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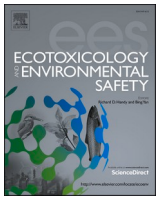

# Behavioural and cyto-genotoxic effects in adult rats and induced congenital anomalies to their embryos, exposed to environmentally relevant concentrations of phthalates

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ABSTRACT

Phthalates are endocrine-disrupting chemicals widely used in everyday plastics and are increasingly recognised as dangerous to humans and ecosystems. In Lithuania, ineffective wastewater treatment has led to rising contamination with phthalates, particularly di(2-ethylhexyl) phthalate (DEHP) and dibutyl phthalate (DBP). As most prior studies used high short-term doses, this study focused on the effects of daily consumption of low environmentally relevant concentrations of DEHP and DBP on female rat physiology, reproduction, embryotoxicity and cyto-genotoxicity. Behavioural observations were included as an exploratory component to assess potential effects. Rats were given environmentally relevant phthalates with food for two months in three groups (200 μg/kg of DEHP, 100 μg/kg of DBP and a mix of both) and two groups for positive control (1000 μg/kg of DEHP and 500 μg/kg of DBP). The negative control group did not receive phthalates. Standard behavioural tests were conducted twice, general blood tests were carried out, and the rats were mated. On the 21st day of gestation, the pregnant rats were euthanised, bone marrow samples were taken for the micronucleus assay, and embryo viability and congenital anomalies were recorded. No significant behavioural or physiological changes were observed in adult rats. However, phthalates increased micronuclei in polychromatic erythrocytes and reduced the PCE/NCE ratio, indicating cyto-genotoxic effects. Long-term exposure to DEHP and DBP at environmentally relevant concentrations induced embryotoxic effects: embryo viability was compromised, with resorptions and morphological abnormalities. In summary, our results suggest that female exposure to phthalates, even in low environmental doses, has hidden cyto-genotoxic, reprotoxic and embryotoxic effects.

## 1. Introduction

Phthalates, commonly used as plasticisers are important to producing everyday plastic products due to their performance and low cost (Anne and Paulauskiene, 2021; Li et al., 2020). Some of them, such as di (2-ethylhexyl) phthalate (DEHP) are found in building materials and furniture, cosmetics and medical devices, toys and baby care products, drinking straws and food containers (Kiralan et al., 2020; Ma et al., 2014; Paluselli and Kim, 2020; Sopheak et al., 2015; Zhang et al., 2020). However, others, such as dibutyl phthalate (DBP), are used in personal care products, paints, glue, and plastic bags and as adjuvants in pesticide formulations for usage in aquatic systems (Paluselli and Kim, 2020; Sopheak et al., 2015; Abdolahnejad et al., 2018). When plastics start to degrade or are exposed to heat, phthalates are easily released into the air, water and soil (Li et al., 2020; Kiralan et al., 2020; Ma et al., 2014; Chen et al., 2019; Dueñas-Moreno et al., 2022; Net et al., 2015; Olujimi et al., 2017). Although phthalates are poorly soluble in water, they are perfectly adsorbed onto various particles floating in water, so they can reach quite high concentrations in sewage water. Consequently, when released into open water, this pollutant reaches only the amount in

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micrograms (Fagbemi et al., 2024), but accumulating in plant biomass and entering the food chain (He et al., 2015; Vannucchi et al., 2019) phthalates are likely to pose a significant health risk to humans and ecosystems.

Phthalates are known as toxic, mutagenic and endocrine-disrupting pollutants, also affecting the mammals development (Paluselli and Kim, 2020; Net et al., 2015; Carbone et al., 2018; Grindler et al., 2018; Ugwor et al., 2022; Wang and Qian, 2021). Studies suggest that phthalates increase anxiety-related behaviours and motor activity and impair cognitive function. For instance, DEHP exposure causes elevated locomotor activity, impairs dopamine system development and heightens anxiety-like behaviours in rats (Carbone et al., 2018; Holahan et al., 2018). Prolonged DEHP and DBP exposure in mice has also been associated with disrupted cognitive functions, depression-like behaviours, reduced learning and memory, and anxiety (Farzanehfar et al., 2016; Kang et al., 2023). In non-rodent species, such as chicks, DEHP and DBP were linked with reduced hatching success, developmental abnormalities, and impaired memory (Abdul-Ghani et al., 2012). In humans, prenatal and childhood phthalate exposure may correlate with developing ADHD traits (Ku et al., 2020).

Phthalates, notably DEHP and DBP, are also widely studied for their effects on fat accumulation, weight gain, and blood parameters in mammals (Apau et al., 2020; Deierlein et al., 2022; Díaz Santana et al., 2019; Hasan et al., 2024; Majeed et al., 2017; Radke et al., 2019). For instance, mice exposed to DEHP exhibited increased weight and fat mass, decreased responsiveness to insulin, changes in adipose tissue function, including altered expression of key genes such as PPARG, and changes in the levels of adiponectin and estrogen. (Klöting et al., 2015). DEHP-treated rats showed altered red blood cell counts, mean corpuscular haemoglobin, and platelet levels (Kwack et al., 2009). The haematological parameters of goats exposed to DBP and other phthalates also changed significantly (Hasan et al., 2024). A study on pregnant women noted that exposure to phthalates was associated with elevated levels of inflammatory indicators in blood cells, including white blood cells, lymphocytes, and neutrophils, reflecting potential inflammatory responses (Yang et al., 2023).

Genotoxic and cytotoxic effects of phthalates, particularly DEHP and DBP, have been investigated through various assays, with the micronucleus test being a routine method. Studies have demonstrated that exposure to DEHP and DBP can lead to increased micronuclei formation in bone marrow cells, indicating damage to chromosomal structures and failure of mitotic processes in both mice and in humans (Balmus et al., 2015; Smith et al., 1990). Elevated frequencies of micronuclei suggest both clastogenic and aneugenic effects, marking these chemicals as genotoxic agents. While some studies, such as Kitamoto et al. (Kitamoto et al., 2015) or Shigano et al. (Shigano et al., 2024), observed no significant genotoxic effects at high doses of DEHP in rats, others (Abdul-Ghani et al., 2012; Hasan et al., 2024; Majeed et al., 2017) have highlighted the potential risks of lower doses or prolonged exposure. Additionally, the cytotoxicity of phthalates has been linked to reduced erythrocyte proliferation, with decreased PCE/NCE (polychromatic erythrocytes/ normochromatic erythrocytes) ratios suggesting disrupted cell division and accelerated ageing of erythrocytes (Krishna and Hayashi, 2000; Venitt and Parry, 1984).

Significant teratogenic effects of phthalates on embryos are evident from various animal studies. A systematic review by Czubacka et al. (Czubacka et al., 2021), which examined studies involving rats, mice, rabbits, and monkeys, highlighted the embryotoxicity of DBP, noting increased foetal resorptions, reduced live births, and skeletal and genital malformations, which were further supported by anti-androgenic and weak estrogenic effects of DBP. In rats, DBP exposure has led to reduced placenta weights, higher resorption rates, and significant skeletal malformations across multiple generations (Mahaboob Basha and Radha, 2017). Saillenfait et al. (Saillenfait et al., 2009) documented dose-related developmental anomalies from di-n-hexyl phthalate (DnHP) exposure in rats, including cleft palates and delayed ossification. Studies on DEHP further emphasised adverse effects; chick models exposed to DEHP or DBP showed reduced hatching rates and abdominal malformations (Abdul-Ghani et al., 2012), while maternal DEHP exposure in humans correlated with reduced birth weight and head circumference (Al-Saleh et al., 2024).

Therefore, some phthalates were determined as pollutants of high priority by the European Union and the US Environmental Protection Agency at the beginning of the 21st century (Net et al., 2015). However, the concentration and dominant compounds of phthalate pollutants in water samples vary significantly across different countries. For example, concentrations range from 1.2 μg/L for DEHP and 0.14 μg/L for DBP in Denmark, to as high as 1000 μg/L for DEHP and 500 μg/L for DBP in Lithuanian wastewater. In Lithuania, due to inadequate clean-up measures, these concentrations end up in environmental waters and may potentially enter the food chain and the surrounding environment, which has become an increasing problem in recent years (Anne and Paulauskiene, 2021; UAB, 2022). As phthalate exposure routes include inhalation, oral, and dermal pathways and vary among individuals, determining the total combined exposure is challenging. Therefore, environmental concentrations are often used as a reference point (Jeon et al., 2023).

