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Do (xeno)estrogens pose a risk to earthworms? Soy isoflavones and estradiol impact gonad structure and induce oxidative stress in *Eisenia fetida*

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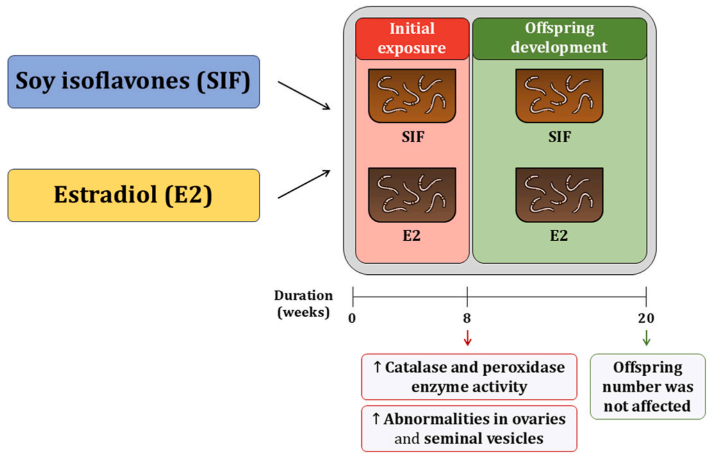
HIGHLIGHTS

- No mortality observed in *E. fetida* after exposure to varying EDC concentrations
- Estradiol and soy isoflavones increased catalase and peroxidase enzyme activities
- Germ cell number was reduced and abnormalities increased in reproductive organs
- Reproductive output was not affected, suggesting possible adaptive mechanisms
- This study highlights the need for future research on EDC effects in soil organisms

GRAPHICAL ABSTRACT



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ABSTRACT

Understanding the impact of endocrine disruptor compounds (EDCs) across a wide range of species is crucial, given their ubiquitous presence. Although invertebrate species lack sex steroid hormone pathways, they exhibit sensitivity to EDCs, which could affect population dynamics. This study assessed reproductive endpoints and oxidative stress parameters in *Eisenia fetida* following exposure to estradiol and soy isoflavones, resembling the concentrations found in livestock manure. The experiment used artificial soil, as recommended by OECD guidelines (7:2:1 sand, kaolin and peat). Adult specimens were randomly divided into seven groups ($n = 11$/replicate): one control, three estradiol (156.1, 283.4 and 633.8 μg/kg of dry soil) and three soy isoflavones (113.0, 215.3 and 405.0 mg/kg of dry soil) concentrations. After eight weeks, samples were collected for cytological, histological and biochemical analysis. Offspring development was assessed after 12 additional weeks. Higher estradiol and isoflavone concentrations led to lower germ cell number and increased abnormalities, especially in the seminal vesicles and ovaries. Catalase and peroxidase activities were significantly increased in all treated groups. The exposure did not significantly affect the number of *E. fetida* offspring. These findings highlight *E. fetida*'s sensitivity to EDCs at a biochemical and tissue level, suggesting its use as a bio-indicator for assessing EDC contamination in soils.

1. Introduction

The increase in population density, industrialisation and the widespread use of pharmaceutical substances have resulted in elevated endocrine-disrupting compounds (EDCs) concentrations in wastewater, surface and drinking waters and soils (Madikizela et al., 2018; Tijani et al., 2013). EDCs encompass a variety of compounds, namely natural and synthetic hormones, phytoestrogens, pesticides, and a variety of industrial chemicals and by-products (Wee and Aris, 2017), disrupting the endocrine system and causing developmental abnormalities and reproductive issues, among others (Basso et al., 2022). Of particular concern is 17β-estradiol (E2), recognised as the principal circulating estrogen in vertebrate females, and isoflavones, plant-derived compounds with estrogenic properties abundant in soy-based products (Jia et al., 2021; Naresh et al., 2019).

Various sources contribute to EDC presence in soil, with livestock manure being a significant supplier (Caron et al., 2012; Grgic et al., 2021; Hama et al., 2021; Yost et al., 2014). Livestock manure, commonly used in agriculture to enhance soil fertility and quality, contains inherent steroidal hormones, notably E2, from animal excretions (Bartelt-Hunt et al., 2013). Manure application introduces variable estradiol levels into the soil matrix (Gudda et al., 2022), as observed for swine manure in France (4–139 μg/kg) (Combalbert and Hernandez-Raquet, 2010) and dairy manure in the United States of America (1416 μg/kg) (Zheng et al., 2008). Livestock fed with plant-based diets excrete isoflavones (Grgic et al., 2021), classified as phytoestrogens and resemble E2 structurally (Patisaul, 2017). Dairy cattle commonly consume feeds rich in phytoestrogens (Hashem and Soltan, 2016), containing significant isoflavone concentrations, such as daidzein (105–560 mg/kg) and formononetin (2500–3000 mg/kg) (Adler et al., 2015). Interestingly, although some isoflavones are hydrolyzed into aglycones in the rumen (Tucker et al., 2010), dairy cattle may exhibit a cumulative faecal isoflavone content of 378 mg/kg dry weight (Njåstad et al., 2014), which can then be applied to soil as an organic fertilizer. However, environmental concentrations of these compounds in soils remain largely unexplored and can only be inferred from levels found in livestock manure used as fertilizer.

Alongside livestock manure, EDCs enter the soil from various other sources. These include pesticides and herbicides used in agriculture (Pironti et al., 2021) and urban landscaping (Müller et al., 2020). Furthermore, airborne EDCs transport occurs from fossil fuel combustion and industrial processes (Metcalfe et al., 2022). Direct effluent discharge from urban, industrial, or agricultural activity further contaminates the soil (Liu et al., 2010). Wastewater treatment plants, ill-equipped to remove micropollutants, produce biosolids (sewage sludges) rich in EDCs, often repurposed as agricultural fertilisers (Liu et al., 2013b; Muscolo et al., 2021). The surging popularity of soy-based products paves the way for phytoestrogen (such as isoflavones) buildup in soil as an industrial by-product (Liu et al., 2013a, 2013b). Additionally, EDCs are present in pharmaceuticals and personal care products, often disposed of improperly (Benotti et al., 2009). Urban runoff transports EDCs from roads, parking lots, and rooftops into soil and water bodies (Kwak et al., 2017).

