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### **Author contribution statement**

**Caide Huang:** Conceptualization, Writing-original draft. **Zhiqiang Shen:** Methodology.

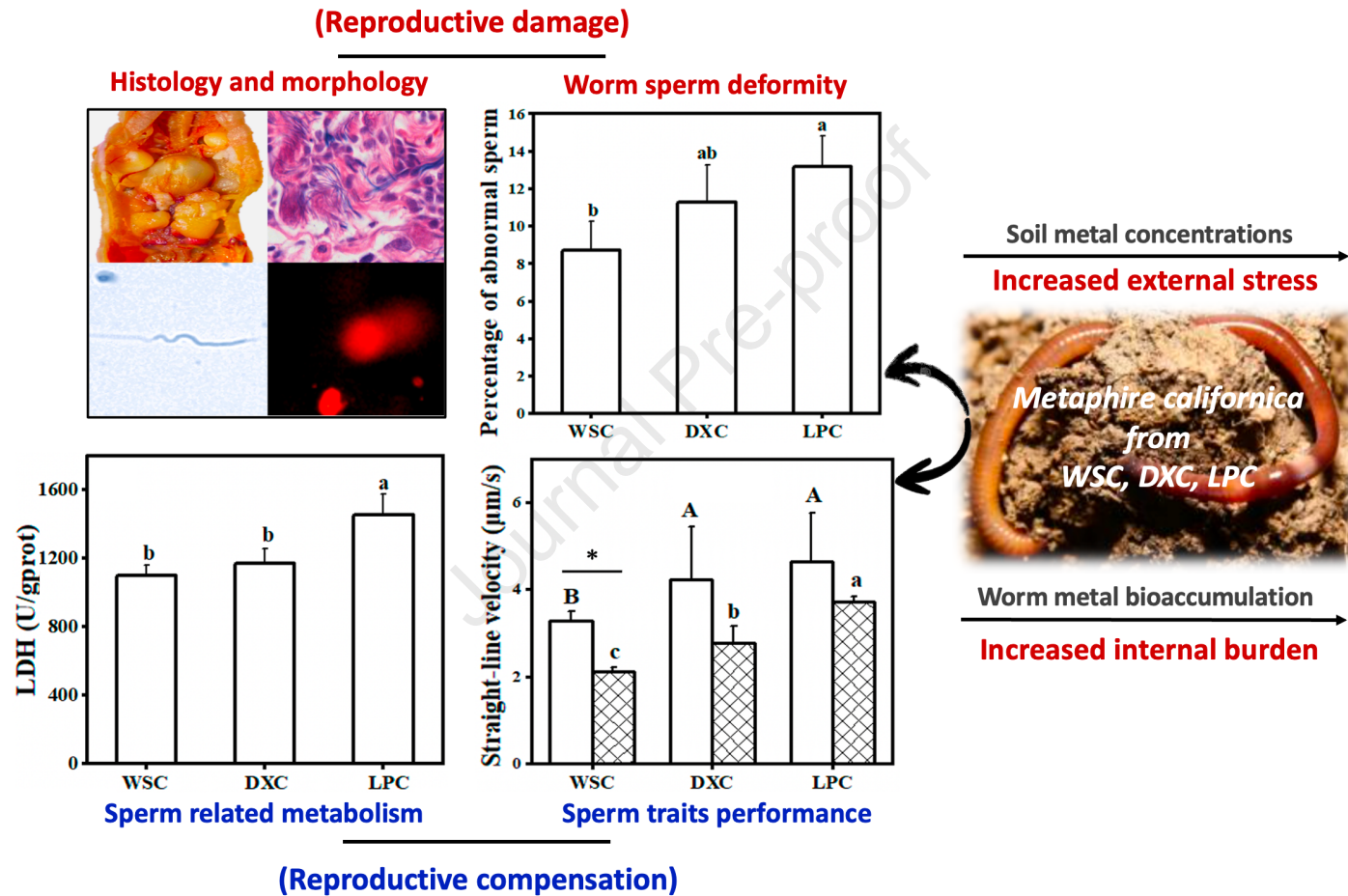
**Liang Li:** Methodology. **Shizhong Yue:** Writing - review & editing. **Li Jia:** Writing - review

& editing. **Kun Wang:** Writing - review & editing. **Wenhao Zhou:** Methodology. **Yuhui Qiao:**

Conceptualization, Funding acquisition, Supervision.

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Graphical abstract:





22 **Abstract**

23       Reproduction is a significant biological process for organisms responding to  
24 environmental stresses, however, little is known about the reproductive strategies of  
25 invertebrates under long-term exposure to contaminations. In this study, earthworm  
26 *Metaphire californica* (Kinberg, 1867) from contaminated fields with an increased  
27 metal gradient were collected to investigate their reproductive responses. The results  
28 showed heavy metals (Cd, Cu, Zn, and Pb) induced histological damage to earthworms'  
29 seminal vesicles, including tissue disorders and cavities, and decreases in mature  
30 spermatozoa. Sperm morphology analysis indicated deformity rates were up to 13.2%  
31 (e.g. head swollen or missing) for worms from the most contaminated site, which  
32 coincided with DNA damages. Furthermore, the computer-assisted sperm analysis  
33 (CASA) system was employed for the evaluation of sperm kinetic traits. Results  
34 suggested earthworms exposed to higher contamination showed a lower sperm viability  
35 rate but faster sperm velocity after re-exposure with Cd solution (like the curvilinear  
36 velocity and straight-line velocity parameters) compared with those from relatively  
37 clean sites. The activities of lactate dehydrogenase and sorbitol dehydrogenase showed  
38 the highest 32.5% and 12.5% up-regulation respectively with the increased metal  
39 gradient. In conclusion, this study elucidated the earthworm reproductive toxicity,  
40 underlying reproductive compensation, metal stress-induced damages, and adaptive  
41 responses caused by heavy metal exposure, while also providing the possibility of  
42 sperm trait analysis (CASA) for related earthworm toxicological studies.

43 **Capsule:** Earthworms under long-term heavy metal stresses coordinate more energy  
44 metabolism for sperm kinetic traits to compensate for reproductive damage.

45 **Keywords:** Earthworms; seminal vesicles; morphology; sperm traits; reproductive  
46 metabolism

47

## 48 **1. Introduction**

49 In recent decades, existing and emerging environmental issues have been  
50 enhanced by rapid urbanization and industrialization, from the pathways of atmospheric  
51 deposition, mining activities, chemical waste emission, pesticide and fertilizer abuses  
52 (Navarro et al., 2016; Gupta et al., 2021). Heavy metals are one of the most consistent  
53 and ubiquitous pollutants posing significant ecological risks and therefore requiring  
54 careful consideration (Wu et al., 2022). Once entering the environment, non-  
55 biodegradable metals can be bioaccumulated, transferred to trophic levels, cause  
56 biological toxicity, and eventually interfere with normal biogenesis and public health  
57 (Huang et al., 2020; Yaashikaa et al., 2022). Consequently, using terrestrial and aquatic  
58 organisms (like earthworms and zebrafishes) to carry out metal risk assessments is  
59 always the foreword research in environmental science and toxicology (Chen et al.,  
60 2021; Sivakumar, 2015).

