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Temperature Modulated Genotoxicity in Coelomocytes of Ecologically Different Earthworm Species Exposed to Sub-lethal Doses of Zinc and Hexavalent Chromium

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Abstract

Ecotoxicology is concerned with the possible long-term impacts of environmental stresses that alter organisms at the genetic level. Earthworm coelomocytes are sensitive environmental stress markers often employed in genotoxicity assessments. In this study, DNA damage was studied in coelomocytes of two different earthworm species, *Eudrilus eugeniae, and Lampito mauritii,* upon exposure to Zn^{2+} and Cr^{6+} spiked soils at variable temperatures viz., 18℃ (Cold), 24℃ (Control), 28℃ (Warm) to measure modulatory actions of temperature stress. In *Eudrilus eugeniae*, Zn^{2+} co-exposures caused a significant augmentation in percentage tail DNA at 28℃ (+106.61 %), indicating severe damage to DNA, while *Lampito mauritii* shown to be sensitive to Cr^{6+} exposures at all temperatures and exhibited exacerbation at 28℃ (+277.09 %). Likewise, Cr^{6+} co-exposures showed consistent percentage tail damage in *Lampito* species at all exposed temperatures, while *Eudrilus eugeniae* exhibited modulation of co-exposure stress. Further, the results of the study infer a clear species-specific response to combined toxicity. Hence, comet assay representing genotoxicity in coelomocytes is the warning signal of temperature and metal stress in earthworms. Therefore,

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temperature should be considered as an important factor in toxicity testing, as it indicates variations in local conditions that could affect the efficacy of heavy metal toxicity in earthworms of different ecological habitats.

Keywords: Temperature, heavy metals, genotoxicity, DNA strand breaks, exacerbation in toxicity

Introduction

The atmosphere and the terrestrial environment are heavily polluted with metals from human sources. Zinc (Zn^{2+}) and Chromium (Cr^{6+}) are essential trace elements for organisms but become toxic beyond certain concentrations and exposure time. Earthworms are well-known biomarkers of soil toxicity and are subject to variations in optimal temperatures during diurnal and seasonal fluctuations. The influence of temperature on metal toxicity in earthworms has not been studied in laboratory tests, though it is standardized in lethal and sub-lethal toxicity tests. The responses of earthworms to these changes are difficult to predict as they reside inside soil layers in given prevailing environmental conditions [1]. Temperature toxicity is explained for various pollutants and toxins, where endpoints of toxicity include mortality, physiological performance, growth rate, and enzyme activity. The study conducted by Demon and Eijsackers revealed that temperature had a mediating effect on pollutant toxicity for soil invertebrates [2]. Specifically, the isopod *Philoscia muscorum* exposed to high temperatures showed increased toxicity of azinphos-methyl and lindane, which was attributed to an increased metabolic rate that resulted in more uptake at higher temperatures.

Based on its sensitivity to sublethal doses of Cr (8 ppm) and Zn (350 ppm), *Eudrilus eugeniae* was used in our previous studies to examine changes in oxidative stress indices and reproductive parameters in the testis. The results suggested that excessive production of free radicals and insufficient antioxidant defenses had caused sperm morphological alterations, which in turn had decreased the reproductive rate [3]. Upon exposure to soils containing Cr, a notable decrease in both hatchability rate and reproductive function was observed. On the other hand, Zn-spiked soils showed higher hatchability [4]. An alternate approach adopted to measure adverse actions of metal

pollution involves genotoxic assessment wherein single and double-strand breaks are studied [5, 6]. Indeed, DNA is known to encode information required for cellular functions, and its damage paves the way for mutations, strand breaks as well as alterations in base pairs that can affect the species individually and ecosystem stability where interactive effects of temperature may have a potential role [7, 8]. Hence, this study has a high rationale for toxicity assessment.

