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1 **Title**

2 One year exposure to Cd- and Pb-contaminated soil causes metal accumulation and
3 alteration of global DNA methylation in rats

4

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18

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26

27 **Abstract**

28 Metal pollution has been associated with anthropogenic activities, such as effluents and
29 emissions from mines. Soil could be exposure route of wild rats to metals, especially in
30 mining areas. The aim of this study was to verify whether soil exposure under
31 environmentally relevant circumstances results in metal accumulation and epigenetic
32 modifications. Wistar rats were divided to three groups: 1) control without soil exposure,
33 2) low-metal exposure group exposed to soil containing low metal levels (Pb: 75 mg/kg;
34 Cd: 0.4), and 3) high-metal exposure group exposed to soil (Pb: 3750; Cd: 6). After 1
35 year of exposure, the metal levels, Pb isotopic values, and molecular indicators were
36 measured. Rats in the high-group showed significantly greater concentrations of Pb and
37 Cd in tissues. Higher accumulation factors (tissue/soil) of Cd than Pb were observed in
38 the liver, kidney, brain, and lung, while the factor of Pb was higher in the tibia. The
39 obtained results of metal accumulation ratios (lung/liver) and stable Pb isotope ratios in
40 the tissues indicated that the respiratory exposure would account for an important share
41 of metal absorption into the body. Genome-wide methylation status and DNA
42 methyltransferase (Dnmt 3a/3b) mRNA expressions in testis were higher in the
43 high-group, suggesting that exposure to soil caused metal accumulation and epigenetic
44 alterations in rats.

45

46 **Keywords:** Cd, DNA methylation, epigenetics, Pb isotope, soil exposure

47

48 **Capsule:** Soil exposure caused metal accumulation and DNA hypermethylation in rats.

49 **1. Introduction**

50 Lead (Pb) and cadmium (Cd) are toxic metals that co-exist ubiquitously in the
51 environment. Mining and smelting activities are among the major sources of these
52 metals, and metal pollution is a matter of worldwide concern. Recently, more than 400
53 children died of Pb poisoning in Zamfara state, Nigeria, where long-term neurological
54 impairment, including blindness and deafness, were also documented (Blacksmith
55 Institute, 2014; Dooyema *et al.*, 2012; Lo *et al.*, 2012). Children in polluted areas are
56 vulnerable to metal exposure because of their inclination to ingest Pb through pica
57 behavior and to assimilate relatively larger amounts of inhaled and ingested Pb than
58 adults (Calabrese *et al.*, 1997; Manton *et al.*, 2000). With regard to Cd, one of the most
59 severe forms of chronic toxicity is *itai itai* disease (a Japanese term meaning
60 “ouch-ouch”), which is characterized by nephrotoxicity, osteoporosis, and
61 cardiovascular disease (Jarup and Akesson, 2009; Uno *et al.*, 2005).

62 To evaluate the toxic effects of Cd and Pb exposure and their mechanisms,
63 many laboratory studies have been performed using *in vitro*, *in vivo*, as well as *in silico*
64 techniques in rodent animal models, such as mice and rats. In addition, in field studies,
65 wild rats (e.g., *Rattus norvegicus*, *Rattus rattus*) have frequently been used as sentinel
66 animals to monitor metal pollution around mining areas (Nakayama *et al.*, 2011;
67 Nakayama *et al.*, 2013). These studies showed that fairly high concentrations of metals
68 were accumulated in the tissues of wild rats collected from mining sites compared to
69 those from control sites, resulting in biological reactions such as metallothionein (MT)
70 upregulation. The authors suggested that soil may be major route of exposure to toxic
71 metals in wild rats, especially in mining areas where soil possesses abundant mineral
72 deposits. However, to our knowledge, there have been no reports of laboratory

73 experiments to verify whether soil exposure under environmentally relevant conditions
74 (i.e., not as oral/gavage administration) could result in metal accumulations in rats. As it
75 is difficult to control the experimental conditions in field studies, laboratory soil
76 exposure experiments should be performed to examine this issue. Many studies have
77 been conducted using earthworms as a model animal to characterize metal accumulation
78 patterns and accumulation factors between soil and terrestrial animals (Qiu *et al.*, 2014),
79 but there have been no such laboratory studies in mammals. To provide new knowledge
80 on soil exposure in terrestrial mammals, we used the laboratory rat (*R. norvegicus*)
81 because of the wealth of toxicological knowledge as well as genomics and epigenetics
82 methodological strategies for this species.

83 We performed prolonged (1-year) exposure of Wistar rats to soil containing Cd
84 and Pb collected in the Kabwe mining area, Zambia (Nakayama *et al.*, 2011), to
85 estimate accumulation factors in tissues of rats. Soil samples from Kabwe were used in
86 this study because high concentrations of Cd and Pb were reported previously in soil, rat,
87 chicken, goat, cattle, and children in this area (Nakata *et al.*, 2016; Nakayama *et al.*,
88 2011; Yabe *et al.*, 2011; Yabe *et al.*, 2015; Yabe *et al.*, 2018). As inhalation of soil and
89 metal accumulation were expected, we collected lung tissue from rats in addition to
90 tissues known to accumulate Cd and Pb, such as the liver and kidney. Neurological
91 effects, including decreased intelligence quotient (IQ), are serious problems associated
92 with Pb exposure in humans, especially children (Manton *et al.*, 2000). Therefore, brain
93 tissues were also collected. The tibiae were collected as Pb accumulation targets
94 because of the very long half-life of this metal in bone (Gerhardsson *et al.*, 1993).

95 Pb isotope ratios of the $^{208}\text{Pb}/^{206}\text{Pb}$ and $^{207}\text{Pb}/^{206}\text{Pb}$ were also measured to
96 clarify the change of those values by accumulation level and the differences among the

97 tissues. A large number of studies has utilized the method of Pb isotopic analysis since
98 an identification of Pb pollution source is highly required to prevent and mitigate the
99 further Pb exposure from the environment. Pb isotopic compositions which consist of
100 four main stable isotopes: ^{208}Pb , ^{207}Pb , ^{206}Pb , and ^{204}Pb are not affected to a measurable
101 extent by physico-chemical fractionation processes (Bollhöfer and Rosman, 2001;
102 Veysseyre et al., 2001). It is thus well known that isotopic ratios of the $^{208}\text{Pb}/^{206}\text{Pb}$ and
103 $^{207}\text{Pb}/^{206}\text{Pb}$ can be used as natural tracers and open up another possibility for tracking
104 the Pb source and pathway. Nevertheless, some previous studies revealed large
105 differences in the isotopic composition of Pb among biological samples within rats
106 (*Rattus norvegicus*), goats and humans (Liu et al., 2014; Nakata et al., 2016; Smith et al.,
107 1996; Wu et al., 2012). It was also suggested the possible biological fractionation
108 system of Pb isotopes and its threshold in the body (Nakata et al., 2016). Given these,
109 we verified the change of Pb isotopic compositions in rat tissues in case of exposure
110 from soil via inhalation.

