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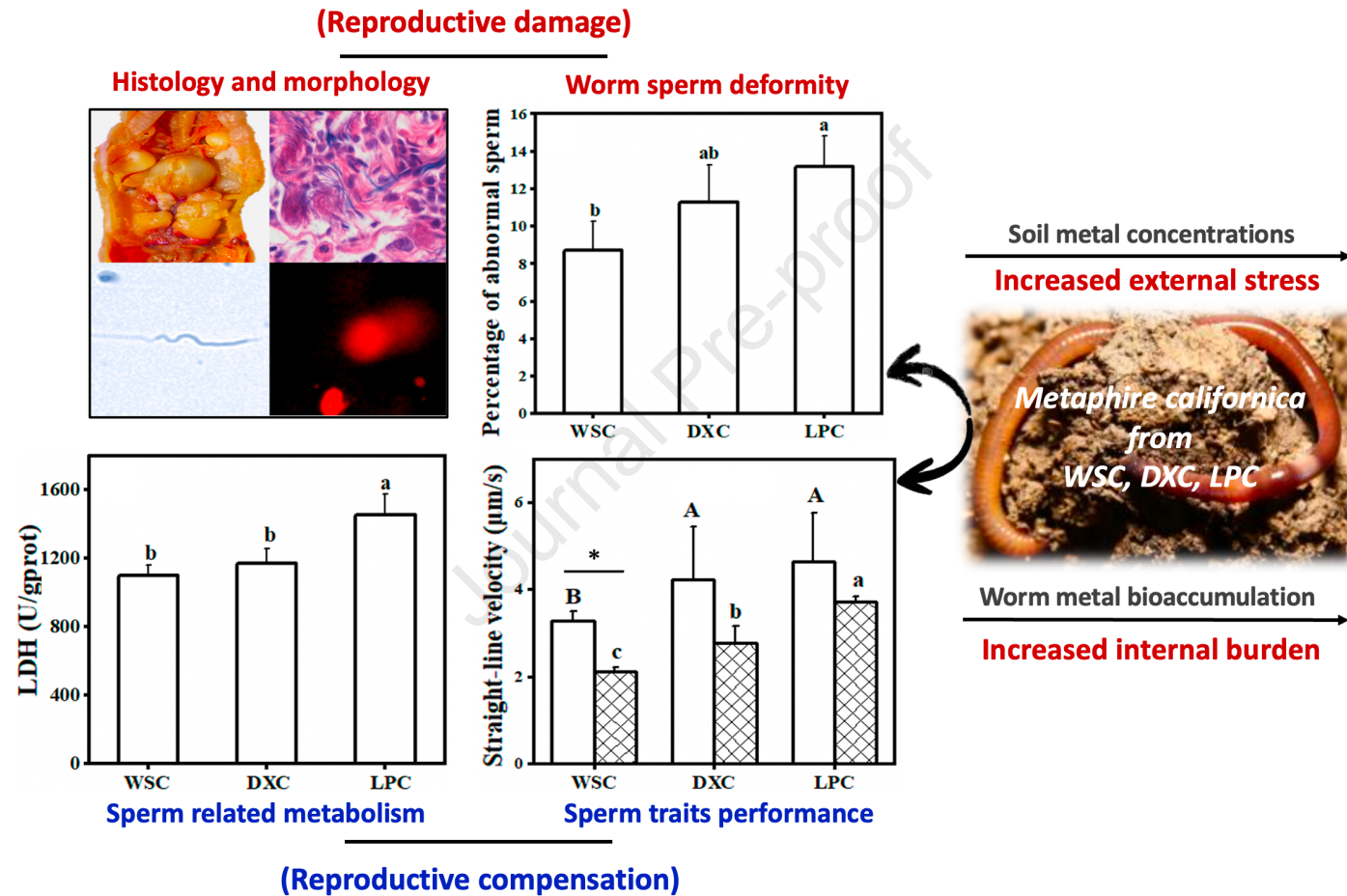
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Author contribution statement

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.

Graphical abstract:



1 **Reproductive damage and compensation of wild earthworm *Metaphire***
2 ***californica* from contaminated fields with long-term heavy metal exposure**

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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.