Earthworm coelomocytes are immune cells involved in internal defense mechanisms, and any damage to these cells results in an impairment in organisms' health [9]. Coelomic fluid plays an interesting role from a toxicological perspective, as it is responsible for the disposition of pollutants and tissue distribution to the whole organism [10,11]. Hence, coelomocytes are widely used as a novel nondestructive pollution biomarker. The preponderance of studies have demonstrated DNA damage in earthworm coelomocytes upon exposure to different toxic chemicals such as pesticides [12], imidacloprid [13], heavy metals [14], polycyclic aromatic hydrocarbons, organic compounds [15], and radiation [16] using single or multiple species to reveal species specificity. The comet assay is performed significantly in different invertebrates, such as springtails [17] and earthworms [18, 19], to explore heavy metal toxicity. Nickel is found to cause single-strand breaks in DNA at very low concentrations in coelomocytes of *E. foetida* [19]. Manerikar *et al.,* reported genotoxicity in coelomocytes of earthworm *Dichogaster curgensis* upon exposure to hexavalent chromium (Cr^{6+}) both in vitro and vivo [20]. Ramdass *et al.,* studied the impact of weathered petroleum hydrocarbon-contaminated soils on earthworms, and their observations revealed two to six-fold higher DNA damage [21]. However, reports on comparative toxicity employing Zn^{2+} and Cr^{6+} with earthworm species of different habitat temperatures are obscure. Thereby, it was hypothesized that the inclusion of temperature in toxicity tests representing the seasonal minima and maxima could exacerbate actions of temperature stress on heavy metal efficacy and expression of sensitivity in earthworms of different ecological habitats.

Material and Methods

Earthworms

Selection of Metal Concentrations and LC_{50}

Zinc (Zn^{2+}) and Chromium (Cr^{6+}) are found in many soils and are essential to the proper operation of biological systems. Numerous physicochemical properties influence the mobility, distribution, and bioavailability of these metals in soil. Regarding soil toxicity and health, the dynamics of these trace elements in soils are crucial [21]. Studies conducted by Schalscha *et al*., showed that wastewater-treated soils in Santiago, Chile contained 228 ppm of Zn^{2+} [22]. In contrast, the concentration of Zn^{2+} in agricultural soils irrigated with sewage was between 2 to 15 ppm. On performing the probit analysis to establish LC_{50} concentrations, 50% lethality in Zn-spiked soils was noted at 1067.64 ppm (R^2=0.8639) and 1159.9 ppm (R^2=0.839) for *E. eugeniae* and *L.* mauritii, respectively, when the 14-day LC_{50} was evaluated following OECD [23] recommendations (Fig 1).

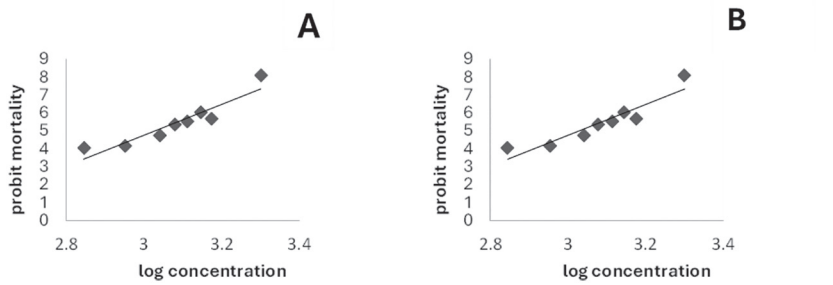

Fig 1: 14-day Probit mortality graphs of *E. eugeniae* (A) and *L.mauritii* (B) exposed to various concentrations of Zn.

Comparably, only two of the chromium's oxidation states—Cr^{3+}, which is non-toxic, and Cr^{6+}, which is toxic [24]. Most Cr^{3+} molecules are not absorbed by cells, and extracellular Cr^{6+} reduction in the gastrointestinal tract—tends to lessen Cr^{6+}'s biological activity. The hexavalent form of chromium ($K_2Cr_2O_7$) used in this investigation is Cr^{6+}, the chromate anion, which, in contrast to Cr^{3+}, uses a general anion transport channel to enter tissue cells [25]. Different amounts of chromium can be found in the atmosphere [5.0 x 10-6 - 1.2 x 10-3μgm^{-3}], seawater (0.1-117 ppb), and soil (10-50 ppm). Between 2000 and 32,000 tons of elemental Cr are released into the environment