111 Biological reactions, such as MT elevation as well as epigenetic alterations
112 regarding global DNA methylation, were examined to provide new insight into
113 epigenetic events associated with chronic metal exposure. This study is significant due
114 to the environmentally relevant soil exposure conditions used to evaluate metal
115 accumulation and biological alterations in rats. In addition, global DNA methylation
116 analysis was performed because DNA 5-methylcytosine (5-mC) modification is
117 increasingly recognized as a key process in the pathogenesis of complex disorders,
118 including cancer, diabetes, and cardiovascular disease (Feinberg 2010; Ordovas and
119 Smith, 2010). This is another significant point of the present study because a recent
120 review (Ray *et al.*, 2014) noted that there have been few studies to assess associations

121 between DNA methylation and Cd or Pb exposure. Alterations of the DNA
122 methyltransferase (Dnmt) family were also examined because these molecules mediate
123 cytosine methylation through the transfer of a single methyl group from S-adenosine
124 methionine (SAM) to cytosine (Feinberg 2010; Ordovas and Smith, 2010).

125 **2. Materials and Methods**

126 *2.1. Soil sampling*

127 We collected soil samples in Kabwe, Zambia (May 2009), because soil in this
128 area is highly polluted with Pb (9 – 51188 mg/kg) and Cd (0.01 – 139 mg/kg)
129 (Nakayama *et al.*, 2011). Soil samples were passed through a 2 mm sieve and
130 transported to the Laboratory of Toxicology, Graduate School of Veterinary Medicine,
131 Hokkaido University, Japan, for laboratory exposure experiments as described in the
132 following section. Details on soil sampling method are mentioned in supporting
133 information of Materials and Methods section.

134

135 *2.2. Animals and experimental design*

136 All animal experiments were performed under the supervision and with the
137 approval of the Institutional Animal Care and Use Committee of Hokkaido University
138 (approval number 09-0220).

139 Thirty male Wistar rats (*R. norvegicus*, 7 weeks old) were purchased from
140 Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). The rats (8 weeks of age) were
141 divided into three groups ($n = 10$ for each group): 1) control without soil exposure, 2)
142 low-metal exposure group exposed to soil containing low metal levels (Pb: 75 mg/kg;
143 Cd: 0.4 mg/kg), and 3) high-metal exposure group exposed to soil containing high metal
144 levels (Pb: 3750 mg/kg; Cd: 6 mg/kg) (Supplementary Table S1). Concentrations of Cd
145 and Pb in the test soil samples were determined prior to exposure experiments by
146 atomic absorption spectrometry (AAS) (Z-2010; Hitachi High-Technologies
147 Corporation, Tokyo, Japan) according to the method described previously (Nakayama *et*
148 *al.*, 2011). Soil samples were spread at the bottom of the cage and rats were exposed to

149 the soil for 1 year (Supplementary Fig. S1). The rats were housed in cages containing
150 either soil for the exposure groups or a bedding of paper chips (Paper Clean; Japan SLC,
151 Hamamatsu, Japan) for the control group (Supplementary Fig. S1). Body weight of the
152 individual rats was measured once every 2 weeks, and no differences were observed
153 among the groups (Tukey's test) during the 1-year exposure period (Supplementary Fig.
154 S2). Details on animal experiment design are also shown in supporting information of
155 Materials and Methods section.

156 To evaluate the effects of metal exposure, behavioral activity of rats was
157 monitored using a Scanet MV-10 (Matys Co., Tokyo, Japan) before starting exposure
158 (day 0) and after 2, 6, and 12 months of exposure. Rats were placed in a box measuring
159 480 mm × 480 mm that had infrared ray detectors set 12.5 cm above the floor. Larger
160 (MOVE 1) and smaller (MOVE 2) horizontal movements and vertical movement
161 (rearing) were recorded every 2 minutes for 20 minutes (Supplementary Fig. S3). This
162 instrument allowed monitoring of the rat behavior by one examiner without special
163 training. Behavioral experiments were performed at night (20:30 – 23:00) as rats are
164 nocturnal animals.

165 After 1 year of soil exposure, rats were euthanized by CO₂ inhalation, and
166 heparinized total blood, liver, kidney, lung, brain, testis, and tibia were collected. Tissue
167 samples other than the tibia were immediately frozen in liquid nitrogen. Plasma was
168 collected after centrifugation (2000 × g, 15 min at room temperature) of total blood with
169 heparin for blood biochemistry analysis. The collected samples were stored at –80°C
170 until analyses.

171

172 *2.3. Blood biochemistry*

173 A conventional blood chemical analyzer (COBAS Ready; Roche Diagnostic
174 Systems, Basel, Switzerland and Spotchem panels I and II; Arkray, Kyoto, Japan) was
175 used to analyze the levels of alanine aminotransferase (ALT), aspartate
176 aminotransferase (AST), gamma glutamyltranspeptidase (GGT), lactase dehydrogenase
177 (LDH), alkaline phosphatase (ALP), total bilirubin (T-Bil), total protein (TP), blood
178 urea nitrogen (BUN), albumin (Alb), urea acid (UA), and creatinine (Cre).

179

180 *2.4. Cd and Pb extraction and concentration analysis*

181 Extraction of metals in tissues were performed as described previously (Yabe
182 *et al.*, 2015) with slight modifications. Details on sample digestion and metal extraction
183 procedures are also described in supporting information of Materials and Methods
184 section.

185 The concentrations of Cd and Pb were determined using an inductively coupled
186 plasma – mass spectrometer (ICP-MS 7700 series; Agilent Technologies, Tokyo, Japan).
187 Analytical quality control was performed using the DORM-3 (fish protein, National
188 Research Council of Canada, Ottawa, Canada) and DOLT-4 (dogfish liver, National
189 Research Council of Canada) certified reference materials. Replicate analysis of these
190 reference materials showed good recoveries (95% – 105%). The instrument detection
191 limit was 0.001 µg/L. The accumulation factor for the high-metal exposure group was
192 calculated using the equation: [metal concentration in rat tissue/metal concentration in
193 soil].

194

195 *2.5. Sample purification and Pb stable isotope analysis*

196 Sample dissolution procedure was similar to the method described by Kuritani
197 and Nakamura (2002). The extracted solutions of liver, kidney, lung, brain, and blood,
198 except for one kidney each of control and low-metal exposure groups whose solution
199 volumes were not enough, were transferred into Teflon tubes after the analyses of Cd
200 and Pb levels. The Pb isotopic data of one kidney sample of each control and low-metal
201 exposure group were not analyzed due to insufficient volume of the solution. Details on
202 sample dissolution and purification procedures are also shown in supporting
203 information of Materials and Methods section.

204 Pb isotopic ratios of the $^{208}\text{Pb}/^{206}\text{Pb}$ and $^{207}\text{Pb}/^{206}\text{Pb}$ were determined on a
205 multiple collector (MC)-ICP-MS (Neptune Plus, Thermo Finnigan, California, USA) in
206 static mode with the Faraday cup configuration. Other general parameters were
207 described in Supplementary Table S2. Details on corrections of fractionation are
208 indicated in supporting information of Materials and Methods section.

