging on flowers in contaminated environments also

454 exhibit cognitive and developmental impairments. Ultimately, these effects can alter
455 colony function, for instance by reducing the collective foraging efficiency and
456 gradually causing population declines, as found with the effects of pesticides at
457 sublethal concentrations (Klein *et al.* 2017). Although our experiment, and recent
458 similar approaches (Hladun *et al.* 2016), did not capture these effects at the population
459 level, differences in colony performances are expected over the longer term. Our results
460 thus call for future studies to better characterize the impact of lead exposure in bee
461 populations, including in combination with other MTE as such cocktails are often found
462 in contaminated areas (Badiou-Bénéteau *et al.* 2013; Goretti *et al.* 2020). More
463 generally, a better assessment of the contribution of heavy metal pollutants to the
464 widespread declines of insects has become an urgent necessity for preserving ecosystem
465 services.

466

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476

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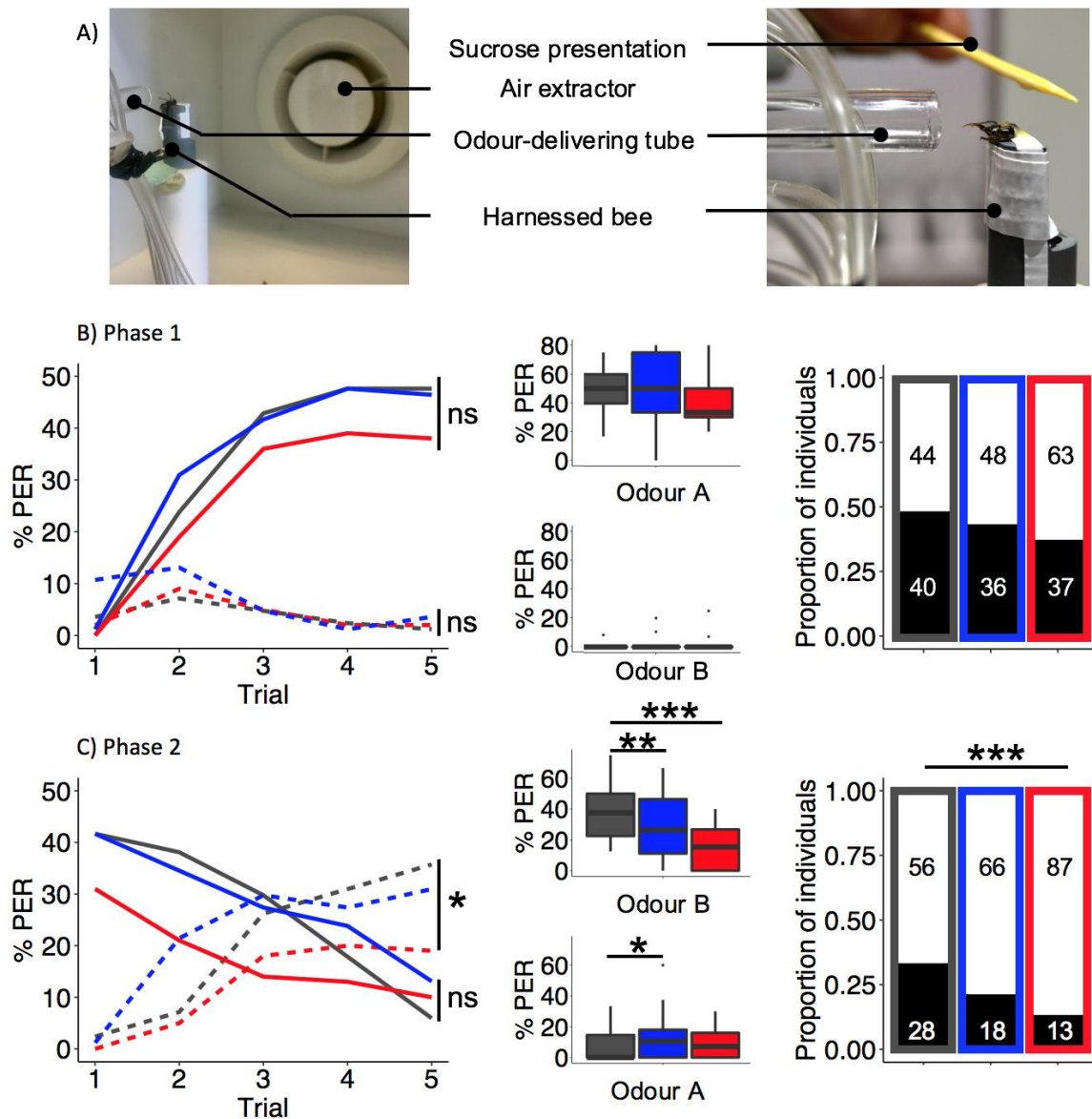
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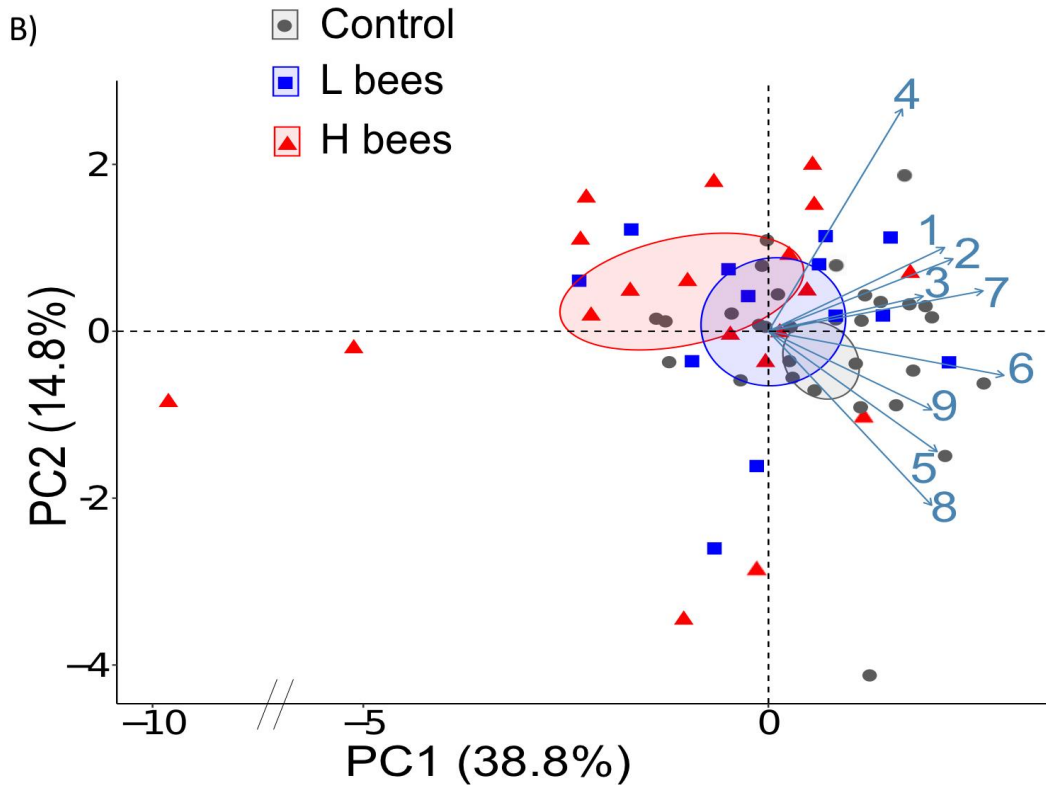
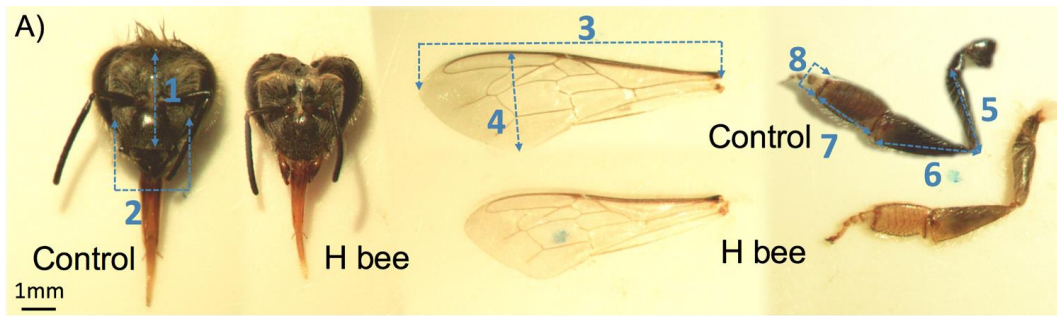
711