each year by the tanning industries in India [26]. The reported total Cr concentration in Bengaluru soils was less than the permissible range (0.1–0.3 ppm). As mandated by the Central Pollution Control Board of India, the maximum allowable concentration of Cr in effluents is 2 ppm. According to Finney's probit mortality [27], the 14-day LC_{50} values of Cr^{6+} for *E. eugeniae* and *L. mauritii* were found to be 22.35 ppm (R^2=0.8393) and 16.57 ppm (R^2=0.9601), respectively (Fig 2). In our study, a sub-lethal dose of 350 ppm Zn and 8 ppm-Cr were used for further studies.

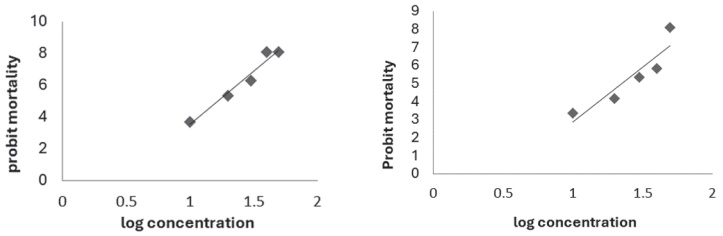

Fig 2: 14-day Probit mortality graphs of *E. eugeniae* (A) and *L.maurutii* (B) exposed to various concentrations of Cr.

Preparation of Experimental Beds

The substrate soils were spiked with specific heavy metals to conduct laboratory-scale tests. Since earthworms tend to accumulate zinc and chromium in their bodies, these elements were chosen for the study. Prior to the experiment, the elemental composition of the experimental beds, which included sun-dried, urine-free cow dung and air-dried soil in a 3:1 ratio (substrate soil), was measured by AAS, with particular attention to Zn (1.29 ppm) and Cr (below detectable levels). The physico-chemical parameters of the substrate soil were also examined (Table 1).

Table 1: The physical and chemical properties of garden soil

pH	Electrical Conduc-tivity	Organic carbon (%)	Available Phosphorus (P2O5) Kg/ ac	Available potassium (K2O5) Kg/ac	Zn (ppm)	Fe (ppm)	Cu (ppm)		Mn (ppm)	Total Cr (ppm)
6.9±0.1	0.07±0.01	0.19± 0.02	20.0±1.2	214±15.8	1.29± 0.3	9.2± 0.8	1.6± 0.2		35.9± 0.5	BDL
	Values are Mean ± SE of 3 values (n=3). BDL-below detectable limits									

Soils were treated individually with solutions of potassium dichromate ($K_2Cr_2O_7$) and zinc chloride ($ZnCl_2$) to attain the necessary concentrations of 350 ppm Zn^{2+} and 8 ppm Cr^{6+}. Thirty experimental boxes with 350 ppm Zn (10 replicates), 8 ppm Cr (10 replicates), and distilled water as control (10 replicates), comprised the entire experimental setup. After adjusting the moisture content in the boxes to 40.0% of the final soil weight, they were covered with fine gauze to keep worms from escaping and a perforated lid to allow for air circulation. The boxes were left to stabilize for a week. Ten gut-cleaned earthworms were put in each box and kept at various soil temperatures (18℃, 24℃, and 28℃) for 14:10 hours. The Agriculture Meteorological Department of India's annual reports on soil temperature changes in Bengaluru soil served as the basis for the temperature selection. Six worms were arbitrarily chosen from ten replicates of each temperature and metal exposure. For further experimentation.

Coelomocytes Extrusion

Earthworm coelomocytes were obtained by a non-invasive extrusion method as reported by Eyambe et al. [28]. The earthworms were rinsed with the extrusion media (pH 7.3), which comprised 5% ethanol, 95% saline, 2.5 mg/ml EDTA, and 10 mg/ml guaiacol glyceryl ether. Following three more washes with Lumbricus balanced solution (LBS), the coelomocytes were extruded in the medium and cold centrifuged at 3000 x g for ten minutes. To ensure the right amount of living cells, the cell count and viability were measured using tryphan blue dye and a hemocytometer. Samples with a cell count of 106 cells/ml and more than 90% viability were used for the comet assay.