209

210 2.6. *Quantitative reverse transcription polymerase chain reaction (qRT-PCR)*

211 Total RNA was extracted using Nucleospin RNA II kit (Takara Bio, Otsu,
212 Japan) from approximately 100 mg of the liver and kidney according to the
213 manufacturer's instructions. Total RNA concentration was measured using NanoDrop
214 ND-1000 (Thermo-Scientific, Newark, DE). A260/280 and A260/230 were generally \geq
215 2. Total RNA (1 μg) was reverse transcribed using ReverTra Ace (Toyobo, Tokyo,
216 Japan) in a final volume of 40 μL , according to the manufacturer's instructions.
217 Gene-specific qRT-PCR primers for MT-1, MT-2, Dnmt 1, Dnmt 3a, Dnmt 3b, and
218 peptidylprolyl isomerase (cyclophilin) genes (Supplementary Table S3) were
219 synthesized by Sigma-Aldrich (Tokyo, Japan). qRT-PCR was performed using the

220 StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). The PCR
221 mixtures consisted of Fast SYBR Green Master Mix (Applied Biosystems), forward and
222 reverse primers (200 nM each), and cDNA derived from 10 ng of total RNA in a total
223 volume of 10 μ L. Details on PCR profile, primer specificity confirmation, internal
224 control and comparative quantification method are presented in supporting information
225 of Materials and Methods section. Eight rats selected at random from each group were
226 used for the qRT-PCR assay, whereas 10 rats from each group were used for all of the
227 other experiments.

228

229 2.7. Genomic DNA extraction and Luminometric Methylation (LUMA) Assay

230 Genomic DNA was extracted from the liver, kidney, and testis samples using a
231 GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) according to the
232 manufacturer's instructions. DNA concentration was measured spectrophotometrically
233 (NanoDrop ND-1000; Thermo-Scientific). Luminometric Methylation Assay (LUMA)
234 assays were performed according to the method of Pilsner *et al.* (2009b). Briefly,
235 methylation-sensitive and methylation-insensitive enzymatic digestion of 300 ng
236 genomic DNA at CCGG sites was completely performed using *HpaII* and *MspI*
237 restriction enzymes (Invitrogen, Carlsbad, CA), respectively. *EcoRI* (Invitrogen) was
238 also used for complete digestion as an internal control. Annealing buffer (Qiagen,
239 Valencia, CA) was added after digestion, and the products were analyzed using the
240 PyroMark Q96 MD system (Qiagen). The *MspI/HpaII* ratios were calculated relative to
241 the *EcoRI* control, and the percent methylation of each sample was calculated using the
242 equation: $[1 - (HpaII/EcoRI)/(MspI/EcoRI)] \times 100$.

243

244 2.8. *Statistical analysis*

245 Tukey's test was used to identify significant differences among the groups and
246 tissues. Principal component analysis (PCA) was performed to characterize the
247 relationships among metal concentrations, methylation status, and mRNA expression of
248 Dnmt 1, Dnmt 3a, and Dnmt 3b in rat testis. JMP ver. 13.0 (SAS Institute Inc., Raleigh,
249 NC) was used for statistical analysis, and $P < 0.05$ was taken to indicate statistical
250 significance.

251 **3. Results**

252 *3.1. Blood biochemistry*

253 Significantly elevated levels of ALP and BUN were detected in the rats from
254 the high-metal exposure group compared to the other two groups (Supplementary Table
255 S4).

256

257 *3.2. Cd and Pb concentrations and accumulation factors*

258 The concentrations of Cd and Pb in rat tissues were determined (Table 1).
259 Significantly higher concentrations of Cd were observed in the blood, testis, lung, liver,
260 and kidney in rats from the high-metal exposure group compared to control and
261 low-metal exposure groups. No significant differences were found in Cd concentrations
262 in the tibia samples. Cd levels in brain samples were below the detection limit (BDL).
263 Significantly higher concentrations of Pb were observed in the blood, testis, brain, lung,
264 liver, kidney, and tibia of rats from the high-metal exposure group compared to the
265 control and low-metal exposure groups.

266 We calculated the accumulation factor between soil and rat tissues
267 (Supplementary Table S5). The unit of blood metal concentration was converted with
268 1.0 of conveniently set the blood specific gravity. The rank order of accumulation factor
269 ($\times 10^{-4}$) for Cd in rat tissues (except the brain) was as follows: kidney (850) > liver (50)
270 > lung (20) > testis (11) > tibia (8.0) > blood (0.2). The rank order of the accumulation
271 factor ($\times 10^{-4}$) for Pb was as follows: tibia (145) > kidney (7.2) > liver (1.4) > lung (0.6)
272 > brain (0.4) > testis (0.3) > blood (0.1).

273

274 *3.3. Pb isotopic compositions*

275 Geographical trends and the values of the Pb isotope ratios ($^{208}\text{Pb}/^{206}\text{Pb}$ and
276 $^{207}\text{Pb}/^{206}\text{Pb}$ ratios) from various tissues of rats are described in Fig. 1 and
277 Supplementary Table S6, respectively. The tissues of control group which was not
278 exposed to Pb recorded large variation in the mean values of $^{208}\text{Pb}/^{206}\text{Pb}$ and $^{207}\text{Pb}/^{206}\text{Pb}$
279 ratios among the different tissues. The average isotopic ratios of low-metal exposure
280 group exhibited relatively small variety among the tissues compared to control group.
281 Moreover, both ratios of $^{208}\text{Pb}/^{206}\text{Pb}$ and $^{207}\text{Pb}/^{206}\text{Pb}$ in the tissues of low-metal exposure
282 group tended to show the values closer to those in Kabwe galena (2.1342 ± 0.0009 of
283 $^{208}\text{Pb}/^{206}\text{Pb}$ and 0.8731 ± 0.0003 of $^{207}\text{Pb}/^{206}\text{Pb}$) reported by Kamona et al. (1999).
284 Compared with those two groups, high-metal exposure group indicated surprisingly
285 small differences of the isotopic compositions in the tissues other than liver, with quite
286 similar isotopic values to those in galena from the deposits of Kabwe. The standard
287 deviation values of isotopic ratios in tissues from high-metal exposure group were also
288 quite small, indicating small individual differences of the isotope ratios in the group.

289

290 *3.4. Behavioral activity*

291 No differences were observed among the groups in MOVE1, MOVE2, or
292 rearing before exposure (Table 2). After 2, 6, and 12 months of exposure, the numbers
293 of horizontal movements (MOVE1 and MOVE2) were significantly reduced in the
294 high-metal exposure group compared to the other two groups, whereas no differences
295 were observed in the number of rearing behaviors (Table 2).

296

297 *3.5. MT-1 and MT-2 mRNA expression in the liver and kidney*

298 Metallothionein (MT)-1 and MT-2 mRNA expression in the kidneys from rats
299 in the high-metal exposure group were significantly higher than those in controls (Fig.
300 2A, 2B). MT-1 mRNA expression in the kidneys of the high-metal exposure group
301 tended to be higher than that the low-metal exposure group, although the difference was
302 not statistically significant. No significant differences were observed among the three
303 groups in MT-1 or MT-2 mRNA expression in the liver (Fig. 2C, 2D).