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

Keywords: Earthworms; seminal vesicles; morphology; sperm traits; reproductive metabolism

1. Introduction

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

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

Furthermore, earthworms are typical sentinel invertebrates and have been widely recommended as model test species in OECD No. 207 and ISO guidelines for risk assessments of various soil pollutants (ISO, 2012; Morgan et al., 2007; OECD, 1984). At present, various individual indexes and biomarkers have been applied to assess the fitness and response of earthworms under hazardous exposure. These include LC₅₀ (50% lethal concentration) and EC₅₀ (50% effective concentration) values for survival rate and individual growth, antioxidant enzyme activities and gene expression for biological processes (Spurgeon et al., 2020; Spurgeon and Hopkin, 1995). Unlike other organisms, earthworms are hermaphrodites, simultaneously keeping male and female reproductive organs and systems, which provides an excellent model to evaluate reproductive toxicity (Domínguez and Velando, 2013; Kwak and An, 2021a). However, up to now related indicators for reproductive studies on earthworms and other invertebrates are very limited (Yesudhasan et al., 2013). For instance, Yang et al. (2022) reported that the earthworm cocoon numbers decrease by 16.7% after exposure to 100 mg/kg carbon nanotubes, whilst other studies also used conventional recordings like juveniles, and hatching rates for reproduction assessments (Huang et al., 2021a).

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

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

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

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

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

2. Materials and methods

2.1 Description of sampling sites

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

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

2.2 Test earthworms

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

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

2.3 The analysis of soil properties and heavy metals

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

Freeze-dried worms were ground into powder and digested in a microwave with a mixture of $\text{HNO}_3\text{-H}_2\text{O}_2$, and metal concentration were analyzed through inductively coupled plasma-mass spectrometry (ICP-MS, 7700ce, USA). Shrimp powder (GBW 10050) was used as standard reference material and all of the recovery rates were over 95%. Besides, the bioaccumulation factor (BAF) value was calculated by dividing worm metal concentration into soil metal contents, which represents the ability of earthworms to bioaccumulate different heavy metals (Huang et al., 2022).

2.4 Histology and morphology examination

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

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

400× magnification (Gao et al., 2015; Yesudhasan et al., 2013; Zheng and Li, 2009).

All earthworms from each site were tested as above and photo-recorded. The sperm deformity rate was calculated by dividing the number of abnormal sperm into total numbers, and over 100 spermatozoa were randomly selected to examine the abnormal rates of each treatment.

2.5 Assessment of earthworm sperm trait performance

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

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

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

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

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

2.6 DNA damage and metabolism evaluation

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

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

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

2.7 Statistical analysis

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

3. Results

3.1 Heavy metal bioaccumulation in *M.californica*

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

3.2 Histological and morphological changes

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

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

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

3.3 Earthworm sperm motility traits

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

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

3.4 Sperm DNA damage and enzyme activities

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

Conclusion

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

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435 **Conflict of interest**

436 There are no conflicts to declare.

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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.

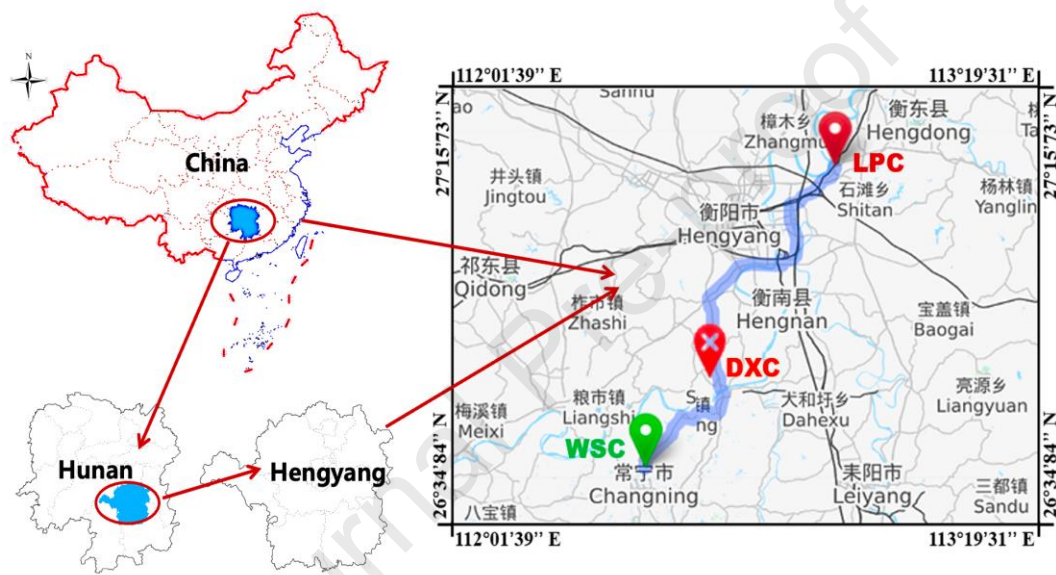
Figures:

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).