Comet Assay

Singh *et al*., used a method to perform the comet assay with slight modifications [29]. After mixing the cell suspension with 100 µl of low melting agarose in PBS at 37°C, the mixture was pipetted onto completely frosted slides that had previously been covered with a coating of 100 µl normal melting agarose. Following ice crystallization, the slides were submerged in a lysis solution containing 100 mM EDTA, 2.5 M NaCl, 10 mM Tris/HCl, and 10% DMSO, and another layer of 85 µl low melting agarose was added. Just before usage, 1% Triton X-100 was added, and the sample was electrophoresed.

Electrophoresis

For DNA unwinding, the slides were incubated in an electrophoretic solution containing 1 mM Na_2EDTA and 300 mM NaOH (pH 13.0) for 20 minutes. Slides were electrophoresed for 15 minutes at 4℃, 25 V, and 300 mA after that. In addition, the slides were maintained at room temperature in the dark, fixed three times in anhydrous ethanol at intervals of five minutes, and neutralized three times in a cold neutralization buffer (0.4 M Tris, pH 7.5). Slides were stained with ethidium bromide for five minutes in dim light. After that, any remaining discoloration was eliminated by submerging the slide in icy distilled water. To assess the state of the comets, roughly 50 cells on each plate were randomly scored using a Zeiss Axioskop-2 Plus microscope.

Statistical Analysis

SPSS software (20.0 version), one-way ANOVA with Duncan's multiple range test (DMRT), and post hoc analysis at $P<0.05$ level of significance were used for the statistical study (Basha and Latha 2016). The mean ± standard error is used to express the results. Values in parenthesis represent percentage changes; increases are denoted by a "+" sign, and decreases are denoted by a "-" sign relative to control. This is how the percentage change was computed:

$$\% \text{ Change} = \frac{\text{Control - Experimental}}{\text{Control}} \times 100$$

Results:

The data (Table 2) illustrate the changes that occurred in the percent (%) tail DNA in two ecologically different earthworm species, *Eudrilus eugeniae* and *Lampito mauritii,* upon co-exposure to heavy metals (Zn^{2+} and Cr^{6+}) and temperature stress (18°C, 24°C, 28°C). The tail length and olive tail moments chosen in the assessment could be used as a parameter of DNA damage.

Table 2: Interactive effects of temperature (18°C, 24°C and 28°C) and heavy metal ($Zn^{2+}$and Cr^{6+}) spiked soil exposure caused changes in % tail DNA in earthworm species '*Eudrilus eugeniae*' and '*Lampito mauritii*' after 30 days of toxic co-exposure.

Species	Groups		
	Control at 24°C	**Temperature stress at 18°C**	**Temperature stress at 28°C**
Eudrilus eugeniae	7.54±0.57 [a]	6.55±0.52 [b] (-13.12)	5.93±0.59 [c] (-21.35)
Lampito mauritii	2.93±0.76 [a]	6.92±2.8 [c] (+136.17)	13.92±2.99 [b] (+375.08)
	Zn toxicity	**Zn toxicity at 18°C**	**Zn toxicity at 28°C**
Eudrilus eugeniae	13.60±1.1 [a]	7.62±0.66 [c] (-43.97)	28.1±1.13 [b] (+106.61)
Lampito mauritii	13.95±2.99[a]	32.81±4.8 [c] (+135.19)	45.63±7.83[b] (+277.09)
	Cr toxicity	**Cr toxicity at 18°C**	**Cr toxicity at 28°C**
Eudrilus eugeniae	26.61±1.0 [a]	26.46±1.01 [a] (-0.56)	27.80±1.04 [a] (+4.47)
Lampito mauritii	13.05±2.5 [a]	34.79±8.9 [c] (+166.59)	36.68±5.01 [b] (+181.07)

Values are mean ± standard error of 50 coelomocytes/ slide. Superscript letters (a, b, c) are statistically significant ($p<0.05$) row-wise as determined by one-way ANOVA, Duncan post hoc (SPSS 20.0). Values in parenthesis indicate percentage change, '+' indicates the extent of DNA damage, and '-' indicates the modulatory effect of temperature and metal toxicity in protecting DNA from damage compared to control.