These EDC sources are pivotal in introducing these compounds to soil and affecting soil biota. Earthworms, comprising a significant portion of the animal biomass in soil (60–80 %) (Sun et al., 2019), significantly enhance soil structure and fertility through their burrowing, feeding, and casting activities (Liu et al., 2017a; Wang et al., 2016). They have been used as bioindicators of soil pollution, namely pesticides, insecticides, and heavy metals, due to their array of behavioural, physiological, and biochemical changes (He et al., 2021; Wang et al., 2022; Yang et al., 2018). Many earthworm species, especially *Eisenia fetida*, are used as model organisms in ecotoxicological studies (Miglani and Bisht, 2019), including international guidelines the effects of chemicals in soil (OECD 1984, 2016; International Standard (ISO) 2008, 2012, 2014, 2023), due to their ecological relevance and fast life cycle; in *E. fetida*, cocoons hatch in three to four weeks and the worms reproduce at a high rate (Teferedegn and Ayele, 2024). These features make earthworms important for life-cycle studies to capture developmental and reproductive endpoints (Scott-Fordsmand et al., 2022). Although sex steroid hormone pathways governing the interaction between EDCs and earthworms are not fully revealed, these species are not free from their adverse impacts (Azevedo et al., 2024; Crane et al., 2022; Heger et al., 2015; Novo et al., 2018; Oliveira et al., 2021; Qian et al., 2023; Yao et al., 2024). Investigating the effects of these compounds on *E. fetida* is of great importance as it provides valuable insights into the potential risks of EDC exposure to soil non-target organisms. Furthermore, it sheds light on the broader ecological implications, considering the dependence of diverse ecosystems on these species.

In this sense, the present study aimed to evaluate the effects of soy isoflavones (SIF) and E2 at concentrations similar to those found in livestock manure, using *E. fetida* as a model organism to investigate the impact of these compounds on reproductive endpoints and oxidative stress parameters.

2. Material and methods

2.1. Earthworms

Earthworms (*E. fetida*) were purchased from EcoGrowing (Quarteira, Portugal). Adult earthworms with developed clitellum and a body weight of 0.40 ± 0.02 g were selected, allowed to acclimatize in the artificial soil for one week, and given 24-h to purge their intestinal contents on wet filter paper. Animals were kept in controlled conditions of temperature (±25 °C), soil humidity (±50 % that corresponded to 40 % of the maximum water holding capacity of the artificial soil) and photoperiod (16h light: 8h dark, 400–800 lux) as recommended by the norms of Organization for Economic Co-operation and Development (OCDE) (OECD, 2016). *E. fetida* was chosen for this study due to its sensitivity to both cutaneous and ingestion exposures, making it a reliable model species for assessing ecotoxicological effects on soil invertebrates as per international testing standards (OECD 1984, 2016; International Standard (ISO) 2008, 2012, 2014, 2023).

2.2. Artificial soil

This experimental work used artificial soil prepared according to the OECD guidelines (2016), consisting of 70 % sand, 20 % kaolin clay and 10 % peat. Soil pH (0.1 M KCl, 1:2.5 ratio) was adjusted with calcium carbonate to 6.0 ± 0.5. A description of selected soil properties is presented in Table 1. Every week, soil moisture was corrected on a weight basis by adding distilled water.

The soil mixture was distributed by polypropylene boxes (20 cm length x 15 cm width x 10 cm height) with perforated lids to allow for adequate aeration ($n = 3$/exposure condition). The soil for each treatment was prepared by adding the compounds with the respective concentrations per dry weight. The cultured earthworms were fed weekly with 2 g of dry sterile crushed oats, and twice a week, the boxes were aerated by opening the lid for a few minutes.

2.3. Experimental design

Three increasing concentrations (low – L, medium – M, and high – H) were used for both E2 and SIF. Promensil® (Promensil, Novogen Ltd., Australia) tablets containing 40 mg of a mixture of isoflavones [genistein (3.7 %), daidzein (2.0 %), formononetin (34.2 %) and biochanin A (56.7 %)] (Setchell et al., 2001) were ground up to provide the nominal concentrations of 134.8 (SIF-L), 269.6 (SIF-M) and 471.7 (SIF–H) mg/kg of dry soil and then applied to the soil. An ethanol stock solution of E2 (Sigma-Aldrich) was diluted in distilled water to the nominal concentrations of 174.4 (E2-L), 348.9 (E2-M) and 697.7 (E2-H) μg/kg of dry soil. A control group was prepared consisting of uncontaminated artificial soil. The experimental design of this study is presented in Fig. 1.

The test started after the preparation of the experimental units with the respective concentrations and compounds. Each unit contained 700 g of moist artificial soil, equivalent to an approximate height of 5 cm, and eleven adult earthworms were weighted and randomly allocated to them. Three replicates were performed for each experimental group, resulting in 21 units. The earthworm reproduction test followed the OECD guideline 222 (OECD, 2016), with the exception of the exposure period. Soil samples were collected at the beginning of the experiment to quantify the concentrations of the compounds and obtain the real concentrations present in the soil.

The exposure to the compounds spanned over 8 weeks. At the end of the assay, animals were weighed, anaesthetized by freezing in ice and then sacrificed. Samples were collected for cytological ($n = 6$/treatment) and histological ($n = 3$/treatment) analysis. Whole earthworms ($n = 6$/treatment) were used for biochemical assays and frozen at −80 °C for further analysis. After the initial 8-week exposure period, the number of unhatched cocoons was also counted. The cocoons were isolated by carefully going through the experimental boxes and selecting them manually. This process was carried out by two researchers to ensure accuracy and consistency. The cocoons were then returned to the experimental box for further development during 12 weeks.

The ponderal growth rate of adult earthworms was calculated using the following formula (Marini et al., 2024):

$$\text{Ponderal growth rate } (\%) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

An additional 12-week period was conducted to evaluate the impact of these compounds on the population growth of *E. fetida*. The individuals in each experimental unit were tallied and categorized into different developmental classes (juveniles, sub-adults, and adults) based on size and the development of the clitellum.

Table 1
Selected properties of the artificial soil used as habitat for *E. fetida*.

Artificial soil properties	
pH (H_2O)	6.4
pH (KCl)	5.8
Organic Matter (g kg^{-1})	64.2
Exchangeable cations ($cmol_{(+)}$/kg)	
Ca^{2+}	9.98
Mg^{2+}	0.77
K^+	0.19
Na^+	0.43
$Al^{3+} + H^+$	0.00
Effective Cation Exchange Capacity	11.37
Particle Size Distribution (g/kg)	
Coarse Sand (0.2–2.0 mm)	737
Fine Sand (0.02–0.20 mm)	36
Silt (0.002–0.020 mm)	74
Clay (<0.002 mm)	153
Texture Classification	Sandy loam