61 Earthworms are regarded as “soil ecological engineers” because of their critical  
62 dwelling and burrowing roles in soil improvements (Morgan and Morgan, 1999).

63 Furthermore, earthworms are typical sentinel invertebrates and have been widely  
64 recommended as model test species in OECD No. 207 and ISO guidelines for risk  
65 assessments of various soil pollutants (ISO, 2012; Morgan et al., 2007; OECD, 1984).  
66 At present, various individual indexes and biomarkers have been applied to assess the  
67 fitness and response of earthworms under hazardous exposure. These include LC<sub>50</sub> (50%  
68 lethal concentration) and EC<sub>50</sub> (50% effective concentration) values for survival rate  
69 and individual growth, antioxidant enzyme activities and gene expression for biological  
70 processes (Spurgeon et al., 2020; Spurgeon and Hopkin, 1995). Unlike other organisms,  
71 earthworms are hermaphrodites, simultaneously keeping male and female reproductive  
72 organs and systems, which provides an excellent model to evaluate reproductive  
73 toxicity (Domínguez and Velando, 2013; Kwak and An, 2021a). However, up to now  
74 related indicators for reproductive studies on earthworms and other invertebrates are  
75 very limited (Yesudhasan et al., 2013). For instance, Yang et al. (2022) reported that the  
76 earthworm cocoon numbers decrease by 16.7% after exposure to 100 mg/kg carbon  
77 nanotubes, whilst other studies also used conventional recordings like juveniles, and  
78 hatching rates for reproduction assessments (Huang et al., 2021a).

79 The reproductive system is essential for organisms under unfavorable conditions  
80 to ensure their specie and population continuation (Taslima et al., 2022). Related  
81 indicators like gene expression, reproductive organ changes, sperm morphology and  
82 mobility have been proven relatively more sensitive for assessing the reproductive  
83 toxicity of some vertebrates exposed to different environmental stressors (Kime et al.,



84 1996; Kime et al., 2001; Kwak and An, 2021b; Yang et al., 2019). Studies using these  
85 novel biomarkers within earthworm toxicological studies are still rare, although the  
86 development of microscopic and computer-assisted sperm analysis (CASA) systems  
87 provides more novel determinations. CASA has been the dominant method for sperm  
88 quality estimation and provides possibilities for rapid *in vivo* monitoring and  
89 comparison of sperm kinetic traits of different animals like rats and birds (Yang et al.,  
90 2019; Waberski et al., 2021). Despite this, as most animal spermatozoa will be rapidly  
91 inactivated *in vitro*, appropriate dilution and capacitation liquid must be maintained to  
92 ensure the activities and movement of spermatozoa before CASA detection (Travis et  
93 al., 2004), presenting one of the main challenges for the detection and assessment of  
94 earthworm sperm quality.

95 Furthermore, most previous reproductive toxicity studies were validated via  
96 laboratory microcosm exposures, while few studies were devoted to considering those  
97 organisms under the sustained stresses of *in-situ* field contaminations (Gao et al., 2015;  
98 Kwak and An, 2021b; Yang et al., 2019). Normally, reproductive regulation is an  
99 alternative response for certain organisms under stress from long-term exposure  
100 through increased reproductive efforts with energy trade-offs to allow reproductive  
101 success (Gowaty et al., 2007; Orton and Tyler, 2015; Yang et al., 2020a). The stress-  
102 induced resource redistribution and reproductive compensation that is driven by  
103 environmental tolerance, phenotypic plasticity, and even parental inheritance have not  
104 been clearly elucidated (Huang et al., 2022). Accordingly, it is of importance to

105 investigate the reproductive damage as well as the strategy of organisms under long-  
106 term stress from *in-situ* exposure. Our previous studies reported a wild oligochaete  
107 earthworm *Metaphire californica* exposed to heavy metal contaminations over several  
108 decades, possessed more considerable population dominance, bioaccumulation, and  
109 better detoxification capacity, especially for Cd resistance and detoxification in  
110 comparison to other wild species, providing an advisable research candidate (Huang et  
111 al., 2021b; Huang et al., 2020; Wang et al., 2020; Wang et al., 2018a).

112 Overall, it could be hypothesized that wild earthworms under persisting  
113 contamination can respond to heavy metal stress by reproductive compensation. The  
114 present study was carried out to systematically investigate the reproductive damage and  
115 compensation in wild earthworm *M.californica* from different long-term heavy metal  
116 exposure histories (Cd, Cu, Zn, and Pb). The detailed purposes of this study were: (1)  
117 to employ the CASA system to evaluate the sperm traits and performance of earthworm  
118 *M.californica*; (2) to determine the reproductive toxicity of heavy metals on  
119 earthworms via histology, morphology and DNA damage analyses; (3) to explore the  
120 reproductive compensation by assessing the sperm traits and metabolic levels. These  
121 findings can shed light on the dynamic reproductive strategies of those organisms living  
122 under persistent stresses.

## 123 **2. Materials and methods**

### 124 *2.1 Description of sampling sites*

125 All three sampling sites Wanshou (WSC, 112° 40' 05"; 26° 44' 49"), Daxing (DXC,  
126 112° 47' 18"; 26° 54' 21"), and Lupu (LPC, 112° 79' 50"; 27° 00' 07") (Fig. 1) were  
127 located in the "hometown of nonferrous metals", Hengyang City, Hunan Province, the  
128 subtropical areas of southern China. These sites showed different distances away from  
129 mining or tailing areas with over 30 years of pollution history, which were all confirmed  
130 as long-term observing and sampling points by our field survey studies began in 2018  
131 (Huang et al., 2020; Wang et al., 2018b; Wang et al., 2018c).

132 The five-spot-sampling method (50 cm × 50 cm × 25 cm) was applied to sample  
133 earthworms and soils (0-25 cm) from each site. The heavy metal contaminations totally  
134 followed an increasing gradient from WSC to DXC and LPC sites, especially for soil  
135 Cd, which was 1.50, 5.08, and 12.6 mg/kg, respectively. According to our previous  
136 studies (Huang et al., 2020; Huang et al., 2021b), only heavy metals of Cd, Cu, Zn, and  
137 Pb exceed the Chinese Environmental Quality Standard for Soils (GB 15618-2018),  
138 which were mainly considered in our study. Detailed site information, soil properties,  
139 and metal concentration are provided in Table S1, and related descriptions of these three  
140 sites also can be found in our previous studies.