Eudrilus eugeniae

In *Eudrilus eugeniae*, exposure to temperature stress alone at 18°C was found to cause a suppression in the % tail DNA (-13.12). A similar suppression was evident at 28°C exposures (-21.35 %) when compared to control. Zn^{2+} exposures at 18°C caused a significant suppression in percent tail DNA (-43.97 %), indicating considerable DNA damage (Fig 3). Exposures carried at 28°C caused further increases in percent tail DNA (+106.61%), representing a higher extent of DNA damage. Cr^{6+} exposures at 18°C and 28°C revealed no significant difference when compared to control (Fig 4).

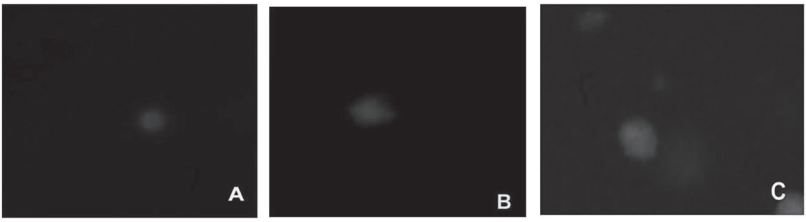

Fig 3: DNA strand breaks (Tail DNA %) in earthworm species '*Eudrilus eugeniae*' upon co-exposure to sub-lethal doses of Zn (350 ppm) and temperature stress (A:18℃, B:24℃, C:28℃).

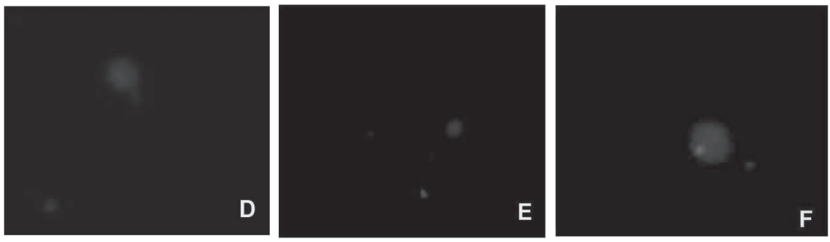

Fig 4: DNA strand breaks (Tail DNA %) in earthworm species '*Eudrilus eugeniae*' upon co-exposure to sub-lethal doses of Cr (8 ppm) and temperature stress (D:18℃, E: 24℃, F:28℃).

Lampito mauritii:

In *Lampito mauritii,* temperature stress at 18℃ caused an augmentation (+136.17 %) in percent tail DNA, while exposures at 28℃ revealed a severe exacerbation (+375.08 %) in percent tail DNA, representing significant DNA damage. While Zn^{2+} exposures at all the studied temperatures showed a severe exacerbation in the percent tail DNA, and the observed percentage change is found to be +135.19 % and +277.09 % at 18℃ and 28℃ respectively, indicating DNA damage at Zn co-exposures (Fig 5). In comparison, Cr^{6+}exposures also revealed a severe exacerbation in percent tail DNA, and the observed % change was found to be +166.59 % at 18℃ and 181.07 % at 28℃ highlighting severe DNA damage in the species *L. mauritii* (Fig 6).

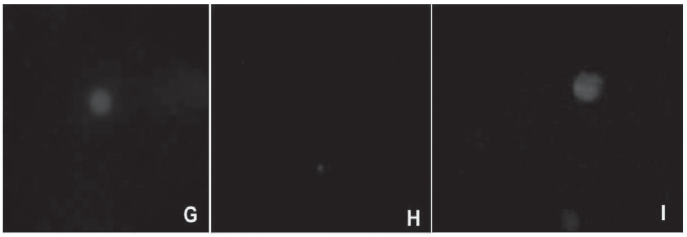

Fig 5: DNA strand breaks (Tail DNA %) in earthworm species '*Lampito mauritii*' upon co-exposure to sub-lethal doses of Zn (350 ppm) and temperature stress (G:18℃, H:24℃, I:28℃).