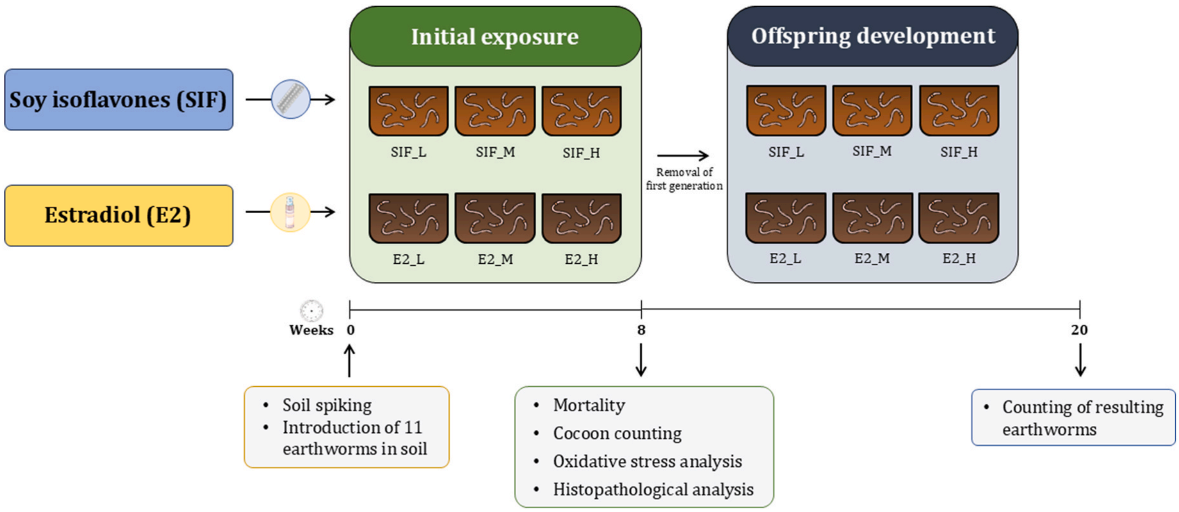

Fig. 1. Schematic representation of the experimental design. Initially, artificial soil was spiked with increasing real concentrations of soy isoflavones (113.0 (SIF-L), 215.3 (SIF-M) and 405.0 (SIF–H) mg/kg dry soil) and estradiol (156.1 (E2-L), 283.4 (E2-M), 633.8 (E2-H) μg/kg dry soil), with 11 adult earthworms introduced per replicate. The initial exposure lasted 8 weeks, after which mortality was assessed, and cocoons were counted. Samples were also collected for histopathology and oxidative stress analysis. Subsequently, cocoons were placed into the soil, allowing offspring to develop for an additional 12 weeks, after which the resulting individuals were counted. This figure has been designed using images from Flaticon.com.

2.4. *Quantification of compounds by gas chromatography-mass spectrometry (GC-MS)*

2.4.1. *Sample preparation*

EDCs in the initial soil samples (0.5 g) were extracted according to Zavala et al. (2016), using 5 mL of methanol (MeOH) and vortexed for 1 min. Subsequently, the samples were sonicated using an ultrasonic cleaner (USE) for 15 min. After sonication, the samples were centrifuged at 4000 rpm for 5 min at 4 °C (VWR, MEGA STAR 600R). The supernatants were carefully decanted, filtered and evaporated to dryness using a gentle stream of nitrogen (N_2) and reconstituted with 200 μL of anhydrous methanol. Due to the low volatility of the present EDCs, 50 μL of each extracted fraction was transferred into GC vials and evaporated to dryness under N_2. Fifty microliters of pyridine were added to the dry residues, which were derivatised by the addition of 50 μL of BSTFA (1 % TMCS) for 30 min in a heated block at 70 °C (Rocha et al., 2013).

2.4.2. *GC-MS analysis*

The quantification of these compounds was carried out using a gas chromatograph (Thermo Scientific GC TRACE 1310, Thermo Finnigan Electron Corporation) connected to an ion trap mass spectrometer (Thermo Scientific ISQ-LT GC-MS), an autosampler (Thermo Scientific Injector Triplus 100LS™), and a Trace GOLD column (TG-5MS, 30 m length, 0.25 mm ID, 0.25 μm film thickness). The GC-MS protocol followed a previously developed technique (Rocha et al., 2013). The ion source and MS transfer line temperature settings were configured to 280 °C. Pure helium (99.9999 %) was used as the carrier gas with a consistent 1.0 mL/min flow and 1 μL of each sample was injected and submitted to the following oven temperatures: a) initial temperature of 100 °C with an equilibrium time of 1 min, followed by an increase to 200 °C at a rate of 10 °C/min; b) a subsequent rise from 200 °C to 260 °C at a rate of 6 °C/min; and c) a final increase from 260 °C to 290 °C at a rate of 1 °C/min, during which the GC oven was held at 290 °C for 5 min (Rocha et al., 2013). Each chromatographic analysis was conducted for approximately 50 min. Working solutions were prepared to dilute the stock solution with methanol at six calibration levels ranging from 10 to 1000 ng/L for all EDCs and 50 ng/L for E2-d_2 (deuterated internal standard, IS). The analytic parameters of the GC-MS method are summarised in Table 2. Since the current EDCs were measured in ng/L, method blanks were used to ensure the absence of contamination by laboratory material. Beyond this, unbiased water samples were spiked with all assayed EDCs at an intermediate concentration (500 ng/L) of the calibration curve and then submitted to the usual analysis.

2.5. *Oxidative stress parameters*

The enzymatic extract was prepared as previously described by Liu et al. with some modifications (Liu et al., 2017b). In brief, the collected earthworms were weighed and inserted in a buffer solution (0.32 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$ and 0.5 mM phenylmethyl sulfonylfluoride (PMSF, 50 mM), pH 7.4) (1:5 w/v) under ice-cold condition and then homogenized using a Potter-Elvehjem homogenizer. Then, the homogenates were obtained after two cycles of centrifugation (Sigma Laborzentrifugen™ 2–16K centrifuge, Osterode am Harz, Germany) at 4 °C: 4000×*g* for 5 min and 16,000×*g* for 20 min. The resulting supernatant was collected and stored at −20 °C for further enzymatic analysis.

Every spectrophotometric measurement was performed in a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, Vermont, WI, USA) at 25–30 °C. To normalize data, protein was quantified at 280 nm using a Take3 Multi-Volume plate (Take3 plate, BioTek Instruments, Vermont, WI, USA).

2.5.1. *Superoxide dismutase*

Superoxide dismutase (SOD) activity was determined based on its ability to reduce and slow the photochemical reduction of a chromophore, nitroblue tetrazolium chloride (NBT). Briefly, the sample (15 μL) was added to a well with 180 μL of the reaction mixture (Potassium phosphate buffer 50 mM (KH_2PO_4 and K_2HPO_4) with hypoxanthine (0.6 mM), EDTA (1 mM), NBT (0.2 mM), pH 7.4). The well plates were monitored spectrophotometrically (560 nm for 2 min) to obtain the blank value. The reaction was then initiated by adding 5 μL of xanthine oxidase (23 mU/mol) to the enzymatic extract and the well plates were read for 3 min. Results were expressed in U/mg protein, where one unit of SOD activity (1 U) is defined as the amount of SOD that inhibits 50 % of the reduction of NBT to formazan.

2.5.2. *Catalase*

The levels of catalase (CAT) activity were determined by adding the reaction mixture consisting of 195 μL sodium buffer 100 mM (NaH_2PO_4 and Na_2HPO_4), pH 7.4, with H_2O_2 (20 mM) to the well plates and reading for 2 min at 240 nm. Following that, 5 μL of the sample was added to each well and read for 3 min at 240 nm. Results were expressed

Table 2
Analytic parameters of the GC-MS method used.