304

305 *3.6. LUMA assay in the liver, kidney, and testis*

306 Global DNA methylation status was analyzed by LUMA assay. Significantly
307 high methylation level (%) was observed in the testis of the high-metal exposure group
308 compared to the other two groups (Fig. 3A). No significant differences were observed
309 among the groups in the liver or kidney (Fig. 3B, 3C).

310

311 *3.7. Dnmt 1, Dnmt 3a, and Dnmt 3b mRNA expression in the testis*

312 Dnmt 1 mRNA expression in the testis did not differ among the groups (Fig.
313 4A). Dnmt 3a mRNA expression levels in the testis of low- and high-metal exposure
314 groups were significantly higher than those in the control group (Fig. 4B). The
315 high-metal exposure group showed significantly elevated Dnmt 3b mRNA expression
316 compared to the control group and this tended to be higher than that in the low-metal
317 exposure group although the difference was not statistically significant (Fig. 4C).

318

319 3.8. PCA

320 Positive associations between metal concentrations, global DNA methylation
321 level, and methyltransferase (Dnmt 1, Dnmt 3a, and Dnmt 3b) mRNA expression in the
322 testis were observed by PCA (Fig. 5).

323 **4. Discussion**

324 In the present study, we exposed laboratory rats to soil containing Cd and Pb
325 via inhalation as well as through ingestion (e.g., grooming, soil adsorbed to food) to
326 represent environmentally relevant conditions. Metals were accumulated in the tissues
327 of rats after 1 year of exposure. To our knowledge, this is the first study to estimate the
328 accumulation factor between soil and rat tissues under conditions of natural exposure
329 and to analyze the biological reactions and epigenetic modifications.

330 Analysis of the tissue distributions of Cd and Pb among the three groups
331 indicated that soil exposure causes metal accumulation in the tissues of rats. As
332 expected, relatively high levels of Cd and Pb were accumulated in the liver and kidneys.
333 The liver and kidneys have been defined as metal accumulating tissues because they
334 express high levels of metal binding proteins (e.g., metallothionein), which play
335 important roles in detoxification of metals and metal elimination (Waalkes and Klaassen,
336 1985). On the other hand, it is well known that the Cd and Pb accumulation levels in the
337 lung are relatively smaller than those in the liver and kidneys because of the low
338 expression levels of metal binding proteins. A recent research reported that the ratio of
339 Cd and Pb accumulation in the lung as compared to the liver were 0.024 and 0.055,
340 respectively, in case of adult male Wister rat which was exposed to both Cd and Pb with
341 solid feed for 12 weeks (Winiarska-Mieczan, 2014). In the sense of those metal
342 accumulation ratios, our results indicated different trends; namely, the accumulation
343 ratios of Cd and Pb in the lung of high-metal exposure group as compared with the liver
344 were 0.401 and 0.432, respectively, whereas those ratios of low-metal exposure group
345 were 0.158 and 0.023, respectively. These greater ratios in the present study suggested
346 that respiratory exposure would account for a large fraction of Cd and Pb accumulation

347 in rats living at highly contaminated soil environment rather than oral exposure
348 although the distribution of absorbed metals through bloodstream should be taken into
349 consideration as well. In fact, the concentration ratios of low- and high-metal exposure
350 groups for Cd and Pb in the lungs were 5.9 and 35.8 times, respectively, which were
351 higher than the values for other tissues except Pb in the tibia for which a ratio of 130
352 was observed.

353 With the intent to consider the exposure route of metals, we also analyzed Pb
354 isotopic ratios of $^{208}\text{Pb}/^{206}\text{Pb}$ and $^{207}\text{Pb}/^{206}\text{Pb}$ in rat tissues. The variety of Pb isotope
355 ratios in rat tissues decreased with an increase in Pb accumulation level. Moreover, as
356 greater the Pb level is, as closer the isotopic compositions of tissues to those of Kabwe
357 galena which is considered as the origin of Pb pollution source in Kabwe (Kamona et al.,
358 1999). These findings are quite similar with those which were indicated by previous
359 studies of rat and goat (Liu et al., 2014 and Nakata et al., 2016). Among the rat tissues
360 of high-metal exposure group, lung tissues showed the isotopic values closer to those of
361 galena compared with other tissues except for kidney, suggesting the possibility that
362 lung could be the tissue absorbing Pb from outside of the body. After the absorption
363 from lung, the isotopic compositions of Pb could be fractionated during the distribution
364 to other tissues as the former research suggested although the exact mechanism is still
365 unknown (Nakata et al., 2016). These findings also support the hypothesis of a large
366 contribution of inhalation to the Pb absorption which was indicated by the result of
367 metal accumulation ratios of lung to liver. Considering the route of exposure in the
368 present study, the contribution of inhalation of soil particles cannot be neglected. Indeed,
369 Cd was accumulated in the lungs of rats due to inhalation of both soluble and insoluble
370 forms (Takenaka *et al.*, 2004). Our results proposed meteorological factors could play

371 an important role of exposure level via inhalation in the field although the
372 meteorological factor was not verified in the present study of a laboratory experiment.
373 When the amount of precipitation is small and soil moisture level is low, the soil
374 containing Pb could easily scatter. Similarly, the strong wind could also increase the
375 amount of scattered dust. The increased amount of dust would contribute to the increase
376 of Pb exposure via inhalation. On the other hand, the wet surface soil could decrease the
377 exposure level via inhalation. Climate change, which is one of the major concerns in the
378 world in recent years, could also affect the environmental exposure level.

379 Although Cd was not detected in any individual rat, significantly higher
380 concentrations of Pb were observed in the brain in the high-metal exposure group. Pb is
381 known to pass through the blood/brain barrier and accumulate in the brain (Struzynska
382 *et al.*, 1997), and this phenomenon was also confirmed here in the case of soil exposure.
383 Among the tissues examined, quite different patterns of accumulation were observed
384 between Cd and Pb in the tibia. Cd in the tibia did not differ among the groups, whereas
385 quite high concentrations of Pb (average: 54.4 mg/kg) were detected. This value was
386 comparable to that reported previously by de Figueiredo *et al.* (2014) who demonstrated
387 that newborn rats exposed to 30 mg Pb/L in drinking water for 60 days after birth
388 accumulated 43.4 mg/kg of Pb in the tibia (Supplementary Table S7). Similarly, rats
389 exposed to 500 mg Pb/L in drinking water for 84 days had 58.2 mg/kg of Pb in the
390 femur (Li *et al.*, 2013). Notably, in the study by Li *et al.* (2013), rats showed
391 hippocampal damage associated with Pb exposure. This accumulation pattern in the
392 tibia can be explained by competition of Pb for Ca²⁺ and deposition in the bone as more
393 than 90% of total Pb burden in the body accumulates in bone tissues. The accumulation
394 factor supports this difference between Cd and Pb; i.e., the highest accumulation factor

395 for Pb and the second lowest accumulation factor for Cd were observed in the tibia. The
396 half-life of Pb in the tibia is estimated as 20 – 30 years in humans (Gerhardsson *et al.*,
397 1993) and lacteal as well as transplacental transfer is one of the most serious exposure
398 routes in infants (Chen *et al.*, 2014). Although the present study used male rats, similar
399 soil exposure experiments using pregnant females to clarify the effects on neonates
400 would be interest because an earlier study indicated that rat pups of dams exposed to Pb
401 via drinking water showed neurochemical alterations in the cerebellum and striatum
402 (Antonio *et al.*, 2002).