The majority of toxicological studies to date have focused primarily on male subjects (Carbone et al., 2018; Kwack et al., 2009; Czubacka et al., 2021; Saillenfait et al., 2009; Zhang et al., 2011), with comparatively little attention given to the potentially distinct effects of phthalates on females and their reproductive health (Grindler et al., 2018; Basso et al., 2022; Lovekamp-Swan and Davis, 2003; Wang et al., 2023). Moreover, most existing studies have focused on short-term exposure to high, often unrealistic concentrations of individual phthalates, which do not reflect environmentally relevant conditions. Few have assessed the combined behavioural, reproductive, and cyto-genotoxic impacts of chronic low-dose exposure to mixtures such as DEHP and DBP, particularly in adult females (Basso et al., 2022). This study addresses that critical gap.

Although behavioural tests were not the primary focus of this research, they were included as part of an exploratory approach to evaluate whether low-dose phthalate exposure could elicit detectable changes in locomotor or anxiety-like behaviours (Gould et al., 2009; Walf and Frye, 2007; Chomiak et al., 2016). These assays were selected based on literature indicating that phthalates may interfere with neurological development. Including them allowed us to capture potential subtle neurotoxic effects and to inform the design of future targeted studies.

We hypothesized that (1) prolonged exposure to low, environmentally relevant doses of DEHP and DBP would lead to measurable alterations in anxiety-like and cognitive behaviours in adult female rats; (2) these exposures would result in significant cyto-genotoxic effects in bone marrow cells; and (3) the developmental outcomes in embryos of exposed females would be adversely affected, even in the absence of overt maternal toxicity. Therefore, this study aimed to determine the effects of low environmentally relevant concentrations of DEHP and DBP on the behaviour and physiology of female rats, as well as their cyto-genotoxic effect on rat bone marrow cells and embryonic development.

## 2. Materials and methods

### 2.1. Chemicals

Di(2-ethylhexyl) phthalate (≥ 99 %, Sigma-Aldrich) and dibutyl phthalate (≥ 99 %, Sigma-Aldrich), were used for this study. The phthalates were dissolved in olive oil (Seville Premium Extra Virgin Olive Oil), while the negative control group of rats received an equivalent amount of olive oil without phthalates. Our study uses environmental concentrations found in Lithuanian wastewater to model potential exposure scenarios, providing valuable insights into the risks associated with phthalate contamination. The exposure concentrations

for DEHP and DBP used in this study were determined based on data obtained from wastewater samples analysed by UAB „Vandens tyrimai", a certified water quality testing facility in Lithuania. According to their report, DEHP and DBP were detected at concentrations of approximately 1000 μg/L and 500 μg/L, respectively (UAB, 2022). The client-specific sources of these samples remain confidential. Environmental concentrations of phthalates in the rat diet were determined by reflecting the actual daily water intake by humans (typically ~2 L/day for adults), which gives a human dose in mg/kg body weight/day. The human dose was converted to an equivalent rat dose using interspecies dose scaling according to the metabolic rate variation between humans and rats (Reagan-Shaw et al., 2008). We used formula: Animal dose (mg/kg) = Human dose (mg/kg) × (Animal Km / Human Km), where Km is a species-specific constant derived from body surface area and weight (Human Km (60 kg adult) ≈ 37 and Rat Km (150–200 g) ≈ 6). So, Rat dose was ≈ Human dose x 6,17. This body surface area normalisation is the most widely accepted method, recommended by the FDA and others (Reagan-Shaw et al., 2008; CDER, 2005). The resulting rat-equivalent doses were then compared against exposure levels reported in existing toxicological studies involving DEHP and DBP in rodent models (Sellinger et al., 2020; Wang et al., 2021). Based on this comparative analysis, we selected two exposure levels for each compound that are both environmentally plausible and toxicologically meaningful. Specifically, rats were exposed to DEHP at 200 μg/kg/day and 1000 μg/kg/day, and to DBP at 100 μg/kg/day and 500 μg/kg/day. These concentrations reflect realistic environmental exposures derived from wastewater analysis, while also aligning with established ranges used in previous experimental studies.

### 2.2. Experimental animals and dosing regimen

In the present study, thirty-six female *Wistar* rats (1–2 months old) from a breeding colony at Vilnius University, Lithuania, were used. The animals were kept in standard, well-ventilated cages with bedding suitable for rats, to ensure comfort and prevent stress. The rats were kept under a 12/12-hour artificial light/dark cycle (lights on at 7:00 a.m.). The room temperature was maintained at a constant 22 ± 1°C. Standard laboratory rat feed (4RF21-GLP, Mucedola srl, Italy) and tap water were freely available throughout the whole experimental period. The animal room was equipped with environmental enrichment (e.g., nesting material, shelters, toys, etc.) to promote natural behaviour and reduce stress.

All rats were divided into six groups: 1) Control (no phthalates); 2) DEHP 200 μg/kg (DEHP_200); 3) DEHP 1000 μg/kg (DEHP_1000); 4) DBP 100 μg/kg (DBP_100); 5) DBP 500 μg/kg (DBP_500); and 6) a mixture of DEHP 200 μg/kg and DBP 100 μg/kg (DEHP_DBP). Rats were weighed twice a week, and the concentration of phthalates was calculated based on each rat's weight. A drop of phthalate, dissolved in olive oil, was placed on a small piece of biscuit ~ 0,25 g (*Lupilu Bio Baby Biscuit*). Each rat received its phthalate dose daily, 7 days per week, at the same time, for two months.

During phthalate administration, rats were temporarily housed in individual small cages without bedding or substrate and were given their pre-weighed biscuit containing the phthalate dose. Each rat was allowed to eat the biscuit at its own pace before being returned to the group cage. Rats were returned to their original cages only after consuming the entire piece. It typically took 3–7 min for a rat to finish the small piece of biscuit. The control group received biscuits treated with olive oil alone, administered in the same manner as the experimental groups.

After 60 days of phthalate exposure, the mating of the female rats with unexposed males was initiated, with some females mating immediately, while others took up to a month to do so. Phthalate exposure continued throughout gestation. Male rats were housed in the same room as the females and maintained under the same conditions, except that they were provided with biscuits containing phthalates. The males and females were only housed together for the purpose of mating. On gestation day 21, rats were euthanised in a $CO_2$ chamber using a gradual $CO_2$ fill, in accordance with the ethical requirements of the European Directive 2010/63/EU on the protection of animals used for scientific purposes (Parliament, 2010). Embryos and bone marrow samples were collected post-mortem, and embryo viability and congenital anomalies were recorded.

All experimental procedures received approval from the State Food and Veterinary Service of the Republic of Lithuania (2022–08/09, No G2–221) and were carried out following the local Animal Welfare Act and the European Communities Council Directive of 22 September 2010 (2010/63/EU).

### 2.3. Sample size determination

Sample size was determined to ensure adequate sensitivity for detecting biologically relevant effects in the primary endpoints: genetic damage (micronucleus frequency, PCE/NCE ratio), embryonic development (embryo weight and length, resorptions, and gross morphological abnormalities), and adult rat physiological parameters (body weight and haematological measures). These outcomes are characterised by low intra-group variability, supporting the detection of moderate to large effects with relatively small sample sizes.

A group size of six pregnant rats per treatment group (with ≥20 embryos per group) was selected based on its consistency with previous studies in developmental and genetic toxicology (Mahaboob Basha and Radha, 2017; Saillenfait et al., 2009; Ahbab et al., 2017; OECD, 2016) Power analysis based on a one-way ANOVA model ($\alpha = 0.05$, two-sided; $\beta = 0.30$, power = 70 %) indicated that this sample size would allow detection of large effect sizes (Cohen's $f \geq 0.6$), which was considered appropriate given the known variability and expected magnitude of biological response in the selected endpoints. (Cohen, 1988).

Behavioural tests (open field, elevated plus maze test, and running wheel) were included as exploratory endpoints to investigate potential neurodevelopmental alterations. These were not formally powered, as they were not the primary focus of the study.