## 141 2.2 Test earthworms

142 Adult *Metaphire californica* with similar weight (500-550 mg) and fully  
143 developed clitellum were collected from each site. Earthworms were kept in *in-situ*  
144 soils and fed with their original surface litter (leaves and humus), and all subsequent  
145 analysis was done within two weeks to reduce the interferences. Part of the worms was

146 deputed on the Petri dish with moist filter paper for 24 h, washed with deionized water,  
147 frozen in liquid nitrogen, and then dried in a freeze dryer (TF-FD-18S, China) to  
148 analyze metal bioaccumulation. Rest living earthworms were used for further dissection  
149 and reproductive analysis. Meanwhile, *M.californica* was ever confirmed in our former  
150 studies for species classification with morphological analysis and the sequencing of  
151 cytochrome oxidase subunit first (COI) genes (Huang et al., 2021b; Xu and Xiao, 2010).

### 152 *2.3 The analysis of soil properties and heavy metals*

153 Soil pH, soil organic carbon (SOC), total nitrogen (TN), cation exchange capacity  
154 (CEC), and heavy metals (Cd, Cu, Zn, and Pb) were analyzed to assess the overall soil  
155 conditions of different sampling sites, following the protocols in our previous study and  
156 meeting the certificated requirements of detection limits and recovery rates (Huang et  
157 al., 2020).

158 Freeze-dried worms were ground into powder and digested in a microwave with  
159 a mixture of HNO<sub>3</sub>-H<sub>2</sub>O<sub>2</sub>, and metal concentration were analyzed through inductively  
160 coupled plasma-mass spectrometry (ICP-MS, 7700ce, USA). Shrimp powder (GBW  
161 10050) was used as standard reference material and all of the recovery rates were over  
162 95%. Besides, the bioaccumulation factor (BAF) value was calculated by dividing  
163 worm metal concentration into soil metal contents, which represents the ability of  
164 earthworms to bioaccumulate different heavy metals (Huang et al., 2022).

### 165 *2.4 Histology and morphology examination*

166 Depurated *M.californica* was fixed with pins on a smooth foam and two pairs of  
167 spermathecal pores were located in the 7th to 9th lateroventral intersegment (Nguyen  
168 et al., 2020; Xu and Xiao, 2010). Dissected and gently tore the septa along the  
169 abdominal midline of worms from anterior to clitellum segments, separated out the  
170 seminal vesicles, then rinsed with ultrapure water to remove adhering mucus, finally  
171 fixed in 10% formaldehyde solution for 48h. Fixed vesicles were dehydrated with a  
172 graded series of ethanol with different concentration and cleared with a xylene agent to  
173 completely displace ethanol. Afterward, all vesicles were embedded with paraffin,  
174 sliced into films, and fully stained with haematoxylineosin (HE). All prepared slides  
175 were finally observed using an optical microscope (ZEISS-AX10, Zeiss, GmbH,  
176 Germany) and image-recorded under different magnifications to determine histological  
177 changes (Adeel et al., 2019; Huang et al., 2021a; Kwak and An, 2021a).

178 Separated seminal vesicles of at least 10 earthworms from the same treatment were  
179 put into a 1.5 ml centrifuge tube to prepare earthworm seminal suspension by exerting  
180 pressure and forcing out the sperm from vesicles with fine-tipped forceps. 10  $\mu$ L  
181 earthworm semen and 10  $\mu$ L phosphate-buffered saline (pH=6.8) was placed in the  
182 center of slides, then dispersed and smeared by overlapping another slide. All slides  
183 were immersed in 10% formaldehyde solution for 15 min fixing and then stained for  
184 25 min using 10% Giemsa staining stock (Solarbio, China), and finally a slow flow was  
185 used to rinse off the excess staining solutions prior to air-drying. Prepared slides were  
186 observed using an optical microscope (ZEISS-AX10, Zeiss, GmbH, Germany) under

187 400× magnification (Gao et al., 2015; Yesudhasan et al., 2013; Zheng and Li, 2009).  
188 All earthworms from each site were tested as above and photo-recorded. The sperm  
189 deformity rate was calculated by dividing the number of abnormal sperm into total  
190 numbers, and over 100 spermatozoa were randomly selected to examine the abnormal  
191 rates of each treatment.

### 192 *2.5 Assessment of earthworm sperm trait performance*

193 In this study, glucose (Glc) was proven as the best sperm capacitation liquid for  
194 *M.californica* compared with more common-used PBS and HBSS (Hank's Balanced  
195 Salt Solution) (Travis et al., 2004; Williams and Ford, 2001; Yang et al., 2020a), which  
196 successfully capacitated earthworm sperms and make them move in straight or curved  
197 lines (Fig. S1, A). Earthworm sperms were diluted by 20 times with 2.5% Glc solutions  
198 (30°C, adjusted pH to 7) to avoid sperm overlap and ensure the appropriate sperm  
199 amount in each viewing field for trait analysis.

200 Specifically, 10 µL seminal suspension was diluted into 190 µL Glc and blown  
201 with a pipette until all sperm was completely diffused into the surrounding medium.  
202 After 10-min dissociation and thermostat, 10 µL suspension was dropped on the slide  
203 center (pre-warmed under 30°C) and then applied with coverslips for later analysis  
204 (Kime et al., 1996; Kime et al., 2001; Niu et al., 2022; Yang et al., 2019). All earthworm  
205 sperm traits were assessed under a computer-assisted sperm analysis (CASA, LINE-  
206 HY, China) system, provided by the College of Animal Science and Technology, China  
207 Agricultural University, which has been successfully applied for sperm analysis on

208 different animals. A total of 300 earthworm sperms from at least 5 nonconsecutive  
209 viewing fields of each treatment were analyzed to comprehensively evaluate the sperm  
210 traits.

211 The reproductive performances of earthworms from WSC, DXC, and LPC sites  
212 were determined by comparing sperm density, sperm viability, and also several  
213 movement parameters: (a) velocity parameters, including curvilinear velocity (VCL),  
214 straight-line velocity (VSL), and average path velocity (VAP); (b) trajectory parameters,  
215 including linearity (LIN) and wobble (WOB) percentages (Kime et al., 2001; Yang et  
216 al., 2019).

217 In order to directly assess the impacts of heavy metals on earthworm sperm traits,  
218 as well as to further confirm the elevated Cd resistance of earthworms reported in our  
219 previous studies (Huang et al., 2020; Huang et al., 2021b), another lab re-exposure was  
220 carried out to conduct the comparative analysis and evaluate the sperm traits continuity  
221 by using 0.1 mg/L Cd-spiked Glc, which could make sperms visibly affected but not  
222 wholly inactivated (Fig. S1).