403 As we found that soil exposure can cause Cd and Pb tissue accumulation, we
404 further analyzed blood biochemistry and behavioral changes in the present study. Some
405 of the items examined suggested that the levels of functional damage in the liver and
406 kidney were not severe. In contrast, decreases in behavioral activity were observed in
407 the soil-exposed groups, suggesting effects of Pb on the central nervous system. In
408 accordance with our study, Pb was reported to cause a significant decrease of locomotor
409 activity in Wistar rats chronically administered 0.5% (v/v) Pb acetate in drinking water
410 for 3 months, with concomitant astrocytic and dopaminergic changes involved in
411 controlling many aspects of brain function (Sansar *et al.*, 2011). Although the molecular
412 mechanism of Pb neurotoxicity is not elucidated well, however some possible pathways
413 such as alterations in genetic regulation and protein synthesis have been reported so far.
414 For instance, the expression, synthesis and conformational maturation of the neuronal
415 cell adhesion molecule (NCAM) are affected by Pb exposure (Breen *et al.*, 1988; Davey
416 *et al.*, 1998). Additionally, voltage-gated calcium channels, which allow the flow in a
417 number of mono- and polyvalent cations, is also affected by Pb. It was observed that Pb
418 is capable of blocking some types of calcium channels, including potassium currents in

419 neurons (Peng *et al.*, 2002; Dai *et al.*, 2001). Oxidative stress is also associated with
420 neurotoxicity of Pb. The former exposure study of rat discovered higher levels of brain
421 2-thiobarbituric acid-reactive substances, an indicator of lipid oxidation, and higher
422 activities of glutathione reductase and glutathione peroxidase compared with controls
423 (Adonaylo and Oteiza; 1999). In the exposure study of the rat to Pb which was done by
424 Villeda-Hernandez *et al.* (2001), revealed Pb accumulation was related with high levels
425 of lipid oxidation products in different brain regions, such as the parietal cortex,
426 striatum, hippocampus, thalamus and cerebellum. Pb is known to be neurotoxic in
427 humans, especially children, because of its ability to compete with Ca²⁺ in nerve
428 functioning (Crosby, 1998). Recently, the Centers for Disease Control and Prevention
429 (CDC, 2012) revised the blood lead “level of concern” from 10 to 5 µg/dL in response
430 to reports that Pb levels of < 10 µg/dL can cause neurological abnormalities, such as
431 decreased IQ in children (Canfield *et al.*, 2003). Therefore, a threshold below which Pb
432 does not result in neurological deficits has not been determined (Needleman *et al.*,
433 2004). The present results were consistent in that behavioral changes were detected at
434 an earlier stage before tissue dysfunctions were observed. In general, biological
435 responses precede the appearance of tissue damage. Therefore, we analyzed the
436 alterations of MT and confirmed the induction of MT-1 as well as MT-2 mRNA in the
437 kidneys of rats in the high-metal exposure group. On the other hand, no significant
438 difference of MT-1 and MT-2 expression in the liver samples was observed among the
439 groups, supporting the former study which revealed the expression levels of MT-1 and
440 MT-2 expression in the livers of rats from Kabwe, where the soil used in the present
441 study was collected, had no significant difference with those from control site
442 (Nakayama *et al.*, 2013). However, in case of acute exposure, the induction of MT-1

443 and MT-2 in kidney of Cd-exposed rat was much lower than in the liver (Vasconcelos *et*
444 *al.*). The major difference between the current study and the former acute exposure
445 study is the accumulated hepatic metal levels. Our study showed approximately 5 times
446 and 2 times greater levels of Cd and Pb in liver of high-metal exposure group compared
447 with those of control, respectively, whereas almost 100 times elevation of the hepatic
448 Cd level were discovered within 24 hours after exposure in the acute exposure
449 experiment. Such the difference of exposure period and accumulation level could be the
450 reason why the significant difference in the hepatic MT expression levels was not
451 observed in our study.

452 Interestingly, global DNA methylation was altered in the testis in the
453 high-metal exposure group, although no changes were detected in the liver or kidneys in
454 the present study. This finding was supported by the higher mRNA expression levels of
455 *de novo* DNA methyltransferases, Dnmt 3a and Dnmt 3b, in the testis and the positive
456 associations among metals, methylation levels, and methyltransferases characterized by
457 PCA. It is unclear why the only testis showed a significant difference in DNA
458 hypermethylation status; however, one of the possible reasons is the rapid cell division
459 in the testis compared to in other organs. The short cycle of cell division could
460 contribute to the high sensitivity to molecular alteration. Ikeda *et al.* (2013) reported
461 testis- or germ cell-specific hypomethylated DNA domains with unique epigenetic
462 features on the mouse X chromosome, suggesting the unique molecular character of the
463 testis. Acute exposure (1 week) of TRL 1215 rat liver cells to Cd inhibited DNA
464 methyltransferase activity and induced global DNA hypomethylation, whereas a
465 relatively longer exposure period (10 weeks) resulted in DNA hypermethylation and
466 enhanced DNA methyltransferase activity, suggesting that the effects of prolonged Cd

467 exposure on DNA methylation may be responsible for its carcinogenicity (Arita and
468 Costa, 2009; Takiguchi *et al.*, 2003). Another study also showed that 10-week exposure
469 to Cd induced malignant transformation associated with global DNA hypermethylation,
470 higher Dnmt 3b protein expression, and increased Dnmt activity, without any change in
471 Dnmt 1 (Arita and Costa, 2009; Benbrahim-Tallaa *et al.*, 2003). In fact, previous studies
472 suggested that Dnmt 3a and Dnmt 3b, but not Dnmt 1, are responsible for *de novo*
473 methylation *in vivo*, as embryonic stem (ES) cells of mice lacking Dnmt 1 are still
474 capable of methylating retroviral DNA *de novo* (Lei *et al.*, 1996; Okano *et al.*, 1999).
475 Taken together, our results and those of these previous reports indicate that chronic Cd
476 exposure can cause global DNA hypermethylation in relation to the elevation of Dnmt
477 3a and Dnmt 3b mRNA expression and activities. It should be noted that Cd induced
478 global DNA hypomethylation, and this could be attributed to the potential facilitator of
479 Cd-stimulated cell proliferation in the chronic myelogenous leukemia K562 cell line
480 (Arita and Costa, 2009; Huang *et al.*, 2008).