Animals were randomly assigned to six experimental groups (n = 6 per group), including one negative control and five treatment groups. All procedures complied with institutional animal welfare policies and adhered to the ethical principles of the 3Rs framework—Replacement, Reduction, and Refinement (Parliament, 2010).

### 2.4. Behavioural tests

During two months of phthalate exposure, three standardised animal behavioural tests were performed twice to evaluate the toxic effect on behaviour: open field test (OFT) (Gould et al., 2009), elevated plus maze (EPM) (Walf and Frye, 2007) and wheel-running test (Chomiak et al., 2016). The first round of tests was conducted at the beginning of the experiment to establish a baseline and determine whether any rats had behavioural issues prior to exposure to phthalates. The results were analysed from the tests conducted after two months of exposure to phthalates. During the behavioural tests, the movement of rats was tracked by EthoVision XT (Ver. 7.1.426, Noldus, Wageningen, Netherlands). Behavioural tests were conducted in a quiet, controlled environment to minimise external stressors. Animals were allowed to acclimatise to the testing room for one hour prior to each procedure. The OFT and EPM were performed between 09:00 and 15:00.

#### 2.4.1. Open field test

Before testing, the brightness of the open field arena was adjusted to approximately 80 lux in the centre, 45 lux in the inner corners, and 65 lux in the outer corners. A 30 % ethanol-based disinfectant solution was used to sanitise the area before and after each trial. The open field apparatus was constructed from black opaque Plexiglass and measured 50 cm (length) × 50 cm (width) × 50 cm (height). Each rat was gently placed in the centre of the arena, and its movements were recorded for

15 min using a high-definition video surveillance camera (DS-2CD1643G0-IZ, Hangzhou Hikvision Digital Technology Co., Ltd.). Four rats were tested simultaneously, and the entire cohort was assessed within a single day. The following behavioural parameters were measured: time spent in the central area and total distance travelled. Time spent in the centre of the open field reflects anxiety-like behaviour, with reduced centre time indicating increased anxiety, while total distance travelled serves as a measure of general locomotor and exploratory activity (Gould et al., 2009). Video recordings were analysed using Viewer III software (Biobserve, Germany).

#### 2.4.2. Elevated plus maze test

The equipment used for the elevated plus maze test was configured in a "+" shape and elevated 50 cm above the floor. The equipment consisted of two closed arms (50 cm long × 10 cm wide × 40 cm high) and two open arms (50 cm long × 10 cm wide) with a centre platform (10 × 10 cm). The closed arms had high walls to enclose the arm, while the open arms had a small border to decrease the number of animal falls. The maze platform and walls were made of black opaque Plexiglass. Light intensity levels were approximately 80 lux in the open arms and 50 lux in the closed arms (measured in the middle of the arms, 50 cm above the floor). Before and after each testing procedure, a disinfectant solution containing 30 % ethanol was used to sterilise the area where the rats were placed. The experimental rat was carefully positioned into a closed right arm, and its motion was tracked for 5 min using a high-definition video surveillance camera (DS-2CD1643G0-IZ, Hangzhou Hikvision Digital Technology Co., Ltd.). The time in the open arms, and the distance travelled were recorded. Time spent in the open arms reflects anxiety-like behaviour, with increased duration indicating reduced anxiety; distance travelled in the open arms captures exploratory drive under anxiety-provoking conditions; and total distance travelled provides a control for general locomotor activity (Walf and Frye, 2007). The ANY-maze computer software (A, NY-maze, Ireland) was utilised for the analysis of the video recordings.

#### 2.4.3. Running wheel test

The running wheel test was carried out as a voluntary running test to investigate the spontaneous motor activity of the animals. Before conducting the running wheel test, the rat underwent a minimum 10-minute acclimatisation period in the behavioural test room. During the experiment, the animals were housed in standard cages with integrated running wheels (35 cm diameter, 2 mm bars, placed 8.8 mm apart) (Ugo Basile, Italy) with their respective food (4RF21-GLP, Mucedola srl, Italy) and supplemented with water *ad libitum*. The rats had unrestricted access to a running wheel. Motor activity was measured by counting the number of revolutions of the wheel during a 24-hour period: a 12-hour day cycle (cage lighting 50–80 lux, light intensity measured at the top of the running wheels) and a 12-hour night cycle (no lighting). The running wheel test enables detailed and objective assessment of locomotor activity, motor skill learning, and behavioural phenotyping in rodent models of neurological and psychiatric disorders (Chomiak et al., 2016). Physical activity was monitored and analysed using a specialised recording system with PowerLab-16/30 hardware and LabChart software (ver. 7–8, ADInstruments Ltd, UK).

### 2.5. General blood test

Blood samples were collected from adult rats via tail tip microsampling one week prior to mating, after two months of phthalate exposure. Each rat was handled by two trained laboratory technicians: one gently restrained the animal, while the other performed a small cut on the tail tip using sterile instruments and collected the blood into a capillary tube. The blood was immediately transferred to an automated analyser (Exigo EOS) for haematological analysis. Haemostasis was achieved by applying gentle pressure to the tail tip with sterile gauze, following best laboratory practices to minimise stress and ensure animal welfare (NC3Rs, 2025). The blood measures analysed included white blood cells (WBC), lymphocytes (LYM), monocytes (MONO), granulocytes (GRAN), haemoglobin (HGB), haematocrit (HCT), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDWa), platelets (PLT), and mean platelet volume (MPV).

### 2.6. Micronucleus assay in rat bone marrow

For the micronucleus assay, rat bone marrow cells were prepared following standard procedures (Schmid, 1975). The femurs were dissected and both ends were cut off. A syringe containing 5 ml of foetal bovine serum (FBS) was inserted into the distal end and the bone marrow was washed. The cells were subsequently centrifuged at 200 x g for 5 min. The supernatant was aspirated with a syringe and the remaining cells were resuspended. At least 2 smears per animal were prepared. The smears were fixed in 96 % ethanol for 10 min. The slides were stained in GIEMSA, coded, and scored blind by the same scorer at a magnification of 1000x (Jenaval microscope, Carl Zeiss, Germany) with immersion oil. The staining procedure allowed to distinguish polychromatic erythrocytes (PCE, blue) and normochromic erythrocytes (NCE, pink) by colour. A total of 2000 polychromatic erythrocytes (PCEs) per animal were assessed for the presence of micronuclei (MNPCE). To evaluate bone marrow toxicity, the ratio of PCEs to total erythrocytes (PCE/NCE) for each animal was determined by examining at least 200 cells (Krishna and Hayashi, 2000; Hayashi et al., 2000; Tinwell and Ashby, 1989). The individual performing the cell counting was blinded to the group assignments of the animals to ensure unbiased results.

### 2.7. Embryo analysis

After two months of consuming phthalates, female rats were mated overnight with unexposed males of the same clone. Pregnancy was ascertained by the detection of sperm in the vaginal smear, and the subsequent 24 h were termed Day 0 of gestation. Mated females were singly housed in clear polycarbonate cages with stainless steel wire lids and hardwood shavings as bedding. On the 21st day of gestation, the pregnant dams were euthanised, and the uterine horns were removed by laparotomy; the number of implantation sites, non-live implants, and viable foetuses were recorded. Embryos were weighed, the crown-rump length was measured, and all body abnormalities were recorded (e.g. hematomas, haemorrhages, changes in placenta) (Harris, 2019). Histopathological and morphological evaluations were conducted visually during dissection. Hematomas were identified as localised, internal accumulations of blood within embryonic tissues. Haemorrhages were defined as clotted blood visibly present on the external surface of the placenta, appearing as protruding masses or attachments. Resorptions were determined macroscopically, based on visibly underdeveloped or degenerated implantation sites with no discernible embryo, typically appearing as small, discoloured or haemorrhagic remnants (OECD, 2018).

### 2.8. Statistical analysis

Separate analyses were conducted for the DEHP and DBP groups. The DEHP groups included Control, DEHP_200, DEHP_1000, and DEHP_DBP, while the DBP groups comprised Control, DBP_100, DBP_500, and DEHP_DBP. For clarity and simplicity, the results from both groups were combined into a single graph. Group differences were assessed using one-way ANOVA followed by Tukey's HSD post-hoc test. When the assumption of equal variances was violated, Welch's ANOVA was used with the Games-Howell post-hoc test. For non-normally distributed data, the Kruskal-Wallis test was applied, followed by Dunn's post-hoc test with Bonferroni correction. Statistical analysis was

conducted using Rstudio version 2023.12.1 + 402.