	Retention time (min)	Quantification ions (*m/z*)	Diagnostic ions (*m/z*)	Limits of detection (ng/L)
E2-d$_2$	24.9	287 (100)	418 (75.2), 328 (72.8)	–
Estradiol	25.0	285 (100)	416 (85.2), 326 (48.4)	2.8
Formononetin	28.6	340 (100)	339 (76.0), 355 (22.6)	8.6
Biochanin A	30.5	356 (100)	341 (34.3)	4.6
Daidzein	30.5	398 (100)	383 (76.0), 355 (22.6)	4.1
Genistein	30.8	471 (100)	473 (19.9)	3.8

in mmol H_2O_2 consumed/min/mg protein.

2.5.3. Glutathione S-transferase

To determine the activity of glutathione S-transferase (GST), the reaction mixture consisting of 180 μL of potassium phosphate buffer 100 mM (KH_2PO_4 and K_2HPO_4), pH 7.4, containing 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM) and 10 μL of the sample were added to the well plates and read for 2 min at 340 nm. After that, 10 μL of Glutathione (GSH, 25 mM) was added to each well plate and read for 3 min at 340 nm. Results were expressed as μmol CDNB/min/mg protein.

2.5.4. Peroxidase

To determine the activity of peroxidase (POD), the reaction mixture consisting of 190 μL of sodium buffer 100 mM (NaH_2PO_4 and Na_2HPO_4), pH 7.0, containing H_2O_2 (10 mM) and guaiacol (20 mM), and then read for 2 min at 470 nm (PowerWave XS2 microplate scanning spectrophotometer, Bio-Tek Instruments, Vermont, WI, USA). After that, 10 μL of the sample was added and read again at 470 nm for 3 min. Results were expressed as U/mg protein, where one unit of POD (1U POD) activity refers to the amount of enzyme that caused an increase in absorbance at 470 nm of 0.001 per minute.

2.6. Cytology

Seminal vesicles were extracted from *E. fetida* by making a dorsal incision between segments 5 and 11 (clitellum) and were subsequently placed on a glass slide. The compression and direct smear techniques were used, and the material was air-dried. A commercial Diff-Quick kit (Rapi-Diff II Stain Kit®, Atom Scientific LTD, Manchester, United Kingdom) was used for staining, sequentially immersing the slides containing the sample in the fixative (methanol), in an acid stain (eosin) and in a basic stain (hematoxylin) for about 3 min. Afterwards, slides were rinsed with distilled water. After drying, *Entellan*™ (Merck Millipore, USA) was used as mounting media and the slides were observed under a light microscope (Eclipse E600 microscope, Nikon, Japan).

2.7. Histology

The earthworms were cut into smaller segments, and the clitellum (segments 5–11) was used for cross-sectioning. These segments were then fixed in 10 % neutral buffered formalin (ITW Reagents, Germany) for 48h. Samples were then routinely processed for paraffin embedding and 3 μm sections were obtained in a rotatory microtome (Leica RM 2135, Wetzlar, Germany) (Luzio et al., 2015). Slides were stained with hematoxylin and eosin (H&E) and mounted with *Entellan*™ (Merck Millipore, USA). Tissue sections were observed under a brightfield light microscope (Eclipse E600 microscope, Nikon, Japan) and histological evaluation was performed.

2.8. Statistical analysis

Data were analyzed using Graphpad Prism version 9.5.0 (Graphpad Software), and the results are expressed as means ± standard deviation (SD). Normality and homogeneity of variance were evaluated using the Shapiro-Wilk test and Levene's test, respectively. A one-way analysis of variance (ANOVA), followed by the Bonferroni post-hoc test, was performed to evaluate the statistical differences between the control and experimental groups. The Chi-Square test was performed to assess differences among results presented as percentages. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Analytical quantification of compounds

The GC-MS analysis of the initial soil samples revealed that no estradiol or isoflavones were detected in the control group. The concentrations of the compounds detected in the soils ranged from 70.4 % (daidzein) to 90.8 % (biochanin A) of the nominal concentration. Therefore, the measured concentrations detected for soy isoflavones, i. e., 113.0 (SIF-L), 215.3 (SIF-M) and 405.0 (SIF–H) mg/kg dry soil, and

Table 3
Nominal and real concentrations of estradiol and isoflavones in the artificial soil quantified by GC-MS.

Experimental conditions			Nominal concentration	Quantified concentration
Low	E2 (μg/kg of dry soil)		174.4	156.1
	Isoflavones (mg/kg dry soil)	Genistein	5.2	4.1
		Daidzein	2.7	2.1
		Formononetin	47.7	35.9
		Biochanin A	79.2	70.8
		SUM	134.8	113.0
Medium	E2 (μg/kg of dry soil)		348.9	283.4
	Isoflavones (mg/kg dry soil)	Genistein	10.4	8.4
		Daidzein	5.5	3.9
		Formononetin	95.4	74.5
		Biochanin A	158.3	128.6
		SUM	269.6	215.3
High	E2 (μg/kg of dry soil)		697.7	633.8
	Isoflavones (mg/kg dry soil)	Genistein	18.1	14.5
		Daidzein	9.6	7.0
		Formononetin	167.0	131.9
		Biochanin A	277.0	251.6
		SUM	471.7	405.0

Table 4
Effects of increasing concentrations of soy isoflavones (113.0 (SIF-L), 215.3 (SIF-M) and 405.0 (SIF–H) mg/kg dry soil) and estradiol (156.1 (E2-L), 283.4 (E2-M), 633.8 (E2-H) μg/kg dry soil) on ponderal growth rate (%) of earthworms (*E. fetida*) and number of cocoons upon an 8-week exposure. Offspring parameters after further 12-week exposure to the contaminants, namely biomass (g) and number of earthworms. Results are reported as mean ± standard deviation (SD).

Group	Initial exposure		Offspring	
	Growth rate (%)	Cocoon number	Biomass (g)	Number
CTRL	96.5 ± 31.7	18.0 ± 3.0	7.7 ± 0.2	80.5 ± 3.5
SIF-L	92.1 ± 9.5	17.3 ± 3.5	10.2 ± 1.1	105.7 ± 23.0
SIF-M	89.5 ± 12.1	20.7 ± 6.7	8.0 ± 4.9	102.0 ± 27.6
SIF–H	87.4 ± 18.2	19.7 ± 4.9	7.5 ± 0.8	89.7 ± 4.1
E2-L	74.0 ± 21.4	17.3 ± 4.0	9.6 ± 1.3	104.3 ± 34.0
E2-M	96.2 ± 20.6	20.3 ± 4.0	8.6 ± 2.4	113.0 ± 42.5
E2-H	110.2 ± 3.5	19.7 ± 1.5	8.9 ± 1.8	92.4 ± 22.9