### 223 *2.6 DNA damage and metabolism evaluation*

224 DNA damage of earthworm sperms was determined by the single-cell gel  
225 electrophoresis assay (also entitled comet assay), which was described in detail in our  
226 and other studies (Huang et al., 2021a; Li et al., 2020; Wang et al., 2018d). Briefly,  
227 dissected seminal vesicles were suspended with cold PBS buffer, then centrifuged to  
228 obtain the sperm cell suspensions for slide preparation by employing “three-steps”

229 agarose and electrophoresis preparing methods. Processed slides were dyed with  
230 propidium iodide buffer, finally observed and photographed under a fluorescence  
231 microscope. Randomly selected DNA cells were analyzed by using Comet Assay  
232 Software Project (CASP) software with the values of tail DNA proportion (Tail DNA %)  
233 and olive tail moment (OTM). Damaged DNA will show typical comet tails with the  
234 Tail DNA% > 5 and OTM > 2 values (Fig. S2).

235 Sperm-related metabolism was evaluated by determining the activities of lactate  
236 dehydrogenase (LDH) and sorbitol dehydrogenase (SDH), which were both significant  
237 biomarker enzymes involved in energy acquisition, sperm production, and germ cell  
238 development (Yang et al., 2020b; Zhu et al., 2019). LDH and SDH were determined at  
239 450 and 600 nm via a microplate reader (XS2, BioTeK, USA), respectively, by using  
240 seminal vesicle cells and commercial enzyme kits (Nanjing Jiancheng Bioengineering  
241 Institute, Nanjing, China), following the manufacturer's specifications.

## 242 *2.7 Statistical analysis*

243 All data analyses were performed with IBM SPSS 24.0 by using Tukey's honest  
244 significant difference (HSD) test. Data were expressed as means  $\pm$  standard deviations  
245 with  $P < 0.05$  representing significant differences. Pearson's correlations analyses were  
246 employed to measure the association between worm metal bioaccumulation and each  
247 reproductive parameter (Fig. S3). Tables and figures were completed in Excel 2016 and  
248 Origin 2020.



### 249 3. Results

#### 250 3.1 Heavy metal bioaccumulation in *M.californica*

251 The internal metal concentration of *M.californica* and bioaccumulation factor  
252 values (BAF) are shown in Table 1. The metal bioaccumulation in earthworms showed  
253 consistency with their exposure history. *M.californica* from the LPC site showed the  
254 highest Zn and Pb bioaccumulation compared with those from WSC and DXC sites,  
255 which were 239 and 38.4 mg/kg, respectively. Furthermore, LPC *M.californica* also  
256 suffered the highest body burden for soil Cd, which was 3.64 and 1.43 times higher  
257 than that of WSC and DXC worms, respectively. *M.californica* also bioaccumulated  
258 other heavy metals (Cu and Pb), but differences were found to be non-significant  
259 between WSC and LPC worms ( $P > 0.05$ ; Table 1). For bioaccumulation factor values,  
260 almost only  $BAF_{Cd}$  values were larger than 1.0, indicating the significant ability of  
261 earthworms to bioaccumulate soil Cd. Specifically, the  $BAF_{Cd}$  values were 11.2, 8.43,  
262 and 4.83 for WSC, DXC, and LPC worms, respectively, showing a significant decrease  
263 with the increase of heavy metal concentration ( $P < 0.05$ ; Table 1).

#### 264 3.2 Histological and morphological changes

265 The histological changes in earthworm seminal vesicles are shown in Fig. 2. Pairs  
266 of the spermathecal were located in the 7/8/9 lateroventral segments within the clitellum  
267 area and the intact one showed a spherical structure and cream color (Fig. 2A, B).  
268 Seminal vesicles aid in sperm nourishment and spermatogenesis, in which the initial

269 development of anucleate spermatocytes occurs and finally separate as sperm bundles  
270 and mature spermatozoa (Fig. 2C - Fig. 2H). The comparison of histological images  
271 revealed that the increased metal concentration from WSC to LPC sites coincided with  
272 the increases in tissue cavities and disordered germ cells and a decrease in bundles of  
273 mature sperms in seminal vesicles.

274 The sperm morphology of *M.californica* and the abnormal sperm rates are  
275 presented in Fig. 3. Unlike human sperm, normal earthworm sperm was more like a  
276 smooth straight or curved line with a coarser nucleus head and a flagellum that is several  
277 times longer. Sperm deformities occur mainly in the head, including swollen heads,  
278 helix heads, bent heads, and missing heads (Fig. 3A). The sperm deformity rates of  
279 WSC, DXC, and LPC worms showed a significant increase from 8.73% to 13.2%, with  
280 the increased gradient of heavy metal contaminations, especially for soil Cd  
281 concentration (Fig. 3B).

### 282 3.3 Earthworm sperm motility traits

283 The sperm trait parameters under Cd and no Cd treatments are shown in Fig. 4.  
284 The density and viability of unprocessed sperms showed a slight decrease but non-  
285 significant difference from WSC to LPC worms ( $P > 0.05$ ; Fig. 3C and Fig. 4A).  
286 Besides, without the Cd re-exposure, no significant difference was found for the sperm  
287 LIN, WOB, VCL, and VAP values among three worms ( $P > 0.05$ ). Only the VSL values  
288 were significantly increased, which were 3.29, 4.23, and 4.64  $\mu\text{m/s}$  for WSC, DXC, and  
289 LPC worms, respectively ( $P < 0.05$ ; Fig. 4). After re-exposure with Cd-spiked Glc, most

290 sperm trait values were significantly inhibited and decreased to varying degrees ( $P <$   
291 0.05; Fig. 4). In contrast, the LIN, WOB, and VSL values showed significant  
292 differences among WSC, DXC, and LPC worms, presenting significant increases with  
293 the increased metal gradient from WSC to LPC and a maximum of 102% increase for  
294 LIN performance ( $P < 0.05$ ; Fig. 4).

#### 295 3.4 Sperm DNA damage and enzyme activities

296 The DNA damage and metabolic enzyme activities of *M.californica* sperms are  
297 shown in Fig. 5. The Tail DNA% and OTM values were 3.46 and 1.24, 3.53 and 2.02  
298 for WSC and DXC worms, respectively. This showed no DNA damage because it didn't  
299 exceed any threshold value limits. Compared with those results, LPC worm sperm  
300 showed slight DNA damage with higher Tail DNA % and OTM values of 5.83 and 3.85,  
301 respectively (Fig. 5A, B; Fig. S2). The LDH activities of LPC worms (1453 U/gprot)  
302 were significantly higher than that of WSC and DXC worms (1097 and 1170 U/gprot,  
303 respectively) ( $P < 0.05$ ; Fig. 5C). The SDH activities were 11.2, 11.9, and 12.6  
304 U/mgprot for WSC, DXC, and LPC worms respectively, and a non-significant  
305 difference was found among them ( $P > 0.05$ ; Fig. 5D).