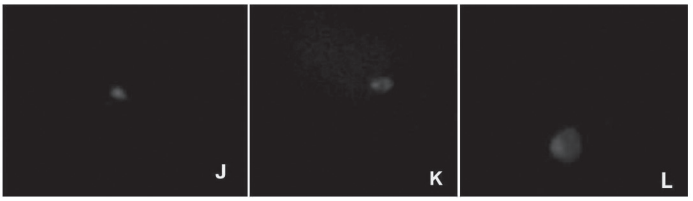

Fig 6: DNA strand breaks (Tail DNA %) in earthworm species '*Lampito mauritii*' upon co-exposure to sub-lethal doses of Cr (8 ppm) and temperature stress (J:18℃, K:24℃, L:28℃).

Discussion

This study demonstrated the potential for genotoxicity when Zn^{2+} and Cr^{6+} were exposed to several earthworm species, using the alkaline unwinding test to measure DNA breaks caused by metal exposure to determine DNA strand breaks [30]. Susceptibility to DNA damage in coelomocytes of ecologically different earthworm species is presumed to be useful in the overall impact of heavy metals; in addition, the main goal of the study was to understand the response of earthworms to temperature stress. The earthworms were acclimatized by changing temperature (1℃/day) for 6 days, then exposed to temperature conditions of 18℃ (cold), 24℃ (control), and 28℃ (warm) over 30 days. There have been reports of several important mechanisms in DNA repair processes that entail the breakdown of brief DNA segments that result in a transient break in a single DNA strand [31]. According to studies, strand breaks can occur as an indirect consequence of repair mechanisms or as a direct result of exposure to xenobiotics [32]. The percentage of tail DNA damage seen in the earthworm control group in this study represents the natural DNA damage or removal of damaged cells by apoptosis.

Similar observations indicating the elimination of damaged cells by apoptotic process in earthworms have been reported [33]. In *E. eugeniae*, the suppressions, as evidenced by Zn2+ exposure at 18°C, could be due to the binding of Zn^{2+} with functional proteins such as Zn-finger proteins [34]. The low deleterious effects of Zn^{2+} at 18°C could also be due to improved DNA repair mechanisms at mild hypothermic conditions in invertebrates [35]. Similar observations were reported for Cadmium toxicity wherein exposures were carried at a lower temperature of 15°C [36]. In contrast, Zn^{2+} exposures carried at 28°C resulted in increased percent tail damage. The observed modulatory effect between temperature and zinc toxicity appears due to physiological interaction with the energy metabolism rather than an increase in zinc buildup at higher temperatures. On the contrary, Cr^{6+} exposures to *E. eugeniae* at 18°C and 28°C didn't show significant differences in the % tail damage compared to Cr^{6+} toxicity at 24°C however, the DNA strand breaks in the earthworms exposed to Cr^{6+} were higher compared with Zn^{2+}.

The ability of Cr^{6+} to permeate cell membranes and produce ROS is the cause of the DNA damage that is seen upon Cr^{6+} exposure in the species *L. mauritii* at all exposed temperatures. Membrane channels designed to transport isoelectric and isostructural anions, like SO^{4-} and H_2PO^{4-}, readily allow Cr^{6+} to enter the cell [37]. It is assumed that chromium's solubility and oxidation state control its toxicity. Cr^{6+} can be reduced to Cr^{5+} by cellular reductants found inside the cells [38]. Molecular oxygen is consumed during the reduction process, resulting in the production of superoxide radicals (•O2), H_2O_2, and hydroxyl (•OH) radicals, which are collectively referred to as reactive oxygen species (ROS) [38]. These radicals can cause base modification, lipid peroxidation, DNA strand breaks, and protein modification, all of which can lead to oxidative stress [39]. The direct oxidizing action of Cr^{6+} and the production of reactive oxygen species (ROS) during the intracellular reduction of Cr^{5+} to Cr^{3+} are the sources of the toxicological effects of Cr^{6+}. The high permeability of Cr^{6+} suggests that it binds to macromolecules, reacts with intermediates, and is hazardous. In addition to oxidative damage, cellular reductants such as reduced glutathione (GSH), NAD(P)H, cysteine, and ascorbate are formed once Cr^{6+} enters the cell. These lesions include Cr–DNA adducts, DNA–protein cross-links, and DNA–DNA cross-links [40].