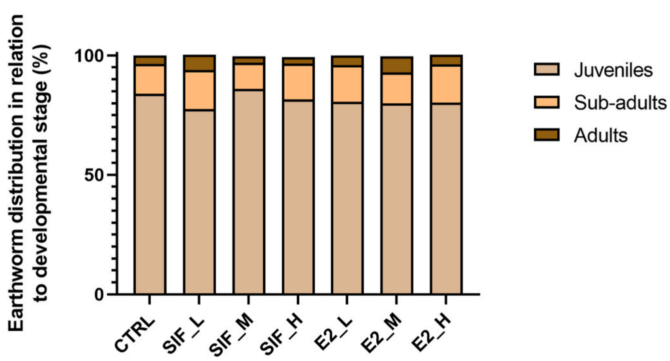

Fig. 2. Effects of increasing concentrations of soy isoflavones (113.0 (SIF-L), 215.3 (SIF-M) and 405.0 (SIF–H) mg/kg dry soil) and estradiol (156.1 (E2-L), 283.4 (E2-M), 633.8 (E2-H) μg/kg dry soil) on earthworm distribution in relation to developmental stage (%) after a further 12-week exposure.

estradiol, i.e., 156.1 (E2-L), 283.4 (E2-M) and 633.8 (E2-H) μg/kg of dry soil, were considered throughout this study as the real concentrations at the start of the experiment instead of the nominal ones (Table 3).

3.2. Earthworm survival, reproductive and biometric parameters

To assess the effects of soy isoflavones and estradiol on soil invertebrates, this study focused on evaluating survival, reproductive and biometric parameters in the earthworm *Eisenia fetida*. No mortality was observed during the course of the study in any experimental group. The various treatments had no major effect on weight (Table 4), as determined by ponderal growth rate assessment ($p > 0.05$).

Reproduction parameters were also evaluated at the end of the 8 weeks, by counting the number of cocoons (Table 4). After an additional 12 weeks of exposure, a comprehensive count of adults, sub-adults, and juveniles was conducted (Fig. 2) and, although there were no statistically significant differences between groups ($p > 0.05$), SIF-M had the greater percentage of adult worms. These results align with the data on earthworm biomass and offspring presented in Table 2.

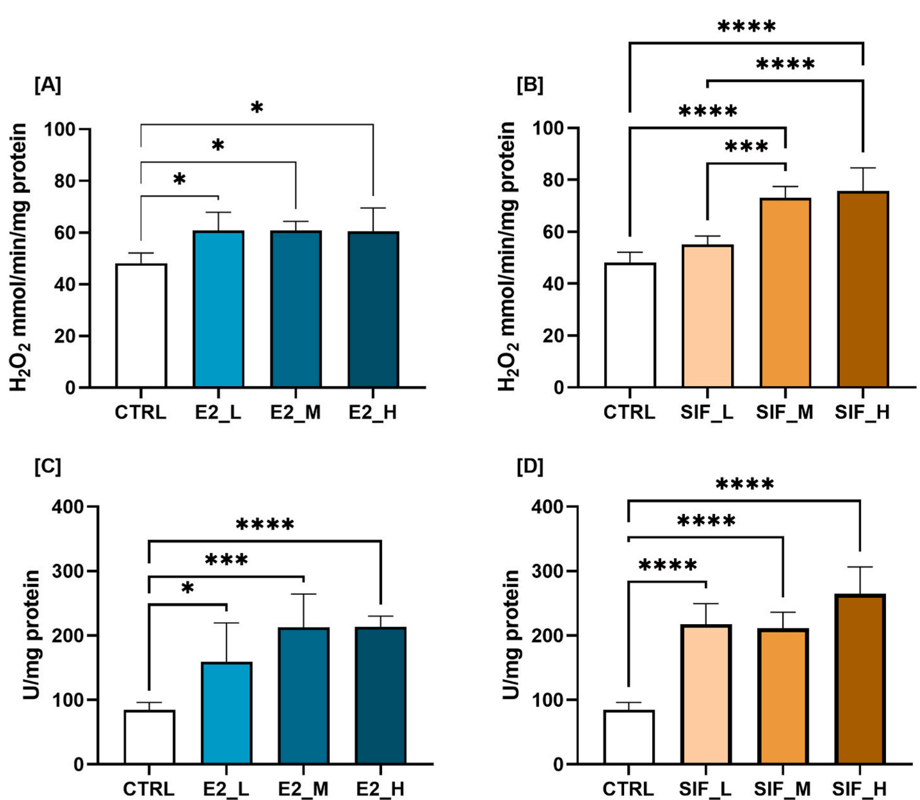

Fig. 3. Effects of increasing concentrations of soy isoflavones (113.0 (SIF-L), 215.3 (SIF-M) and 405.0 (SIF–H) mg/kg dry soil) and estradiol (156.1 (E2-L), 283.4 (E2-M), 633.8 (E2-H) μg/kg dry soil) on stress oxidative enzymatic activity of *E. fetida* upon an 8-week exposure: catalase ([A] estradiol and [B] soy isoflavones) and peroxidase ([C] estradiol and [D] soy isoflavones). Results are reported as mean ± SD. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

3.3. Oxidative stress enzyme activity

Estradiol and soy isoflavone treatments lead to a clear impact on the oxidative stress parameters evaluated, particularly catalase and peroxidase activity levels.

Catalase activity (Fig. 3A and B) was significantly superior in the two highest concentrations of SIF in comparison with the control group ($p < 0.0001$), while in the case of estradiol treatment, every group was significantly superior in comparison with the control group (E2-L: $p = 0.0206$, E2-M: $p = 0.0198$, E2-H: $p = 0.0422$). In terms of peroxidase activity (Fig. 3C and D), all treated groups exhibited significantly higher levels compared to the control group (SIF-L, SIF-M, SIF–H: $p < 0.0001$; E2-L: $p = 0.0284$, E2-M: $p = 0.0001$, E2-H: $p < 0.0001$).

SOD and GST activities were not significantly affected by both exposures (Table 5). Although the results show that SOD activity was observed lower in the control group compared to the E2-L group, this difference is not significant ($p = 0.5283$). GST activity also presented its lowest value in the control group, and highest value in the S1 group, although this difference was not statistically significant ($p = 0.2100$).

Table 5
Effects of increasing concentrations of soy isoflavones (113.0 (SIF-L), 215.3 (SIF-M) and 405.0 (SIF–H) mg/kg dry soil) and estradiol (156.1 (E2-L), 283.4 (E2-M), 633.8 (E2-H) μg/kg dry soil) on oxidative stress enzymatic activity of *E. fetida* upon an 8-week exposure: superoxide dismutase (SOD) and glutathione S-transferase (GST). Results are reported as mean ± SD.