## 3. Results

### 3.1. Long-term exposure to low-dose DEHP and DBP diets did not disturb the normal behaviour of adult female rats

The open field test results of test II showed that the DEHP_200 and DEHP_1000 groups spent twice as long in the open field centre ($F_{(2, 13)} = 5.437$, $p < 0.05$; post hoc Tukey's HSD for both groups, $p < 0.05$) and travelled twice as far compared to the negative control group ($F_{(2, 13)} = 12.681$, $p < 0.001$; post hoc Tukey's HSD for both groups, $p < 0.05$). (Fig. 1A, B). The open field test results for the DBP_100 and DEHP_DBP groups were unavailable due to a technical error, in which the recordings were not saved by the tracking software and the issue was discovered only after the completion of data collection. The elevated plus maze test showed the opposite results (Fig. 1C) to the open field test; experimental groups appeared to have travelled shorter distances and spent reduced time in the open arms of the maze. However, no significant differences were detected between the experimental groups and the negative control group in the time spent and distance travelled in the open arm, nor in the total distance travelled during an elevated plus maze test (see Supplementary Fig. S1; Fig. S2). Additionally, no significant differences were detected between groups in the rotation count during a running wheel test (Fig. 1D).

### 3.2. Long-term exposure to low-dose DEHP and DBP diets did not affect the normal physiology of adult female rats

Individual and group-level body weight data for adult female rats at the beginning and end of the months of phthalate exposure are presented in Supplementary Table S1. There were no significant differences detected in the adult female rats' weights between the experimental groups and the negative control, nor between the experimental groups themselves after the two-month phthalate exposure.

The detailed blood test results can be found in Table 1. A notable increase in white blood cell (WBC) count was observed in the DBP_500 group compared to the control group ($F_{(5, 30)} = 2.931$, $p < 0.05$; post hoc Tukey's HSD test $p < 0.05$); however, the results were within the normal range for this measure. Mean corpuscular haemoglobin concentration (MCHC) was also significantly higher in the DEHP_DBP group ($F_{(5, 30)} = 4.559$, $p < 0.01$; post hoc Tukey's HSD test $p < 0.05$). LYM, MONO, GRAN, HGB, RBC, MCV, MCH, RDWa, and PLT values were within the normal range and showed no significant differences when compared to the negative control group. HCT and MPV values for all groups were below the normal range, with no significant differences when compared to the negative control.

### 3.3. Long-term exposure to low-dose DEHP and DBP diets has a cyto-genotoxic effect in rat bone marrow cells

The results of the phthalate effect on bone marrow cells (Fig. 2) indicated a cyto-genotoxic effect in all groups receiving DEHP and DBP, with the results being quite firm. The DEHP_200, DEHP_1000, and DEHP_DBP groups had at least twice the number of polychromatic erythrocytes with micronuclei compared to the negative control ($F_{(3, 20)} = 21.655$, $p < 0.0001$; post hoc Tukey's HSD test for all groups, $p < 0.05$). No significant differences in means were observed between the different DEHP concentration groups in the MNPCE count. The results of the PCE/NCE ratio in the DEHP groups showed a significant reduction in the ratio in the DEHP_200 and DEHP_DBP groups compared to the negative control group ($F_{(3, 20)} = 10.004$, $p < 0.001$; post hoc Tukey's HSD test for both groups, $p < 0.05$). There were no statistically meaningful differences in the ratio between the negative control and DEHP_1000 groups and among the experimental groups.

The results for the DBP groups showed a significant increase in the MNPCE count in the DBP_100, DBP_500, and DEHP_DBP groups compared to the negative control. Moreover, the DBP_500 group had almost four times the number of MNPCEs compared to the negative control and twice as many compared to the DBP_100 and DEHP_DBP groups ($F_{(3, 20)} = 188.776$, $p < 0.0001$; post hoc Tukey's HSD test for all groups, $p < 0.05$). The PCE/NCE ratio was significantly reduced in the DBP_100, DBP_500, and DEHP_DBP groups compared to the negative control ($F_{(3, 20)} = 19.445$, $p < 0.001$; post hoc Games-Howell test for all groups, $p < 0.05$). There were no significant differences among the DBP groups in the PCE/NCE ratio.

### 3.4. Long-term exposure to low-dose DEHP and DBP diets had a differential effect on the weights and lengths of rat embryos, disturbed embryo viability in the rat uterus and induced some morphological abnormalities in the embryos

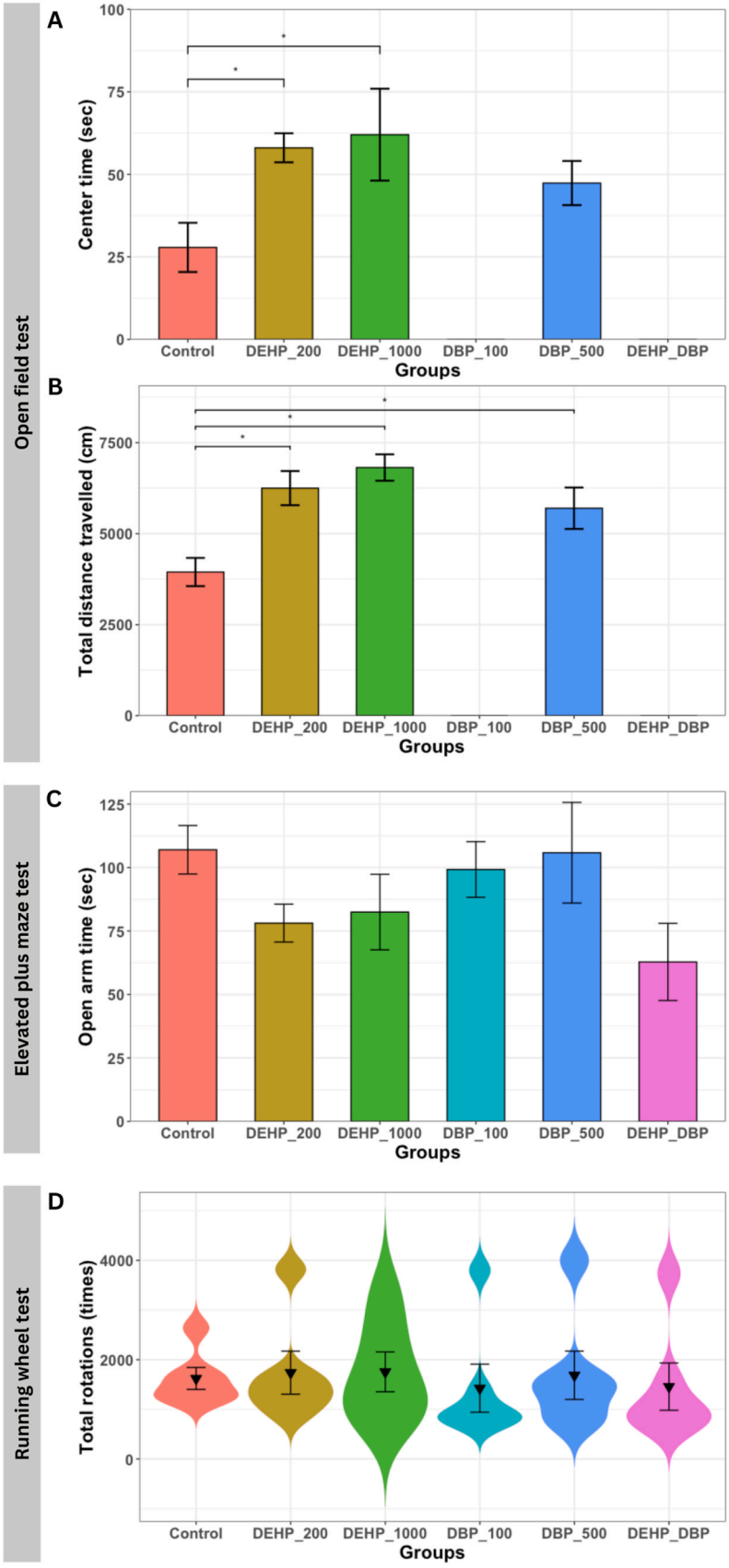
The average weights and lengths of the embryos and the number of embryo resorptions and gross morphological abnormalities (GMA) of the embryos were recorded during the dissection (Table 2). The weight of embryos in the DBP_100 group was significantly lower than that of the negative control group. Additionally, these embryos demonstrated a significant decrease in weight compared to those in the DBP_500 group, which acted as the positive control, and the DEHP_DBP group ($X^2_{(3)} = 16.717$, $p < 0.001$, post hoc Dunn's test for all groups, $p < 0.05$). Embryos in the DEHP_200 group also weighed less than those in the DEHP_1000 group, indicating a potential dose-response relationship. Conversely, the embryo weight in the DEHP_1000 group was significantly greater than that in the negative control group and DEHP_200 group ($X^2_{(3)} = 16.419$, $p < 0.001$, post hoc Dunn's test for all groups, $p < 0.05$).