481 In a human epidemiological study, significant associations were observed
482 between urinary Cd concentrations and global DNA methylation as well as between
483 arsenic metabolism (measured as percentage of dimethylarsinate) and global DNA
484 hydroxymethylation (Tellez-Plaza *et al.*, 2014). The major limitation of the present
485 study was that we did not measure hydroxymethylation levels. Histone modification is
486 also one of the key factors of epigenetics while the limited number of studies has
487 reported an effect of Cd and Pb on histone modification. It was suggested that Cd
488 exposure could make heritable change in chromatin structure for rapid transcription
489 activation, resulting the establishment and maintenance of a bivalent chromatin domain
490 at the MT-3 promoter as well as histone modifications (Martinez-Zamudio and Ha,

491 2011). In Cd-transformed urothelial cells, levels of H3K4me3, H3K27me3 and
492 H3K9me3 occupancy at the MT-3 promoter were increased compared to untransformed
493 cells, indicating chronic Cd exposure may alter transcriptional responses through
494 histone modification (Somji *et al.*, 2011). Relating to Pb, Cantone *et al.* (2011) reported
495 that the levels of H3K4me2 and H3K9ac on histones from blood leukocytes of steel
496 production plant workers were not significantly associated with the Pb exposure level.
497 Another factor of epigenetic alteration is miRNA expression. After 3 days exposure to
498 particulate matter (PM) containing Cd, the expression of miR-146a in peripheral blood
499 leukocytes from electric furnace steel plant workers was not statistically increased
500 whereas miR-146a was negatively associated with exposure (Bollati *et al.*, 2010). Same
501 research group also reported that miR-222 expression showed a positive association
502 with Pb exposure, while miR-146a expression was negatively correlated with Pb
503 (Bollati *et al.*, 2010). Further studies of the association between metal exposure and
504 hydroxymethylation status, histone modification and miRNA expression are required to
505 reveal the molecular alteration mechanisms.

506 In contrast to the elevated methylation observed in the present study, the
507 methylation status of Long Interdispersed Nuclear Element 1 (LINE-1) in
508 pheochromocytoma (PC12) cells decreased after acute exposure to 500 nM Pb for 2 and
509 7 days (Li *et al.*, 2012). In addition, a dose-dependent decrease in global DNA
510 methylation in PC12 cells was observed with decreasing Dnmt 1 mRNA expression (Li
511 *et al.*, 2012). These discrepancies may be explained by differences in exposure duration,
512 as we used 1-year prolonged chronic exposure. Similar phenomena (i.e.,
513 hypomethylation with acute exposure and hypermethylation with chronic exposure)
514 observed in the case of Cd exposure were discussed above (Arita and Costa, 2009;

515 Takiguchi *et al.*, 2003). Long-term chronic exposure experiments are necessary to
516 elucidate the effects of Pb on epigenetics.

517 Another interesting observed in the present study was that we found alterations
518 in DNA methylation in the male testis as a recent study indicated that aberrant DNA
519 methylation of the H19-DMR (differentially methylated region) and the DAZL (deleted
520 in azoospermia-like) gene promoters is associated with defects in sperm
521 production/function in infertile men (Li *et al.*, 2013). Recently, our research team
522 reported severe Pb accumulation in the blood of children in Kabwe mining site, Zambia,
523 from which our soil samples were collected, and all children examined under the age of
524 7 years ($n = 246$) had blood Pb levels exceeding 5 $\mu\text{g}/\text{dL}$ with a maximum of 427.8
525 $\mu\text{g}/\text{dL}$ (Yabe *et al.*, 2015). An earlier report showed that maternal bone Pb levels in
526 humans were inversely associated with cord blood methylation levels in LINE-1 and a
527 short interspersed element (Alu-1), which are frequently analyzed in genomic DNA
528 methylation studies (Pilsner *et al.*, 2009a). However, little is known about the
529 relationship between Pb exposure and DNA methylation (and hydroxymethylation) in
530 humans. Therefore, the effects of prolonged Pb exposure on epigenetics modifications
531 in children at this site should be examined in future studies.

532

533 **5. Conclusions**

534 The aim of this study was to verify whether soil exposure under
535 environmentally relevant circumstances results in metal accumulation and epigenetic
536 modifications in the rats. Our present results suggested that soil contaminated with Cd
537 and Pb caused tissue metal accumulation and epigenetic alterations, such as elevation of
538 global DNA methylation, in rats. From the view of metal accumulation ratios

539 (lung/liver) and Pb isotopic ratios in the tissues, it was suggested that the respiratory
540 exposure would make up a significant proportion of metal absorption into the body.
541 Although we found elevation of methylation status in the rat testis, contradictory results
542 have also been documented in the literature. Further studies are required to gain a better
543 understanding of this issue.
544

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562

563 **Declarations of interest**

564 None.

565

566 **Figure Legends**

567

568 Fig. 1. Analysis of Pb isotope ratios ($^{208}\text{Pb}/^{206}\text{Pb}$ and $^{207}\text{Pb}/^{206}\text{Pb}$) in the tissues of rats
569 and Kabwe galena (Kamona et al., 1999). The mean values and SD values are shown
570 with error bars. Bold dash = blood of control group, x mark = blood of low-metal
571 exposure group, asterisk = blood of high-metal exposure group, white diamond = brain
572 of control group, grey diamond = brain of low-metal exposure group, black diamond =
573 brain of high-metal exposure group, white square = lung of control group, grey square =
574 lung of low-metal exposure group, black square = lung of high-metal exposure group,
575 white triangle = liver of control group, grey triangle = liver of low-metal exposure
576 group, black triangle = liver of high-metal exposure group, white circle = kidney of
577 control group, grey circle = kidney of low-metal exposure group, black circle = kidney
578 of high-metal exposure group, white cross in black square = Kabwe galena.

579

580 Fig. 2. Analysis of mRNA expression levels ($n = 8$ for each group) for kidney MT-1 (A),
581 kidney MT-2 (B), liver MT-1 (C), and liver MT-2 (D). Data are shown in box and
582 whiskers plots: box limits represent 25 and 75 percentiles; lines within the boxes
583 indicate the medians; whisker ends indicate minimum and maximum values. Different
584 characters (a, b) indicate significant differences (Tukey's test).

585

586 Fig. 3. Analysis of global methylation levels ($n = 10$ for each group) in the testis (A),
587 liver (B), and kidney (C). Data are shown in box and whiskers plots: box limits
588 represent 25 and 75 percentiles; lines within the boxes indicate the medians; whisker

589 ends indicate minimum and maximum values. Different characters (a, b) indicate
590 significant differences (Tukey's test).

591

592 Fig. 4. Analysis of mRNA expression levels ($n = 8$ for each group) of Dnmt 1 (A),
593 Dnmt 3a (B), and Dnmt 3b (C) in the testis. Data are shown in box and whiskers plots:
594 box limits represent 25 and 75 percentiles; lines within the boxes indicate the medians;
595 whisker ends indicate minimum and maximum values. Different characters (a, b)
596 indicate significant differences (Tukey's test).

597

598 Fig. 5. Principal component analysis among metal concentrations, global DNA
599 methylation, and of Dnmt 1, Dnmt 3a and Dnmt 3b mRNA expression in the rat testis.
600 The letters C, L, and H indicate individual rats of control, low-, and high-metal
601 exposure groups, respectively.

602

603

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910 lead-zinc mine in Kabwe, Zambia. Chemosphere 202, 48-55, PMID: 29554507,
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912 Table 1. Comparison of Cd and Pb concentrations (mean \pm standard deviation) in rat
 913 tissues.