#### 306 4. Discussion

307 With the increasing emergence of environmental pollutants and developments in  
308 chemical risk assessment, more test endpoints are used to conduct toxicological studies  
309 (Madia et al., 2021; Robinson et al., 2008). Various indicators represent different

310 biological processes of the test model organisms, while among which, reproduction is  
311 one of the most important evaluations because it's decisive in population continuation  
312 for all organisms (Mirmonsef et al., 2017). In the present study, several different  
313 endpoints including individual, histological, and biological ones were comprehensively  
314 used to determine the effects of *in situ* metals-contaminated soils on the reproductive  
315 system in wild earthworm *M.californica*. The following discussion detailly organized  
316 and talked about the reproductive toxicity and the relationship with exposure histories,  
317 then the influence of reproductive compensation on earthworm adaptation and  
318 evolution, and finally prospects and disadvantages of the CASA method.

319 In this work, test worms were collected from an increasing gradient of metals-  
320 contaminated soils with decades of pollution histories (Huang et al., 2020). Indubitably,  
321 earthworms under such persistent stresses suffered different adverse effects including  
322 reproductive damages. Analyses here showed the ultrastructural changes in earthworms'  
323 seminal vesicles and significant internal spermatocyte decrease based on histological  
324 observation (Fig. 2). These results were in agreement with the study of Kwak and An.  
325 (2021a) which reported that the endocrine-disrupting chemicals induced significant  
326 damage to seminal vesicle cells and also inhibited normal internal spermatogenesis after  
327 a 7-day exposure.

328 According to previous studies, soil metals bioaccumulated by earthworms via the  
329 epidermal and intestinal pathways can suppress cell proliferation, alter osmotic pressure  
330 and affect the synthesis and secretion of reproductive hormones (Orton and Tyler, 2015;

331 Warner et al., 2020). Additionally, the statistical data on sperm deformity and DNA  
332 damage provided more direct evidence for reproductive toxicity (Fig. 3; Fig. 5A, B; Fig.  
333 S2). With the increase of heavy metal contamination, larger abnormal sperm rates and  
334 DNA damage levels were revealed here. These findings were also formerly proved by  
335 several studies with various chemicals and different earthworm species (Gao et al.,  
336 2015; Huang et al., 2021a; Yesudhasan et al., 2013; Zheng and Li, 2009).

337 Arguably, it may be easier to use some above reproductive parameters, such as  
338 cocoon and offspring recordings to assess the reproductive toxicity impacts, which have  
339 been confirmed and widely applied in toxicological fields (Sivakumar, 2015; Zheng  
340 and Li, 2009). Nevertheless, it is almost impossible to generalize these in field studies  
341 because of the complexity of *in-situ* fields and the difficulty of breeding wild organisms  
342 in the laboratory. It is also challenging sometimes to extrapolate laboratory results of  
343 model organisms to fields and universal species, which may under- or overestimate the  
344 acute toxicity (Jaskulak et al., 2021; Spurgeon et al., 2020). Therefore, it is necessary  
345 to develop individual test metrics rather than those across generations. This was also  
346 one of our main motivations to employ the CASA system to evaluate sperm traits, while  
347 the current study successfully demonstrated the feasibility of this approach in  
348 earthworm reproductive studies.

349 The mobility and velocity of earthworm sperms were affected to different degrees  
350 and showed especially significant inhibition in our “re-exposure” tests, while adverse  
351 effects of the lab Cd compound on the sperm traits also supported the other field

352 findings of sperm deformities and DNA damage. However, the results concurrently  
353 confirmed that earthworms from DXC and LPC sites showed good sperm traits  
354 continuity, with better movement trends and faster swimming traits compared to those  
355 from relatively clean WSC sites under the same re-exposure conditions (Fig. 4; Fig.S1).  
356 Existing studies have pointed out that sperm quality and kinetic performance directly  
357 determine whether they can overcome reproductive barriers and successfully fertilize  
358 within the entire reproductive process (Kime et al., 2001; Yang et al., 2020b). In  
359 addition, available biochemical energy resources also determine sperm traits. Some  
360 involved enzymes, such as LDH and SDH, have been reported as essential metabolism  
361 enzymes that participate in glycolysis and oxidative phosphorylation processes (van der  
362 Horst, 2020; Zhu et al., 2019). The results of the current study also showed that the  
363 LDH and SDH activities of LPC worms were slightly higher than that of WSC and  
364 DXC ones.

365 Earthworms under long-term stress tend to coordinate more resources for sperm  
366 kinetics to compensate for morphological damage, while these changes were probably  
367 highly-depend on their exposure histories. The correlation analysis between the  
368 reproductive changes and the initial exposure history implied some positive  
369 relationships between sperm parameters (including trait values and enzyme activities)  
370 and metal concentration in earthworms (Fig.S3). Increased body burdens of heavy  
371 metals may promote the occurrence of phenotypic plasticity in earthworms, which  
372 regulates their physiological distribution in coping with environmental stresses. In

373 addition, these “adaptive changes” may also be explained by the reproductive  
374 compensation hypothesis proposed by Gowaty et al. (2007), which suggests that parents  
375 attempt to increase reproductive effort for producing competitive offspring in order to  
376 make up for lowered offspring viability. Similarly, Yang et al. (2020a) found that tree  
377 sparrows under persistent exposure evolved to grow longer flagella to obtain greater  
378 mitochondrial load volumes and achieve better reproductive efforts. However, such  
379 compensation is also the result of long-term exposure, while acute toxicity experiments  
380 in the laboratory may induce higher adaptive costs and result in challenges needed to  
381 make related adaptive responses and changes (Huang et al., 2022). The reproductive  
382 compensation of *M.californica* also supports our previous results and further confirmed  
383 its metal tolerance based on population surveys and bioaccumulation analyses (Huang  
384 et al., 2021a; Huang et al., 2020; Wang et al., 2020). Certainly, such compensation may  
385 also attribute to the ability to evolutionary genetic adaptation after decades of exposure.  
386 The study of population genetics and omics mechanisms is further explored by  
387 ourselves now in pursuit of more proof.

388 Previous studies also frequently used other different biomarkers, like oxidative  
389 stress, membrane integrity, and 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium  
390 bromide (MTT) measurements to indirectly assess the sperm quality and reproductive  
391 toxicity induced by chemicals (Cuccaro et al., 2021; De Marchi et al., 2020). Related  
392 studies also establish some molecular indicators, like the earthworm annetocin gene  
393 that shows significant down-regulation after Cd exposure, and in turn influencing the

394 cocoon production processes (Mo et al., 2012). Nevertheless, the CASA system was  
395 gradually applied to gain more direct data for sperm evaluation on various animals,  
396 such as zebrafish, sparrows, and domestic animals, highlighting such possibilities for  
397 earthworm sperm analysis (Hong et al., 2022; van der Horst, 2020; Yang et al., 2020a;  
398 Yang et al., 2019).