Group	Oxidative stress enzyme activity	
	SOD (U act/mg protein)	GST (μmol CDNB conjugated/min/mg protein)
CTRL	4.4 ± 0.9	262.0 ± 58.0
SIF-L	4.9 ± 1.2	293.6 ± 44.7
SIF-M	5.3 ± 0.8	324.5 ± 29.5
SIF–H	4.6 ± 0.7	276.2 ± 21.8
E2-L	6.0 ± 1.3	346.6 ± 72.9
E2-M	4.9 ± 1.1	278.1 ± 22.3
E2-H	4.5 ± 1.1	332.1 ± 57.7

3.4. Cytology

Cytological evaluation of *E. fetida* seminal vesicles (Fig. 4) allowed the observation of a large amount of germline cells in their various stages of differentiation, with sperm morulae, spermatocytes, spermatids and spermatozoa. The cells displayed normal phenotypes in the control, E2-L, SIF-L and SIF-M groups (Fig. 4A, B and C). In groups E2-M, E2-H and SIF–H there was a decrease in the relative proportion of spermatids and spermatozoa (Fig. 4D), a focal disorganization of sperm morulae that also showed variable sizes and shapes of nuclei and

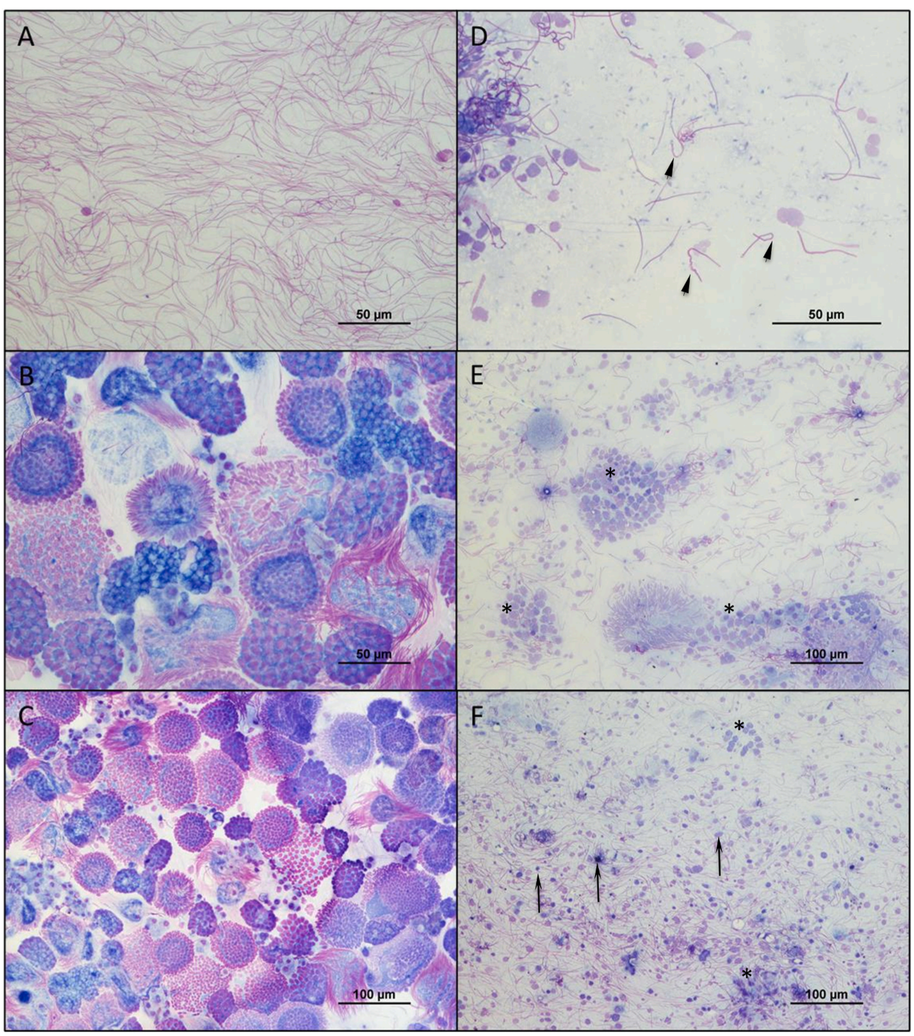

Fig. 4. Micrographs (Diff Quick staining) of the seminal vesicles from *E. fetida*. A – abundant and normal spermatozoa observed in animals from the control group; D- spermatozoa with morphological changes (block arrows) from the animals of the E2-M group; B and C- abundant and normal sperm morulae, spermatocytes and spermatids in specimens from the E2-L group. E and F- decreased number of germ cells, focal disorganization of sperm morulae (*) and anisokaryosis and anisocytosis (black arrows) in animals from groups E2-H and SIF–H.

cytoplasm (anisokaryosis and anisocytosis) (Fig. 4E and F). Morphological changes in the spermatozoa such as tail, and head curling or swelling were also observed (Fig. 4D).

3.5. Histology

The evaluation of the tissue sections allowed the observation of the seminal vesicles (Fig. 5A, B, D and E) and ovaries (Fig. 5E and F), both with germ cells in different phases of differentiation. Normal phenotypes were observed in the control group (Fig. 5A, B and C) and in the SIF-L group. In the remaining groups, architectural changes were observed, some of which involved an asymmetry between both organs, associated with a decrease in the number of germ cells in both seminal vesicles and ovaries, leading to gonadal atrophy (Fig. 5D and F). In the seminal vesicles the changes already described in the cytological evaluation were confirmed, with less and disorganized sperm morulae, deposition of abnormal proteinaceous material and conspicuous stroma (Fig. 5E). In the ovaries there was a relative increase in the number of granulosa cells when compared to the number of visible oocytes (Fig. 5F). The severity of these changes was higher in the E2-H and SIF–H groups.

4. Discussion

EDCs are an ever-growing threat to the environment and wild organisms, considered ubiquitous (Kelly et al., 2020). These compounds can be found in various products, such as plastics, pharmaceuticals and personal hygiene products (Gao and Kannan, 2020). These compounds are generally not removed by wastewater treatment systems and can be found in streams, rivers, and soils, posing a risk to human and ecosystem health (García-Fernández et al., 2020). While numerous studies have evaluated the ecotoxicity of these compounds in aquatic systems (Pironti et al., 2021; Surana et al., 2022), research on their effects on soil biota remains limited despite their presence in soil (Saha et al., 2022). Therefore, it is crucial to shift attention towards the ecotoxicological impacts of these compounds on soil organisms and enhance knowledge in this area. As such, this work aimed to evaluate the effect of estradiol and soy isoflavones, known as EDCs, in *E. fetida* to understand their toxicological impact on a soil organism.