712 **Figures**



713 **Figure 1: Learning and memory performances of bees from caged hives.** A) Photo
 714 of conditioning set-up and harnessed bee. B)-C) Control bees (N=84, *dark grey*), bees
 715 exposed to a low concentration of lead (L bees: 0.075 mg.L⁻¹; N=84, *blue*) or a high
 716 concentration of lead (H bees: 0.75 mg.L⁻¹; N=100, *red*). Line plots show the percentage
 717 of proboscis extension responses (PER) elicited by odour A (solid line) and odour B
 718 (dashed line) during phase 1 (B) and phase 2 (C) of reversal learning. Statistical
 719 comparisons were obtained with a LMM on the learning slopes. Boxplots show the
 720 percentage of PER elicited by each odorant during the last trial of each phase. Statistical

721 comparisons were obtained with p-values from the model average following MMI
722 procedure (see Table 1 and S2). Bar plots show the proportions of learners (*black*) and
723 non-learners (*white*) in the last trial of phase 1 (**B**) and phase 2 (**C**), with sample size
724 displayed. Statistical comparisons were obtained with p-values from the model average
725 following MMI procedure (Table 1). (ns: non-significant, $p > 0.05$; * $p < 0.05$; ** $p < 0.01$;
726 *** $p < 0.001$)