Embryo lengths were similarly affected, as smaller concentration groups had shorter embryo lengths, and higher concentration groups had increased embryo lengths. The DEHP_200 and DEHP_DBP groups had significantly shorter lengths than the embryos from the DEHP_1000 group. The DEHP_1000 group also showed a significant increase in embryo length compared to the negative control group ($X^2_{(3)} = 17.178$, $p < 0.001$, post hoc Dunn's test for all groups, $p < 0.05$). Similar tendencies were observed in the DBP groups regarding embryo length; however, the differences were not significant.

The number of embryo resorptions and/or gross morphological abnormalities (GMA) in the embryos was recorded during dissection in all groups exposed to phthalates, with no pathologies observed in the negative control group (Table 2). The incidence of overall pathologies in the environmentally relevant groups, such as DEHP_200 (3.45 %) and DEHP_DBP (4.55 %), was similar. However, the DBP_100 group had three times more pathologies (12.50 %). The higher concentration groups had an increased incidence of pathologies, with 16.00 % in DBP_500 and the highest incidence of all groups at 32.76 % in the DEHP_1000 group. GMA included haematomas, haemorrhages, and placentomegaly, which were found in embryos or the placenta (see Supplementary Fig. S3; Fig. S4).

## 4. Discussion

Phthalates are recognised as endocrine-disrupting chemicals that impact multiple physiological systems by interfering with hormonal regulation, including those of the reproductive, nervous, and circulatory systems (Wang and Qian, 2021; Flaws et al., 2020). To reduce animal use in separate tests, we conducted a comprehensive study on the long-term effects of environmentally relevant DEHP and DBP exposure in female rats and their embryos. We assessed physiological, behavioural, genetic, and epigenetic endpoints. Our results show significant genetic and developmental disruptions, even at low doses. Unlike previous studies on high-dose or male-specific effects, our findings highlight the risks of prolonged low-level exposure in females, helping to fill a key knowledge gap.




*(caption on next page)*

**Fig. 1.** Behavioural test results during the open field test II, elevated plus maze test II, and running wheel test II. (A) Mean time spent (sec) in the central part of the open field area and (B) mean total distance travelled (cm). The DBP_100 and DEHP_DBP groups are not shown in (A) and (B) due to missing data resulting from a technical error in which recordings were not saved by the tracking software and the issue was discovered only after testing was completed. (C) Mean time spent (sec) in the open arm of the elevated plus maze. (D) Distribution of total rotation counts (times) during running wheel test, displayed as violin plots with overlaid group means (black triangles) and standard error of the mean (error bars). Statistical analysis: A, B and C - one-way ANOVA with Tukey's HSD post hoc test (*$p < 0.05$). D - Kruskal-Wallis test with Dunn's post hoc test and Bonferroni correction (*$p < 0.05$). Overall, no consistent effect across all tests was observed.

**Table 1**
General blood test results for adult rats across study groups. Values are presented as mean ± SD (p-value). Statistical comparisons between the experimental and control groups were performed using one-way ANOVA with Tukey's HSD post hoc test or Welch's ANOVA with Games-Howell post hoc test, as appropriate. Significant differences compared to the control group are indicated by p-values in brackets (*$p < 0.05$). Abbreviations: White Blood Cell count (WBC), Lymphocytes (LYM), Monocytes (MONO), Granulocytes (GRAN), Hemoglobin (HGB), Hematocrit (HCT), Red Blood Cell count (RBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Red Cell Distribution Width – Anisocytosis (RDWa), Platelet count (PLT), and Mean Platelet Volume (MPV).

| Parameter | Units | Control | DEHP_200 | DBP_100 | DEHP_DBP | DEHP_1000 | DBP_500 | Normal Range |
|---|---|---|---|---|---|---|---|---|
| **WBC** | $10^9$/l | 7.7 ± 4.9 | 8.2 ± 1.6 (0.998) | 10.2 ± 1.1 (0.476) | 9.7 ± 1.8 (0.698) | 11 ± 1.3 (0.199) | 12.2 ± 1.6 (0.0313)* | 7.2 – 12.6 |
| **LYM** | $10^9$/l | 5.9 ± 4.4 | 6.6 ± 1 (0.981) | 8.1 ± 0.8 (0.840) | 7.8 ± 1.4 (0.745) | 8.3 ± 0.9 (0.589) | 9.5 ± 1 (0.467) | 5.0 – 9.1 |
| **MONO** | $10^9$/l | 0.2 ± 0.1 | 0.2 ± 0.1 (1) | 0.3 ± 0.1 (0.742) | 0.2 ± 0.1 (1) | 0.4 ± 0.1 (0.0525) | 0.4 ± 0.1 (0.165) | 0.1 – 0.5 |
| **GRAN** | $10^9$/l | 1.7 ± 0.7 | 1.4 ± 0.5 (0.763) | 1.8 ± 0.5 | 1.6 ± 0.3 (0.983) | 2.2 ± 0.5 (0.462) | 2.3 ± 0.6 | 1.3 – 4.1 |
| **HGB** | g/dl | 14.1 ± 1.1 | 14.3 ± 0.8 (0.991) | 13.8 ± 0.5 (0.991) | 14.6 ± 0.6 (0.828) | 14 ± 0.9 (1) | 14.1 ± 0.7 (1) | 13.2 – 16.4 |
| **HCT** | % | 31.6 ± 2.3 | 31.8 ± 2 (1) | 30.2 ± 1 (0.726) | 31.4 ± 1.3 (1) | 31 ± 1.9 (0.989) | 31 ± 1.5 (0.991) | 43.6 – 48.6 |
| **RBC** | $10^{-2}$/l | 7.4 ± 0.5 | 7.4 ± 0.4 (0.999) | 7.3 ± 0.3 (0.998) | 7.4 ± 0.3 (0.999) | 7.3 ± 0.4 (1) | 7.2 ± 0.3 (0.972) | 7.21 – 8.45 |
| **MCV** | fl | 42.9 ± 1 | 42.6 ± 1.1 (0.996) | 41.5 ± 1 (0.185) | 42.1 ± 0.9 (0.787) | 42.5 ± 0.6 (0.98) | 43.1 ± 1.2 (0.999) | 39.0 – 50.0 |
| **MCH** | pg | 19.1 ± 0.5 | 19.3 ± 0.3 (0.985) | 19 ± 0.5 (0.985) | 19.6 ± 0.4 (0.383) | 19.2 ± 0.5 (1) | 19.5 ± 0.3 (0.579) | 17.7 – 20.1 |
| **MCHC** | g/dl | 44.6 ± 0.8 | 45.3 ± 0.5 (0.584) | 45.7 ± 0.7 (0.178) | 46.6 ± 0.3 (0.00094)* | 45.2 ± 1 (0.678) | 45.4 ± 1 (0.513) | 31.4 – 33.6 |
| **RDWa** | fl | 26.1 ± 0.9 | 26 ± 0.8 (1) | 25.6 ± 0.6 (0.709) | 25.9 ± 0.4 (0.995) | 26.1 ± 0.7 (1) | 26.3 ± 0.9 (0.999) | 0.0 – 99.9 |
| **PLT** | $10^9$/l | 314.5 ± 81.8 | 320 ± 63.7 (1) | 272.2 ± 24.1 (0.878) | 309.3 ± 60.3 (1) | 315.3 ± 79.8 (1) | 311.3 ± 73.6 (1) | 250 – 1200 |
| **MPV** | fl | 6.1 ± 0.2 | 6 ± 0.3 (0.977) | 5.9 ± 0.2 (0.829) | 5.9 ± 0.2 (0.766) | 5.8 ± 0.2 (0.473) | 6 ± 0.3 (0.996) | 8.0 – 12.0 |

### *4.1. Behavioural effect*

In this study, we conducted exploratory behavioural assessments as a supplementary component to our primary investigation of genetic, embryotoxic, and physiological effects of DEHP and DBP exposure in female rats using the open field test, elevated plus maze test, and running wheel test.