In this study, the selected concentrations of estradiol and soy isoflavones mirror the levels typically found in livestock manure (Adler et al., 2015; Combalbert and Hernandez-Raquet, 2010; Njåstad et al., 2014; Zheng et al., 2008), a common component in agricultural practice aimed at soil improvement (Rayne and Aula, 2020). This approach assesses whether these concentrations induce sub-lethal effects on

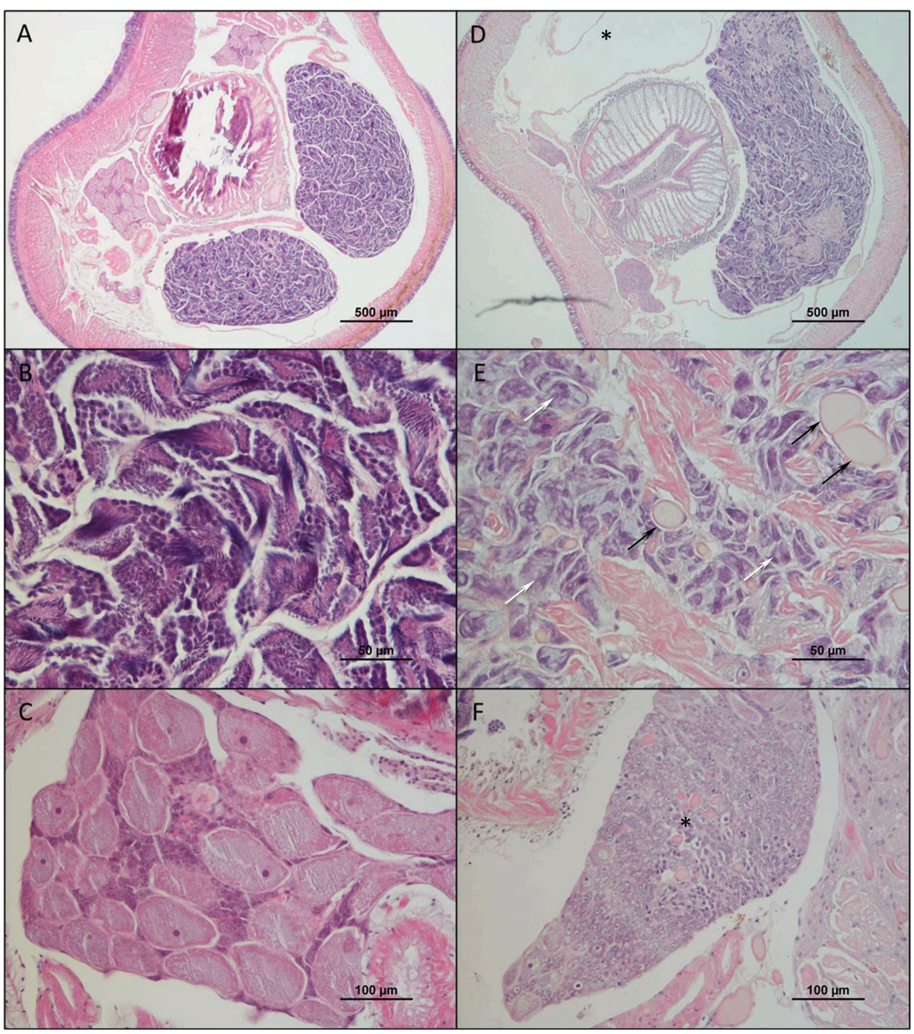

Fig. 5. Micrographs (H&E staining) from *E. fetida* specimens: complete transverse section at the level of segments 5 to 11. Normal seminal vesicles (A and B), and ovaries (C) of an individual from the control group; D - cross-section of an individual from the SIF-M group, showing a clear asymmetry in the seminal vesicles due to loss of germ cells and subsequent atrophy of the contralateral seminal vesicle (*); E – seminal vesicle from an individual from the SIF–H group displaying low cellularity of sperm morulae (white arrows) and deposition of proteinaceous material (black arrows); F – ovary atrophy (*) in an individual from the SIF–H group.

E. fetida. Mortality and ponderal growth rate revealed no significant differences between the treatment and control groups. These findings suggest that, at these concentrations, these compounds do not adversely affect the overall physiology of *E. fetida*. The number of cocoons was also counted at the end of the 8 weeks, and no significant differences were found between the groups. A study by Heger et al. reported an increase in the number of juveniles after 8-week exposure to estradiol concentrations of 10–50 μg/kg dry soil (Heger et al., 2015); it is worth noting that these authors did not count the number of cocoons. Future research should investigate the interaction between these compounds and hatching success, as it is a crucial factor for reproduction (Maboeta and Rensburg, 2003). Interestingly, our findings regarding the further 12-week exposure indicate that the population number remains unaffected at the end of the experimental study, which can be interpreted as not affecting the reproductive activity of exposed earthworms. These compounds also did not affect the development of the offspring.

Despite discovering a few differences in the first generation across some parameters in our study, we did not find a significant impact on the overall population of *E. fetida* that developed after an extra 12-week period, which contradicts other findings (Heger et al., 2015) and prompts further exploration. Earthworms have proven to be effective bioindicators of EDC contamination in their surroundings, as they can accumulate these compounds in their tissues (Markman et al., 2007). However, our study aimed to investigate how the population of *E. fetida* would respond to prolonged exposure to EDCs, providing valuable insights into the potential effects of EDC-contaminated soil on population dynamics. Interestingly, the treated groups in our study displayed more descendants than the control group, although this difference was not statistically significant. While not reaching statistical significance, our findings indicate intriguing trends in certain treatments, notably SIF-L, where there was a higher percentage of individuals in the sub-adult and adult stages. This could be attributed to an accelerated reproductive pattern initiated by the F0 generation in response to exposure to these EDCs. This phenomenon could be attributed to the hormetic effect, whereby a harmful substance demonstrates stimulating and beneficial effects on organisms as an overcompensation strategy for mild environmental stress (Ray and Stick, 2015). Hormetic effects have been described in many other organisms, namely *Danio rerio* (Barros et al., 2020, 2022, 2023; Santos et al., 2014), *Myzus persicae* (Ayyanath et al., 2013), *Rattus norvegicus* (Docea et al., 2019), and *Rissa tridactyla* (Blévin et al., 2017), exposed to various chemicals (Hashmi et al., 2014).

In vertebrates, EDCs interfere with the cellular organization of the central nervous system and gonads, leading to persistent alterations to the reproductive axis (Caporale et al., 2022; Carvalho Henriques et al., 2020; Ghosh et al., 2022; Lopez-Rodriguez et al., 2021; Santos et al., 2017; Vosges et al., 2010). Considering this, we aimed to investigate the impact of estradiol and soy isoflavones on the reproductive organs and germ line of *E. fetida*. In our study, we observed that the groups exposed to higher concentrations of these compounds exhibited the greatest number of changes in organ and cell morphology and quantity. Theoretically, these changes should lead to fertility problems and, therefore, a decrease in the population of *E. fetida* in the ecosystem, which would affect soil quality, disrupt the food chain, and impact the ability of the soil to provide ecosystem services (Medina-Sauza et al., 2019). Histological and cytological findings from other studies also support our observations, indicating that the evaluation of oogenesis and spermatogenesis in earthworms' reproductive organs can serve as useful early indicators for investigating the endocrine-disrupting properties of chemicals (Babić et al., 2016; Kwak and An, 2021). Interestingly, continuing this study beyond the initial 8 weeks revealed that, while some changes were noted in the gonads of the initial individuals, these alterations did not translate into significant differences in the overall offspring populations. The obtained results suggest that the current OECD Test 222 - Earthworm reproduction test (*E. fetida/E. andrei*) may be disregarding effects in reproduction below determined EC_{50}, as the endpoint used is an effect on the offspring number. Considering that the compounds were only introduced at the beginning of the experiment and, due to transformations occurring in the soil matrix, their concentrations are expected to decline during the process, the current study did not evaluate a continuous long-term exposure response. However, alterations on the gonads' organization were observed, suggesting that in natural environments where earthworms are exposed for extended periods, alteration in populations might occur. The prolonged evaluation period used in this study (20 weeks) only effectively captured the multigenerational effects of EDCs. This extended duration permitted the observation of F1 offspring maturation (approximately 12 weeks post-hatching) and potential F2 reproduction, thereby providing insights into transgenerational effects.