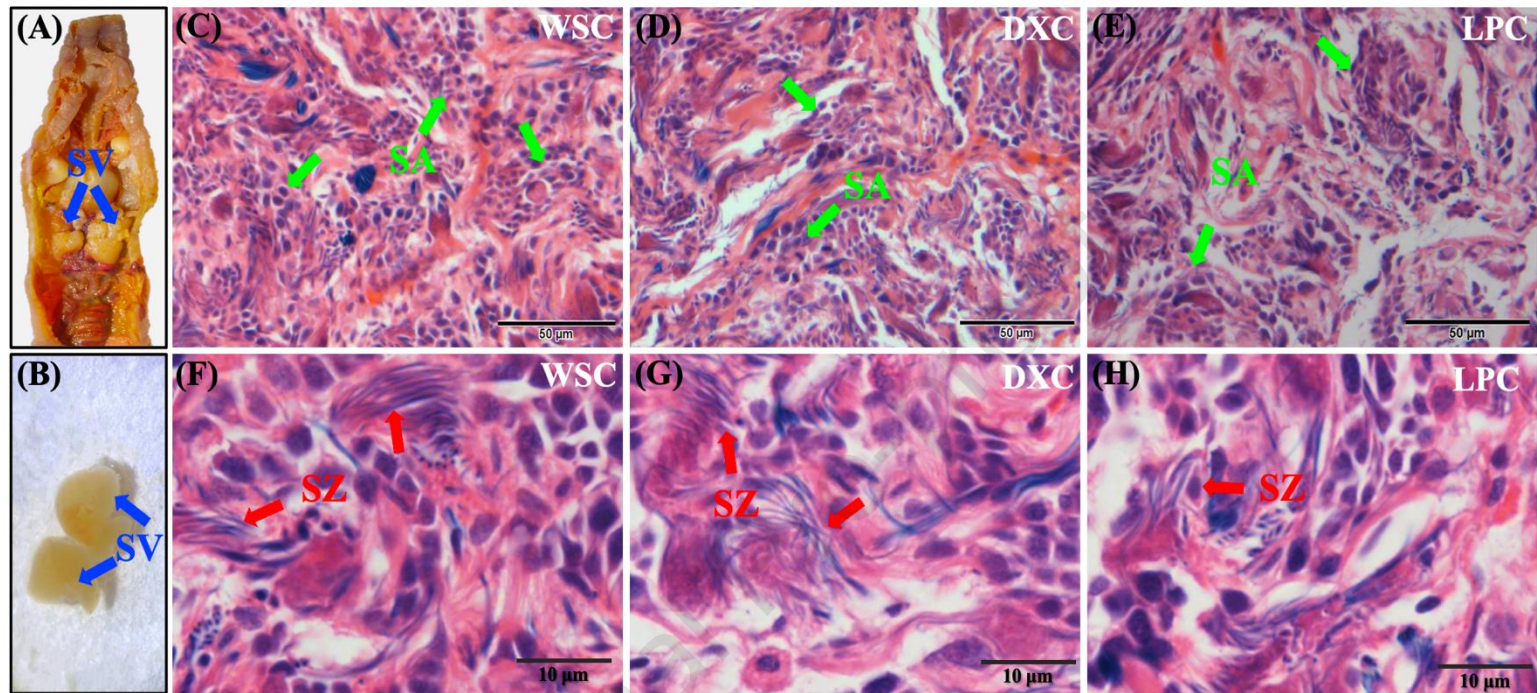


Fig. 2. Ultrastructural histology changes in the seminal vesicles of *M. californica*. (A-B) abdominal dissection near clitellum for worms, SV (blue arrows): seminal vesicles; (C-E) spermatocyte morphology in seminal vesicles, SA (green arrows): spermatocyte; (F-H) mature sperms within the spermatogenesis 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.