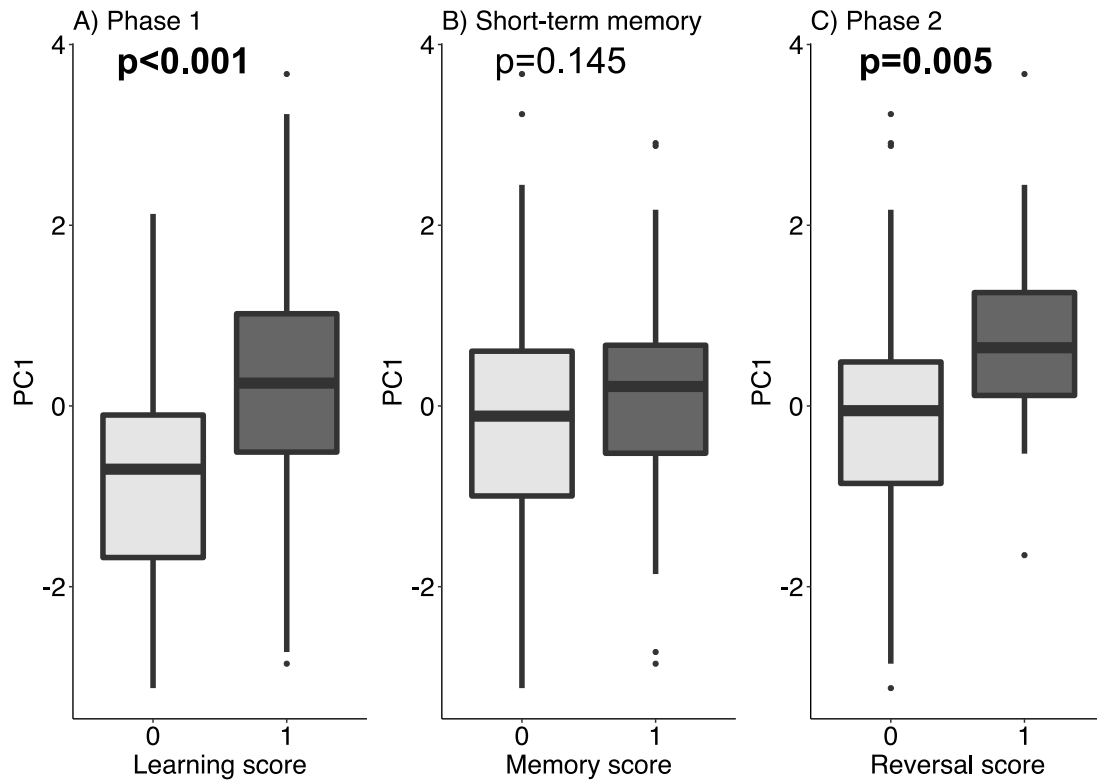
728

729 **Figure 2: Morphometric analysis of bees from caged hives.** A) Details of the
 730 parameters measured. This example shows morphological differences in emerging
 731 bees. (1) Head length, (2) Head width, (3) Wing length, (4) Wing width, (5) Femur
 732 length, (6) Tibia length, (7) Basitarsus length, (8) Basitarsus width, (9) Bee weight (not
 733 shown). B) Principal component analysis (PCA) map shows the relationship among the
 734 morphometric measures (same number code as in A). 95% confidence ellipses of the
 735 mean are displayed for each treatment. Controls: bees unexposed to lead (N=30); L

736 bees: bees exposed to the low concentration of lead (0.075 mg.L^{-1}) (N=13); H bees:

737 bees exposed to the high concentration of lead (0.75 mg.L^{-1}) (N=19).

738



740

741 **Figure 3: Relationship between head size and cognitive performance in unexposed**742 **bees from uncaged hives (N=149).** Boxplots show the head size variation of learners743 (*dark grey*) and non-learners (*light grey*), as defined by their individual scores. **A)**744 Learning score at the end of phase 1; **B)** Short-term memory score at the beginning of745 phase 2; **C)** Reversal score at the end of phase 2. Horizontal line represents median and

746 box encompasses 25th to 75th percentiles. Error bars encompass the smallest to highest

747 value that is within 1.5*inter-quartile range of the hinge. Any value outside these

748 whiskers is considered as an outlier and displayed as a dot. Statistical comparisons were

749 obtained with α -values from the binomial GLMM testing bees coordinates in PC1 on750 cognitive scores, significant values (<0.05) are shown in bold. Increasing head size

751 significantly enhanced the learning performances in phase 1 (Binomial GLMM:

752 estimate \pm SE, 0.908 ± 0.230 , $p < 0.001$) and phase 2 (0.661 ± 0.238 , $p = 0.005$), while753 memory remained unaffected (0.243 ± 0.167 , $p = 0.145$).

754 **Tables**

755 **Table 1: Parameter estimates from the conditional model average for response**
756 **levels at the end of both learning phases, and for learning, reversal and memory**
757 **score models in bees from caged hives.** Significant p-values (<0.05) are shown in
758 bold. SE=conditional standard errors.

759

Conditional average	Estimate	SE	p-value
<i>PER response at the end of Phase 1</i>			
Intercept	-0.2542	0.1769	0.152
Low concentration	-0.0015	0.3115	0.996
High concentration	-0.4330	0.3101	0.164
Time	0.4098	0.2602	0.116
Odour B-	-3.6150	0.4646	<0.001
Time:Odour B-	-0.9143	0.8817	0.301
<i>PER response at the end of Phase 2</i>			
Intercept	-3.8148	0.5670	<0.001
Low concentration	0.9861	0.4637	0.034
High concentration	0.5224	0.4568	0.254
Time	0.1398	0.7126	0.845
Odour B+	2.8546	0.4173	<0.001
Low concentration:Odour B+	-1.3166	0.4873	0.007
High concentration:Odour B+	-1.8589	0.5010	<0.001
Low concentration:Time	1.4253	0.9128	0.119
High concentration:Time	1.3869	0.8986	0.124
Time:Odour B+	-0.3685	0.5862	0.531
<i>Learning score</i>			
Intercept	-0.2466	0.1965	0.211
Low concentration	-0.2494	0.3141	0.429
High concentration	-0.5370	0.3082	0.083
Time	0.4480	0.2551	0.081
<i>Reversal score</i>			
Intercept	-0.6940	0.2321	0.003
Low concentration	-0.6053	0.3529	0.088

High concentration	-1.2064	0.3780	0.001
Time	-0.0292	0.3088	0.925
<i>Memory score</i>			
Intercept	-0.6467	0.2175	0.003
Low concentration	0.0083	0.3249	0.980
High concentration	-0.5906	0.3337	0.078
Time	0.5077	0.2754	0.066

760

761 **Supporting materials**

762

763 **Table S1: Parameter estimates of fixed effects after conditional model average on**

764 **colony dynamics parameters of caged hives.** Significant p-values (<0.05) are shown

765 in bold.