The role of EDCs in *E. fetida* is particularly complex, with differing perspectives among researchers. Some authors proposed it as a non-estrogenic organism (Heger et al., 2015), while others indicate the contrary (Novo et al., 2019). Invertebrate endocrinology still remains an area of limited scientific understanding (Crane et al., 2022), and disparities and inconsistencies exist in the known phylogenetic distribution of the estrogen receptor (ER) in invertebrates (Jones et al., 2017). Interestingly, in 2009, the first report of hormone-activated ERs in annelids (*Platynereis dumerilii* and *Capitella capitata*) emerged, suggesting the potential presence of similar receptors in *E. fetida* (Keay and Thornton, 2009). More recently, a study by Novo et al. (2019) identified a new sequence encoding the ER protein of *E. fetida*. The work of these authors has been followed by Yao et al. which found that, after a 28-day exposure of *E. fetida* to E2 (0.1–1 mg/kg), *ER* gene expression increment was associated with lower cocoon and juvenile numbers (Yao et al., 2024). The present study, with E2 concentrations within the same range, showed that cocoon and offspring numbers are not affected after 56 days of exposure, suggesting that adaptative and cope mechanisms may have been activated. However, these compounds have the ability to influence the expression of genes associated with endocrine function, such as *EcR*, *MAPR*, and *AdipoR*, as evidenced by a study in which earthworms (*E. fetida*) were exposed to bisphenol A (Novo et al., 2018). In addition to these genes, epigenetic mechanisms (*DNMTs*), genotoxicity (*PARP1*), and stress responses (*SOD*, *CAT*, *GST*, *Hsp70*, *HSC70 4*), among others, can also be affected by EDCs, emphasizing their potential impact on crucial regulatory pathways (Novo et al., 2018, 2019; Xu et al., 2015).

To explore some potential mechanisms of toxicity of the selected compounds, we evaluated oxidative stress parameters. Oxidative stress refers to an imbalance between the formation and the removal of free radicals, arising as a result of increased ROS levels, reduced number of scavenger molecules, and depletion of the activities of antioxidant enzymes (Pizzino et al., 2017). Our results indicated several significant changes, namely increased catalase (CAT) and peroxidase (POD) enzyme activity. These enzymes play a crucial role in protecting cells from oxidative damage caused by ROS and other harmful compounds (Bhattacharyya et al., 2014). The observed increase in CAT and POD activity suggests a significant production of hydrogen peroxide (H_2O_2) in response to exposure to estradiol and soy isoflavones. Previous studies have also reported an activation of the earthworms' antioxidant defense system in response to exposure to various toxic substances (Dos Santos Gonçalves Nascimento et al., 2023; He et al., 2021; Liu et al., 2020; Wang et al., 2022), particularly EDCs (Sicińska et al., 2020; Song et al., 2019). In one study, the authors found that phthalates induced oxidative stress through increased superoxide dismutase (SOD), POD, and CAT activity, and DNA damage in earthworms (Song et al., 2019). As previously mentioned, the diffusion of estrogen hormones, capable of undergoing redox cycling, freely into the cellular microenvironment leads to the generation of ROS (Heger et al., 2015). The increased levels of H_2O_2, as indicated by the elevated CAT and POD activities, may result from an initial, and prior to our sampling time, increase in SOD activity, which converts the superoxide radical ($O_2^{\bullet -}$) into H_2O_2 and molecular oxygen (O_2) (Maurya and Namdeo, 2021), not detected in the current study. Although using rodents, a previous study on ovariectomized C57BL-6N female mice supplemented with 1 μg estradiol/day showed an increase in H_2O_2 production, corroborating our findings (Torres et al., 2018); these elevated H_2O_2 levels subsequently led to an increase in CAT and POD activity levels. It is important to note that the effects of oxidative stress observed in earthworms exposed to EDCs can have broader implications. Specifically, compromised earthworm health can alter nutrient bioavailability (Rehman et al., 2023), biodiversity (Angst et al., 2022), soil structure (Bertrand et al., 2015) and water retention capacity (Bertrand et al., 2015; Rehman et al., 2023). These changes, in turn, may result in less healthy soils, more susceptible to erosion.

Overall, our study shows that *E. fetida* is sensitive to EDCs, which are commonly found in livestock manure. This sensitivity makes it a valuable bioindicator for assessing EDC presence in terrestrial environments and understand their effects soil fauna, biodiversity, and ecosystem quality. While the long-term population dynamics of *E. fetida* were not significantly affected by the studied EDCs, even though gonad organization was affected, the organisms were affected at the biochemical and tissue level, raising questions about the long-term ecological consequences of EDCs. Further research is necessary to unveil underlying mechanisms and develop mitigation strategies to safeguard soil health and ecosystem functioning. To better understand the impact of these compounds, it would be beneficial to measure the chemical concentrations at multiple time points throughout the experiment, due to potential degradation or transformation over time, particularly considering the influence of soil microorganisms.

CRediT authorship contribution statement

Tiago Azevedo: Writing – review & editing, Writing – original draft, Methodology. **Rita Silva-Reis:** Writing – review & editing, Methodology. **Beatriz Medeiros-Fonseca:** Writing – review & editing, Methodology. **Mariana Gonçalves:** Writing – review & editing, Methodology. **Gabriel Mendes:** Methodology. **Marta Roboredo:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Maria J. Rocha:** Writing – review & editing, Methodology. **Francisco Peixoto:** Writing – review & editing, Methodology. **Maria de Lurdes Pinto:** Writing – review & editing, Methodology. **Manuela Matos:** Writing – review & editing, Methodology. **João R. Sousa:** Writing – review & editing, Methodology. **Paula A. Oliveira:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Ana M. Coimbra:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ana Maria Coimbra reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Abbreviations

CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
E2	17β-estradiol
EDC	Endocrine Disruptor Compound
EDTA	Ethylenediaminetetraacetic Acid
ER	Estrogen Receptor
GSH	Glutathione
GST	Glutathione S-Transferase
H&E	Hematoxylin and Eosin
H_2O_2	Hydrogen Peroxide;
NBT	Nitroblue Tetrazolium Chloride;
OCDE	Organization for Economic Co-Operation and Development
POD	Peroxidase
ROS	Reactive Oxygen Species
SI	Soy Isoflavones
SOD	Superoxide Dismutase

Data availability

Data will be made available on request.

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