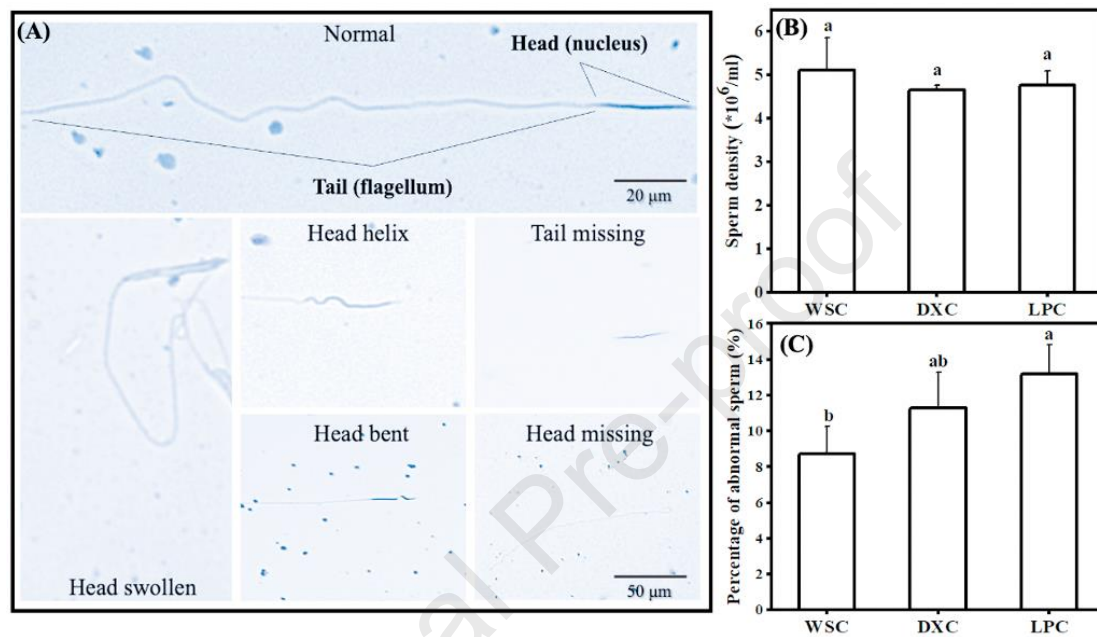


Fig. 3. Sperm morphology, density, and the abnormal sperm rates of *M. californica*. (A) the morphology of normal and abnormal earthworm sperms, the abnormal performance including the head swollen, head helix, head bent, head or tail missing; (B) sperm density (determined using a 20-fold dilution); (C) 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 ($P < 0.05$).