399 To the best of our knowledge, the present study is the first to successfully employ  
400 the CASA system for earthworm reproduction analysis, providing direct evidence for  
401 the evaluation of reproductive strategies in earthworms. However, it is important to note  
402 that some limitations still remain. We employed the most appropriate system conditions  
403 (30°C, pH=7, and 10-min dissociation) integrated from several previous references for  
404 the CASA tests (Travis et al., 2004; Yang et al., 2020b; Zhu et al., 2019) because of the  
405 difficulty to raise wild earthworms in a laboratory as well as minimizing the impacts of  
406 environmental changes. Future studies could further explore the best system for model  
407 earthworm sperm tests and different sperm capability liquids for different earthworm  
408 species considering the potential species-specific limitations. To date, the  
409 histopathological examination can only be achieved by image-overlay and step-  
410 zooming comparison as in previous studies. Hence, more likewise artificial-intelligence  
411 technology and tools should be developed for more accurate analysis. Moreover, further  
412 research should aim to study the reproductive impacts of environmental stress on  
413 invertebrates, from the phenotype, physiology, genes and whole perspective in the  
414 context of biodiversity loss.



**415 Conclusion**

416 In summary, various endpoints were determined to explore the reproductive  
417 strategies of wild earthworms *M.californica* under the long-term stress of heavy metals.  
418 Continued exposure indeed induced different reproductive damages to earthworms,  
419 however, earthworms also showed elevated reproductive compensation effects. These  
420 trade-offs of earthworm reproduction are likely the processes of adaptive regulation and  
421 phenotypic plasticity. The current study developed a novel bioassay, which employed  
422 CASA to determine the kinetic traits of earthworm sperms, and further confirmed the  
423 metal tolerance of *M.californica* from reproductive perspectives together with our  
424 previous results, providing the information and data for related toxicological studies,  
425 especially for reproductive evaluations. In addition, the reproductive study also aimed  
426 to comprehensively understand the stress responses and promote biodiversity  
427 conservation in view of increasing genetic erosion, and even biodiversity extinction of  
428 existing organisms.

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434 great support of the CASA system.

435 **Conflict of interest**

436       There are no conflicts to declare.

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Journal Pre-proof

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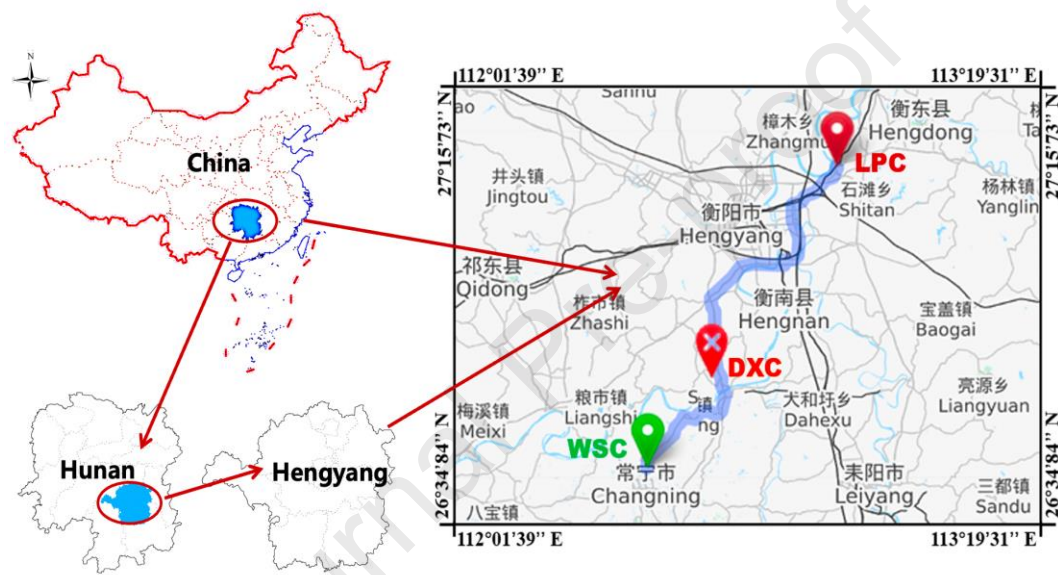
## Tables:

**Table 1.** Internal metal concentrations in earthworms (mg/kg) and bioaccumulation factors (means  $\pm$  SD, n=3)

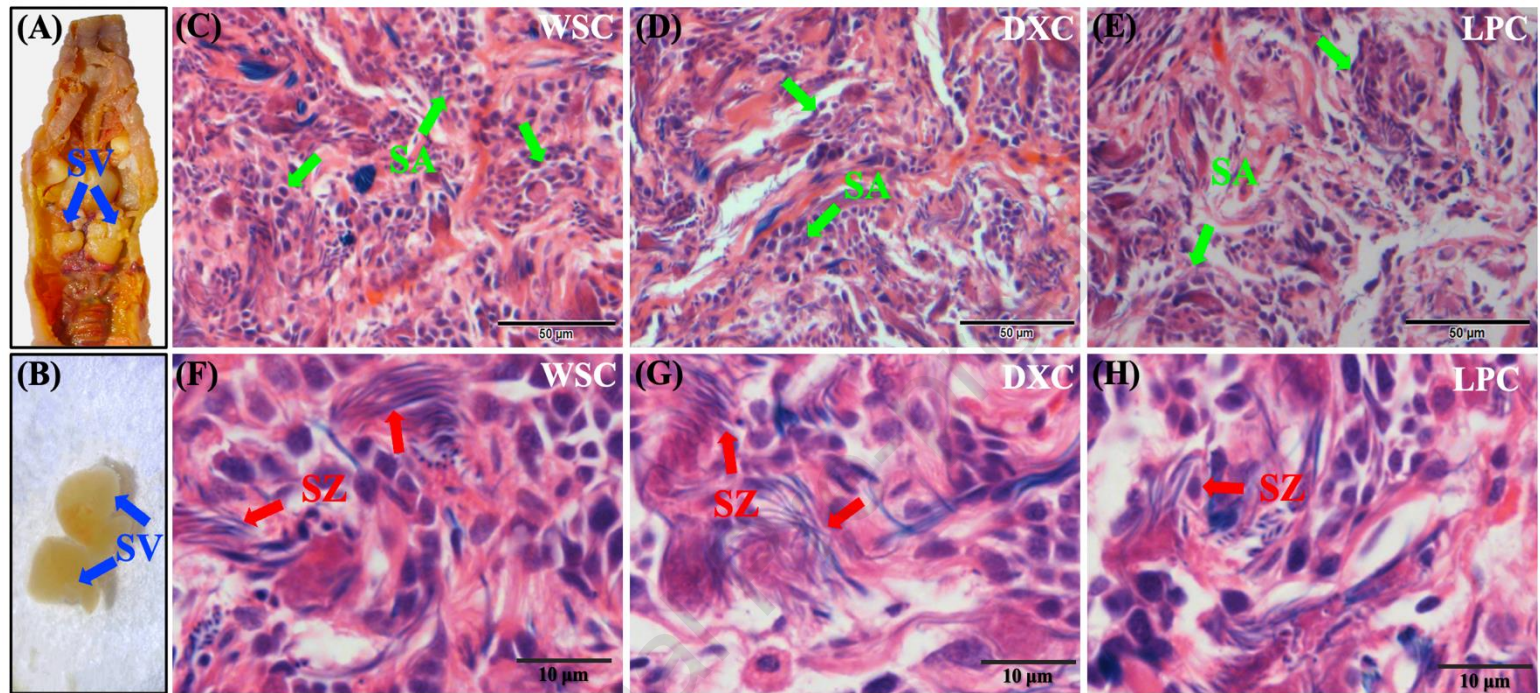
Sites	Internal concentrations				Bioaccumulation factor			
	Cd	Cu	Zn	Pb	Cd	Cu	Zn	Pb
WSC	16.8 $\pm$ 1.12c	26.0 $\pm$ 2.82b	166 $\pm$ 6.48b	34.0 $\pm$ 6.13a	11.2 $\pm$ 0.75a	0.57 $\pm$ 0.06b	1.13 $\pm$ 0.04a	0.32 $\pm$ 0.06a
DXC	42.8 $\pm$ 8.08b	43.0 $\pm$ 6.73a	156 $\pm$ 16.7b	19.9 $\pm$ 3.90b	8.43 $\pm$ 1.59b	0.52 $\pm$ 0.08b	0.88 $\pm$ 0.09b	0.28 $\pm$ 0.06a
LPC	61.1 $\pm$ 8.56a	31.7 $\pm$ 1.82b	239 $\pm$ 10.8a	38.4 $\pm$ 10.3a	4.83 $\pm$ 0.68c	0.85 $\pm$ 0.05a	0.83 $\pm$ 0.04b	0.40 $\pm$ 0.11a

Note: Different letters (a-c) indicate significant differences ( $P < 0.05$ ) among WSC, DXC, and LPC sampling plots.

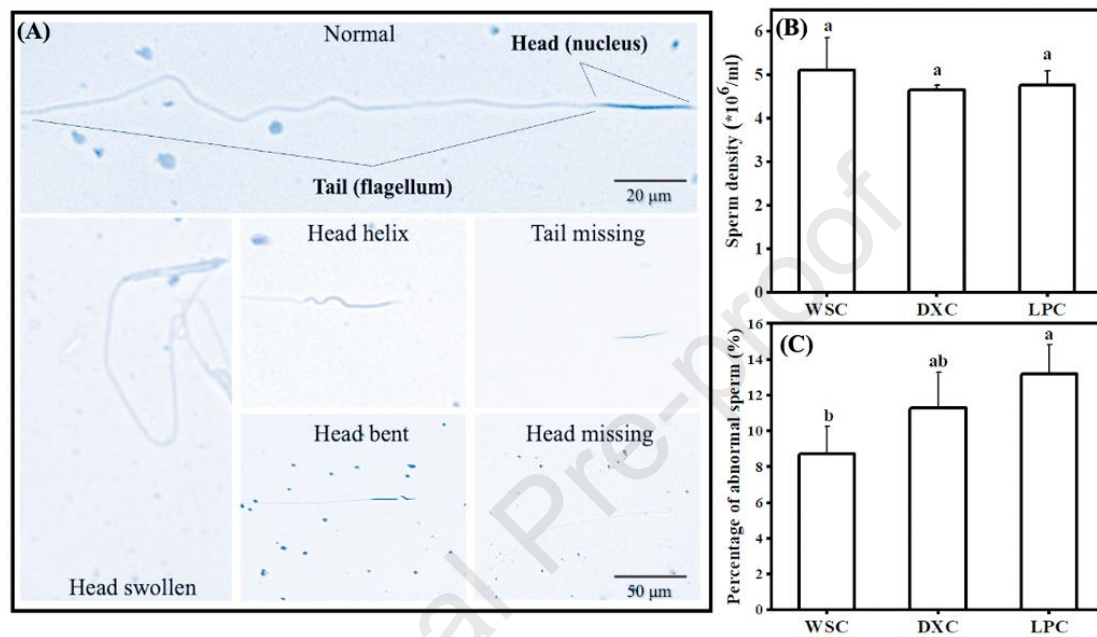
## Figures:



**Fig. 1.** Site location of the earthworm sampling in Hengyang, Hunan province, China. The green to red sampling site labels represents the increased gradient of soil metal contaminations (WSC: Wangshou; DXC: Daxing; LPC: Lupu). The five-spot sampling method was applied for each sampling site (n=5).