Conditional average	Estimate	SE	p-value
<i>Brood area</i>			
Intercept	96.2608	6.3228	<0.001
Time	-1.2920	0.1550	<0.001
<i>Varroa sp.</i>	-8.8562	4.3048	0.046
Time: <i>Varroa sp.</i>	0.1581	0.0699	0.028
<i>Food stores area</i>			
Intercept	4069.53	707.33	<0.001
Time	20.955	15.008	0.172
<i>Varroa sp.</i>	658.83	386.58	0.099
Time: <i>Varroa sp.</i>	-13.528	6.292	0.037
<i>Adult bee mass</i>			
Intercept	1.5367	0.2467	<0.001
Time	-0.0182	0.0041	<0.001
<i>Varroa sp.</i>	-0.2374	0.1258	0.068
Time: <i>Varroa sp.</i>	0.0042	0.0020	0.046

766

767 **Table S2: Model selection with Akaike's information criterion corrected for small**
768 **sample size (AICc) for the response level at the end of both learning phases, as well**
769 **as for the learning, reversal and memory scores for bees in caged hives. Full and**
770 **null models are presented with the three best models considered for each analysis.**

Models	df	AICc	ΔAICc	Wi
<i>PER response at the end of Phase 1</i>				
Time + Odour	5	432.1	0	0.250
Odour	4	432.3	0.20	0.227
Time + Odour + Time:Odour	6	433.1	0.95	0.156
Treatment + Time + Odour + Treatment:Time +	14	445.0	12.88	0
Null model	3	583.5	151.35	0
<i>PER response at the end of Phase 2</i>				
Treatment + Odour + Treatment:Odour	8	481.6	0	0.179
Treatment + Odour + Time + Treatment:Odour +	11	481.7	0.12	0.169
Treatment + Odour + Time + Treatment:Odour	9	481.7	0.15	0.166
Treatment + Time + Odour + Treatment:Time +	14	486.9	5.35	0.012
Null model	3	519.0	37.41	0
<i>Learning score</i>				
Time	3	368.2	0	0.387
Treatment + Time	5	369.2	1.05	0.228
Treatment	4	370.9	2.77	0.097
Treatment + Time + Treatment:Time	7	373.3	5.17	0.029
Null model	2	369.0	0.80	0.259
<i>Reversal score</i>				
Treatment	4	279.7	0	0.640
Treatment + Time	5	281.7	2.07	0.228
Time	3	287.3	7.62	0.014
Treatment + Time + Treatment:Time	7	283.8	4.11	0.082
Null model	2	285.4	5.78	0.036
<i>Memory score</i>				
Treatment + Time	5	338.8	0	0.370
Time	3	339.4	0.65	0.267
Treatment	4	340.7	1.94	0.140
Treatment + Time + Treatment:Time	7	342.5	3.72	0.058

Null model	2	340.4	1.62	0.165
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771 df=degree of freedom; AICc=Akaike's information criterion corrected for small sample
772 size; Δ AICc=differences in AIC between all the models and the most parsimonious one;
773 W_i =relative importance values of each model.

774 **Table S3: Analysis of the morphological parameters of forager bees from caged**
 775 **hives.** Estimated regression parameters, standard errors (SE) and p-values of the linear
 776 mixed effects models. Significant differences with control group ($p < 0.05$) are shown
 777 in bold.

Morphological parameters	Treatment	Estimate \pm SE	p-value
Head length	Low concentration	-0.1054 \pm 0.0432	0.017
	High concentration	-0.1877 \pm 0.0395	<0.001
Head width	Low concentration	-0.0294 \pm 0.0354	0.456
	High concentration	-0.0990 \pm 0.0324	0.040

778

779

780 **Table S4: Principal component analysis (PCA) on the morphometry of emerging**
781 **bees from caged hives.** Correlation coefficients >0.4 in absolute value are shown in
782 bold.