Tissue	Group	Cd		Pb	
Blood ($\mu\text{g/dL}$)	Control	0.005 \pm 0.001	b	0.24 \pm 0.03	b
	Low	0.005 \pm 0.002	b	0.37 \pm 0.14	b
	High	0.009 \pm 0.002	a	4.22 \pm 0.82	a
	Ratio *	1.7		11.3	
Testis ($\mu\text{g/kg}$)	Control	2.1 \pm 0.5	b	19.1 \pm 11.3	b
	Low	2.0 \pm 0.3	b	13.1 \pm 4.9	b
	High	7.2 \pm 0.7	a	98.2 \pm 79.5	a
	Ratio *	3.6		7.5	
Brain ($\mu\text{g/kg}$)	Control	BDL		40.2 \pm 39.1	b
	Low	BDL		20.8 \pm 9.9	b
	High	BDL		128.0 \pm 100.4	a
	Ratio *	-		6.1	
Lung ($\mu\text{g/kg}$)	Control	1.9 \pm 1.0	b	4.7 \pm 2.3	b
	Low	2.1 \pm 1.0	b	6.4 \pm 4.8	b
	High	12.1 \pm 2.8	a	227.7 \pm 132.7	a
	Ratio *	5.9		35.8	
Liver ($\mu\text{g/kg}$)	Control	5.9 \pm 5.0	b	252.1 \pm 139.4	b
	Low	13.1 \pm 5.2	b	279.7 \pm 89.5	b
	High	30.2 \pm 16.2	a	527.2 \pm 97.0	a
	Ratio *	2.3		1.9	
Kidney ($\mu\text{g/kg}$)	Control	155.3 \pm 19.8	b	833.6 \pm 150.3	b
	Low	122.8 \pm 16.9	b	1016.5 \pm 242.1	b
	High	508.0 \pm 88.0	a	2690.7 \pm 464.2	a
	Ratio *	4.1		2.6	
Tibia ($\mu\text{g/kg}$)	Control	5.6 \pm 2.0	a	12.6 \pm 165	b
	Low	5.0 \pm 0.6	a	419.5 \pm 56.3	b
	High	5.2 \pm 1.5	a	54444 \pm 5831	a
	Ratio *	1.0		130	

Note: * and BDL indicate the concentration ratio (High/Low) and the below detection limit, respectively.

Note: Different characters (a, b) indicate significant difference (Tukey's test).

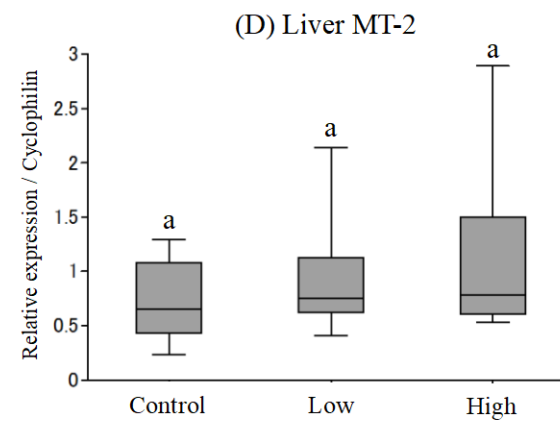
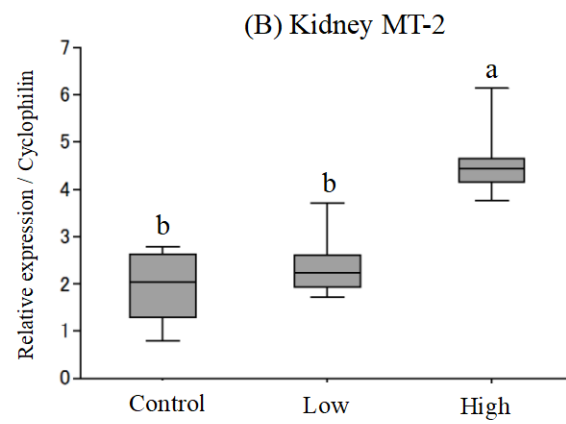
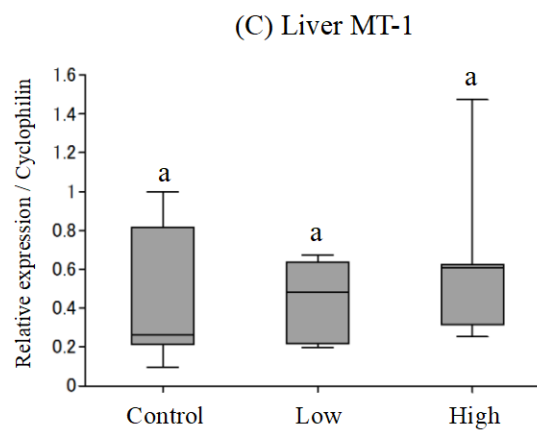
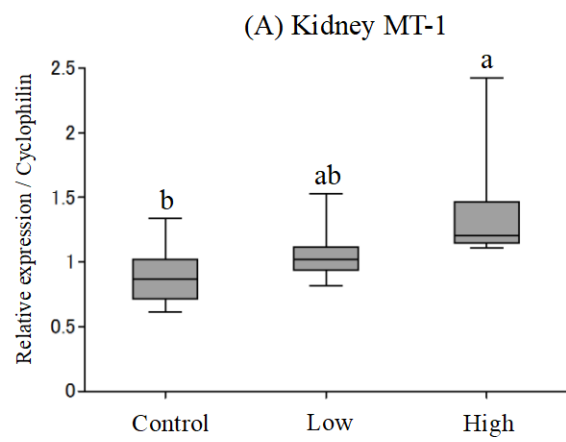
914 Table 2. Comparison of behavioral activity (mean \pm standard deviation) among the groups.

915

Period	Group	MOVE 1		MOVE 2		Rearing	
Before exposure	Control	5535 \pm 1678	a	3745 \pm 1264	a	164 \pm 43	a
	Low	5263 \pm 1385	a	3557 \pm 1022	a	161 \pm 42	a
	High	4313 \pm 690	a	2861 \pm 491	a	159 \pm 32	a
2 month	Control	10522 \pm 1535	a	6647 \pm 1193	a	200 \pm 37	a
	Low	7465 \pm 1461	b	4795 \pm 1036	b	163 \pm 24	b
	High	8244 \pm 1548	b	5452 \pm 1145	ab	203 \pm 17	a
6 month	Control	9159 \pm 1782	a	5663 \pm 1219	a	164 \pm 28	a
	Low	7852 \pm 989	ab	4852 \pm 702	ab	165 \pm 10	a
	High	7268 \pm 1821	b	4379 \pm 1249	b	151 \pm 33	a
12 month	Control	6852 \pm 1582	a	4281 \pm 1174	a	117 \pm 24	a
	Low	4894 \pm 1755	b	2967 \pm 1119	b	98 \pm 30	a
	High	4798 \pm 1442	b	2928 \pm 988	b	104 \pm 16	a