In the open field test, our study observed a significant increase in locomotor activity and reduced anxiety-like behaviour in adult female rats exposed to DEHP (200 and 1000 μg/kg) and DBP (500 μg/kg) via dietary administration. These results align with findings by Kaimal et al. (Kaimal et al., 2023), who administered 5 μg/kg body weight/day of DEHP orally to pregnant Sprague-Dawley rats during gestational days 6–21. Their female offspring demonstrated anxiolytic-like behaviours, including significantly fewer perimeter entries during the open field test, implying a relative increase in time spent in the central area. However, no significant changes in overall locomotor activity were reported. Although both studies reported anxiolytic outcomes, important methodological differences exist: our exposures were postnatal, long-term and oral via dietary intake, whereas Kaimal et al. used prenatal short-term exposure via gavage. Differences in strain (Sprague-Dawley vs. Wistar rats), concentration and the developmental window of exposure likely contributed to some differences in behavioural outcomes.

In contrast, Holahan et al. (Holahan et al., 2018), who tested the effects of DEHP at 0, 1, 10, and 20 mg/kg from postnatal days 16–22 in Long-Evans rats, found that the 20 mg/kg dose was associated with heightened motor activity in both males and females. While this hyperactivity mirrors our findings, the effective dose in that study was approximately 20 times higher than our highest DEHP dose, suggesting that even lower, environmentally relevant concentrations may exert subtle behavioural effects over longer exposure durations. Additionally, epidemiological evidence from Ku et al. (Ku et al., 2020) found that increased doses of phthalates in children's urine were associated with a higher risk of developing attention deficit hyperactivity disorder, which supports our findings.

However, contrasting results were observed in the elevated plus maze test. In this test, the experimental groups spent less time and travelled shorter distances in the open arms of the maze compared to the negative control group, suggesting a possibility of an increase in anxious behaviour. However, these results were not statistically significant. These findings are consistent with those of Sellinger et al. (Sellinger et al., 2020), who used similar concentrations (200 μg/kg and 1000 μg/kg) of a phthalate mixture—including DEHP and DBP—administered via diet for 30 days from gestational day 2 to postnatal day 10. Their study also reported no significant differences in EPM performance in male and female Long-Evans rats exposed during the perinatal or adolescent periods. In contrast, several other studies found statistically significant results for males, including Carbone et al. (Carbone et al., 2018), where increased anxiety-like behaviour was noted in adult male rats exposed to 30 mg/kg body weight/day of DEHP from birth to day 60. Other studies with mice have found that oral exposure to DBP (12.5, 25, 50, 100, and 200 mg/kg) for 14 days, or DEHP (2 mg/kg and 20 mg/kg) for 100 days, also induced anxiety-related behaviours (Farzanehfar et al., 2016; Kang et al., 2023). It is important to emphasise that the concentrations used in these studies were approximately 100 times higher than those used in our study and well above the typical human exposure levels. The differences in dosage, sex and animal models used in previous studies may have contributed to the divergent findings observed in comparison to our study.

Furthermore, the running wheel test did not reveal an increase in motor activity in the experimental groups compared to the negative control, unlike the open-field test. This divergence likely comes from the fact that the OFT primarily measures a rodent's exploratory locomotor response to novelty and stress, whereas the wheel-running test captures a motivated, self-reinforcing behaviour—one that involves reward systems, endocrine changes, and neuroplastic adaptations—rather than routine activity (Careau et al., 2012). Notably, there is a lack of existing

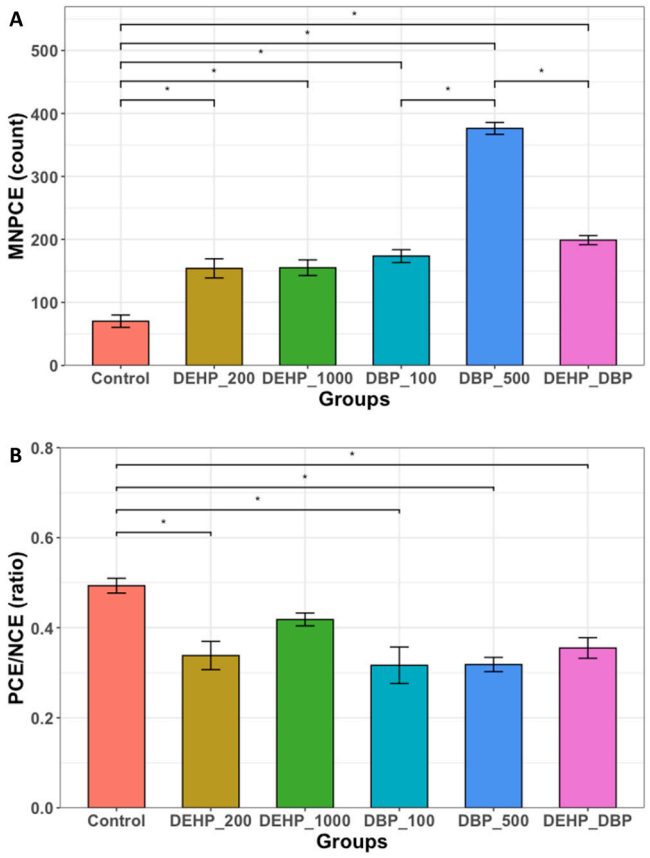



**Fig. 2.** Distribution of micronucleated PCEs (MNPCE) (A) and the PCE/NCE ratio (B) across study groups. Significant differences were observed between the experimental groups and the negative control group for both MNPCE count and PCE/NCE ratio. Statistical analysis: (A) One-way ANOVA with Tukey's HSD post hoc test (*$p < 0.05$); (B) One-way ANOVA with Tukey's HSD post hoc test (*$p < 0.05$) for the DEHP groups, and Welch's ANOVA with Games-Howell post hoc test for unequal variances (*$p < 0.05$) for the DBP groups.

literature on the use of voluntary wheel running to assess behavioural outcomes following DEHP or DBP exposure, which limits direct comparison but also highlights the novelty of this approach in toxicological research.

After evaluating all three behavioural tests, we conclude that there is no convincing evidence that DEHP and DBP, at concentrations reflective of typical exposure levels in Lithuania or even at five times higher concentrations (as used in positive controls), have significant effects on behavioural changes in adult female rats. However, it is important to highlight that behaviour testing was not the main focus of this study, and that it was done for exploratory purposes. As a result, the sample size for behaviour assessments was not optimised, and the results of this study should be interpreted with caution.

### 4.2. Impact on rat physiology

During our study, we did not find a significant effect of DEHP or DBP on weight gain in adult female rats after 2 months of daily phthalate exposure. There is extensive literature and numerous experiments on phthalates' effects on fat and weight gain in animals and humans, particularly concerning DEHP and DBP. However, the evidence is not entirely clear (Apau et al., 2020; Deierlein et al., 2022; Díaz Santana et al., 2019; Majeed et al., 2017; Radke et al., 2019). For example, a study reported increased weight gain and fat mass after 10 weeks of DEHP consumption at a concentration of 0.05 mg/kg via chow in female mice (Klöting et al., 2015). The difference between these findings and our own may be attributable to differences in animal models, exposure duration, or DEHP dosage.

Moreover, it has been noted in the literature that body weight is not always a reliable indicator of adiposity. Changes in fat mass can be obscured by simultaneous alterations in lean body mass, such as muscle loss. Since our study did not include direct measures of fat mass, it is difficult to determine whether DEHP or DBP may have influenced body composition in the absence of weight gain (Pohjanvirta et al., 2008).