On the contrary, the observation of reduced DNA damage in *E. eugeniae* with exposure to Cr^{6+} at a higher temperature of 28℃ could potentially be attributed to the creation of distinct forms of DNA cross-links. It is believed that a variety of metals and environmental contaminants might cause genotoxicity by covalently binding with DNA to produce DNA adducts [41]. When an electrophilic, positively charged metabolite, or active chemical species, bonds covalently to negatively charged DNA molecules, adducts are produced. DNA adducts are byproducts of covalently attaching a metal molecule, whole or in part, to chemical moieties inside DNA. Further, they indicate the quantity of a specific metal that has interacted with important macromolecules in the cell, like proteins or DNA. The biologically effective dose of a metal that reaches the DNA in cells can be determined by quantifying DNA adducts. Therefore, according to Ehrenberg et al., they stand for the quantity of metal that has entered the body, undergone metabolic activation, attached itself to cellular DNA, and not been restored [42]. Similar findings in freshwater mussels show that when temperature rises, DNA damage increases. Buschini et al. found *Dreissena polymorpha* after exposure to 4, 18, 28, and 37℃ [43]. Similarly, research conducted by Anitha et al. revealed that goldfish *Carassius auratus* were induced to break their DNA strands, highlighting the role of increased temperature in inducing genotoxicity [44].

The observations on the species show a distinct reaction, with the larger species, *E. eugeniae*, exhibiting less damage to its DNA than the smaller species, *L. mauritii*. This is most likely caused by variations in coelomocyte metabolic rate(s), metal absorption, and retention capabilities. Because different species have varying capacities for binding metal, Spurgeon and Hopkin suggest that species physiology is a more important factor in predicting sensitivity differences in earthworms when exposed to metal-contaminated soils than ecological groupings [45]. In a similar vein, Morgan et al. showed that distinct earthworm species (*D. rubidus* and *L. rubellus*) had varying isomers of metallothioneins that sequester metals, which can, therefore, influence the sensitivity of the species [46]. Consequently, the species sensitivity variations found in this study may be connected to the phylogeny of the species. Martin et al. found that apoptosis

is responsible for the activation of endogenous nucleases that cleave nuclear DNA into oligo-nucleosomal-sized fragments [47].

Conclusion

The findings of this study indicate an increasing trend in percent tail DNA at all exposed temperatures. Though Zn^{2+}exposures exhibited different intensities of percent tail DNA damage, Cr^{6+} exposures exhibited consistent percentage tail damage in both the species at all temperatures. Species-specific responses, as evidenced in *E. eugeniae* and *L. mauritii* to combined stress, emphasise *L. mauritii* as the most vulnerable species to combined toxic stress. It can be inferred from the above observations that DNA alterations have the potential to predict deleterious effects in exposed species. Thereby, comet assay representing genotoxicity in coelomocytes can be considered as a warning signal of sub-lethal doses of metal stress and interactive effects of temperature in earthworms as it closely represents the field conditions in environmental biomonitoring studies.

Ethical Committee Approval: The Institutional Animal Ethics Committee (IAEC), Bangalore University, Bengaluru, India (CPCSEA registration No:402) has approved the protocol of the study.

Author's contribution statement: Latha. V: Experimentation, analysis of data, preparation of draft manuscript, Reviewing and editing of manuscript. **Mahaboob Basha**: Methodology and overall supervision; Reviewing and editing of the manuscript.

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Conflict of Interest: The authors declare that they have no competing interests.

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