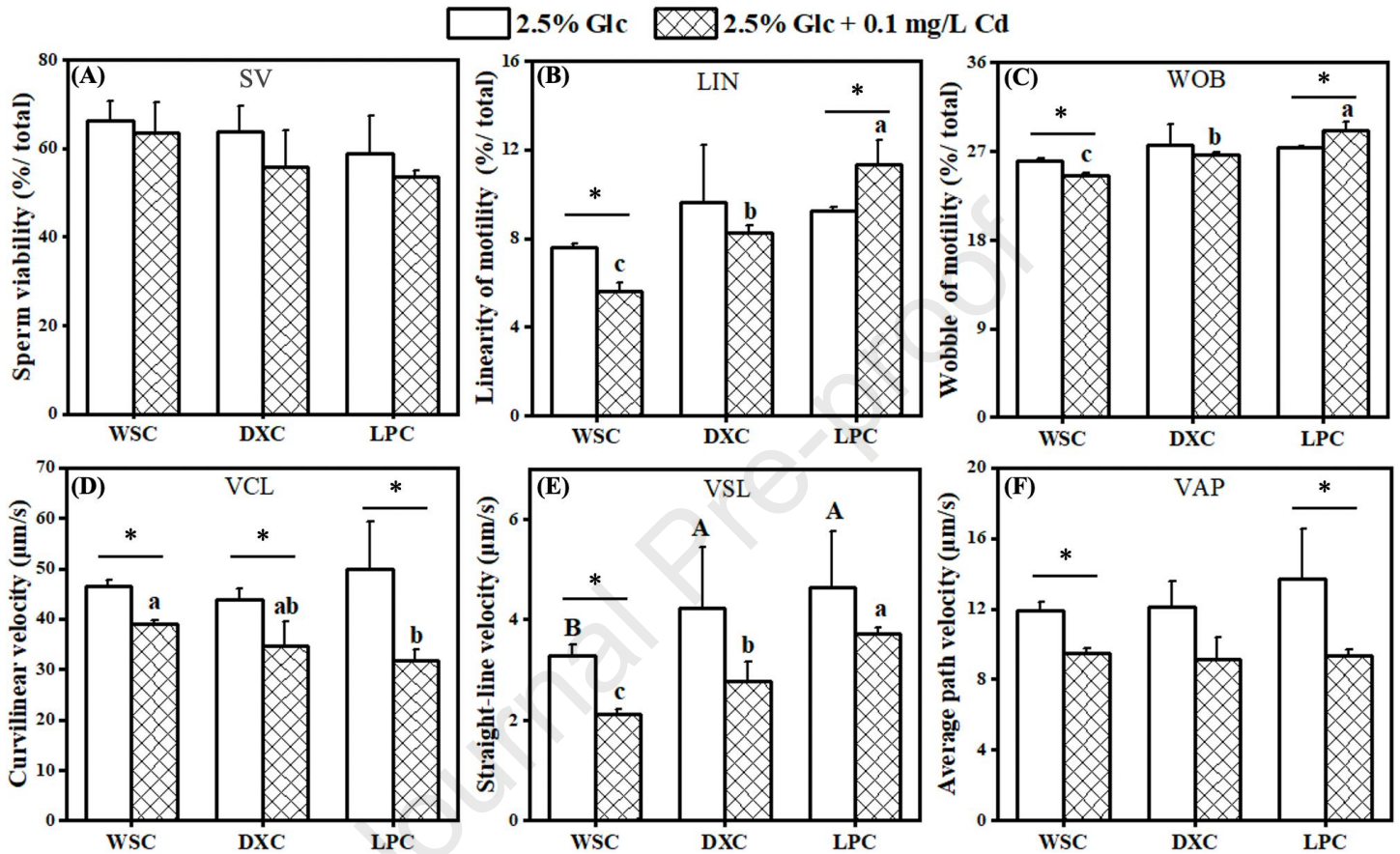


**Fig. 2.** Ultrastructural histology changes in the seminal vesicles of *M. californica*. (A-B) abdominal dissection near clitellum for worms, SV (blue arrows): seminal vesicles; (C-E) spermatocyte morphology in seminal vesicles, SA (green arrows): spermatocyte; (F-H) mature sperms within the spermatogenesis processes in seminal vesicles, SZ (red arrows): spermatozoon. The hematoxylin-eosin staining showed increases in disordered germ cells and tissue cavities and a decrease in mature sperm in the SV of worms from WSC to LPC sites.

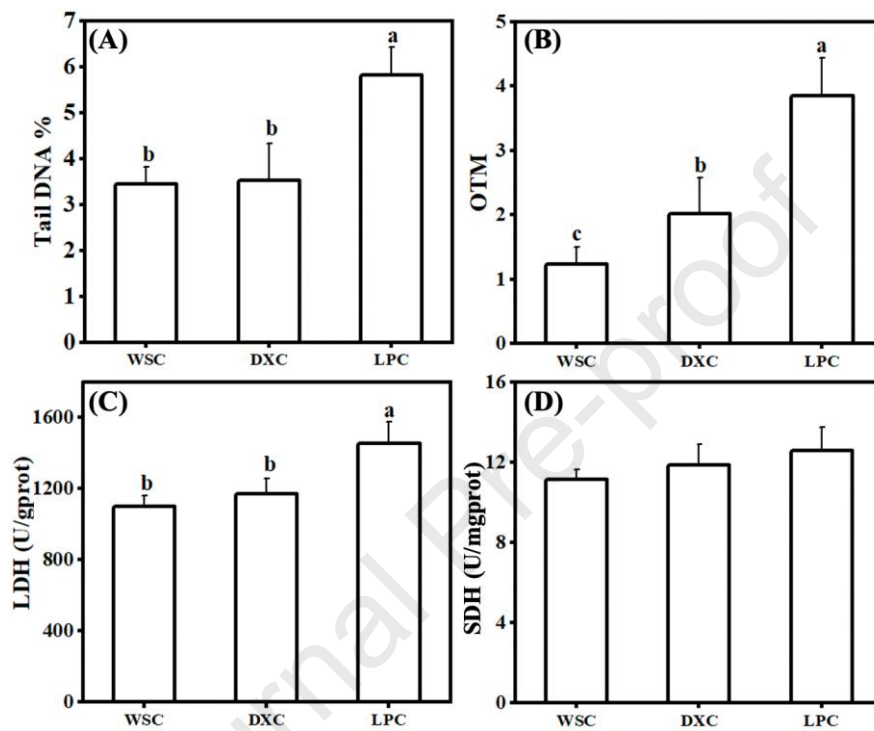


**Fig. 3.** Sperm morphology, density, and the abnormal sperm rates of *M. californica*. (A) the morphology of normal and abnormal earthworm sperms, the abnormal performance including the head swollen, head helix, head bent, head or tail missing; (B) sperm density (determined using a 20-fold dilution); (C) percentage of abnormal sperms (n=100). Bars are the mean  $\pm$  standard deviation (SD), and different letters (a-b) indicate significant differences among the treatments ( $P < 0.05$ ).





**Fig. 4.** Parameters of sperm performance traits under the treatments with pure sperm capacitation liquid (Glucose, Glc) or Cd-spiked ones reported by the computer-assisted sperm analysis (CASA) system ( $n = 300$ ). Bars are the mean  $\pm$  standard deviation (SD), and different letters (A-C) indicate significant differences among treatments with pure capacitation liquid while (a-b) indicate significant differences among treatments with Cd-spiked liquid, \* represents significant differences between treatments with or without Cd spiking ( $P < 0.05$ ), and unmarked ones represent no significance.



**Fig. 5.** DNA damage and sperm-related enzyme activities of *M. californica* seminal vesicles. (A) and (B) are Tail DNA% and OTM values, which represent the levels of sperm DNA damage ( $n = 50$ ); (C) and (D) are the activities of enzyme LDH and SDH, which are both related to the metabolism of sperms ( $n = 4$ ). Bars are the mean  $\pm$  standard deviation (SD), different letters (a-b) indicate significant differences among the treatments ( $P < 0.05$ ), and unmarked one represents no significance.



## Highlights

- (1) Earthworms with different exposure history were collected for reproduction tests.
- (2) The CASA system was employed for assessing the earthworm sperm kinetic traits.
- (3) Histological changes, sperm deformities, and DNA damage were observed.
- (4) Sperm velocity and metabolism were increased for reproductive compensation.

Journal Pre-proof

**Declaration of interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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