783

Variable	PC1	PC2
Bee weight	0.568	0.253
Head length	0.618	-0.239
Head width	0.645	-0.203
Wing length	0.774	-0.220
Wing width	0.499	-0.732
Femur length	0.562	0.436
Tibia length	0.805	0.203
Basitarsus length	0.742	-0.092
Basitarsus width	0.541	0.615
% Total variance	42.11	15.16
Cumulative proportion of total variance	42.11	57.27

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789 **Table S5: Median, minimum and maximal values of each morphological**
790 **parameter of emerging bees from caged hives, per treatment and percentage of**
791 **variation between medians compared to unexposed bees.** Percentage of variation
792 for each parameter was the percentage of variation of median values between
793 treatments. We considered variation of the same order of magnitude as isometric
794 scaling, if not as allometric scaling.

795

Variable	Control	Low concentration	High concentration	% variation low concentration	% variation high concentration
Bee weight	0.12	0.11	0.1	-5.41%	-12.5%
Head length	2.99	3.04	2.97	+1.64%	-0.67%
Head width	2.41	2.42	2.34	+0.41%	-2.99%
Wing length	8.78	8.89	8.73	+1.24%	-0.57%
Wing width	3.12	3.15	3.23	+0.95%	+0.96%
Femur length	2.30	2.28	2.27	-0.88%	-1.32%
Tibia length	3.04	3.04	3.02	0%	0%
Basitarsus length	2.07	2.02	2.03	-2.23%	-1.72%
Basitarsus width	1.16	1.16	1.1	0%	-5.45%

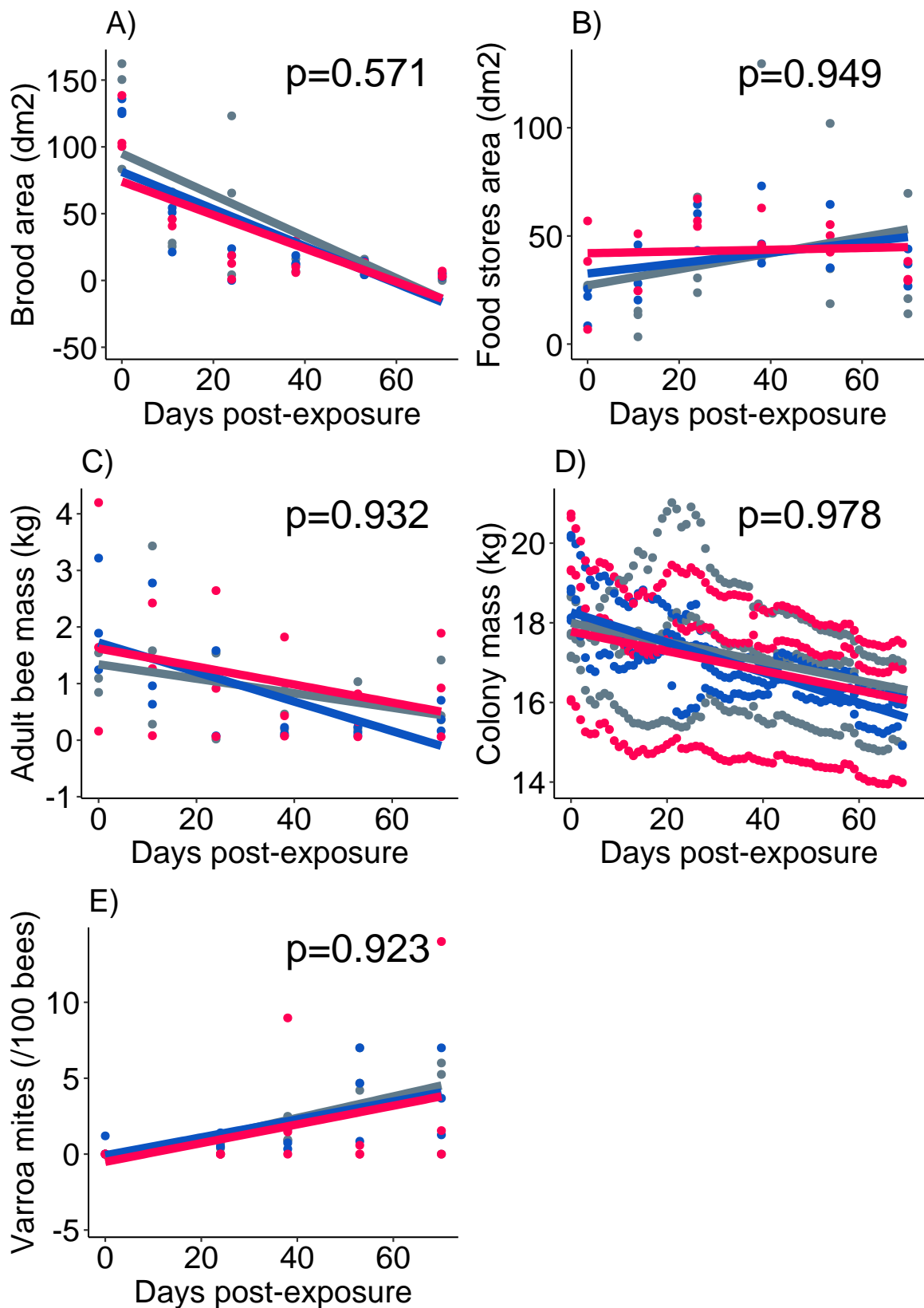
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797