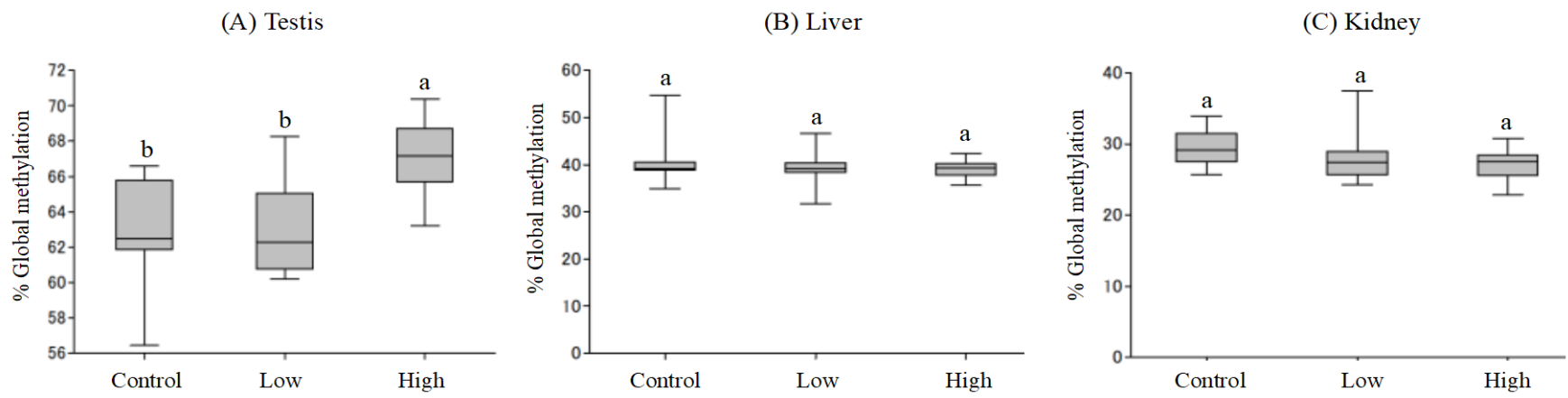
Note: MOVE 1 and MOVE 2 indicate large and small horizontal movement, respectively.

Note: Different characters (a, b) indicate significant difference (Tukey's test).



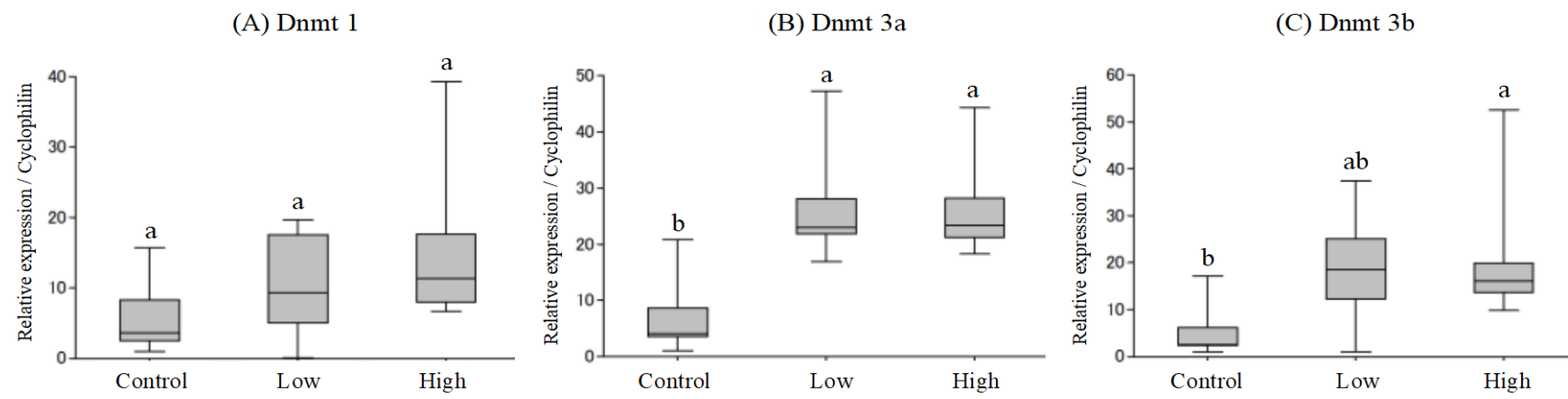
918

919 Fig. 2.



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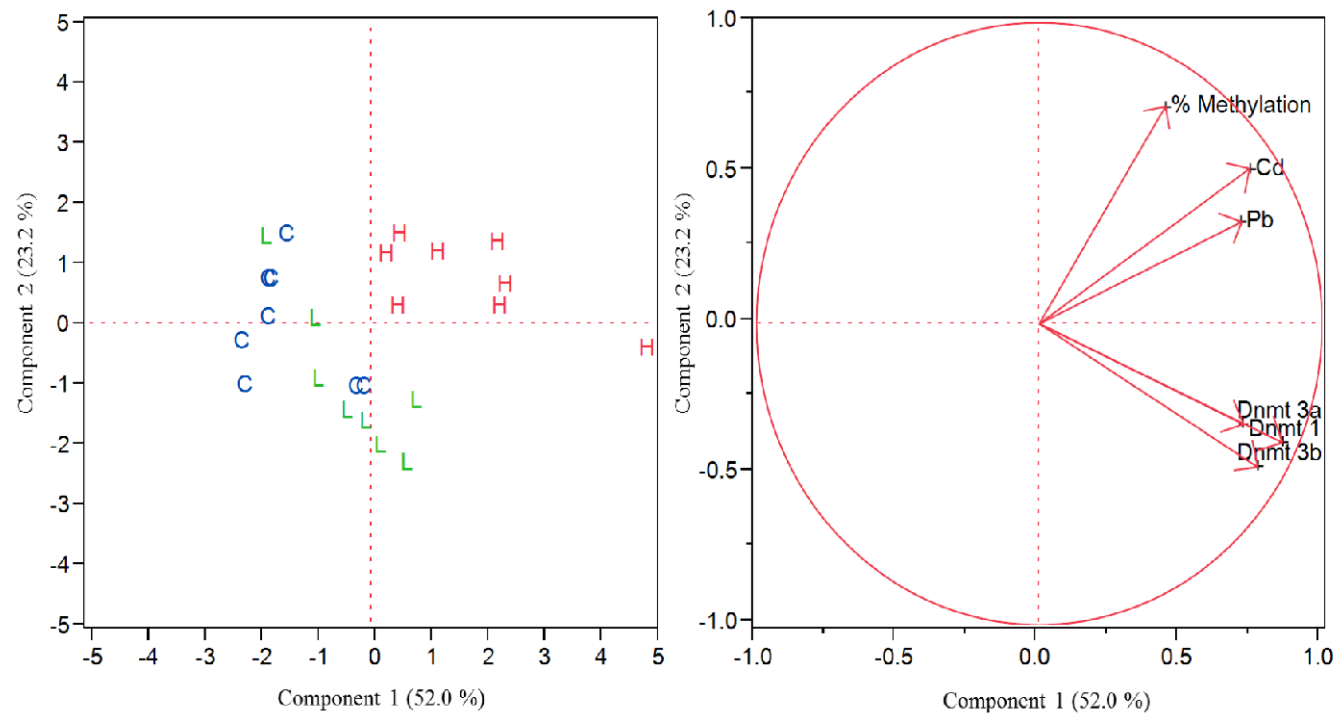
921 Fig. 3.



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923

924 Fig. 4.



925

926 Fig. 5.

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