A possible explanation for fat gain is that some phthalate metabolites induce adipocyte differentiation, thus stimulating adipogenesis through the activation of peroxisome proliferators. Another study with mice showed that the DEHP metabolite MEHP (mono(2-ethylhexyl) phthalate) enhanced PPARγ mRNA expression for adipocyte differentiation in mice receiving DEHP (1, 10, 100 mg/kg) for 8 weeks, although this did not correspond with an increase in body weight (Chiu et al., 2018). Similarly, a longitudinal observational study with women found changes in fat gain and body fat composition without an increase in body weight in midlife women (Peng et al., 2023). Future studies should focus on measuring fat mass directly to better understand how phthalates affect body fat, even when body weight does not change.

We analysed general blood parameters, and only two—WBC and MCHC—were significantly different from the control in two of the five experimental groups. The WBC value was increased in the DBP_500 group, although it remained within the normal range. A recent study involving pregnant women found that exposure to phthalates was associated with elevated levels of inflammatory blood cells (WBC, lymphocytes, neutrophils, and monocytes) (Yang et al., 2023). However, in our study, only WBC was increased, while LYM and MONO were not. Given the number of comparisons made, this significant difference could be attributable to chance or could represent the early stages of inflammation. Additionally, MCHC was significantly increased in the DEHP_DBP group. In a study with Sprague-Dawley male rats, which were administered phthalates at 500 mg/kg body weight (bw)/d, a

**Table 2**
Average embryo body weights and lengths, as well as the number of embryo resorptions and gross morphological abnormalities (GMA) per group, expressed as a percentage of total pathologies. Data are presented as mean ± SD or n (%). Percentages rounded to the nearest whole number. Significant differences were observed between some experimental groups and the negative control and between certain experimental groups for embryo weight and length. Statistical analysis: Kruskal-Wallis test with Dunn's post hoc test and Bonferroni correction ($p < 0.05$) for non-normally distributed data (embryo length in the DEHP groups), and one-way ANOVA with Tukey's HSD post hoc test ($p < 0.05$) for the analysis of all other results. * - significantly different from the control ($p < 0.05$). Values with the same superscript letter in a row differ significantly from each other ($p < 0.05$).

| Variable | Control | DBP_100 | DEHP_200 | DEHP_DBP | DBP_500 | DEHP_1000 |
|---|---|---|---|---|---|---|
| Live embryos, n | 38 | 37 | 28 | 22 | 21 | 42 |
| Weight, g | 5.57 ± 0.43 | 5.20 ± 0.56$^{*bc}$ | 5.23 ± 0.54$^{a}$ | 5.43 ± 0.59$^{c}$ | 5.62 ± 0.40$^{b}$ | 6.00 ± 0.85$^{*a}$ |
| Length, cm | 3.86 ± 0.27 | 3.77 ± 0.18 | 3.76 ± 0.27$^{a}$ | 3.76 ± 0.21$^{b}$ | 3.90 ± 0.22 | 4.02 ± 0.34$^{*ab}$ |
| Resorptions, n | 0 | 3 | 1 | 0 | 4 | 16 |
| GMA, n | 0 | 2 (hematoma, haemorrhage) | 0 | 1 (haematoma) | 0 | 3 (2- haemorrhages, placentomegaly) |
| Overall pathologies, n (%) | 0 (0.00) | 5 (13) | 1 (4) | 1 (5) | 4 (16) | 19 (33) |

significant increase in MCH, MCHC, and PLT values was found. However, the concentrations in that study were 1000 times higher and do not reflect actual concentrations found in the environment (Kwack et al., 2009). The observed change in MCHC may indicate a minor or early response to exposure, which has not yet resulted in noticeable changes in other RBC indices (MCV, MCH, etc.). This finding requires further investigation to eliminate statistical anomalies or the onset of inflammatory processes.

Overall, the literature is not entirely clear about the effects of phthalates on weight gain or changes in general blood parameters. Our findings contribute to this body of knowledge by demonstrating that after 2 months of daily phthalate consumption, we did not detect any substantial weight change or concerning blood parameter change in adult female rats.

### 4.3. Cyto-genotoxic effects

A micronucleus test on bone marrow cells yielded alarming results. The micronucleus assay demonstrated that DEHP and DBP have genotoxic and cytotoxic effects on rat bone marrow cells, resulting in a significant increase in the number of micronuclei in PCEs and a notable decrease in the ratio of PCEs to total erythrocytes (PCE/NCE).

In mature rats, the frequency of micronuclei in young erythrocytes is less than 0.1 %, and elevated frequencies indicate toxic effects on the body (OECD, 2016). Micronuclei formation results from the failure of the mitotic spindle, kinetochore, or other components of the mitotic apparatus, as well as damage to chromosomal substructures, changes in cell physiology, and mechanical disorders. Consequently, an increased frequency of micronucleated cells serves as a biomarker of genotoxicity, reflecting the effects of clastogenic (chromosome breakage: DNA is the target) or aneugenic (effects on chromosome number: non-DNA is the most common target) agents (Balmus et al., 2015; Smith et al., 1990; Borkotoky et al., 2014).

There are few studies on the potential genotoxic effects of phthalates on rat bone marrow cells. In one study (Kitamoto et al., 2015), the effect of DEHP on rat bone marrow cells was observed after administering three doses of 2000 mg/kg. The results indicated no significant increase in micronuclei in the cells. Conversely, no studies have investigated the long-term effects of DEHP phthalate concentrations on bone marrow cells. Our experiment showed that rats receiving 1000 μg/kg DEHP and 200 μg/kg DEHP had significantly more micronuclei in polychromatic erythrocytes compared to the control group. Therefore, it can be concluded that prolonged exposure of female rats to DEHP phthalate leads to an increase in micronuclei in polychromatic erythrocytes, indicating the genotoxic effect of di(2-ethylhexyl) phthalate on rat bone marrow cells.

The effects of DBP on rodent bone marrow cells have not been thoroughly investigated. Some genotoxicity studies of DBPs have been conducted on bone marrow cells in other organisms, such as the Nile tilapia (*Oreochromis niloticus*) (Benli et al., 2016). In our genotoxic experiment, DBP was shown to be genotoxic and induced micronuclei formation in PCE cells. Both doses of 500 μg/kg DBP and 100 μg/kg DBP significantly increased the micronucleus number in young erythrocytes. The phthalate mixture of 200 μg/kg DEHP + 100 μg/kg DBP was also genotoxic to rat bone marrow cells.

The genotoxicity of phthalates was further assessed by examining their effect on erythrocyte proliferation. Proliferation is the growth of a population of cells by division. Typically, the PCE/NCE ratio is 1:1. An increase in the NCE population and a decrease in the PCE population signal the cytotoxicity of the substance (Venitt and Parry, 1984). Our results indicated that most concentrations of phthalates (200 μg/kg DEHP, 100 μg/kg DBP, 500 μg/kg DBP, and 200 μg/kg DEHP + 100 μg/kg DBP) reduced the PCE/NCE ratio, indicating that both phthalates are cytotoxic to rat bone marrow cells. The statistically significant decrease implies that these phthalates accelerated erythrocyte ageing (Krishna and Hayashi, 2000).

The results of this study underscore how little we still understand about the health effects of these pollutants. Even low doses of phthalates found in Lithuanian nature have strong genotoxic and cytotoxic effects on bone marrow cells. Unfortunately, research in this area is very sparse and needs to be expanded to get a better understanding of the effects of phthalates on DNA.

### 4.4. Effect on reproductive outcomes and embryo development

Interesting findings emerge from the analysis of embryo development. It appears that phthalates indeed affect embryo development, but the effect varies across different concentrations. Higher concentration groups (1000 μg/kg of DEHP; 500 μg/kg of DBP) had heavier and longer embryos compared to the negative control. In contrast, the smaller – 200 μg/kg of DEHP and 100 μg/kg of DBP (environmentally relevant) concentrations showed the opposite effect, with embryos being lighter and shorter than the negative control.