798 **Table S6: Analysis of the morphological parameters of emerging bees from**
799 **caged hives.** Estimated regression parameters, standard errors (SE) and p-values of
800 the linear mixed effects models. Significant differences with control group ($p < 0.05$)
801 are shown in bold.
802

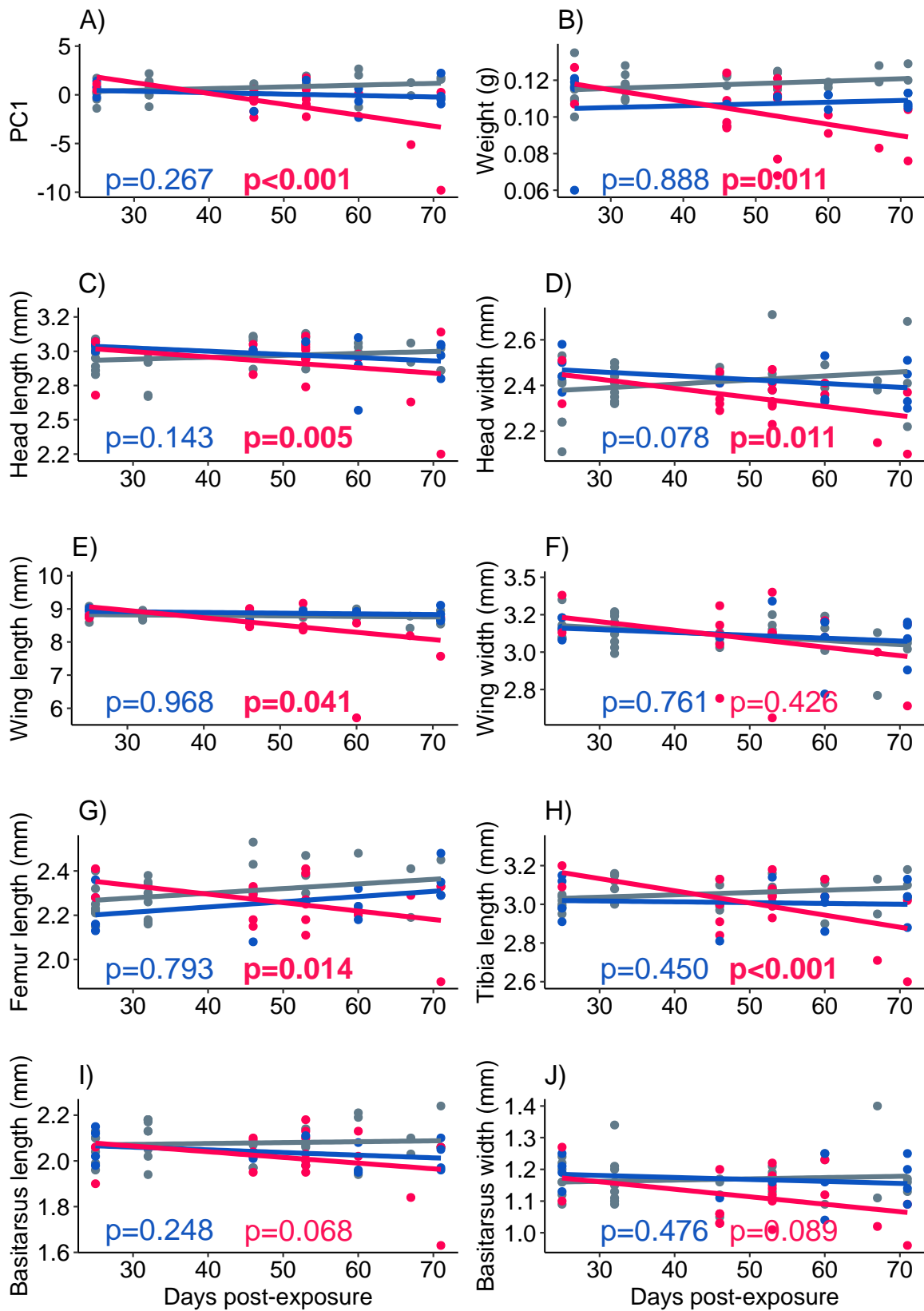
Morphological parameter	Treatment:Time	Estimate \pm SE	p-value
Weight	Low concentration	-0.0001 \pm 0.0002	0.888
	High concentration	-0.0007 \pm 0.0003	0.011
Head length	Low concentration	-0.0044 \pm 0.0030	0.143
	High concentration	-0.0104 \pm 0.0036	0.005
Head width	Low concentration	-0.0034 \pm 0.0019	0.078
	High concentration	-0.0059 \pm 0.0023	0.011
Wing length	Low concentration	0.0001 \pm 0.0043	0.992
	High concentration	-0.0127 \pm 0.0052	0.018
Wing width	Low concentration	0.0009 \pm 0.0030	0.761
	High concentration	-0.0029 \pm 0.0036	0.426
Femur length	Low concentration	0.0005 \pm 0.0019	0.793
	High concentration	-0.0057 \pm 0.0023	0.014
Tibia length	Low concentration	-0.0012 \pm 0.0016	0.450
	High concentration	-0.0082 \pm 0.0020	<0.001
Basitarsus length	Low concentration	-0.0018 \pm 0.0016	0.248
	High concentration	-0.0035 \pm 0.0019	0.068
Basitarsus width	Low concentration	-0.0010 \pm 0.0013	0.476
	High concentration	-0.0028 \pm 0.0016	0.089
Bees coordinates in PC1	Low concentration	-0.1972 \pm 0.2170	0.368
	High concentration	-1.0158 \pm 0.2564	<0.001