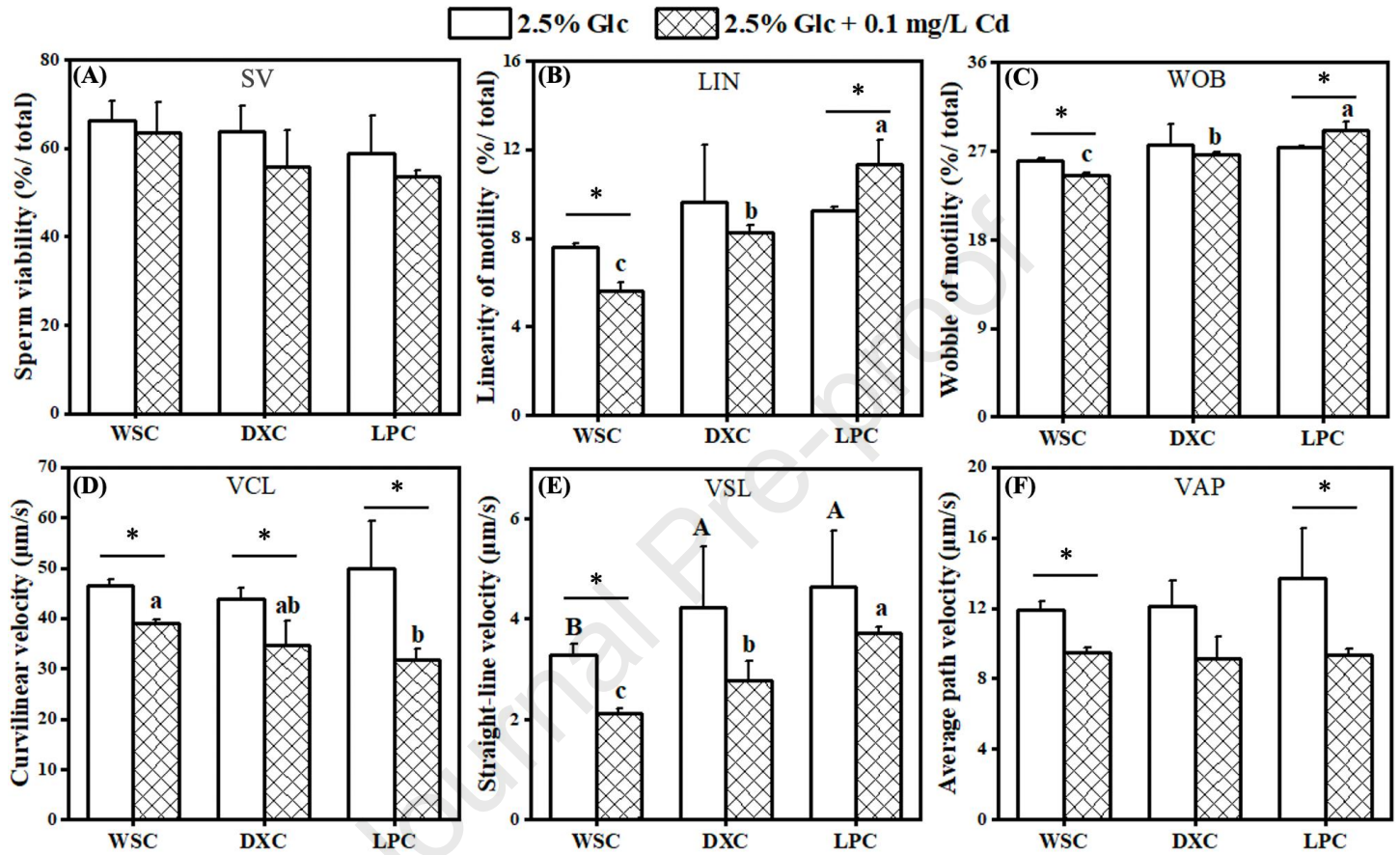


Fig. 4. Parameters of sperm performance traits under the treatments with pure sperm capacitation liquid (Glucose, Glc) or Cd-spiked ones reported by the computer-assisted sperm analysis (CASA) system ($n = 300$). Bars are the mean \pm standard deviation (SD), and different letters (A-C) indicate significant 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.

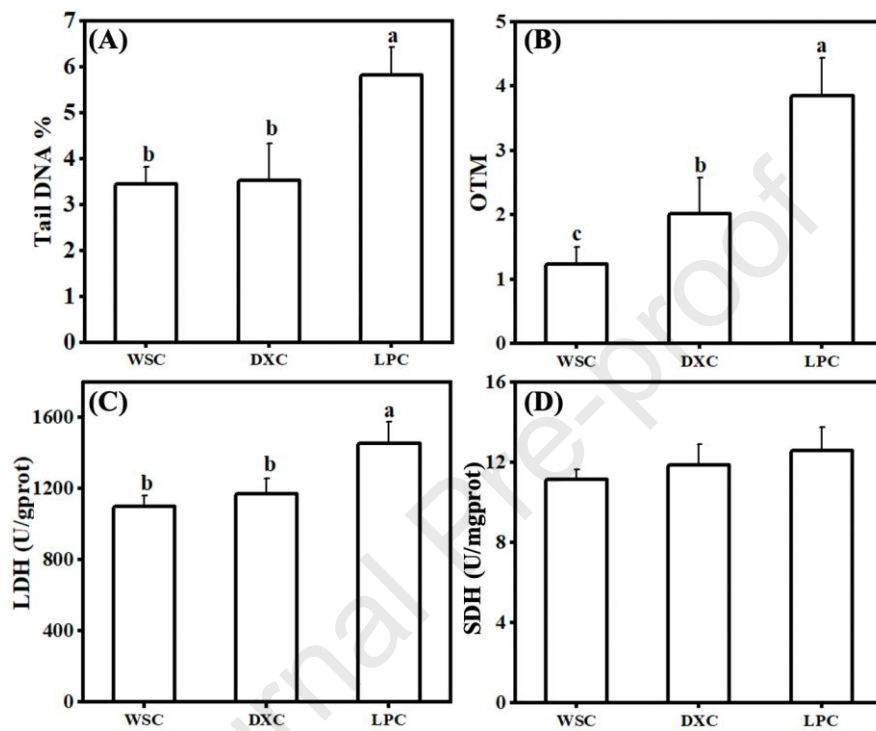


Fig. 5. DNA damage and sperm-related enzyme activities of *M.californica* seminal vesicles. (A) and (B) are Tail DNA% and OTM values, which represent the levels of sperm DNA damage (n = 50); (C) and (D) are the activities of enzyme LDH and SDH, which are both related to the metabolism of sperms (n = 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.

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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