These observations are suggestive of a possible non-monotonic dose response (NMDR) of phthalates as documented in the literature (Scientific Committee et al., 2021; Hill et al., 2018). While the endpoints assessed in our study (embryo weight and length) have not been specifically reported in existing NMDR studies, a recent systematic review on the reproductive toxicology of DBP, particularly its effects on male reproductive parameters, is supportive of our findings, showing that DBP exhibits a non-linear dose response, with more robust physiological responses observed at lower concentrations ($\leq$ 10 mg/kg bw/day) (Czubacka et al., 2021). However, only a few studies were done with environmentally relevant concentrations of $<$ 1 mg/kg dw/day (Czubacka et al., 2021). This underscores the importance of researching environmentally relevant concentrations to better understand the effects of phthalates on embryo development.

The implications of NMDR effects are significant from both toxicological and regulatory perspectives. As highlighted by EFSA (Scientific Committee et al., 2021), NMDRs are particularly relevant for receptor-mediated mechanisms and may not be adequately captured by traditional testing strategies, which often assume linearity and rely on limited dose ranges. Moreover, Hill et al. (Hill et al., 2018) emphasise that NMDRs can occur at doses below the NOAEL and even below reference doses, thereby challenging the assumption that high-dose testing can reliably predict safety at low exposure levels. This raises concerns about the adequacy of current risk assessment frameworks in protecting human health, especially when environmentally relevant concentrations—such as those assessed in our study—are rarely included in standard toxicological evaluations. Incorporating NMDR considerations into regulatory guidelines is thus essential for improving the accuracy and relevance of health-based guidance values for endocrine-disrupting chemicals like phthalates.

Phthalates have demonstrated not only an impact on embryo growth but also embryotoxic effects, with embryo resorptions and gross morphological pathologies observed in all experimental groups except the negative control. The most prevalent pathologies appeared in the positive control groups (DEHP_1000 – 32.76 %; DBP_500 – 16.00 %). Notably, even environmentally relevant DBP concentrations of 100 μg/kg resulted in a substantial percentage (12.50 %) of pathologies, including embryo resorptions and gross morphological changes (hematomas, haemorrhages in embryos and placentamegaly). Other researchers have reported similar findings (Mahaboob Basha and Radha, 2017; Saillenfait et al., 2009; Al-Saleh et al., 2024), which have observed increased resorptions, implantation losses, alterations in foetal body measurements, and skeletal abnormalities following exposure to DBP, DEHP, or other phthalates in animal or human studies. For instance, a study conducted with rats receiving DBP at 500 mg/kg bw/day found a reduced number of live births, lower foetal body weights, increased mortality, and implantation loss. Furthermore, skeletal abnormalities involving the eyes and face were identified (Mahaboob Basha and Radha, 2017). Another study observed decreased foetal weights and

malformations, including cleft palate, eye abnormalities, and defects in the spinal skeleton in rats at concentrations of 750 mg/kg of di-n-hexyl phthalate (DnHP). Foetal growth was also reduced in rats receiving 750 mg/kg of dicyclohexyl phthalate (DCHP) (Saillenfait et al., 2009). Notably, the concentrations used in these studies were up to 1000 times higher and administered at specific gestational days, which does not fully reflect the typical human exposure (Abdul-Ghani et al., 2012; Czubacka et al., 2021; Mahaboob Basha and Radha, 2017; Saillenfait et al., 2009). A study analysing maternal exposure to phthalates also supports these *in vivo* findings, showing decreased birth weight in women exposed to DEHP (Al-Saleh et al., 2024).

Previous research (Lovekamp-Swan and Davis, 2003) has demonstrated that mono-(2-ethylhexyl) phthalate (MEHP), a metabolite of DEHP, activates Peroxisome Proliferator-Activated Receptors (PPARα and PPARγ) in granulosa cells, which are essential in regulating gene expression. The activation of PPARs leads to a reduction in the transcription of aromatase, an enzyme that converts androgens (such as testosterone) into oestrogens (like estradiol). This disruption impairs follicle development and ovulation, ultimately leading to infertility (Lovekamp-Swan and Davis, 2003). Although we did not detect infertility in our study, we observed the genotoxic effects of phthalates, which may have contributed to increased incidences of embryo resorptions and abnormalities.

Additionally, a recent study discovered that MEHP may impact the Wnt/β-catenin pathway in mouse embryos, a pathway critical for cell proliferation and differentiation, thus affecting processes such as gastrulation, axis formation, organogenesis, and placental formation, consequently impairing proper embryo development (Wang et al., 2023). An earlier study detected downregulation of the Wnt/β-catenin pathway in foetal male rats after their mothers' exposure to DBP (Zhang et al., 2011). These findings regarding the mechanism of action of phthalates could potentially explain the pathological effects observed in embryonic development.

The results of our study align with other research, suggesting that DEHP and DBP might affect embryo development and could be embryotoxic, even at lower, environmentally relevant concentrations faced daily over a longer period, which is of high concern. New studies are only now beginning to reveal the pathways behind these developmental pathologies.

### 4.5. Limitations and future directions

This study includes several limitations that should be acknowledged. The experiment was conducted exclusively on female rats. Although rats are frequently used in toxicology studies as models for human biology, the results might only partially represent the effects on humans. The focus on female rats also introduces variability due to their oestrous cycles, which can influence physiological and behavioural responses. Importantly, oestrous cycle stages were not monitored or controlled for in this study, which could have contributed to within-group variability and may have masked subtle effects, particularly in behavioural measures. Future studies should incorporate monitoring or synchronisation of oestrous phases to reduce biological noise and clarify hormone-sensitive endpoints. However, since numerous studies have already been conducted on males, our focus was to expand the knowledge of phthalate effects on female health.

Furthermore, the sample size was relatively small, which may limit the statistical power of our findings and the ability to detect subtle effects. We chose the minimum number of animals for statistical validity to balance ethical considerations. Nevertheless, the limited group sizes increase the risk of Type II errors and reduce the robustness of intergroup comparisons. This constraint is particularly relevant in the context of non-monotonic dose-response patterns, where detection of non-linear effects may require finer resolution and greater sensitivity. A small sample size can also lead to biased estimates of effect sizes, potentially underestimating mild or variable responses, such as behavioural changes. This limitation reduces the precision of our estimates and weakens the ability to detect heterogeneity among individuals. Moreover, it constrains the extrapolation of our findings to broader populations or environmental contexts. It should also be noted that behavioural testing in this study was performed as an exploratory analysis; the primary focus of the research was on embryonic, genetic, and physiological outcomes.

Future studies should increase the sample size to strengthen the statistical reliability of the results. Larger cohorts would also enable stratification by additional biological factors, including oestrous cycle phase or developmental stage, which may interact with phthalate exposure. Additionally, researchers should account for and control for the different stages of the oestrous cycle in female rats, which could give insights into how these cycles influence the observed outcomes. By addressing these factors, future research could expand on the current study, providing a more thorough understanding of the effects and their potential implications for human health.

## 5. Conclusions

This study showed that environmentally relevant concentrations of DEHP and DBP, when consumed daily over the long term, do not significantly impact the neurological behaviour or physiology of adult female rats. However, it should be noted that behavioural testing was conducted for exploratory purposes and was not the primary focus of this study; as such, the sample size was not optimised for behavioural endpoints, and these results should be interpreted with caution. In contrast, these concentrations caused a cyto-genotoxic effect on female rat bone marrow cells and adversely affected embryo development by affecting embryo length and weight and inducing pathologies and resorptions. These findings suggest that even low concentrations of phthalates could harm the mammalian body, raising significant concerns about their potential impact on human health.

## CRediT authorship contribution statement

**Valdas Šimčikas:** Methodology. **Rasa Aukštikalnienė:** Writing – review & editing, Investigation. **Rokas Buišas:** Methodology. **Grita Skujienė:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Violeta Žalgevičienė:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Laurynas Orla:** Writing – review & editing, Investigation. **Edita Paulikaitė-Bivainė:** Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Evita Sėrikovaitė:** Investigation. **Justina Alčauskaitė:** Investigation. **Vaidotas Valskys:** Funding acquisition. **Rokas Zalūba:** Investigation.

## Ethical Statement

All experimental procedures were approved by the State Food and Veterinary Service of the Republic of Lithuania (2022–08/09, No G2–221) and were carried out following the local Animal Welfare Act and the European Communities Council Directive of 22 September 2010 (2010/63/EU).

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## Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used ChatGPT-3.5

and Grammarly in order to improve readability and language. After using these tools/services, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

## Declaration of Competing Interest

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.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2025.118736.

## Data Availability

Data will be made available on request.

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