803



804 **Figure S1: Amount of brood, food stores, bees, colony mass and *Varroa sp.***
 805 **pressure for caged hives throughout the experiment. Control colonies (N=3, *grey*),**
 806 **colonies exposed to a low concentration (0.075 mg.L⁻¹; N=3, *blue*) or a high**

807 concentration (0.75 mg.L^{-1} ; N=3, *red*) of lead. Evaluations for brood, food stores and
808 bees were conducted every 15 days for all hives. Mass was recorded every hour and
809 averaged on a daily basis. A) Area of capped brood cells. B) Area of food (honey and
810 pollen) stores. C) Adult bee mass. D) Colony mass. E) *Varroa sp.* load over time.
811 Estimate trends are displayed in solid lines. 95% confidence level interval are displayed
812 in the same colour code as treatment. P-values were obtained from LMMs and are
813 displayed for the treatment effect.



815 **Figure S2: Morphological measurements of bees from caged hives upon**
 816 **emergence throughout the experiment. Control bees (N=30, dark grey), bees exposed**

817 to a low concentration (L bees: 0.075 mg.L⁻¹; N=13, *blue*) or a high concentration (H
818 bees: 0.75 mg.L⁻¹; N=19, *red*) of lead. A) Bees coordinate on PCA Dimension 1. B)
819 Bee weight. C) Head length. D) Head width. E) Wing length. F) Wing width. G) Femur
820 length. H) Tibia length. I) Basitarsus length. J) Basitarsus width over time. Estimate
821 trends are displayed in solid lines. 95% confidence level interval are displayed in the
822 same colour code as treatment. P-values obtained from LMMs (Table S6) are given for
823 the interaction between treatment and time, significant results (p<0.05) are shown in
824 bold.